An Investigation into the Diagnosis, Prediction and Management of Oral Potentially Malignant Disorders

A Thesis Submitted to Newcastle University for the Degree of Doctor of Philosophy in the Dental Sciences

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Abstract

Oral cancer may be proceeded by dysplastic PMDs mainly presenting as leukoplakia or erythroplakia and which can carry an increased risk of malignant transformation. Therefore, early recognition of PMDs with a high potential for cancer development is important to improve patient outcome.

100 patients with dysplastic PMDs presenting in Newcastle underwent a standardised interventional management protocol based on risk factor assessment, laser excision of dysplastic lesions and long-term clinical follow-up at regular intervals. This patient cohort was studied in detail to examine the clinicopathological features that may influence disease progression. Single dysplastic PMDs were mainly observed in the floor of mouth, with 92% presenting as leukoplakia and 8% as erythroplakia. Follow-up revealed that 62% of patients remained disease-free following laser surgery, 17% showed recurrent-disease, 14% new-site dysplasia, with 5% malignant transformation and 2% developed OSCC at a site distant from the primary dysplasia.

Clinical appearance, high grade dysplasia, larger sized PMDs, high risk sites and positive excision margins were shown to increase the risk of unfavourable clinical outcome. Malignant transformation was mainly seen in non-smokers and non-alcohol users, whilst new-site OSCC was only seen in non-smokers and light drinkers.

The use of Raman spectroscopy in the detection and classification of dysplasia within the human oral tissue was investigated. Currently, histopathology is considered the diagnostic gold standard. Consensus opinion on dysplasia grading of individual PMD lesions using two classification systems (WHO and binary grading) was obtained and a spectral diagnostic model then correlated with the results. The ability of Raman spectroscopy to differentiate between dysplasia and morphologically normal tissue was shown, with an 81% sensitivity and specificity. This supports the suitability of the Raman system in clinical use to distinguish morphologically normal from dysplastic tissue. This work has also shown the efficacy of Raman spectroscopy in identifying early biochemical changes in epithelial dysplastic tissue before morphological/histological change becomes apparent.

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Dedication

This thesis is dedicated to the spirit of my mother who taught me how to be strong and how to love all people

Declaration

I declare that the work presented in this thesis is original, has been carried out by the author and has never been presented in full or in part in the same or in different form on this or any other university in support of any application for any degree.

Published Abstracts (Appendix 1-G):

1. **Risk Factors and Oral Precancer–Developing a High/Low Risk Profiling System**. Ameena Diajil, Michaela Goodson and PJ. Thomson. *Oto-Rhino-Laryngology and Head and Neck*. March 2010; 267 (Supp. 1).

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3. **Smoking Behaviour Influences Outcome in Oral Potentially Malignant Disorder Patients**. Ameena Diajil and PJ Thomson *UAE International Dental Conference & Arab Dental exhibition AEEDC Dubai*. February 2011.

4. **Smoking Behaviour and Oral Potentially Malignant Disorders**. Ameena Diajil and PJ Thomson. *J Dent Res* 90 (Spec Iss A): 879, 2011.

5. **Clinical Outcomes Following Oral Potentially Malignant Disorder Treatment- The Newcastle 100 patient study**. PJ Thomson and Ameena Diajil. *British Association of Head & Neck Oncologists*. April 2012 London.

6. **Time to Treatment - Dose Time to Treatment Influence Clinical Outcome Following Laser Excision of Oral Potentially Malignant Disorders?** Michaela Goodson, Ameena Diajil, Max Robinson, PJ Thomson. *British Association of Head & Neck Oncologists*. April 2012 London.

Abbreviations

4NQO	4 Nitroquinoline 1-oxide
ANNs	Artificial neural networks
ANOVA	Analysis of variance
A. u	Arbitrary unit
BaF_2	Barium fluoride
BCC	Basal cell carcinoma
CASRS	Coherent anti-Stokes Raman scattering
CCD	Charge-coupled device
Cig/day	Cigarettes per day
CIS	Carcinoma *in situ*
Cnts/sec	Counts per second
DA	Disease active
DDT	Definitive diagnosis time
DF	Disease free
DM	Diabetes mellitus
EBV	Epstein-Barr virus
ESS	Elastic scattering spectroscopy
FCA	Fussy c-means cluster analysis
FFPE	Formalin-fixed paraffin embedded
FOM	Floor of the mouth
FS	Fluorescence spectroscopy
H and E	Haematoxylin and eosin stain
HCA	Hierarchical clustering analysis
HGD	High grade dysplasia
HGN	Morphologically normal from high grade dysplasia
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
IARC	International Agency for Research on Cancer
ICD	International Classification of Disease
ICD-DA	International Classification of Diseases Application to Dentistry and Stomatology
IR	Infrared
IRS	Infrared spectroscopy
ISCO	International Standard Classification of Occupations
KMCA	K-mean cluster analysis
LDA	Linear discriminant analysis
LGD	Low grade dysplasia
LGN	Morphologically normal from low grade dysplasia
MD	Mahalanobis distance
Md	Mild dysplasia
Mn	Morphologically normal from mild dysplasia
Modd	Moderate dysplasia

Modn	Morphologically normal from moderate dysplasia
MT	Malignant transformation
N	Number
NIR	Near-infrared
OE	Oral erythroplakia
OED	Oral epithelial dysplasia
OL	Oral leukoplakia
OLP	Oral lichen planus
OR	Odd-ratio
OSCC	Oral squamous cell carcinoma
OSF	Oral submucous fibrosis
PCA	Principal component analysis
PCs	Principal components
PDT	Provisional diagnostic time
PLSCs	Partial least square analysis components
PLS-DA	Partial Least Squares Discriminant Analysis
PMD	Potentially malignant disorder
PVL	Proliferative verrucous leukoplakia
Q^2	Quality of model fit
RRS	Resonance Raman spectroscopy
RS	Raman spectroscopy
S	Scores
SD	Standard deviation
Sd	Severe dysplasia
SERS	Surfaces enhance Raman spectroscopy
SG	Savitzky-Golay
SIL	Squamous intraepithelial lesions
SIMCA	Soft Independent Modeling of Class Analogy
SIN	Squamous intraepithelial neoplasia
Sn	Morphologically normal from severe dysplasia
SNV	Standard normal variate
THC	Tetrahydrocannabinol
U/W	Units per week
UV	Ultra violet

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General Introduction

Oral cancer is a potentially fatal disease that usually presents late and has a poor prognosis. In the UK in 2009, 6236 persons were diagnosed with oral cancer and there were approximately 1985 deaths in 2010 (Oral cancer statistics - UK, 2012). The most common oral cancer is squamous cell carcinoma (OSCC) (Chen and Hunter, 2005; Lee et al., 2006; Epstein et al., 2008; Rapidis et al., 2009).

OSCC affects significant numbers of people around the world and represents more than 90% of head and neck cancers (Arduino et al., 2008; Warnakulasuriya, 2009). Recently, 4-10% of cases have been reported in patients below the age of 40 years (Bachar et al., 2011); however, other studies suggest that the incidence in this age group may actually be higher (Myers et al., 2000; Gillison, 2007). Approximately two thirds of OSCCs are diagnosed at advanced stages (Epstein et al., 2007; Ahmed et al., 2009). The late diagnosis of a significant number of OSCCs is mostly attributable to delays in patients seeking treatment, insufficient patient awareness, asymptomatic clinical states and/or inappropriate investigation (Mehrotra et al., 2006; Morse et al., 2009).

Despite the different treatment modalities for OSCCs, such as surgery, radiotherapy, chemotherapy, chemo-radiation and immunotherapy (Bagan and Scully, 2008), the five-year survival rate has not improved in recent years (Chen and Hunter, 2005). There is thus a vital need for an effective and reliable diagnostic procedure for early detection and subsequent effective management and eventually improvement in quality of life for patients.

OSCCs may arise from Potentially Malignant Disorders (PMDs), a term that has been recently introduced by the WHO to be used instead of premalignant or precancerous lesions/conditions (van der Waal, 2009), which incorrectly imply cancer development is a simple two-step process (Reibel, 2003). PMDs are mainly leukoplakia, erythroplakia, erythroleukoplakia, lichen planus, submucous fibrosis and actinic cheilitis as well as inherited cancer syndromes (van der Waal, 2009). Most oral PMDs are asymptomatic or present with few symptoms and they are regarded as an intermediate stage between normal and malignant tissues (Kujan et al., 2006).

PMDs may exhibit epithelial dysplasia, a histological term that has no specific clinical association, but tends to increase the risk of malignant transformation (MT) of these disorders

(Reibel, 2003; Hsue et al., 2007). Histologically, epithelial dysplasia is characterised by cellular atypia and tissue dysmaturation and it ranges from hyperplasia to intraepithelial dysplasia (Kujan et al., 2007). MT of normal oral epithelium into a carcinoma may be caused by carcinogens and this includes changes in genes regulating cell division, cell cycle progression, DNA synthesis and repair (Bettendorf et al., 2004; Thomson et al., 2006).

There are several classification systems for epithelial dysplasia and this causes difficulties in routine application (Kujan et al., 2007). As a result, the WHO Consensus Group made efforts to establish a universal classification of oral epithelial dysplasia. This classification depends on architectural and cellular features as well as the dysplastic layer thickness in relation to full epithelial thickness (Gale et al., 2005). Five histological grades have been identified: hyperplasia, mild, moderate or severe dysplasia, and carcinoma *in situ*. Using the same WHO cellular and architectural criteria, a binary grading system of 'high risk' and 'low risk' classification has been suggested and evaluated by Kujan *et al.* (2006) who indicated that a two-tier classification may produce an improvement in observer agreement compared with the five-point WHO classification system.

Early diagnosis and treatment of oral cancer and PMDs requires assessment of potential predisposing risk factors (Mawardi et al., 2011) and necessitates a partnership between clinicians, pathologists and surgeons. Taking a biopsy from a PMD at the first visit is preferable to delaying biopsy until after elimination of possible causative factors and observation for possible regression (van der Waal, 2009). However, an observation period of 2-4 weeks for regression has been suggested and if no regression occurs, a biopsy for histopathological diagnosis should then be carried out to confirm the presence and also the degree of epithelial dysplasia (van der Waal, 2009).

Surgical biopsy is the conventional method of diagnosis for PMDs and suspected cancers. Although histopathological examination is the gold standard in the diagnosis of dysplastic and malignant tissues (Bremmer et al., 2009), it suffers from inter- and intra-pathological observer variation. Hence, there is a vital need for an objective tool which is able to detect early dysplastic tissue changes in a standardised and universal way.

Recently, optical spectroscopy has been employed to analyse different aspects of clinical disease and has been used for PMD diagnosis (Malini et al., 2006; Sharwani et al., 2006). Optical spectroscopy is a general term encompassing three methods: fluorescence, Raman and infra-red spectroscopy (Swinson et al., 2006). These techniques have different principles but they are all sensitive to changes in biochemical tissue structure which has been found to play a role in differentiating between normal and dysplastic tissues (de Veld et al., 2005b; Krishna et al., 2005b) potentially aiding early, objective diagnosis and targeting interventional treatments.

Chapter One: Potentially Malignant Disorders

Potentially Malignant Disorders

PMD is the latest WHO recommend term for a group of disorders that carry an unpredictable risk of MT; leukoplakia and erythroplakia are the most common lesions (van der Waal, 2009). PMDs have been defined as a morphologically altered tissue in which cancer is more likely to develop than apparently normal counterpart (Kramer et al., 1978a; Pindborg et al., 1997). They vary from a small well-defined white or red mucosal patch to a widespread and extensive involvement of oral mucosa (van der Waal and Axell, 2002; Sloan, 2011).
Examples of white, predominantly white and/or red disorders of the oral mucosa that carry an increased risk for oral cancer development are listed in Table 1.1.

Table 1.1: Potentially malignant disorders.

(Pindborg et al., 1997; Napier and Speight, 2008; Scully and Bagan, 2009; van der Waal, 2009)

Disorders
Leukoplakia
Erythroplakia
Erythroleukoplakia
Lichen planus
Submucous fibrosis
Actinic cheilitis
Dyskeratosis congenita
Xeroderma pigmentosum
Epidermolysis bullosa
Discoid lupus erythematousus
Fanconi syndrome
Paterson–Kelly-Brown syndrome (sideropenic dysphasia; Plummer–Vinson syndrome)
Immunosuppression
Chronic candidiasis
Scleroderma
Palatal lesion of reverse cigar smoking

1.1. Oral Leukoplakia

Oral leukoplakia (OL) is defined as ‘‘a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer” (Warnakulasuriya et al., 2007). OL is the most common PMD (Axell et al., 1996; Pindborg et al., 1997; Dietrich et al., 2004; Mithani et al., 2007) affecting 0.2- 4.9% of the world population (Gupta et al., 1980; van der Waal et al., 1997), depending on country, population, associated risk factors and clinical definition (Napier and Speight, 2008). Epidemiological data over the past 30 years showed a prevalence of OL ranging from 1.1% to 11.7% depending on age and sex (Campo-Trapero et al., 2008a). In a systematic review of 23 studies from 17 countries, an overall 2.6% prevalence of OL was calculated in spite of considerable differences noted between studies (Petti, 2003).

Generally, OL is more common in males than females with a ratio of 5: 3 (Petti, 2003). However, the sex distribution may vary greatly depending on geographical variations and lifestyle habits (Reichart, 2001). Usually OL is not diagnosed before the age of 30 years, the highest prevalence is in the fifth decade (van der Waal et al., 1997). In developed countries, leukoplakias are more common in middle age and elderly patients with the majority seen in patients aged between forty and seventy, whilst in developing countries it may appear five to ten years earlier (Napier and Speight, 2008).

The most common site affected by OLs varies in different studies and this variation may similarly be related to geographical differences, race, and individual habits (Ishii et al., 2004). For example, the buccal mucosa, alveolar mucosa and lower lip were the common sites observed in a study conducted by Waldron and Shafer (1975), whilst buccal mucosa and the floor of mouth (FOM) were the most commonly affected oral sites, followed by lateral border of tongue, with gingiva and labial commissures least affected in a study carried out by Jaber *et al.* (2003). Ishii *et al.* (2004) showed that OL is mainly observed on gingiva, followed by tongue, and palatal mucosa. Another study, performed by Holmstrup *et al.* (2006), showed that the FOM and the buccal mucosa were the most common sites. A retrospective hospital-based study demonstrated that the lateral border of the tongue was the commonest affected site, followed by the buccal mucosae (Arduino et al., 2009).

In the same year, (Hamadah and Thomson, 2009) reported that the FOM and the ventro-lateral surface of the tongue, followed by the buccal mucosae were the most common involved sites. In more recent studies, Lim *et al.* (2010) reported that the FOM, lip, tongue and buccal mucosa were the most common oral subsites affected by OL, whereas the palate and alveolus were less commonly affected. Jerjes *et al.* (2012) showed that primary affected sites were mainly the ventro-lateral tongue and FOM, followed by buccal mucosa.

1.1.1. Oral Leukoplakia: Clinical Presentation

OL is a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion (Axell et al., 1996) and may be seen in any part of the oral cavity and oropharynx. Clinically, OL is of two main types: homogenous and non-homogenous. The differentiation between them is entirely clinical, based upon surface, colour and morphological features (Warnakulasuriya et al., 2007; Winter et al., 2011).

Homogenous leukoplakia, which represents approximately 90% of all leukoplakia cases, is predominantly white in colour, flat, thin, with shallow cracks, wrinkled or corrugated surface keratin with a uniform texture throughout (Axell et al., 1996; Warnakulasuriya et al., 2007; van der Waal, 2009).

Non-homogenous leukoplakia is predominantly white or mixed white and red. It is either irregular flat, nodular with small polypoid outgrowths or excrescences, speckled (erythroleukoplakia) which is mixed white and red, ulcerated or verrucous leukoplakia (Pindborg et al., 1997; Warnakulasuriya et al., 2007). However, individual leukoplakias may have a varied clinical appearance which can change over time (Neville et al., 1995).

OL may present as single localized lesion, or as multiple widespread areas with diffuse outlines (van der Waal and Axell, 2002). Classification and/or staging of OL recommend recording the size of a single leukoplakic lesion and to add together sizes of multiple leukoplakias (van der Waal and Axell, 2002).

Also, the oral subsite should be specified according to the International Classification of Disease (ICD) topographical codes for localization in the oral cavity (van der Waal and Axell, 2002); Table 1.2.

Table 1.2: Topographical codes of disease localization in the oral cavity.

International Classification of Disease (ICD-10)	
Oral subsites	**Topographical code**
Lips	C00.3, C00.4, C00.5, C006
Tongue	C02.0, C02.1, C02.2
Gum	C03.0, C03.9
FOM	C04.0, C04.1
Hard palate	C05.0
Soft palate	C05.1
Cheek mucosa	C06.0
Retromolar area	C06.2
Mouth unspecified	C06.9
Tonsillar pillar	C09.1

Proliferative verrucous leukoplakia (PVL), which was first described by Hansen *et al.* (1985), is more prevalent among elderly women with or without history of tobacco use (van der Waal, 2009). It is four times more common in women than men and is typically diagnosed after the sixth decade of life (Hansen et al., 1985; Silverman and Gorsky, 1997; Bagan et al., 2011). Tobacco use, the presence of candida or immunodeficiency are not associated with PVL (Suarez et al., 1998) and the majority (63%) of PVL patients do not use tobacco products according to a study conducted by Cabay *et al.* (2007).

PVL is of uncertain aetiology, although some authors have reported an association with HPV (Eversole, 2000), whilst others have found no association (Bagan et al., 2007; Stokes et al., 2012). The verrucous texture of PVL is a distinguishing feature from homogeneous leukoplakia; however, verrucous leukoplakia may be indistinguishable from verrucous carcinoma (Cabay et al., 2007).

PVL may develop on any soft tissue surface of the oral cavity and may be either a single, discrete lesion or less commonly scattered, multifocal growths involving multiple oral sites (Morton et al., 2007). Buccal mucosa and tongue are the most common sites associated with PVL, with palatal mucosa, alveolar mucosa, gingiva, FOM and lip showing a much lower incidence (Hansen et al., 1985; Batsakis et al., 1999). According to Silverman and Gorsky (1997), buccal mucosa is the most common site for PVL in women, whilst in men it is the tongue.

Clinically, PVL mostly appears as a flat white keratotic lesion with a verrucous surface and may be associated with an erythematous component. As the lesion progresses it becomes more exophytic, granular and verruciform ultimately becoming multifocal and developing a warty-type appearance (Hansen et al., 1985).

1.1.2. Leukoplakia and Level of Certainty

The term leukoplakia can be used at different levels of certainty (C-factor). According to van der Waal (2009), four steps of certainty may be useful in the diagnosis of OL: C_1 and C_2 which are clinical terms and C_3 and C_4 which are clinicopathological terms.

1) C_1: a single visit, provisional clinical diagnosis involving inspection and palpation.
2) C_2: a definitive clinical diagnosis when there is no evidence of resolution following elimination of etiological factors (mechanical irritation) over 2-4 weeks of follow-up or in the absence of any other etiological factors.
3) C_3: a provisional histopathological diagnosis similar to C_2 but with incisional biopsy data.
4) C_4: the definitive histopathological diagnosis with evidence from histopathological examination of completely excised lesions.

Leukoplakia is mostly used as a clinical term and a provisional diagnosis is made when a predominantly white area at clinical examination cannot be clearly diagnosed as any other disease in the oral mucosa (Axell et al., 1996; Pindborg et al., 1997).

The diagnosis of OL is thus made after exclusion of other disorders and biopsy is recommended when other disorders cannot be identified. The disorder is diagnosed as an OL with or without epithelial dysplasia.

Table 1.3 shows the 'excluded disorders' with their diagnostic criteria and whether there is a need for biopsy or not.

Figure 1.1 demonstrates a schematic diagram of the most common steps of OL diagnosis.

All leukoplakias should be viewed with suspicion because even small leukoplakia can manifest significant dysplasia or unexpected carcinoma; therefore conventional biopsy is recommended for any true leukoplakia.

Table 1.3: The "excluded disorders" in relation to diagnosing of oral leukoplakia.

(Warnakulasuriya et al., 2008; van der Waal, 2009)

Disorders	Diagnostic criteria	Biopsy need
White spongy nevus	Seen in early life, family history, larger area, may affect genital mucosa as well as oral mucosa	Not required
Lichen planus	plaque type or reticular form	Yes
Discoid lupus erythematousus	Circumscribed central erythema with white radiating striae	Yes, with Immunofluorescence
Frictional keratosis	Associated with trauma, along the occlusal line, mostly reversible by removing the cause	Yes
Morsicatio buccarum	Irregular white scale due to habitual biting of lip and cheek	Not required
Chemical injury	Related to chemical substance, painful, rapidly reversible	Not required
Acute pseudomembranous candidiasis	The membrane can be removed by scraping, resulting in an erythematous bleeding surface	Swab for culture
Leukoedema	Bilateral on buccal mucosa	Not required
Hairy leukoplakia	Hyperkeratosis of lateral tongue bilaterally	Yes
Skin graft	From the history	Not required
leukokeratosis nicotina palate	Related to smoking history, greyish, white palate	Not required
Lichenoid reaction	Related to drug history or sited near amalgam restoration	Yes

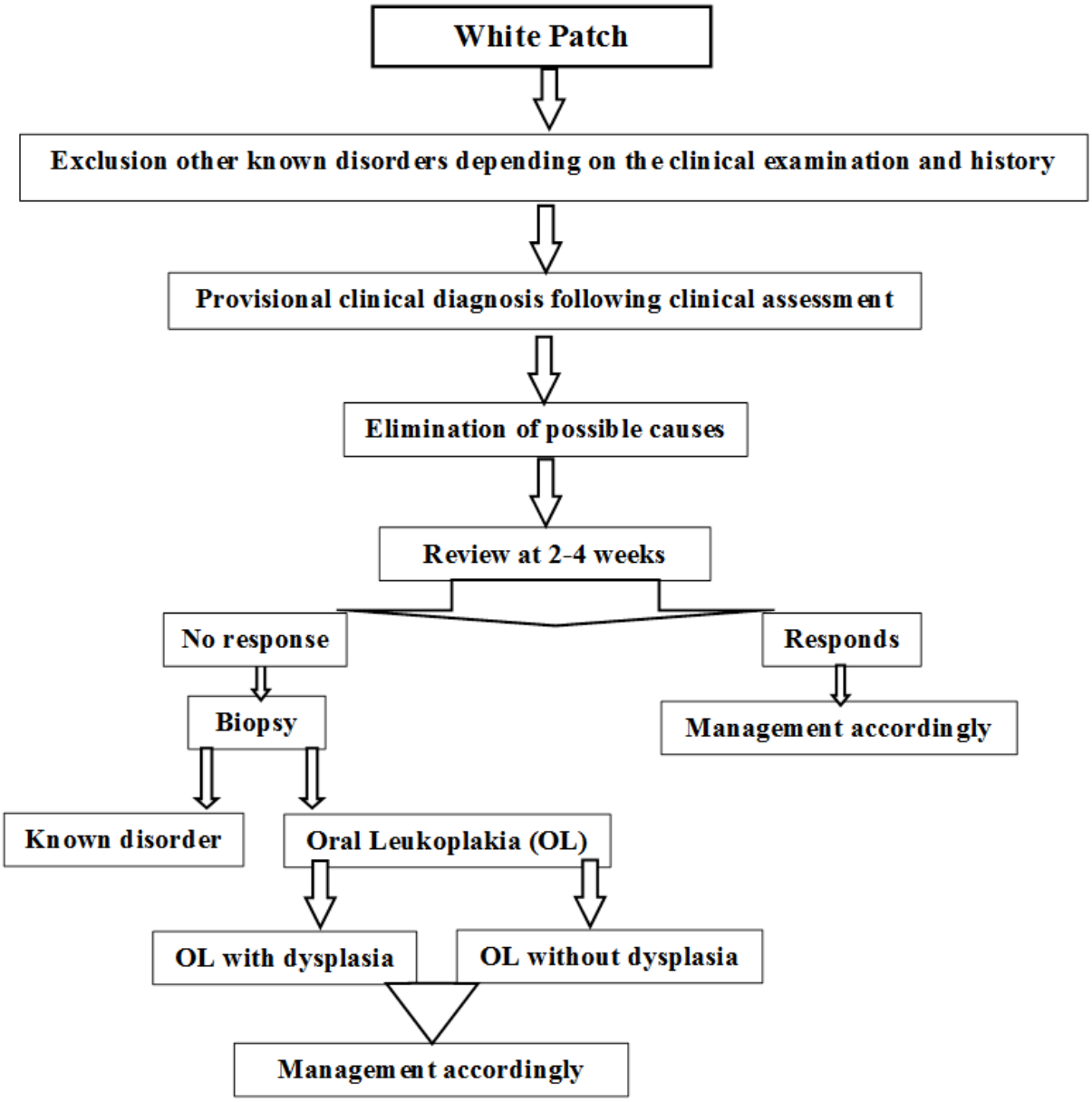

Figure 1.1: Steps in diagnosis of oral leukoplakia.

(Warnakulasuriya et al., 2007; van der Waal, 2009)

1.1.3. Histopathology of Oral Leukoplakia

OL is entirely a clinical term and has no definitive histopathological connection (Reibel, 2003), it may present with atrophy or hyperplasia and may or may not show epithelial dysplasia (Warnakulasuriya et al., 2007). Microscopically, differentiation can be made between dysplastic and non-dysplastic OLs and a pathological report should always include a statement of the presence or absence of epithelial dysplasia (Pindborg et al., 1997).

The histopathological changes observed in leukoplakia range from hyperkeratosis, hyperplasia, mild to severe dysplasia or carcinoma *in situ* (CIS) (Chattopadhyay and Ray, 2008). According to Speight (2007), histological assessment shows approximately 50% of leukoplakias display epithelial dysplasia, whilst the other 50% shows hyperplasia and/or hyperkeratosis. In dysplastic leukoplakias, 50% of severe, 30% of moderate and less than 5% of mild dysplasias transform to oral cancer (Speight, 2007).

The rates of MT vary in different parts of the world, probably due to differences in ethnic and environmental factors, particularly oral and dietary habits (Gupta et al., 1989; van der Waal et al., 1997; Shiu et al., 2000; Reibel, 2003). The MT rate of leukoplakia has been reported to range from 0.13 to 17.5% (Reibel, 2003; Amagasa et al., 2006). According to epidemiological data from European patients, the annual rate of MT probably does not exceed 1% (Scheifele and Reichart, 2003).

Regarding the risk of MT, it is generally accepted that the non-homogeneous subtypes of leukoplakia carry an increased risk of transformation compared to homogenous leukoplakia (Silverman et al., 1984; Pindborg et al., 1997; Reibel, 2003; Warnakulasuriya et al., 2007; van der Waal, 2009).

Data have also shown that speckled leukoplakias are more often associated with high histological grade of dysplasia or CIS (Arduino et al., 2009) and have a high potential for MT (Hosni et al., 2009).

Regarding PVL, it is characterized by both a resistance to treatment with a high recurrence rate and a high rate of MT into verrucous carcinoma or OSCC (Cabay et al., 2007; van der Waal and Reichart, 2008; Bagan et al., 2010). It has been reported that PVL lesions may undergo MT to OSCC in 40-100% of cases (Hansen et al., 1985; Silverman and Gorsky, 1997; Cabay et al., 2007; Gandolfo et al., 2009).

Histologically, PVL has been reported to show progressive histological features over time with lymphocytic infiltrate within the superficial lamina propria that can be mistaken for lichen planus. This lymphocytic infiltrate may be intense and can mask visualization of the basement membrane (Cabay et al., 2007). In advanced cases, the deeply folded tissue may erode and infiltrate the underlying bone developing pseudocysts that may be mistaken for odontogenic cysts (Morton et al., 2007). In spite of the initially benign histological features, PVL has a poor clinical outcome with high rates of recurrence and a high risk MT reflecting both under-treatment and ineffective management (Cabay et al., 2007). It probably requires, therefore early and aggressive treatment.

1.1.4. Staging and Classification of Oral Leukoplakia

For the purpose of a standardised reporting system of OL management, staging and classification is highly recommended. The size, histopathology and localization of OL should be taken in consideration as should, sex, age and possible causative factors at the time of diagnosis (van der Waal et al., 2000; van der Waal and Axell, 2002; van der Waal, 2009).

Depending on the treatment plan, if the decision to manage leukoplakia is by clinical observation alone, then the important feature to be evaluated is the size, colour and/or texture of the individual lesions. However, because the clinical appearance of OL may be variable and does not always allow clear classifications, clinical subtyping may be not useful (van der Waal and Axell, 2002).

Considering assessment after surgical intervention, biopsy reveals that the staging system is an important consideration. Van der Waal (2009) indicated that the staging system of OL is mainly dependent on size and pathology. The system takes into account the highest pathological score in case of multiple biopsies of single or multiple leukoplakias and the lowest size if there is any uncertainty in considering size category.

For classification and staging purposes it is recommended to record the size of a single leukoplakia, and to add together the sizes of multiple leukoplakias. The oral subsite should be specified according to the International Classification of Diseases Application to Dentistry and Stomatology (ICD-DA) codes for the oral cavity (van der Waal and Axell, 2002).

In this system, three size categories have been proposed, analogous to the TNM system of oral cancer. L represents the size of single or multiple leukoplakias as follows:

L_1 < 2 cm

L_2 =2-4 cm

L_3 > 4 cm

L_x size not specified.

P represents the pathology of leukoplakias as follows:

P_0 No epithelial dysplasia

P_1 Mild or moderate dysplasia

P_2 Severe dysplasia

P_x Absence or presence of dysplasia not specified

Accordingly, four stages have been proposed in this system which includes:

Stage 1	L_1 P_0
Stage 11	L_2 P_0
Stage 111	L_3 P_0 or L_1 L_2 P_1
Stage 1V	L_3 P_1 or any L P_2

1.2. Oral Erythroplakia

The current widely accepted WHO definition of oral erythroplakia (OE) is: "a fiery red patch that cannot be characterized clinically or pathologically as any other definable disease" (Kramer et al., 1978b; Pindborg et al., 1997).

Research on the incidence and prevalence of OE is limited and depends on retrospective analysis. Previous studies have shown a prevalence range between 0.02% and 0.83% (Shafer and Waldron, 1975; Lay et al., 1982; Zain et al., 1997; Hashibe et al., 2000b). These data were only derived from research performed in South and Southeast Asia, however, and no published studies were reported from other geographical areas (Reichart and Philipsen, 2005).

OE is a rare disorder (Hosni et al., 2009) and is much less common than leukoplakia (Neville and Day, 2002). It is a disease of middle age and elderly and more common in men (Scully, 2004). However, previous studies showed no sex predilection; female to male ratio was 1: 1.15 and 1: 1.04 in studies conducted by Shafer and Waldron (1975) and Hashibe *et al.* (2000b), respectively.

1.2.1. Oral Erythroplakia: Clinical Presentation

Clinically, erythroplakias may have flat or depressed surfaces which may be smooth, granular or nodular with a well-defined demarcation adjacent to mucosa of normal appearance (Pindborg et al., 1997; Reichart and Philipsen, 2005). OE tends to present as solitary lesions and rarely affects widespread areas (Reichart and Philipsen, 2005). These features are quite useful clinically in discrimination OE from other multicentre, bilateral or symmetrical oral disorders, such as erosive lichen planus, erythematous candidiasis and lupus erythematous (Reichart and Philipsen, 2005; van der Waal, 2009).

Erythroplakias may affect any part of the oral mucosa and different localizations have been reported. The soft palate, ventral tongue surfaces and the FOM (Pindborg et al., 1997; Scully, 2004) together with buccal mucosa (Scully, 2004) are the most commonly OE affected sites.

Regarding size of OE, it is typically less than 1.5 cm in diameter, but lesions larger than 4 cm have been reported (Bouquot and Ephros, 1995).

1.2.2. Oral Erythroplakia: Histopathology

Erythroplakia as a clinical term does not carry any histological connotation; however, histological biopsy of OE may show epithelial dysplasia, CIS or invasive carcinoma (Shafer and Waldron, 1975). According to Reichart and Philipsen (2005), all erythroplakias showed some degree of epithelial dysplasia: 51% showed invasive squamous cell carcinoma, 40% CIS or severe dysplasia and the remaining 9% demonstrated mild to moderate dysplasia. Interestingly, since all OEs are therefore high risk, there is no need for high risk and low risk site classification, contrary to OL.

Although OE is rare, its MT rate is the highest among all of the oral PMDs (Shafer and Waldron, 1975; Bouquot and Whitaker, 1994; Reichart and Philipsen, 2005). Dysplasia and CIS or invasive carcinoma may be seen in more than 90% of OE cases (Shafer and Waldron, 1975; Mashberg, 2000). However, no annual transformation rate is available due to a lack of clinical case series which allow calculation of this rate (van der Waal, 2009).

1.2.3. Oral Erythroplakia: Diagnosis and Management.

Similar to leukoplakia, OE diagnosis is based on the principle of exclusion of numerous other red patches on the oral mucosa that may be confused with erythroplakia and should thus be excluded to confirmed the diagnosis (Pindborg et al., 1997).
Table 1.4 lists a number of erythematous disorders which have a similar appearance to OE and need to be excluded when making a diagnosis of OE (Reichart and Philipsen, 2005).

There is no specific study that considers the treatment of erythroplakia in the literature. Since OE may be symptomatic and biopsy may show severe dysplasia, CIS or microinvasive carcinoma (Reichart and Philipsen, 2005) and since erythroplakia is characterized by a high rate of MT, surgical removal is highly recommended either by conventional scalpel or by laser surgery (van der Waal, 2009).
However, no guidelines are available for the width of resection margins and data on recurrence rate after surgical treatment are limited. OE recurrence rate seems to be high and

this may be due to the high rate of MT and the presence of epithelial dysplasia (Reichart and Philipsen, 2005).

One can conclude that studies demonstrating that OE are the most dangerous PMDs are limited and further wider studies are needed to answers many question that are related to its behaviour, clinical outcome and treatment modalities. This will require either a meta-analysis of published series or longitudinal multicentre studies.

Table 1.4: Disorders that should be excluded to diagnose oral erythroplakia.
(Reichart and Philipsen, 2005)

Red lesions resembling oral erythroplakia
Infections
Erythematous candidiasis **Denture-induced stomatitis** **Histoplasmosis** **Tuberculosis**
Inflammatory-Immune disorders
Erosive / Atrophic oral lichen planus **Discoid Lupus Erythematosis** **Pemphigus** **Pemphigoid**
Others
Haemangioma **Telangiectasia** **Lingual varices** **Oral purpura** **Amelanotic melanoma** **Kaposi's sarcoma**

1.3. Oral Lichen Planus

Oral lichen planus (OLP) is a chronic inflammatory disorder of unknown aetiology affection up to 2% of the middle aged and elderly (Axell and Rundquist, 1987). The pathogenesis of OLP is not fully understood; however, it is a cell mediated autoimmune condition associated with accumulation of an inflammatory infiltrate composed predominantly of T-lymphocytes beneath the epithelium of the oral mucosa resulting in cell-mediated damage to basal keratinocytes (Pindborg et al., 1997; Thornhill et al., 2006; Warnakulasuriya et al., 2007; Danielsson et al., 2011), which can be recognized by the immune system as antigenically foreign, causing the release of cytokines, chemokines and other proinflammatory mediators (Thornhill et al., 2006).

There is an increased rate of differentiation of stratified squamous epithelium, resulting in hyperkeratosis and erythema with or without ulceration (Epstein et al., 2003).

OLP has six clinical types: papular, reticular, plaque-like, atrophic, erosive (ulcerative) and bullous (Pindborg et al., 1997). It has characteristic clinical and histological appearance which usually allows distinction from OL. However, the plaque type of lichen planus may often resemble leukoplakia, emphasising the importance of biopsy in diagnosis (Warnakulasuriya et al., 2007).

There is considerable debate in the literature about the potential malignant nature of OLP (van der Meij et al., 2003). However, previous studies have stated and accepted that OLP carries a risk of MT (Thornhill et al., 2006; Hsue et al., 2007; van der Meij et al., 2007).

Although MT in lichen planus is distorted by the lack of clinicopathologic correlation in the diagnosis (van der Meij and van der Waal, 2003), in all clinical types of lichen planus, the reported annual MT rate is usually below 1% (Gandolfo et al., 2004). However, there are no possibilities to truly prevent MT of OLP and regular follow-up of patients has thus been recommended by many authors (Eisen, 2002; Al-Hashimi et al., 2007).

1.4. Oral Submucous Fibrosis (OSF)

OSF is a chronic disorder characterized by fibrosis of the lining mucosa of the upper aerodigestive tract and frequently the upper third of the oesophagus (Warnakulasuriya et al., 2007). It is limited to Southeast Asia, but a number of cases have been reported in other parts of the world including South Africa, Greece and the United Kingdom (van der Waal, 2009). The main aetiological factor of OSF is chewing areca and betel quid (Tilakaratne et al., 2006), however, in some cases genetic traits may be implemented as predisposing factors (Pindborg et al., 1997).

Within the oral cavity, different sites may be affected in different populations (Warnakulasuriya et al., 2007). Clinically, with the exception of early stage disease, OSF has characteristic clinical features due to fibrosis of the lamina propria and submucosa with an increasing loss of tissue mobility (Warnakulasuriya et al., 2007).
Early stages of OSF are characterized by burning sensations, blanching of mucosa, vesiculation, and leathery mucosa, while fibrous bands within the mucosa appear in the cheeks, faucial of pillars, and encircle the lips resulting in stiffening of the affected mucosa and trismus in advanced stages (Warnakulasuriya et al., 2007; van der Waal, 2009).

Histologically, fibrosis and hyalinization occur in the lamina propria, causing severe epithelial atrophy with underlying dense collagenous tissue fibre formation associated with varying degree of hyperkeratosis and epithelial dysplasia (Pindborg et al., 1997).
The epithelial atrophy may predispose to cancer development in the presence of carcinogens. According to Murti *et al.* (1985), the MT rate of OSF was 4.5% over a 15-year observation period with an approximately 0.5% annual MT rate.
Since OSF has a tendency to develop to oral cancer (Pindborg et al., 1997) regular follow-up of affected patients should be considered.

1.5. Actinic Cheilitis

Actinic cheilitis is a clinical term for an ulcerative lesion, sometimes with crust formation on the mucosa of part of or the entire vermilion border of the lip (van der Waal, 2009). This PMD of the lip is mainly seen in men, although no figures for incidence rate are available in the literature (van der Waal, 2009).
Histologically, the squamous epithelium of the lip vermilion may show hyperplastic or atrophic changes with disordered maturation, varying degrees of keratinization, cytological atypia and increased mitotic activity with the underlying connective tissue showing basophilic degeneration of collagen and elastosis (Pindborg et al., 1997).

Depending on the histology and clinical signs and symptoms, the treatment of this disorder is usually by superficial surgical excision (lip shave) or laser ablation (Satorres Nieto et al., 2001). Photodynamic therapy may be also used but it is less effective (Berking et al., 2007).

Squamous cell carcinoma often develops in untreated patients. There are, however no follow-up studies of untreated actinic cheilitis that allow calculation of annual MT rates (van der Waal, 2009).

1.6. Hereditary Disorders

There are a few known inherited disorders associated with an increased risk of malignancy in the oral cavity, such as Fanconi's anemia, Dyskeratosis congenita, Epidermolysis bullosa, Xeroderma pigmentosum and Bloom's syndrome (Prime et al., 2001).

1.6.1. Fanconi's anaemia

Fanconi's anaemia is a rare autosomal recessive syndrome caused by defects in approximately 11 genes involved in the detection and repair of DNA (Thompson, 2005). The incidence of the disease is unusually high in the Afrikaner population of South Africa (Saleh and Stephen, 2008). Those types of patients are characterized by aplastic anaemia with progressive bone marrow failure, congenital abnormalities, and a high tendency to malignancies including head and neck cancer (Otan et al., 2004; Masserot et al., 2008; Saleh

and Stephen, 2008). In the absence of alcohol and tobacco exposure, 14% of anaemic patients develop head and neck squamous cell carcinomas by the age of 40s (Kutler et al., 2003).
Of the 800 cases of Fanconi's anaemia available for a study conducted by Lustig *et al.* (1995), 17 cases with head and neck cancer arose in young patients less than 30 years and were seen equally in females and males mainly affecting the tongue, followed by the gingivae.
Orally, there is a manifestation of generalized black hyperpigmentation on the buccal mucosa, tongue and palate associated with severe generalized periodontitis reported in patients with Fanconi's anaemia (Saleh and Stephen, 2008).

1.6.2. Dyskeratosis Congenita

Dyskeratosis congenita is a rare inherited syndrome exhibiting marked clinical and genetic heterogeneity. It is characterised by mucocutaneous abnormalities, bone marrow failure and a predisposition to cancer (Walne and Dokal, 2009).
Most cases of dyskeratosis congenita are X-linked and affect males (Handley et al., 2006). Leukoplakia has been reported in 80% of dyskeratosis congenita patients (Dokal, 2000) and it may affect any mucosal surface, but the oral mucosa is most commonly affected with the tongue the most frequently affected site (Ling et al., 1985). Patients with dyskeratosis congenita often develop white plaques on the dorsal surface of the tongue which may be confused with OL, but the absence of habits and the young age of patients indicate the hereditary nature of this disorder (Handley et al., 2006).

Diagnosis of dyskeratosis congenita is relatively straight forward when all the classical mucocutaneous features of the disease exist. However, genetic analysis can confirm the diagnosis in the majority of dyskeratosis congenita patients (Handley et al., 2006).

Patients with dyskeratosis congenita have a recognised increased risk of malignancy from pre-existing mucosal leukoplakia (Drachtman and Alter, 1995), with an incidence of approximately 35% with a peak in the third decade of life (Sirinavin and Trowbridge, 1975).

1.6.3. Epidermolysis Bullosa

Epidermolysis bullosa is a hereditary disorder of skin and oral mucosa which is either autosomal dominant or recessive (Das and Sahoo, 2004). Clinically, epidermolysis bullosa is characterized by an exceptional tendency of the skin and mucosae to form bullae and vesicles after minor friction and trauma (Sedano and Gorlin, 1989), such as routine dental care or even normal tooth brushing.

Oral features include repeated blistering and oral tissue scarring leading to limited oral opening, ankyloglossia, obliteration of the oral vestibule, perioral stricture (microstomia), severe periodontal disease and alveolar bone resorption, atrophy of the maxilla with mandibular prognathism and an increased mandibular angle. Patients with epidermolysis bullosa have an increased risk for squamous cell carcinoma (Wright and Fine, 1994; Pindborg et al., 1997; Das and Sahoo, 2004).

1.6.4. Xeroderma Pigmentosum

Xeroderma pigmentosum is a rare neurocutaneous disease with a recessive mode of inheritance which may affect all races worldwide and has an equal sex incidence (Mehta et al., 1996). It is characterized by skin changes and neoplasia in exposed parts of the body due to UV radiation. The lips are the most frequently affected and show epithelia atrophy, telangiectasia and hyperpigmentation and this may occasionally be seen in oral mucosa (Pindborg et al., 1997).

Most cases of Xeroderma pigmentosum start in early childhood and are fatal by 20 years of age due to multiple metastasis of squamous cell carcinoma and melanoma, infection or neurological complications (Mehta et al., 1996). There is an increased incidence of malignancies including oral cancer (Prime et al., 2001).

1.6.5. Bloom's Syndrome

Bloom's syndrome is an autosomal recessive chromosomal disorder. Patients with Bloom's syndrome are hypersensitive to UV radiation, suffering from sun-sensitive, facial erythema, and show predisposition to solid tumour development in a wide variety of anatomical sites; 8% of these tumours are seen in the tongue (Prime et al., 2001).

1.7. Oral Epithelial Dysplasia (OED)

Dysplasia as a term has been used for many years and has been implemented again in the latest version of WHO Classification of Tumour in the Oral Cavity and Oropharynx (Barnes et al., 2005). Dysplastic features of a stratified squamous epithelium are characterized by cellular atypia and loss of normal maturation and stratification; a combination of architectural and cytological changes that are associated with an increased risk of MT compared to normal mucosa (Pindborg et al., 1997; Reibel, 2003; Warnakulasuriya et al., 2008).

Epithelial dysplasia represents a spectrum of changes and does not refer to a precise diagnostic category (Kujan et al., 2007). No existing criteria can precisely divide this spectrum definitively into mild, moderate or severe dysplasia (Gale et al., 2005). Therefore, pathologists frequently face difficulties in the accurate assessment of dysplasia grade, mainly due to a lack of specific classification criteria, limited objectivity for evaluation of the diagnostic criteria and insufficient information about the important criteria that may be used to predict MT (Kujan et al., 2006).

Although some cancers may develop from non-dysplastic tissue (Warnakulasuriya et al., 2008), there is support for the view of greater risk of cancer development in more severe dysplasias (Reibel, 2003). This is possibly because of the greater accumulation of chromosomal, genomic and molecular alterations (Warnakulasuriya et al., 2008). Approximately 50% of OL biopsies demonstrate OED, although not all of them progress to oral cancer (Speight, 2007).

Since OED is considered a precursor for MT, pathologists need to evaluate accurately the dysplastic changes of the PMD for accurate (error-free) prediction and effective management (Kujan et al., 2006).

It is well-recognized that the classification system is not perfect, however it is highly recommended for the selection of treatment options to help and improve clinical outcomes.

1.7.1. Classification Systems

Different classification schemes have been proposed to detail OED: Squamous Intraepithelial Neoplasia (SIN), Ljubljana classification/squamous intraepithelial lesions (SIL) and WHO epithelial dysplasia grading (Warnakulasuriya et al., 2008).
Table 1.5 displays the three classification systems for the purpose of evaluation and relation.

Table 1.5: Classification systems for epithelial dysplasia.
(Gale et al., 2005)

Oral Epithelial Dysplasia 2005 WHO Classification (OED)	Squamous Intraepithelial Neoplasia (SIN)	Ljubljana Classification Squamous Intraepithelial Lesions (SIL)
Epithelial hyperplasia	**-**	**Simple hyperplasia (squamous cell)**
Mild dysplasia	**SIN 1**	**Basal/parabasal hyperplasia**
Moderate dysplasia	**SIN 2**	**Atypical hyperplasia**
Severe dysplasia	**SIN 3**	**Atypical hyperplasia**
Carcinoma *in situ*	**SIN 3**	**Carcinoma *in situ***

Squamous Intraepithelial Neoplasia (SIN)

Squamous intraepithelial neoplasia (SIN) or oral intraepithelial neoplasia (OEN) is a modification of a classification of cervical pre-malignant lesions which has been suggested previously by Richard in 1968 (Warnakulasuriya et al., 2008). This system was not accepted by the WHO consensus group because it is unable to explain the concept of epithelial dysplasia internationally (Warnakulasuriya et al., 2008) and also there is no evidence to suggest that all PMDs will transfer to malignancy (Warnakulasuriya et al., 2007; Napier and Speight, 2008).
Squamous intraepithelial neoplasia (SIN) is of three grades: SIN1, SIN2 and SIN3, where the first grade corresponds to mild dysplasia and the second grade corresponds to moderate dysplasia, while SIN3 refers to a combination of severe dysplasia and CIS in the WHO classification system; Table 1.5.

Ljubljana Classification-Squamous Intraepithelial Lesions (SIL)

The Ljubljana classification was developed to be used for special clinical and histological laryngeal abnormalities (Hellquist et al., 1999; Gale et al., 2000). This system is more complicated even for the experience pathologist to use when compared to the WHO concept of dysplasia (Warnakulasuriya et al., 2008).

The Ljubljana grading system identifies four categories: simple and abnormal hyperplasia both of which are regarded as benign, atypical hyperplasia which is pre-malignant, and CIS which is malignant without detectable invasion (Hellquist et al., 1999; Zerdoner, 2003).

Table 1.5 demonstrates Ljubljana grading system in relation to WHO and SIN.

1) Simple hyperplasia is characterized by increased thickness of the spinosum (prickle) cells layer without cellular atypia.
2) Abnormal hyperplasia shows hyperplasia of basal and parabasal cell layers which constitutes up to one- half of full epithelial thickness.
3) Atypical hyperplasia or risky hyperplasia is characterized by recognizable changes toward malignancy but epithelial stratification is unchanged.
4) Carcinoma *in situ* shows marked cellular atypia, abnormal mitotic figures and complete loss of epithelial stratification.

Although this system is quite difficult even for well-trained pathologists, it focuses on clinical decision though its scores. Simple and abnormal hyperplasia does not require close follow-up, while close follow-up is required for risky hyperplasia, and CIS needs intervention (Zerdoner, 2003).

A classification system with an easy routine daily application is required for an accurate diagnosis and subsequent successful management, with a universal grading system requires a high level of agreement between pathologists (Warnakulasuriya et al., 2008).

Epithelial Dysplasia /WHO Classification System (2005)

The WHO Consensus Group made great efforts to establish a universal classification of OED by updating and improving the grading system used for OED (Kujan et al., 2007).
The WHO classification system is mainly based on two main characteristic features: cellular atypia and architectural deregulation of epithelial tissue as well as the dysplastic layer thickness in relation to full epithelial thickness (Gale et al., 2005).

WHO Five-Grade System

The 5-score grading system has been proposed in the latest WHO Working Group (Gale et al., 2005). This classification system recognizes five-point ordinal scale grading system depending on nine-cellular and seven-architectural criteria, listed in Table 1.6.

Table 1.6: Architectural and cytological features used in grading of OED in 2005 WHO classification system.

Architecture	Cytology
1. Irregular epithelial stratification 2. Loss of polarity of basal cells 3. Drop- shaped rete ridges 4. Increased number of mitotic figures 5. Abnormal superficial mitoses 6. Premature keratinisation in single cell (dyskeratosis) 7. Keratin pearls within rete pegs	1. Abnormal variation in nuclear size (anisonucleosis) 2. Abnormal variation in nuclear shape (nuclear pleomorphism) 3. Abnormal variation in cell size (anisocytosis) 4. Abnormal variation in cell shape (cellular pleomorphism) 5. Increased nuclear- cytoplasm ratio 6. Increased nuclear size 7. Atypical mitotic figures 8. Increased number and size of nucleoli 9. Hyperchromasia

The 5 grades of the 2005 classification system include: hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia and CIS (Gale et al., 2005).

1) Squamous hyperplasia is characterized by increased cell numbers in the spinous layer without cellular atypia and with a regular stratification. The hyperplasia may also be seen in the basal or parabasal cell layers.

2) Mild dysplasia: the architectural disturbances are limited to the lower third of the epithelium associated with minimum cellular atypia.

3) Moderate dysplasia shows a considerable degree of atypia with architectural disturbances extending to the middle third of the epithelium.

4) Severe dysplasia shows a significant degree of atypia with architectural disturbances affecting more than two-thirds of the epithelium.

5) Carcinoma *in situ*: the architectural disturbances involve the full or almost the full epithelial thickness, with marked cytological atypia.

Binary Grading System

It should be emphasised that using five point system is found to be associated with inter- and intra-examiner variability in the assessment of OED and a better agreement may be achieved by reducing this number into a two-point classification system (Kujan et al., 2007).

Kujan and co-workers (2006) evaluated the scheme of a "high-risk" and "low-risk" binary grading system based on the same architecture and cytology criteria used by the WHO classification 2005 for grading epithelial dysplasia. The high-risk grade which has the potential for MT is assigned using a minimum of four architectural changes and five cellular alterations, whilst the low-risk grade which has low tendency for MT was assessed using less than four architectural changes and less than five cellular alterations (Kujan et al., 2006). According to this system all hyperplasias and mild dysplasias are classified as low-risk, whereas, all cases with severe dysplasia and CIS are considered as high-risk. With respect to moderate dysplasia, the binary grading system can classify moderate dysplasia cases into either low-risk or high-risk grade.

The evaluation of this system suggested that a binary scoring system reduced observer variability and increased agreement between pathologists, compared with the five-point system and that it may be easier to use, less subjective and have better discriminatory powers. Kujan *et al.'s* (2006) study demonstrated reasonable values of sensitivity and specificity (85% and 80%, respectively) with a test accuracy of 82% for predicting MT.

1.7.2. Pathologists' Observer Variability in Grading Oral Epithelial Dysplasia

The histopathological grading of epithelial dysplasia remains the most clinically applicable predictor of potential MT (Lumerman et al., 1995; Cowan et al., 2001; Reibel, 2003). According to Guillaud *et al.* (2008), the significance of dysplasia phenotypes as cancer risk prognosticators is well documented and the strong association of higher grade dysplasia with increased risk of cancer progression has been observed and confirmed in multiple body sites; this is why histopathologic diagnosis of dysplasia remains the current gold standard. However, this standard is subjective with low intra-and inter-observer agreement in grading epithelial dysplasia, lack of knowledge of the weight characteristics used to assess histopathologic grades and the problem of reactive changes in oral epithelial term which can cause changes similar to low-grade dysplasia, making differential diagnosis in early disease problematic.

So, assessment of epithelial dysplasia by histological examination remains subjective and is greatly dependent upon individual pathologist's experience. Therefore, disagreement on grading between pathologists is not uncommon.

The degree of agreement between pathologists is measured by kappa statistics (weighted and un-weighted) which takes the value zero when there is no agreement or the value one for perfect agreement. A kappa value lower than 0.4 represents fair agreement, between 0.4 and 0.6 moderate agreement, between 0.6- 0.8 substantial agreement and between 0.8-1.0 almost perfect or excellent agreements (Thomsen et al., 2002; Malpica et al., 2005).

Several studies on inter-and intra-observer variability have demonstrated considerable variations in the assessment of the presence, absence and degree of OED using different scoring systems (Kujan et al., 2006). These variations may be due to the lack of objectivity in the evaluation of established criteria, arbitrary division of the grading, lack of calibration of criteria and grading, and lack of sufficient knowledge of which criteria are important for the prediction of MT (Pindborg et al., 1985; Warnakulasuriya, 2001).

Abbey *et al.* (1995) used six American board-certified oral pathologists to evaluate 120 oral biopsies, with pathologies ranging from simple hyperkeratosis to severe dysplasia. The agreement range amongst the pathologists was from 35.8- 55.8%; however, the kappa values

improved from 0.70 to 0.88 for inter-observers comparisons when the grading was considered to within one histological grade.

A more recent study conducted by Brothwell *et al.* (2003) described a higher inter-observer agreement and showed substantial agreement using a 5-point ordinal scale with a group-weighted kappa of 0.74. However, similar to the other studies, the group un-weighted kappa showed a fair to moderate agreement of 0.37.

Equally, Fischer *et al.* (2004) reported an overall kappa weighted value of 0.59 and an improvement in the kappa measure agreement of 0.70 was observed when the histological diagnosis was simplified into three general classes of no abnormality- hyperplasia, mild-moderate- severe dysplasia and CIS.

In a study conducted by Kujan *et al.* (2006), all pathologists demonstrated satisfactory agreement on discriminating mild from severe dysplasia or from CIS using the WHO classification. Nonetheless, evaluation of moderate dysplasia remained problematic. The same group showed that 46.7% of moderate dysplasia cases (WHO grading) showed malignant changes, whilst the binary grading system distinguished between two subtypes of moderate dysplasia (high/low risk) according to the clinical outcomes, showed 87.5% of high-risk moderate dysplasia progressed to OSCC.

Kujan *et al.* (2006) showed better agreement in the histological assessment of the presence or absence and the degree of OED between inter-and intra-observer variation of a novel binary grading system compared with the 2005 WHO system. This improvement was mainly due to the reduction in the number of grading points from five grades in the WHO to two grades in the binary system making decisions simpler (Kujan et al., 2007).

Although reducing the points for a grading system may improve the agreement among pathologists, the subjectivity in evaluation the histopathological criteria remains the main cause of observer variability.

1.7.3. Factors Influencing the Epithelial Dysplasia Status

Studies have shown that many variables may affect the status of epithelial dysplasia, and that these variables are often seen in PMDs which have a higher tendency for MT. However, the majority of these variables are not researched sufficiently but include patient age, sex, lesion duration, site, size, clinical appearance and the presence of associated risk factors.

1) Age of Patient

There is some evidence that the prevalence of OL closely follows the incidence of oral cancer (Napier and Speight, 2008). Usually leukoplakia is not diagnosed before the age of 30-years with the highest prevalence in the fifth decade (van der Waal et al., 1997). OED commonly affects old-age patients with a peak incidence in their 50s and 60s (Lumerman et al., 1995; Arduino et al., 2009), with the higher risk for OSCC reported in individuals over 45-years (Hay et al., 2002). An important relation between age of patients and MT rates has also been reported, with a higher MT rate in patients over 50-years old compared to those under 50 (Amagasa et al., 2006). This is supported by the findings of Chiesa *et al.* (1993) and Banoczy (1977) who reported a higher risk of MT among 60-70 year old patients.

2) Sex

The sex distribution of PMDs is variable depending on geographical variations and lifestyle habits (Reichart, 2001). An earlier study conducted by Lind (1987) showed that dysplasia was more frequent in females and that females were more prone to undergo MT than males. Also, previous reports showed an increased risk of MT of oral PMDs in women without smoking habits (Silverman et al., 1984; Schepman et al., 1998). Silverman *et al.* (1984) showed an equal sex ratio in leukoplakias (125 males: 132 females), but slightly more females developed cancer (19 males: 26 females). Schepman *et al.* (1998) studied 76 male and 90 female patients with leukoplakia, but found oral squamous cell carcinoma (OSCC) developed in 4 males and 16 females. This is also supported by the finding of Amagasa *et al.* (2006) who reported a MT rate of 11.2% in females with leukoplakia, which was higher than the 6.2% observed in males. However, the reason for a higher risk of MT in females remains unclear.

3) Duration

All studies emphasize that when PMD disorder is present for years, there is a chance to develop dysplasia and subsequently transform to malignancy (Napier and Speight, 2008; Warnakulasuriya et al., 2008). The chronicity of the oral PMD increases the tendency for malignant changes in the first five year, after that, the rate may decrease but the risk may not completely disappear (Napier and Speight, 2008). It was noted that the longer the time of follow-up, the higher the number of cases which undergo MT (Schepman et al., 1998); Kaplan-Meier survival curves in a group of 166 patients showed that 50% of patients with leukoplakia develop cancer with 200 months.
Evidence suggests that large percentage of OSCCs are associated with or preceded by pre-existent, long-standing OL (Gupta et al., 1989; Bouquot, 1994; Reibel, 2003).

4) Site-Intraoral Location

The localization of PMDs, mainly OLs, may have a profound influence on the risk of MT (Napier and Speight, 2008). Most pathologists take oral sites into consideration during their pathological reporting and may thus adjust the degree of risk (Warnakulasuriya et al., 2008). For example, the FOM, ventral and lateral borders of the tongue with thin or atrophic lesions may show a higher rate of malignancy, thus pathologists may enhance basic histological features with known clinical high risk behaviour in their reports.

Leukoplakias located in the anatomic oral subsites such as FOM, ventral and lateral surfaces of the tongue, soft palate, and tonsillar pillar are considered high-risk sites, with an increased risk of cancer development compared to other oral subsites which are considered to be at lower risk of transformation (Mashberg and Meyers, 1976; Boffetta et al., 1992; Zhang et al., 2001a; Neville and Day, 2002).
In one recent study, for example, the chance of exhibiting a carcinoma and OED were 2.72-fold and 1.84-fold higher, respectively, in leukoplakias on the tongue or FOM, compared to buccal mucosa (Lee et al., 2006). Similar to the previous study, which showed that OED especially severe dysplasia was more liable to occur on the lateral surface of tongue and FOM (Jaber et al., 1999), leukoplakias in these oral sites also have an increased rate of MT (Mashberg and Meyers, 1976; Kramer et al., 1978b; Silverman et al., 1984; Gupta et al.,

1989; Zhang et al., 2001b). According to Reibel (2003), 24% of patients with FOM and/or ventral surface of the tongue followed-up from 1 to 19 years developed carcinoma. In a cross-sectional study of OED and OSCC, the highest prevalence of severe dysplasia or CIS was found in the FOM and tongue, 13.5% and 5% respectively, emphasising the high-risk at these sites (Waldron and Shafer, 1975).

Although the majority of OLs (63%) were seen on the buccal mucosa or buccal commissures in another study, only 2.9% and 1.1% respectively, progressed to OSCC, whilst 13% of FOM leukoplakias developed OSCC again emphasising that the FOM is a high-risk site for MT (Banoczy, 1977).
Similarly, a study from England showed that the FOM was particularly at risk because 24% of leukoplakias eventually became malignant (Kramer et al., 1978b). Furthermore, in a study conducted by Ishii *et al.* (2004) whilst OL was mainly observed on the gingiva, followed by the tongue and palatal mucosa, MT was observed in 13.6% of tongue but only 1.8% of gingival cases.

In contrast, however, Holmstrup *et al.* (2006) showed in a follow-up study of 166 leukoplakias that although the majority of OLs were situated in the FOM and in the buccal mucosa, which were considered high-risk sites, these sites exhibited no increased risk of malignant development as compared with other oral sites.
This is also supported by Schepman *et al.'s* (1998) findings which showed that no specific oral subsites were associated with an increased risk of malignant conversion.

Different studies have thus shown differences in the most common affected oral site by OLs and variable rates of MT. These differences in the most common oral subsites among different studies may be related to geographical differences, race and associated oral habits (Ishii et al., 2004). In a study conducted by Lim *et al.* (2010) the FOM, lip, tongue, and buccal mucosa were the most common sites affected by leukoplakia, while the palate and alveolus were less commonly affected.
A retrospective hospital-based study conducted by Arduino *et al.* (2009) demonstrated that the lateral border of the tongue was the most commonly affected site followed by the buccal mucosa. A further study showed that the buccal mucosa and the FOM were the most

commonly affected oral sites, followed by the lateral border of tongue with the gingiva and commissure the least commonly affected (Jaber et al., 2003).

5) Clinical Appearance

Although OED is not associated with any specific clinical appearance (Arduino et al., 2009; Jaber, 2010), leukoplakia and erythroplakia are classically associated with dysplastic changes (Reibel, 2003).

OL can show a variety of clinical appearances with some containing nodular or red area. Speckled leukoplakias have been shown to carry a greater risk of MT than uniformly white types (Napier and Speight, 2008). Therefore, the clinical appearance of OL may be considered as one of the major indicators for malignant transformation; non-homogeneous lesions are considered at a more significant risk for MT compared to homogeneous leukoplakias (Lee et al., 2006).

This is in agreement with a study conducted by Holmstrup *et al.* (2006) in which 73% of non-homogeneous lesions exhibited dysplasia, compared to 33% for homogeneous leukoplakia; non-homogenous leukoplakia showed a seven-fold increased risk of developing OSCC compared with homogenous types. Also, this is supported by another study conducted by Arduino *et al*. (2009) in which mild dysplasias present clinically as homogenous or verrucous leukoplakias, whilst speckled leukoplakias are more often associated with increased degrees of OED. In a previous study conducted by Kramer *et al.* (1978a), 57% of 63 FOM nodular leukoplakias eventually transformed to OSCC compared to only 13.6% of other clinical subtypes. This is also consistent with a study of 157 leukoplakias conducted by Lind (1987) in which leukoplakias were divided into homogenous, verrucous, erosive and nodular; OSCC developed in a single homogenous, two verrucous, three erosive and eight nodular leukoplakia subtypes. Moreover, an earlier study showed that OSCC eventually developed in 74% of erosive leukoplakias and 26% of verrucous types (Banoczy and Sugar, 1972).

In contrary to the aforementioned studies, Jaber *et al.* (2003) in their study found that 50% of OED manifested as homogeneous white patches, 45% as speckled, 2.4% as ulcers and 2% as erythroplakia. They suggested that clinical appearance was not a significant predictor of the degree of dysplasia; erythroplakia showed more moderate dysplasia than leukoplakia, but both had the same frequency of severe dysplasia.

These findings contrast totally with previous studies which demonstrated that speckled leukoplakias and erythroplakias are predominantly severely dysplastic or carcinomas (Axell et al., 1996) and carry a higher risk of both severe dysplasia and malignancy.

In spite of some conflicting results, it is clear that non-homogenous leukoplakias, nodular, speckled and erosive subtypes show a higher tendency for higher grade OED and are more liable for MT compared to homogenous and other non- homogenous subtypes.

6) Size

Although very few studies have investigated the size of PMDs and its significance in cancer development, the individual size of a PMD may be relevant in oral carcinogenesis. Previously, it was reported by Saito *et al.* (1999) that widespread, multiple leukoplakias have a higher potential for developing carcinoma compared to localized lesions, regardless of their dysplastic status. Saito *et al*. investigated series of 111 patients with OLs, 12 patients had large and widely spread leukoplakias with three of them (25%) developed OSCC compared with 5% (5/99) of localized leukoplakias.
More recently, Holmstrup *et al.* (2006) reported a 5.4 odds ratio for cancer to occur in oral lesions exceeding 200 mm^2 in size compared with smaller ones. In Holmstrup *et al.'s* study, the size was recorded by multiplying the length by the width of the excised specimens taking from the pathological reports.

In a retrospective study to investigate the clinicopathological features of OL in smokers and non-smokers, Freitas *et al.* (2006) reported that the size of OLs in smokers at the time of diagnosis was 2 cm in 62%, 2-4 cm in 31%, and 4 cm in 8%, while leukoplakia in non-smokers showed 55% with 2 cm and 45% with 2-4 cm.

In another study of 50 patients conducted by Napier *et al.* (2003), multivariate survival analysis highlighted the link between the clinical extent of PMD and the risk of OSCC development, with the risk of malignant progression approximately six-times greater in patients with large, confluent PMDs. Also, the same group found that the risk of OSCC was approximately four-times greater in patients affecting with a single anatomical site lesion than those with multiple sites lesions.

According to Napier *et al.* (2003), the high risk of larger size PMDs may be explained on the basis that PMDs with a larger size may have been present for a longer time compared to smaller lesions, so the transformation of these PMDs may be seen sooner compared to small sizes. Also, larger PMDs may be at higher risk of acquiring the critical genetic hits from oncogenic agents, or they may be biologically different with clonal expansion replacing the surrounded normal epithelial tissue. In addition, there may be a greater chance of sampling errors with non-representative incisional biopsies in large PMDs, resulting in underestimates of the severity of dysplasia.

One can conclude that the size of PMDs should be taken into consideration as an important clinical parameter in patients' follow-up, due to its potential importance in MT.

7) Idiopathic Leukoplakias

While tobacco use is the most important risk factor in developing OL, so-called "idiopathic leukoplakias" in non-tobacco users seem to be at increased risk of MT (Banoczy, 1977; Silverman et al., 1984; Schepman et al., 1998; Napier and Speight, 2008). Studies showed that idiopathic leukoplakias, where no associated aetiological factors have been identified, have a significantly increased risk of MT compared with leukoplakias that where associated with obvious causative agents (Napier and Speight, 2008).

According to Silverman *et al.* (1984), out of 74 non-smokers in a group of 257 leukoplakia patients, 18 (24%) developed OSCC, whilst in contrast 21 carcinomas (16%) arose in the 133 individuals who continued to smoke after diagnosis and only 6 (12%) arose in the 50 individuals who stopped smoking after diagnosis of leukoplakia.

In a study conducted by Schepman *et al.* (1998), three patients who developed OSCC were non-smokers. Also, the same study found that non-smoking female patients with leukoplakia were at higher risk of MT compared with female smokers, although no such relationship could be identified for men.

This is supported by the results of a multifactorial statistical analysis which was unable to determine an association between tobacco use and MT of PMD (Holmstrup et al., 2006).

To conclude, the factors associated with the development of PMDs and dysplasia are more or less the same that are associated with increased risk of MT. Female-sex, widespread and

long-standing disease, anatomical site, clinical appearance, idiopathic leukoplakia and associated risk factors have been demonstrated in several studies as important factors in OED development and potential malignant changes (Silverman et al., 1984; Gupta et al., 1989; van der Waal et al., 1997; Jaber et al., 1999; Reibel, 2003; Napier and Speight, 2008).

1.8. Aetiology-Risk Factors

Table 1.7 demonstrates 14 risk factors associated with oral cancer and PMDs, which will be discussed in this section.

Table 1.7: Risk factors of oral cancer and PMDs identified from the literature.

Risk Factors
1. Tobacco smoking
2. Smokeless tobacco
3. Alcohol drinking and alcoholic mouthwash
4. Betel quid and Areca nut
5. Marijuana
6. Family cancer history/ Genetic factors
7. Old age
8. Dietary factors
9. Immunodeficiency
10. Oral health
11. Socioeconomic factors
12. Human Papilloma Virus (HPV)
13. Oral candida infection
14. Diabetes mellitus

1.8.1. Tobacco Smoking

Tobacco use has been identified as probably the most important risk factor for oral cancer (Llewellyn et al., 2004b) and PMDs (Dietrich et al., 2004), particularly OLs (Gupta, 1984; Evstifeeva and Zaridze, 1992; Dietrich et al., 2004), in a strong dose- response relationship (Reichart, 2001). A linear relation between the number of cigarettes smoked per day and the risk of oral cancer has been observed. However, quitting cigarette smoking considerably reduces oral cancer risk after 1-3 years but patients may need 17 years or more to be at the same risk of never smokers (Castellsague et al., 2004).

It is evident that heavy smokers who smoke more than 20 cigarettes daily and with more than 20 years smoking history are at higher risk of oral cancer, whereas mild smokers do not show such an enhanced risk for oral cancer compared with non-smokers (Applebaum et al., 2007).

Also, in a follow-up study on smokers, Khuri *et al.* (2001) found that treated cancer patients who continued smoking had a 2 to 6-times greater risk of developing a second primary tumour than those who stopped smoking.

Regarding sex predisposition, it has been reported by Muscat *et al.* (1996) that the risk of oral cancer is higher for women than for men after adjustment for alcohol and other confounding variables; 40 pack-years of smoking approximately increased the risk of oral cancer 2-fold in men and 5-fold in women relative to non-smokers. The possible biological explanations for females higher susceptibility to tobacco carcinogens with a higher risk of oral cancer compared with men, include nutritional deficiencies and metabolism (Muscat et al., 1996).

It has been reported that approximately two-thirds of oral cancer patients use tobacco regularly (Khuri et al., 2001; Williams et al., 2008). This is supported by findings from a cohort study conducted by Lee *et al.* (2007b) who showed that heavy smoking tends to increase the rate of leukoplakia development to five-times compared with non-smokers.

It has been found that both the type and amount of tobacco consumption have an influence on the location, size and number of PMDs (Schepman et al., 2001; Freitas et al., 2006; Napier and Speight, 2008). Tobacco smoking is associated with an increased risk of oral epithelial dysplasia (Morse et al., 2007) an important key stage in oral carcinogenesis preceding MT (Morse et al., 2007) and is considered as one of the most important predictors of MT in PMDs (Reibel, 2003).

Although cigarette smoking is the commonest form of tobacco use, tobacco may be chewed or snuffed (Gupta et al., 1996). Smoking products are mainly prepared from Nicotiana tabacum, while smokeless or chewing tobacco is prepared from Nicotiana rustica (Reichart, 2001). Processed tobacco contains approximately 3050 different compounds (Hoffmann and Hoffmann, 1998) and many of these compounds have been recognized as toxic, tumorigenic and carcinogenic (Reichart, 2001).

Smoked tobacco releases a complicated mixture of thousands of chemicals; of these, more than 60 known chemical carcinogens, and a further 16 chemicals in unburned tobacco, have been identified by the International Agency for Research on Cancer (IARC) (IARC, 2004b; IARC, 2007). Polycyclic aromatic hydrocarbons such as benzo(a)pyrene together with tobacco-specific nitrosamines and aromatic amines are the most important tobacco related carcinogens (Hecht, 2003). These carcinogens may be considered as the causative agents for PMDs in both smoking and chewing tobacco products (Reichart, 2001).

Tobacco use is suggested to play an important role during oral carcinogenesis which precedes MT of oral epithelial dysplasia to oral cancer (Morse et al., 2007). Tobacco specific nitrosamines have been regarded by IARC as carcinogenic to humans (IARC, 2007) because of their direct mutagenic effect on the exposed epithelia of the upper aerodigestive tract. This nitrosamines react with DNA forming DNA adducts which subsequently cause mutational effect on important oncogenes and tumour suppressor genes (P53) resulting in cancer development (Pfeifer et al., 2002).

Nearly all carcinogens and procarcinogens require activation by metabolizing enzymes named xenobiotic-metabolizing enzymes which are found in the liver and mucosa of the upper aerodigestive tract (Ho et al., 2007). Similarly, detoxifying enzymes exist to deactivate carcinogens as well as their intermediate by-products (Ho et al., 2007). Both biotransformation and detoxification processes are essential for the metabolism and subsequent excretion of chemical carcinogens of tobacco smoke and these take place in two phases: phase I oxidative enzymes such as cytochrome P450 transform lipophilic compounds into more polar molecules and phase II xenobiotic-metabolizing enzymes such as glutathione-S-transferases (Ho et al., 2007) then convert them into water-soluble compounds making them less biologically active and facilitating the excretion of the toxins and carcinogens from the body (Pelkonen and Nebert, 1982).

Genetic polymorphisms of these enzymes may play an important role in individual's response to carcinogens and also determine inter-individual differences in the metabolism and excretion of tobacco smoke carcinogens (Ho et al., 2007).

It has been suggested that OLs in tobacco smokers occur preferentially in particular locations in the oral cavity (Schepman et al., 2001). The buccal mucosa and FOM were the most frequent locations among smokers whereas; leukoplakias at the borders of the tongue are more common among non-smokers (Schepman et al., 2001; Freitas et al., 2006). This is in agreement with a pervious study investigated the role of tobacco in relation to the anatomical subsites for the development of OSCC, with a highest relative risk associated with tobacco smoking in the retromolar area followed by the FOM (Jovanovic et al., 1993).
These findings are remarkable, since smoking is the most important etiologic factor for carcinoma of oral mucosa with possibility of accumulation of tobacco-smoke toxins in saliva (Lederman, 1964). Also, spatial variations in the degree of keratinization and permeability of oral mucosa may modulate the local effect of tobacco-smoke toxins (Mashberg and Meyers, 1976; Lesch et al., 1989).

With respect to the site predilection for leukoplakia in male and female smokers, it has been reported that leukoplakia in the cheek mucosa in men and in the FOM in women were the most commonly affected site (Schepman et al., 2001). However, there is no explanation for the sex differences in site for leukoplakia and it may be that men and women use a different way of placing cigarettes between their lips; men keep their cigarette perhaps more to the side of their lips, whilst women might keep the cigarette more centered (Schepman et al., 2001).

One can conclude that tobacco is the principle risk factor for oral carcinogenesis associated with a site preferential localisation and inter-individual variation in the activity of enzymes involved in the detoxification of tobacco smoke.

1.8.2. Smokeless Tobacco

According to Warnakulasuriya and Ralhan (2007), there are two main types of smokeless tobacco: chewing tobacco and snuff. Chewing tobacco is either loose leaf, cut or shredded, while snuff is for applying, dipping or sucking. Worldwide, several names are used to represent different products of smokeless tobacco: shamma, toombak, plug, gutkha, khiwam, khaini, iq'milk, zarda, naswar, nass, chimo, gudhaku, gul, mishri, maras and moist snus (Warnakulasuriya and Ralhan, 2007).

Smokeless tobacco is widely used in south and Southeast Asia, the USA and Scandinavia (Axell, 1993; Warnakulasuriya, 2004). It is more commonly used by males and it is mainly placed in the buccal or labial vestibule (Idris et al., 1994; Warnakulasuriya, 2004).
Oral mucosal disorders are mainly developed at the site where smokeless tobacco is usually placed, and mostly appeared as white plaque with wrinkled surface (Warnakulasuriya et al., 2010). There is clear site-specific involvement connected to the pattern of use, chewing and dipping and placement of smokeless tobacco in different oral sites, which may be different from tobacco smoking associated leukoplakia. For example, in India, buccal site is more common in tobacco chewers (Warnakulasuriya and Ralhan, 2007).

Although various commercial products may cause population differences, epidemiological studies have shown a significant risk from chronic daily use of smokeless tobacco (Warnakulasuriya and Ralhan, 2007), with the length and frequency of used were highly significant for leukoplakia (Wolfe and Carlos, 1987). In the United States studies that were reviewed by the Surgeon Advisory Committee on the health consequences of using smokeless tobacco, between 8-59% of smokeless tobacco consumers were found to have OL (Cullen et al., 1986), and the wide range may be due to different products, duration of use, and inconsistent terminology used for OL in some studies.
Also, in an earlier study on 280 English coal miners who were tobacco chewers, leukoplakia were reported in 10 (3.6%) (Tyldesley, 1971).

The IARC working group on smokeless tobacco has reported that there is sufficient evidence of the oral carcinogenicity of smokeless tobacco (Cogliano et al., 2004). This carcinogenesis may be due to direct contact with the lower lip, lower vestibule and the FOM (Allard et al.,

1999), with histopathological appearance of smokeless-tobacco induced disorder may include epithelial hyperplasia, dysplasia, and CIS (Salem et al., 1984; Zhang et al., 2001c). Further, multiple oral PMDs associated with leukoplakia, notably erythroplakia, and submucous fibrosis were described in a cohort of tobacco chewers in Kerala, India. The presence of such multiple oral lesions is consistent with the concept of field cancerization due to prolonged chewing tobacco (Thomas et al., 2003).

Smokeless tobacco products contain a large number of carcinogens (Hoffmann and Djordjevic, 1997) although it is fewer than in cigarette smoke (Hecht, 2003). Benzo(a) pyrene and other polycyclic aromatic carcinogens are the most important carcinogenic agents in tobacco smoke but in unburnt tobacco, nitrosamines are the strongest carcinogens (Cogliano et al., 2004). These carcinogens in experimental in vitro systems affect oral keratinocytes and cause alterations in cell proliferation, apoptosis and activation of inflammatory mediators (Warnakulasuriya and Ralhan, 2007).

1.8.3. Alcohol Use

It is well established that alcohol consumption is the second major risk factor for oral cancer (IARC, 1988) and oral PMDs (Maserejian et al., 2006a) including leukoplakia (Hashibe et al., 2000a) and erythroplakia (Hashibe et al., 2000b). In a prospective study on men, Maserejian *et al.* (2006a) demonstrated that alcohol is an independent risk factor for oral precancer in non-tobacco users regardless of the type, pattern and frequency of drinking status. Also, according to the same group, one additional drink per day (12.5 g) is associated with a 22% increase in risk of oral cancer. Recently, Rooban and co-workers (2009) reported a higher prevalence of oral mucosal disorders among alcohol misusers.

The risk for oral precancer increases with increased ethanol content of each type of drink therefore, drinking of spirits may increase the risk compared with wine or beer drinking (Castellsague et al., 2004). According to the same group, drinking alcohol only at meals is associated with a reduction of risk compared to drinking between or without food. This may be due to the washing effect of a meal minimizing alcohol effects on the oral mucosa or mixing with food may diminish its topical effect on oral epithelia. The extended analysis of the same group demonstrated that the risk progressively and significantly increased with a longer history of drinking, for example after 20 years, while stopping the consumption of

alcohol may considerably reduce that risk after at least 3 years of cessation. However, the overall risk may need 14 years of cessation to be close to that of non-drinkers (Castellsague et al., 2004).

Alcohol can induce carcinogenesis in different organs either by local or systemic effects (Toriola et al., 2010). The metabolism of alcohol is carried out in the liver, although some metabolism takes place in the stomach and upper digestive tract and gut (Homann et al., 1997). Ethanol is a general component for all kinds of alcoholic beverages (Castellsague et al., 2004), but by itself ethanol is not carcinogenic and there is no clear experimental evidence of pure ethanol carcinogenicity (Wight and Ogden, 1998). However, there is strong epidemiological evidence supporting the role of the first metabolite of alcohol oxidation, acetaldehyde, in oral carcinogenesis (Kurkivuori et al., 2007; Salaspuro, 2007; Meurman and Uittamo, 2008).

Whilst the exact carcinogenic effect of alcohol is not fully understood, there are many possible hypothetical explanations of alcohol's effect on oral mucosa. Firstly, in the oral cavity, alcohol is metabolized in saliva either by oral microflora via bacterial alcohol dehydrogenase (Jokelainen et al., 1996b) or by alcohol dehydrogenase of oral and oesophageal mucosa. (Homann et al., 1997), into its main carcinogenic substances 'acetaldehydes' (Harris, 1997; Burton, 2005) which act directly to damage the mucosa (Castellsague et al., 2004).

Acetaldehydes are unstable substances which produce free toxic radicals damaging the DNA or they may covalently bond to DNA forming DNA adducts (Baez, 2008). This may interfere with DNA synthesis and repair (Poschl and Seitz, 2004; Brooks and Theruvathu, 2005), thus initiating carcinogenesis. It is believed that ethanol may exert a direct effect on the bilayer phospholipid cell membrane (Axford et al., 1999) by dissolving some of the lipid content of cell membrane in the superficial regions of the epithelium, thus increasing oral mucosal permeability. Increased oral mucosal permeability may play a role in carcinogenesis (Hindle et al., 2000) by affecting intercellular mucosal transport, and enhancing the penetration of carcinogens through oral epithelia (Squier et al., 1986; Castellsague et al., 2004) for example, solvent effects on tobacco (Riedel et al., 2003; Poschl et al., 2004) and this may explain the greater risk in heavy drinkers (Kato and Nomura, 1994). Furthermore, Ogden (2005) studied

the intracellular mucosal transport and found a significant reduction in endocytosis (membrane transport) of oral cells in patients with heavy alcohol consumption. This may reflect a reduction in their ability to eliminate local carcinogens leading to an increased exposure time to a particular carcinogen and this may explain the interaction between alcohol and tobacco use (Axford et al., 1999).

Interestingly, dilute ethanol (15%) was found to be more effective than higher concentrations of ethanol (40%) which might be because higher ethanol concentrations act as chemical fixatives reducing tissue permeability (Squier, 1991) and eliminating the subsequent reduction of local carcinogens, exposing the oral tissue to long term effects of possible carcinogens.

Secondly, in addition to ethanol, alcoholic beverages contain other possible carcinogens such as volatile and non-volatile flavour components and additives; of these components nitrosamines, acrylamide and oxidized polyphenols have been classified as possible carcinogens in humans, and animal experiments have shown mutagenic activity on oral epithelial cells (Jagerstad and Skog, 2005).

The third possibility of alcohol carcinogenicity is interference with detoxifying enzymes. Normally alcohol oxidized to acetaldehyde by alcohol dehydrogenase enzyme which then converted to acetate by the catalysing action of aldehyde-dehydrogenase. Acetate is oxidized into carbon dioxide, fatty acids, and water as a final step in ethanol metabolization (Wight and Ogden, 1998; Ho et al., 2007).

The fourth possible mechanism is interference with diet bioavailability (Wight and Ogden, 1998; Blot, 1999) by impairment of nutrient metabolism causing nutritional deficiency (Homann et al., 1997; Riedel et al., 2003). Fifthly, reduce the immune function (Wight and Ogden, 1998; Blot, 1999; Riedel et al., 2003) potentiating the genotoxicity or activate carcinogenic agents, interfering with DNA repair (Wight and Ogden, 1998; Blot, 1999).

According to Gillison (2007), the solvent effects of alcohol may be the most important factor of its carcinogenicity rather than acetaldehyde genotoxicity influenced by local habits such as diet and presence of other risk factors.

Considering the use of alcohol containing mouthwashes, many commercial products contain alcohol in the range of 10-25% (Bhatti et al., 1994) or even more. Frequent alcohol mouthwash users may expose their oral mucosal tissues to levels of alcohol equivalent to alcoholic beverages, which have been implicated in oral cancer development (Winn et al., 2001).

The potential relationship between mouthwash use and the risk of oropharyngeal cancers has been considered by a number of epidemiological studies which have been reviewed by La Vecchia (2009) who concluded that the majority of these studies have shown inconsistent results or negative relations. This may either suggest no significant risk associated with the use of alcohol containing mouth wash or small sample size, differences in the methodologies, difficulties in measuring exposures or individual variations, difficulties in separating the independent effects of mouthwash from smoking, drinking effects and also to the exact effects of other mouthwash constituents (Winn et al., 2001). Furthermore, these studies suffer from limitations such as underreporting of mouthwash use by individuals and the use of different mouthwash types, varying alcohol content, duration of use and time retained in the mouth leading to inaccurate results. The role of alcoholic mouthwash in oral carcinogenicity requires more accurate longitudinal studies with larger sample sizes and more detailed parameters to investigate the potential mechanisms of oral carcinogenesis with and without alcohol.

Alcohol consumption often coexists with tobacco use, thus a combination of tobacco smoking and alcohol drinking are considered as a major risk factors for oral cancer and PMDs (Harris et al., 2004) in a dose-response and synergistic manner rather than in an additive mode (Castellsague et al., 2004). To date, numerous studies have shown that a combination of tobacco smoking and alcohol drinking increase the risk of oral cancer and together they are associated with approximately 75% of upper aerodigestive tract cancers (Llewellyn et al., 2003; Dobrossy, 2005; Ogden, 2005). According to Warnakulasuriya *et al.* (2008) this combination is a strong intensifying risk factor for PMDs and subsequent MT. The possibility of strong combined effects of alcohol and tobacco may be explained on the basis of high levels of acetaldehyde production from both in a synergistic and multiplicative risk effect (Salaspuro and Salaspuro, 2004; Salaspuro, 2007). After a dose of ethanol, salivary

acetaldehyde was 2 times higher in smokers without smoking and 7-times higher with active smoking (Salaspuro and Salaspuro, 2004).

1.8.4. Betel Quid and Areca Nut

Betel quid with or without tobacco has been identified as an oral carcinogen in humans by the IARC with evidence of a dose-response relationship (IARC, 2004a; Jacob et al., 2004; Secretan et al., 2009). It has been estimated that between 10-20% of the world's population chew betel quid or areca nut in some form (Gupta and Warnakulasuriya, 2002). Betel quid is regarded as the fourth most frequently consumed psychoactive substance after nicotine, ethanol, and caffeine (Gupta and Ray, 2004). It is mostly used by people in the developing countries as dried husks or fresh nut (Bagan and Scully, 2008), chewed alone as in China and Taiwan, or used with tobacco as in India and Pakistan (Reichart and Zhang, 2007).

Betel quid, with or without tobacco has been found to increase the risk of oral PMDs, although this relation is not as significant as with cigarette smoking or alcohol drinking (Yen et al., 2007). A significant association has been found between the frequency and duration of chewing betel quid and the risk of PMDs (Yen et al., 2007), such as OL (Hashibe et al., 2000a; Lee et al., 2003; Jacob et al., 2004), submucous fibrosis (Hashibe et al., 2002a; Lee et al., 2003; Jacob et al., 2004), erythroplakia (Hashibe et al., 2000b; Jacob et al., 2004) and multiple PMDs (Thomas et al., 2003). However, the most significant association is with oral submucous fibrosis (Jacob et al., 2004). According to Reichart and Nguyen (2008), oral submucous fibrosis in betel quid chewers in Vietnam was observed in 13%, OL in 3.8% with lichen planus in 5.2%.

Betel quid ingredients have been shown to exert cytotoxicity, mutagenicity and genotoxicity toward different types of cells including oral epithelial cells, bone marrow cells and peripheral blood mononuclear cells (Jeng et al., 2001; Kumpawat et al., 2003). The carcinogenicity of both betel quid and areca nut may refer to nitrosation with consequent production of potentially carcinogenic nitrosamines such as 3-methylnitrosopropionitrile and also to the generation of reactive oxygen species in the oral cavity due to auto-oxidation of polyphenols contained in areca nut enhanced by the alkaline pH from slaked lime (IARC, 2004a; Nair et al., 2004; Subapriya et al., 2007).

With respect to the affected oral sites, in betel quid chewing, the buccal mucosa, followed by tongue and lips are the most commonly affected sites (Reichart and Nguyen, 2008), reflecting the effect of direct contact with these locations.

1.8.5. Marijuana (Marihuana/Cannabis)

Marijuana is one of the most commonly misused psychoactive unlicensed drugs (Scully, 2007) which is mainly smoked or may be taken orally either directly or mixed with food (Hall et al., 2005). However, smoked marijuana is the predominant form of cannabis used (Agrawal and Lynskey, 2009). Marijuana preparations, which contains more than 60 types of cannabinoids, are derived from the plant Cannabis sativa particularly from small glands on the surface of the plant (ElSohly, 2007). Delta-9-tetrahydrocannabioid (THC) is the principle psychoactive chemical ingredient which is found in all types of cannabis (Ashton, 2001; Cho et al., 2005; Scully, 2007).

Due to the similarity in carcinogens and co-carcinogens between marijuana and tobacco smoke, except for nicotine (Tashkin et al., 2002), it is important to include marijuana use in the medical history of patients and it should be included within risk factor assessment for oral PMDs (Hashibe et al., 2005). Smoking a low number of marijuana cigarettes per day has been described to have similar histopathological effects on tracheobronchial epithelium as smoking more than 20 tobacco cigarettes (Fligiel et al., 1997). This might be due to a different smoking pattern with deep inhalation and more tar retention in the respiratory tract from marijuana compared with tobacco smoke (Tashkin et al., 2002).

Patients using marijuana may develop PMDs, such as OL, erythroplakia and oral submucous fibrosis which are found to have higher prevalence rates in marijuana users compared with the general population (Thavarajah et al., 2006). The higher prevalence of PMDs in marijuana users necessitates periodic oral examination of such patients for early identification of PMDs.

Within the oral cavity, cannabis smoking and/or chewing are associated with changes in the oral epithelia forming cannabis stomatitis (Cho et al., 2005) which results in chronic inflammation and leukoplakia, with the possibility of progression to neoplasia (Darling and Arendorf, 1992). Epidemiological studies have shown that marijuana smoking raises the risk

of oral and head and neck cancers by 2.6-fold compared with non-users (Zhang et al., 1999) and in a dose-response relationship for both frequency of use per day and duration (Hashibe et al., 2002b). However, other studies have shown a conflicting results; Llewellyn *et al.* (2004a) in a small case-control study and a community-based study conducted by Rosenblatt *et al.* (2004) showed no association between regular cannabis use and OSCC. The absence of association may be due to a small sample size (small number of heavy cannabis users) or due to differences in marijuana consumption.

Recently, cannabis and other derivatives have been suggested to have various therapeutic uses and efforts have been made to provide non-smokable, safe therapeutic agents without psychoactive effects.

Regarding such medicinal use which is under intensive investigation (Ware et al., 2005), delta-9-tetrahydrocannabioid (THC) may be used in cancer care to improve appetite, reduce nausea and vomiting and to relieve moderate neuropathic pain (Hall et al., 2005). It is designed as a spray under the tongue or on the buccal mucosa in alcohol with a peppermint flavouring to relieve chronic pain in multiple sclerosis, arthritis and neuropathy (Scully, 2007). Although it is efficient in pain relief, patients may develop white lesions, an oral burning sensation and stinging which might be due to the high alcohol concentration in addition to peppermint (Scully, 2007); this increases the concern in chronic oral users.

The interactions between different risk factors may increase the risk of developing potentially malignant disorders such as leukoplakia, erythroplakia and oral submucous fibrosis which are found to be higher in psychoactive substances users who smoked tobacco, chewed areca nut, drank alcohol and smoked cannabis (Thavarajah et al., 2006). The synergistic effect between tobacco and marijuana smoke has been observed, suggesting that tobacco and cannabis smoking may enhance the inflammatory responses possibly promoting their carcinogenicities (Melamede, 2005).

1.8.6. Family Cancer History/Individual Susceptibility

A positive family history is usually defined as one or more first degree relatives with a history of cancer (Brown et al., 2001). In families with known inheritance syndromes, first-

degree relatives have a 50% chance of inheriting gene mutations which increases the risk of developing associated cancers and passing the causative gene to their offspring compared with families with no familial mutations (Trepanier et al., 2004).

The genetic factors may include chromosomal aberrations, single nucleotide polymorphisms, matrix metalloproteinase-1 and TP53 (Vairaktaris et al., 2006; Kuroda et al., 2007). In addition to a germline mutation in the p16 gene, all of these may affect the normal function of genes, regulation of cell growth and DNA integrity (Yu et al., 2002).

Although few epidemiologic studies have assessed the importance of familial hereditary factors in oral carcinogenicity, there is growing evidence from case control studies that consider family history as a risk factor for oral cancer (Jefferies et al., 2008). The evidence is mainly based on the fact that more than 50% of oral cancer patients have not been exposed to any major identifiable risk factors such as tobacco and alcohol or other risk factors (Campo-Trapero et al., 2008b). Also, a young age of onset of oral cancer is more likely to support the concept of inherited disorders associated with an increased risk of cancer (Jefferies et al., 2008; Scully and Bagan, 2008).

It has been found that the risk of cancer for patients with a positive family history increased after adjustment for the age, sex, alcohol and tobacco exposure (Brown et al., 2001). However, it is difficult to differentiate between the effects of genetic susceptibility, associated lifestyle exposures or a combination of these factors.

In a multicentre case-control study conducted in Italy and Switzerland it was found that first-degree relatives' family history of oral and pharyngeal cancer is directly and significantly associated with the risk of oral and pharyngeal cancer independent of tobacco smoking and alcohol consumption (Garavello et al., 2008b). Also, Garavello *et al.* indicated that the risk is increased in subjects with a family history of lung cancer or skin melanoma and it is higher in families with two or more affected relatives.

Furthermore, considering individual genetic variations in the development of oral cancer, one needs to understand that exogenous carcinogens usually require activation by metabolic enzymes, whilst deactivation of carcinogens or their by-products is by detoxifying enzymes

(Gillison, 2007). Hereditary polymorphisms in these enzymes may affect the balance between both metabolic activation and detoxification of carcinogens leading to an increase or decrease in carcinogenic potential (Chen and Hunter, 2005). Therefore, polymorphisms of carcinogen-metabolizing enzymes may affect an individual's susceptibility to risk factors and subsequent progression to cancer (Baez, 2008). It is believed that the influence of a positive family history is also due to an increased sensitivity to the genotoxic effects of mutagens in tobacco smoke and metabolites of alcohol which represent an inherited sensitivity (Gillison, 2007). However, the majority of hereditary cancer syndromes are characterized by variable expressivity in cancer development, age of onset, site and number of tumours (Trepanier et al., 2004).

Recently, there is increasing interest in "Genetic Risk Assessment" which involves a multistep process commencing with a detailed personal medical and family history and ending with hereditary cancer risk assessment by Genetic Counselling (Trepanier et al., 2004). This risk assessment requires an accurate and comprehensive family history "genetic pedigree" or family tree which is useful in the management plan including prevention, risk reduction and cancer screening.

1.8.7. Old Age

Ageing is a complex physiological and biochemical process with molecular changes manifested at a cellular level as well as in the organism as a whole (Malaguarnera et al., 2010). A considerable body of data suggest that the human lifespan is continuously growing with an increased prevalence of many diseases including oral cancers and PMDs. The majority of OSCCs and PMDs are observed in patients between 50 and 80 years old (Jemal et al., 2006; Hirota et al., 2008; Goldenberg et al., 2009) with a peak in the 70s (Kurtz et al., 2010) and less common in patients under the age of 50s (Goldenberg et al., 2009). Investigating the risk factors for OL, Dietrich *et al.* (2004) demonstrated that age is one of the independent predictors for this most common PMD.

The relation between cancer and age may be explained as a result of long periods of carcinogenic exposure with mixed genetic and environmental components (Finkel et al., 2007); long cumulative exposure to different carcinogens mainly smoking and alcohol misuse

in old individuals compared to younger subjects with relatively less exposure time (Hirota et al., 2008).

Furthermore, increasing knowledge regarding telomeres which are defined as "DNA protein complexes that cap the chromosomal ends promoting chromosomal stability" (Epel et al., 2004) may help to understand and explain the relation between ageing and risk of tumour development (Malaguarnera et al., 2010). Normally with ageing, telomere length gradually decreases in dividing somatic cells and this may contribute to neoplastic transformation, replicative senescence or apoptosis resulting in early death and early onset of disease (Cawthon et al., 2003). It has been documented that the mortality rate for patients with short telomeres is two-fold more than those with long telomeres (Cawthon et al., 2003) and this is also supported by a study performed by Warnakulasuriya *et al.* (2007) to compare the survival rate of patients less than 45 years with patients who had been 45 years and older ~~who~~ when diagnosed with oral cancer; they showed a higher 5-year survival rate in young patients who have long telomeres.

Due to many changes associated with the ageing process, one may conclude that age is one of the risk factors for oral cancer and PMDs and a periodic screening for all adult patients over the age of 40-years is recommended every 6 months for early detection and diagnosis of oral cancer and PMDs.

1.8.8. Dietary Factors

Dietary factors play a substantial role in the aetiology of many chronic diseases and cancers including oral cancer (Ronco et al., 2006; Gillison, 2007). In the United Kingdom, evidence has shown that the consumption of fat and refined carbohydrates have increased by 5 to 10-fold, whilst there has been a reduction in fibre-rich grain intake (Petti, 2009).
Epidemiological studies have shown that an unhealthy diet rich in saturated fats, sugar, with less dietary fibre, lack of physical activity and resultant obesity are responsible for 30-40% of cancer cases worldwide (Petti, 2009).

Food diversity has a protective effect on health due to variation in dietary components and is often associated with a favourable lifestyle (Garavello et al., 2008a). Fruit and vegetable

intake appears to be the most consistent dietary component influencing the risk of oropharyngeal cancer, with most studies highlighting the protective effects of fruit and vegetables compared with other factors such as cereals, meat, fish, milk and dairy product (Amtha et al., 2009). These latter products have been investigated in many case-control and cohort studies, but results are inconsistent and may be due to variations in food preparation in different populations (Amtha et al., 2009).

In chemoprevention studies, supplements of nutrients commonly obtained from fruit and vegetables, such as beta-carotene and lycopene have been shown to encourage regression of OLs (Singh et al., 2004). In a study to investigate the risk of oral PMDs, Maserejian and co-workers (2006b) showed that increased consumption of fruit, particularly citrus fruits in men reduced the risk of oral PMDs even with the presence of tobacco smoking, whilst the results for vegetable consumption were inconsistent. Similarly, Boccia *et al.* (2008) found that even in presence of high alcohol consumption or cigarette smoking, a higher intake of fruit and vegetables might prevent the development of approximately one quarter of head and neck cancer cases. Llewellyn *et al.* (2004a) in a case-control study in Southern England showed that daily consumption of three or more portions of fresh fruit and vegetables showed a considerable risk reduction compared with two portions or less.

Studies have also shown that deficiencies in fruit, non-starchy vegetables and carotenoid food may be associated with 10-15% of oral cancer cases (Popkin, 2007). This is in agreement with a previous study conducted by Levi *et al.* (1998) who showed higher percentages of oral and pharyngeal cancers with low fruit and vegetables intake. Furthermore, fast, fermented and canned processed food have been found to double the risk for oral cancer, PMDs and other diseases due to their high fat content that generates polycyclic aromatic hydrocarbons during high temperature cooking; these hydrocarbons have been shown to cause cancer in laboratory animals (Grosvenor and Smolin, 2002). Moreover, an association between the risk of oral cancer and heterocyclic amines, such as benzopyrene generated from burned amino acids and other substance in meat, has been reported by the same group.

Neither the mechanism of action nor the relation between diet and the risk of oral cancer and PMDs is fully understood and this is partly due to the fact that not all chemical compounds of food are well known or measured. Also, the role of different micronutrients are not

completely clear (Petti, 2009). Nevertheless, this may be explained on the basis that raw fruit and vegetables provide a mechanical cleansing effect for the oral cavity with the benefit of many properties in plant food such as a dilution action, anti–oxidant and anti-carcinogenic properties of micronutrients such as vitamins A, C, E, carotenoid, flavonoids, phytosterol, folates and fibres which are crucial for neutralizing the carcinogenic effects of tobacco, alcohol, and betel quid (Potter and Steinmetz, 1996; Kane, 2005; Rossi et al., 2007; Boccia et al., 2008; Lucenteforte et al., 2009).

Further research is required to investigate the relationship between oral cancer and PMDs with micro/macro-nutrients and ultimately a dietary pattern may be established for a better dietary cancer protection.

1.8.9. Immunodeficiency

Immunodeficiency is a condition in which the immune system is either compromised or absent. It may be either a primary (genetic) or secondary (acquired) immunodeficiency resulting from disease or external influence such as drugs, environmental or iatrogenic factors (Rosen et al., 1995). Anti-tumour immune responses by both innate and adaptive immunity are essential for clearance and elimination of malignancies. However, accumulated evidence reveals that these responses may themselves be suppressed by the properties and function of cancer cells (Jewett et al., 2006).

Genetic immunodeficiency has been implicated in the aetiology of oral cancer and PMDs in young individuals. Inherited cancer syndromes such as xeroderma pigmentosum, Fanconi's anaemia and Bloom’s syndrome are associated with an increased incidence of oral cancer (Prime et al., 2001).

Acquired, induced immunodeficiency such as in organ transplant patients where the drugs are used to prevent organ rejection is greatly improving the quality of life and survival rate of many patients. This improvement is partly due to the use of modern immunosuppressive regimens to control rejection after transplantations. However, an increased incidence of both benign and malignant post-transplantation tumours as side effects of long-term immunosuppression is recognised. Azathioprine, cyclosporine and steroids are the main

immunosuppressive agents that are used to avoid tissue rejection in transplanted patients (Curtis et al., 2005).

Several studies have shown an increased incidence of post-organ transplantation tumours involving head and neck cancers by 2 to 4-fold compared with non-transplanted populations (Vajdic et al., 2006; Vegso and Jaray, 2007). In kidney transplantation patients, lip and skin cancers increase by 35-fold and head and neck cancers by 4-fold (Makitie et al., 2008). Also, OL has been found to be the third most commonly diagnosed disorder in patients who underwent solid organ transplantation with prevalence of 10.7% (King et al., 1994). According to the same group, in renal transplant recipients, leukoplakia of the lip occurred 22 times more often than in normal subjects. Furthermore, the presence of leukoplakia in normal patients increases the risk of oral cancer development by 5-fold compared with a 50-fold higher risk in immunosuppressed transplant patients (Hernandez et al., 2003).

In Human Immunodeficiency Virus (HIV) infection in AIDS patients, Human Papillomavirus (HPV), Epstein-Barr virus (EBV) and Human Herpes Virus-8 in which the precursors of the associated tumours are usually removed in immune competent individuals, but grow into tumours in congenitally or immunodefective individuals with immune impairment (Klein and Klein, 2005).

The main role of the immune system is to protect the body's integrity; however, our genome contains a number of proto-oncogenes continuously exposed to mutagens and this may explain why cancer increased following organ transplantation. Impaired or lack immunosurveillance due to prolonged immunosuppression resulting from several years treatment for chronic graft versus host disease, emerges as a major factor in the elevated incidence of tumours in transplanted patients (Gourin and Terris, 2004; Giese et al., 2009). Further, a rapid transformation of OL in liver transplant recipient four months after the transplantation (Hernandez et al., 2003), the diagnosis of OSCCs in organ transplant patients on immunosuppressive therapy in the absence of other risk factors, such as tobacco and alcohol (Meng and Jiamei, 2000). These are clearly assigned to the carcinogenic effect of immunosuppressive drugs (Hernandez et al., 2003).

A study performed by Gourin and Terris (2004) showed that patients post-liver transplant due to alcoholic liver cirrhosis suffer from more malignancies compared with patients who underwent liver transplant for other causes. This is probably due to the fact that both alcohol and tobacco, as well documented risk factors for oral cancer, synergies with the effect of immunosuppressive in this group. This is also agreeing with Scheifele *et al.* (2005) who found that the incidence of oral, pharyngeal and laryngeal carcinomas as late complications of liver transplantation is 3.6% in patients with alcoholic liver transplants compared with only 0.17% incidence in patients with non-alcoholic liver transplants.

Immunosuppression must be considered as an important risk factor for oral cancer and PMDs. Therefore, controlling or reduction of carcinogenic agents such as tobacco, alcohol, sun exposure and viral infections along with early detection and treatment of PMDs with low and efficient dose of anti-proliferative action of immunosuppressive therapy are more likely to reduce the risk of post-transplant tumour formation (Vegso and Jaray, 2007).

1.8.10. Oral Health

In the oral cavity, one milligram of dental plaque contains approximately 10^{11} bacteria, and between 750 to 1000 bacterial species (Aas et al., 2005; Jenkinson and Lamont, 2005). Oral streptococci, a gram-positive bacteria, forms the largest proportion of normal oral microflora (Jenkinson and Lamont, 2005). In high density plaques, these microbial species adhere to tooth surfaces and to other surfaces in the mouth such as oral epithelia, restorative and prosthetic dental materials (Meurman and Uittamo, 2008).

Patients with poor oral hygiene show higher numbers of microorganisms from supragingival dental plaque which may increase carcinogenic acetaldehyde production in saliva (Bloching et al., 2007; Meurman and Uittamo, 2008) compared to those with better oral health (Homann et al., 1997). In saliva, several oral streptococci have alcohol dehydrogenase enzymes which enable these micro-organisms to oxidize ethanol into acetaldehyde (Meurman and Uittamo, 2008). It is therefore understandable that such micro-organisms in the mouth may increase the production of carcinogenic acetaldehyde with a consequent increased potential for oral cancer (Meurman and Uittamo, 2008).

Bacterial production of acetaldehyde from ethanol depends on the activity and characteristics of alcohol dehydrogenase enzymes (Kurkivuori et al., 2007). This may contribute to the individual variation of acetaldehyde salivary level seen after alcoholic drinks, with subsequent variation in risk of oral PMD and cancer formation (Kurkivuori et al., 2007).

In patients with poor oral health, higher salivary acetaldehyde concentrations during and immediately after drinking alcoholic beverages and smoking has been observed probably due to direct dissolution into saliva (Kurkivuori et al., 2007). The derived acetaldehyde appears to act as a local carcinogen and in a dose-dependent and synergistic way (Salaspuro, 2007).
In a case control study, Macigo *et al.* (2006) investigated the influence of oral hygiene and development of OL and they found that failure to brush teeth and non-use of toothpastes was significantly associated with the development of OL, emphasising the need for proper daily oral hygiene in patients, particularly those who are using alcohol.

Regarding carcinogenicity, acetaldehyde may cause point mutations in human lymphocytes, sister chromatid exchanges and cross chromosomal aberration and interference with DNA repair (Dellarco, 1988). Also, acetaldehyde may interact with DNA forming adducts which can lead to mutation (Brooks and Theruvathu, 2005). Furthermore, formation of endogenous nitrosamines in the oral cavity by nitrate-reducing bacteria in patients with poor oral health may enhance carcinogenicity (Shapiro et al., 1991).

Interestingly, researchers have observed that the alcohol dehydrogenase enzyme can be inhibited by 4-methylpyrazole which subsequently reduces the acetaldehyde production. Also, using antiplaque, antimicrobial mouthwashes such as chlorhexidine may reduce oral microbiota responsible for acetaldehyde production and thus reduce local acetaldehyde production, subsequently protecting the subjects from acetaldehyde carcinogenesis (Meurman and Uittamo, 2008). However, there is no clinical study in that field due to chlorhexidine side effects (Muller and Kramer, 2008).

Meurman and Uittamo (2008) suggested future studies for elimination of acetaldehyde production in the oral cavity either by using cysteine (α-amino acid), which supports the second step in acetaldehyde metabolism into relatively harmless acetic acid, or by using

probiotics (live naturally occurring microorganisms) which have an inhibitory effect on acetaldehyde production from oral flora.

It is clear that routine dental care with regular dental visits as an indicator of a good oral health may reduce or prevent the exposure to some carcinogens, in addition to regular screening programs for prevention (Guha et al., 2007) and treatment as a part of the long-term standardised care (Rooban et al., 2009).
Oral hygiene status should be involved among the strategies of oral PMDs preventive and control programmes.

1.8.11. Socioeconomic Factors

Socioeconomic status is mainly based on the three important parameters of income, education and occupation and comprises three classes: high, middle and low level (Greenwood et al., 2003).
Although socioeconomic factors have been regarded of importance in the aetiology of oral cancer and PMDs, they are time changeable factors (Conway et al., 2008). The relation between socioeconomic status and oral cancer is complex and has received little attention. Lifestyle risk factors, particularly tobacco smoking and alcohol consumption, are directly affected by socioeconomic circumstances which influence the effects of these factors (Hashibe et al., 2003). These risk factors have been described as coping mechanisms for stress, which is associated with a variety of factors including deprivation, unemployment, low income and insecurity of work (Marmot, 1997; Stead et al., 2001).

However, the biological basis for the stresses associated with low socioeconomic status and cancer development is not entirely clear. However, emerging hypotheses from “biological ageing” demonstrate that psychological stresses are associated with significant increased oxidative stress which can shorten telomeres, a known determinants of cell senescence and longevity, leading to premature ageing and earlier onset of disease with early death (Epel et al., 2004). Gradual loss of telomeric DNA (telomere shortening) in dividing somatic cells contributes to replicative senescence, apoptosis and neoplastic transformation (Cawthon et al., 2003) and may all result from low socioeconomic circumstances (Adams and White, 2004).

Individuals in low socioeconomic groups with low income are almost always under stress which may require coping mechanisms such as tobacco chewing and/or smoking and alcohol drinking (Conway et al., 2008). So, those individuals are subjected to potential lifestyle risk factors besides a low consumption of fruit, vegetable and vitamins (protective agents) and their resultant low body mass index and less access to medical care may lead to a higher prevalence of oral PMDs (Brennan et al., 2004); they can be regarded as high risk patients.

Furthermore, low socioeconomic status may lead a person to detrimental environmental and housing conditions, with physical, social and psychological effects in turn affecting overall human health (Conway et al., 2008). Large numbers of household residents increase human contact affecting hygienic living conditions, which may increase the possibility of fungal, bacterial or viral infections predisposing to the development of oral PMDs (Hashibe et al., 2003).

Analytical studies have shown that oral cancer is more common in the most deprived people in society (Greenwood et al., 2003; Conway et al., 2007; Conway et al., 2010) and low income may also contribute to cancer diagnosis delay (Hansen et al., 2008) which may have direct effects on the prognosis and survival rate for patients. Speight *et al.* (2006) proposed that patients at higher risk may be determined by their sex, age, smoking and drinking status and Conway *et al.* (2007) suggested that socioeconomic status should be considered as key for 'priority risk grouping'.

In contrast, individuals from high socioeconomic groups having higher incomes may be protected from oral cancer and PMDs through their higher consumption of vitamins from fruit and vegetables. In addition, they have more access to medical care at early stages of their diseases, such as treatment of oral PMDs before transformation to oral cancer. Indeed, such groups of people are more likely to be classified as low risk patients (Hashibe et al., 2003).

As preventive measures, all the socioeconomic factors that can be identified as real risks for oral cancer and PMDs need to be improved by effective measures to reduce inequalities between people in society through either local or national authorities or the WHO commission.

True prevention and early detection of oral cancer and PMDs requires addressing the underlying poverty and inequalities that affect the socioeconomic status of all peoples.

1.8.12. Human Papillomavirus

It has been reported that even in the absence of well-known risk factors or predisposing genetic factors, between 15–20% of patients may develop OSCCs (Gillison and Shah, 2001). This suggests the presence of other aetiological risk factors, such as viral infectious agents (Scully 2002).

HPV is a small epitheliotropic DNA virus which may be considered as either an independent risk factor or as an intensifier to tobacco and alcohol effects in oral carcinogenesis (Nair and Pillai, 2005). More than 118 HPVs have been identified which can infect various human epithelial tissues (de Villiers et al., 2004); the mucosal type can infect human genital and oral epithelial cells and may cause disorders ranging in severity from benign growth to malignant features (Wise-Draper and Wells, 2008).

HPVs are divided into low risk and high risk subtypes according to their clinical association. Low-risk subtypes include HPV 6 and 11 and are mostly associated with benign or mildly dysplastic disorders, whereas high-risk subtypes such as HPV 16 and 18 are mainly identified during MT and in established cancers (Munger and Howley, 2002; Slebos et al., 2006; Acay et al., 2008; Wise-Draper and Wells, 2008). HPV 16 is the most commonly recognized HPV in OSCCs and both HPV 16 and 18 play very important roles in MT (Boulet et al., 2007).

It is well documented that HPVs are implicated in the pathogenesis of cervical cancer and although more than 95% of human cervical cancers are associated with HPV 16 and 18 (Yuan et al., 2005); the association with the head and neck cancer remains controversial.

Some studies could not demonstrate an association between HPV infection and oral PMDs or oral cancer and suggest HPV may represent a transient infection rather than an essential prerequisite during the step-wise carcinogenesis of OSCCs (Ha and Califano, 2004). However, more recently HPV infection as a possible etiologic factor for head and neck cancer has emerged indicating that HPV positive tumours may represent a distinct subset and

this is supported by the similarity between the HPV oncogenic types detected in cervical carcinomas and those seen in head and neck cancers (Khovidhunkit et al., 2008; Chocolatewala and Chaturvedi, 2009).

According to a study conducted by D'Souza *et al.* (2007), oral HPV infection is strongly associated with oropharyngeal cancer with or without the established risk factors of tobacco and alcohol use. From different studies, a wide variation in prevalence of between 20-75% for HPVs in OSCCs has been reported (Smith et al., 2004; Kreimer et al., 2005; Syrjanen, 2005; Chocolatewala and Chaturvedi, 2009).

Furthermore, in a meta-analysis, high-risk HPV genotypes have been detected in normal oral mucosa, precancerous oral mucosal tissue and oral carcinoma, with increased frequency in oral epithelial dysplasia and carcinoma compared with normal oral mucosa (Miller and Johnstone, 2001). The virus was identified in precancerous oral mucosa at levels 2-3 times higher than in normal mucosa. Such findings may provide evidence for including HPV in the risk factors for oral PMDs. HPV has been identified in primary tumours of the tonsil, larynx, hypopharynx, oral cavity, tongue, and nasopharynx; however, the highest rates were found in tonsillar cancer followed by tongue and buccal mucosa (D'Souza et al., 2007; Chocolatewala and Chaturvedi, 2009).

Molecular research has shown that carcinogenesis involving high risk HPVs acts mainly through two viral oncogenes E6 and E7 which are both regarded as indicators of HPV positive cancers, affecting apoptosis which is essential for HPV infection to avoid the immunological response (Mantovani and Banks, 2001). These two proteins have no intrinsic activities of enzymes, but they are able to interact directly and indirectly with two-key tumour suppresser proteins p53 and retinoblastoma. This may affect their ability to stimulate DNA repair or apoptosis interfering with the cell cycle control and promote carcinogenic processes (Wise-Draper and Wells, 2008).

The knowledge of the detection of HPV in a patient with oral dysplastic disorders may be of benefit for deciding upon treatment plans and selection of treatment modalities which may be different for HPV associated disorders compared with non-HPV associated dysplastic disorders. According to Tomson *et al.* (2004), the identification of HPV as a cause of cervical

cancer indicates that HPV vaccines can potentially be used to prevent or treat cervical cancer and other HPV-associated malignancies and if these prophylactic and therapeutic vaccines prove as successful in patients as they have in animal models, HPV vaccines may play a role in the control of HPV infection and HPV-associated disease.

1.8.13. Candida Infection

Candida albicans is a normal flora of the aerodigestive and vaginal tracts but it may become pathogenic and produce dermatological, mucous membrane and internal infections in certain circumstances such as pregnancy, oral contraception, antibiotic therapy, diabetes, skin maceration, topical steroid therapy, certain endocrinopathies, and factors related to depression of cell-mediated immunity (Vuckovic et al., 2004).

There is an increasing interest to investigate oral Candida infection, not only because of the association with Human Immunodeficiency Virus (HIV) but also because of an important association with oral PMDs (Vuckovic et al., 2004). Within the oral cavity, Candida infections are regarded by many studies as a significant risk factor enhancing epithelial dysplasia and subsequent MT of OL. In a study conducted by Cao and co-workers (2007) to estimate the rate of Candida infection in different oral epithelial disorders using human salivary samples, they found that Candida infection is one of the important factors inducing epithelial dysplasia and MT of OL. Also, the same group suggested using salivary cultures as routine tests for patients exhibiting OL with Candida infection and regular follow-up was encouraged for such patients.

Vuckovic *et al.* (2004) isolated Candida from PMDs, mainly OL and lichen planus, with an apparent increased incidence in lesions with marked epithelial dysplasia. This is supported by a study performed by McCullough *et al.* (2002) who found significantly higher number of oral yeast in histopathologically confirmed oral epithelial dysplasia with more isolated yeast in advanced levels of dysplasia. These results are in agreement with the previous study performed on 223 oral mucosal biopsies to investigate the prevalence of fungal infection using periodic acid-Schiff stain; the study showed a significant positive association between fungal infection with both moderate and severe epithelial dysplasias (Barrett et al., 1998).

The same group suggested the use of anti-fungal therapy as a part of treatment plan in these types of disorders.

However, it could be argued that the high numbers of oral yeast associated with dysplasia are completely coincidental with environmental changes contributing to the proliferation of these microorganisms, otherwise many more patients with chronic candidiasis should develop oral carcinomas (Sitheeque and Samaranayake, 2003).

More recently, a study was carried out to test the ability of Candida albicans to promote epithelial dysplasia using 4 Nitroquinoline 1-oxide (4NQO) as an initiator of neoplasia in a mouse model. Candida albicans was found to play a promotion role in oral dysplasia in this model (Dwivedi et al., 2009) and this may provide more evidence of an association between Candida albicans and oral epithelial dysplasia. This is also supported by a previous study conducted by Field *et al.* (1989) who proposed that nitrosamine compounds produced by Candida species may act directly on oral mucosa, or interact with other chemical carcinogens to activate specific proto-oncogenes which initiate the development of oral neoplasia.

In spite of the general acceptance of an association between Candida infection and the occurrence of oral epithelial dysplasia, the possible role of yeast in oral carcinogenesis is still unclear and further studies in this area of research are warranted.

1.8.14. Diabetes Mellitus

Diabetes mellitus (DM) is a rapidly growing metabolic disorder that parallels obesity and poor lifestyle behaviours (Auluck, 2007; Skamagas et al., 2008). It accounts for approximately 5% of world-wide deaths (Roglic et al., 2005). DM is a group of metabolic diseases characterized by hyperglycaemia resulting from a defect either in insulin secretion, action or both (American Diabetes Association, 2009). It is mainly of 2 types: type I or insulin dependent because of its clinical need for insulin or Juvenile onset diabetes due to its early age of onset (Jahromi and Eisenbarth, 2007). Type II or non-insulin dependent forms 85-90% of DM cases (Skamagas et al., 2008).

Research has shown that DM increases the risk of oral pathologies, such as acute infections, periodontitis, candidiasis, PMD (lichen planus) and malignancies (Ujpal et al., 2004; Dikshit et al., 2006; Goutzanis et al., 2007; Skamagas et al., 2008). Recently, epidemiological studies have involved DM in the aetiopathogenesis of OSCCs and PMDs (Ujpal et al., 2002; Dietrich et al., 2004; Ujpal et al., 2004; Dikshit et al., 2006). However, more recent studies have shown no association between DM and oral PMDs (Saini et al., 2010). According to the same group, this contradictory result may be related to the sample size and adjustment of the confounding factors, and a relation between PMD and DM may still be possible. Although a more recent study conducted by de Souza Bastos *et al.* (2011) also found no significant association between OL and DM type II possibly due to a reduced number of smokers in this study group.

A significant association between the development of OL and DM has been reported in a study conducted by Dietrich *et al.* (2004) who found that diabetic patients were 3-times more likely to develop OL compared to non-diabetics. Similar relations have been previously reported between DM and OL (Albrecht et al., 1992; Ujpal et al., 2002). Albrecht *et al.* (1992) reported a prevalence of 6.2% among diabetics compared with 2.2% in a control group. Also, Dikshit *et al.* (2006) reported that the odds ratio of OL in women with a history of DM was 2 and 3.2 for erythroplakia after adjusting for potential confounders. The association is stronger in females with a history of diabetes compared with males which may relate to poorer metabolic control and higher levels of insulin in females with DM leading to more oxidative damage to DNA (Dikshit et al., 2006).

Normally, oral epithelial tissue provides a protective barrier against external carcinogens. In diabetic patients, and as part of the disease process, a progressive atrophy of oral mucosa may occur due to reduction in both salivary secretion and pH (Taylor et al., 1996). This may increase oral disorders, such as glossitis and cheilitis (Ujpal et al., 2004) as well as increasing the permeability of oral mucosa to different carcinogens as a result of loss of the normal protective barrier of oral epithelium (Auluck, 2007).

There are several explanations for the potential tumorigenic effects of DM. This tumorigenicity may be directly mediated by insulin receptors in target cells or might be due to related changes in endogenous hormone metabolism (Auluck, 2007). Insulin deficiency

results in reduction of insulin receptor substrate-1 (Goutzanis et al., 2007) and changes in the cytoskeleton leading to reduction in cell adhesion by affecting focal adhesion kinase pathways (Vairaktaris et al., 2007). This is probably a starting step towards neoplastic or dysplastic change (Goutzanis et al., 2007). Further, insulin can stimulate the synthesis and biologic activity of insulin-like growth factor-1 which promotes cell proliferation and inhibits apoptosis (Calle and Kaaks, 2004).

However, limited evidence indicates that the effect of insulin-like growth factor-1 might be connected to p53 mutations, which are frequently seen in head and neck tumours (Dikshit et al., 2006). Also, elevated blood glucose levels and protein breakdown may lead to excessive formation of free radicals (Ujpal et al., 2004) causing imbalance between production of free toxic radicals and biological systems due to reduction of antioxidant activity of enzymes (Auluck, 2007). This may cause DNA damage and subsequent promotion of carcinogenesis (Seril et al., 2003).

From the aforementioned points, the role of DM in the aetiopathogenesis of PMDs needs more accurate study to determine if the association is related to characteristics of the diabetic state or to the treatment agents or to other associated risk factors. To explain the exact associated mechanisms, future studies should take in consideration information such as the type of diabetes, type of treatment, age of onset of diabetes, duration between the onset of diabetes and the development of PMDs (Dikshit et al., 2006).

1.9. Management of Patients with Oral PMDs

Management of PMDs includes both diagnostic and treatment processes (Marley et al., 1996). The intention of clinical management of oral PMDs is early detection, treatment, and prevention of MT by initiating an adequate intervention (Holmstrup et al., 2006).
Since treatment of patients with advanced stages of disease is often associated with more damaging interventions, more cost and are more liable to failure (Heintzelman et al., 2000), early detection of oral PMDs is essential. However, there is no universally reliable method for early detection of PMD tissue changes (Heintzelman et al., 2000). This may be due to inexperienced practitioners who are unable to detect early dysplastic tissue changes or discriminate them from benign tissue changes or the whole mouth lining may be unstable and it is difficult even for experienced clinicians to decide the location and the time for biopsy. Also, performing repeated surgical and follow-up biopsies are often unpopular for both patients and practitioners (Heintzelman et al., 2000).

At the present time, there are no molecular markers which enable a researcher to distinguish disorders that progress to cancer from those that will not (Napier and Speight, 2008; Warnakulasuriya et al., 2008). Therefore, the major challenge for the early diagnosis and treatment of patients with PMDs is the limited ability to discriminate between disorders at risk of progressing into OSCC and those at low-risk of such progression (Scully et al., 2003; Guillaud et al., 2008). However, currently, the most valuable prognostic indicators for the potential progression is the severity of oral epithelial dysplasia, with the most severe dysplasia at higher-risk of MT (Gupta et al., 1980; Silverman et al., 1984; Lumerman et al., 1995; van der Waal et al., 1997; Schepman et al., 1998; Reibel, 2003).
Epithelial dysplasia, however, is not always a reliable predictor in individual patient management.

Currently, there is no consensus on the most effective treatment for complete cure, or prevention of recurrence or MT (Tradati et al., 1997; Zhang et al., 2001a; Schaaij-Visser et al., 2010). Also, there have been no large trials to show an ultimate, reliable intervention (Jerjes et al., 2012) nor any meaningful randomized controlled trials, leaving the management of oral PMDs controversial (Goodson and Thomson, 2011).

Several management modalities for the treatment of oral PMDs have been suggested, which include a change of patient lifestyle factors, such as tobacco use and alcohol consumption (Silverman et al., 1984; Gupta et al., 1995), medical management with vitamin A, retinoids or antimycotics (Boisnic et al., 1994; Tradati et al., 1994; Scully, 1995), surgical excision (Sako et al., 1972; Vedtofte et al., 1987; Lumerman et al., 1995; van der Waal et al., 1997; Neville and Day, 2002), cryosurgery (Sako et al., 1972; Tal et al., 1982; Saito et al., 2001), laser vaporization (Gooris et al., 1999; Ishii et al., 2004; van der Hem et al., 2005) or laser excision (Frame, 1985; Thomson and Wylie, 2002; Ishii et al., 2004). Although there are numerous treatment regimens, no strong scientific evidence base exists.

Modification of lifestyle risk factors such as elimination of tobacco and alcohol intake and correction of local irritation are considered as essential first steps in the management of PMDs patients. However, risk factor assessment in individual patients is frequently based on subjective self-reporting of tobacco smoking or alcohol consumption. Patients may inaccurately report tobacco and alcohol intake, making both the assessment and the modification of risk factors of limited benefit which may affect the accurate identification of patients at high-risk of MT (Goodson et al., 2010).

Generally, the clinical management of OL consists of initial biopsy to assess the grade of epithelial dysplasia followed by either excision or laser treatment, or observation (Schaaij-Visser et al., 2010). Surgical intervention is predominantly recommended in the presence of moderate or severe epithelial dysplasia (Reibel, 2003). However, mild to moderate dysplastic leukoplakia may be either completely excised or left *in situ* depending on other factors that influence the treatment decision, such as site and size of OL, smoking habits and if the patient is engaged in a smoking cessation program or not (van der Waal et al., 1997; van der Waal and Axell, 2002; Reibel, 2003). Recently, Goodson and Thomson (2011) recommended the excision of mild oral epithelial dysplastic tissue at high-risk sites and in young patients mostly for those who continue to smoke or who are unlikely to attend the follow-up appointments on a regular basis.

Surgical excision seems to decrease the risk of MT by more than a half, but does not eliminate it. Hence, continuous surveillance, especially for high-grade dysplasia cases, even after surgical excision is required (Mehanna et al., 2009). Also, van der Waal (2010)

recommended excision or laser intervention of any oropharyngeal leukoplakia or erythroplakia, irrespective of the presence or absence of dysplasia. However, it is unknown whether such removal truly prevents the potential MT, thus lifelong follow-up is recommended at intervals of no more than 6 months.

Regarding non-surgical treatment of OL, randomized controlled trials have shown no evidence of effective treatment in preventing MT or lesion recurrence and this also necessitates close and regular follow-up of patients after any clinical resolution (Ribeiro et al., 2010).

Both treated and untreated patients may be followed-up every 3 months or less frequently at 6-month intervals depending on the presence of epithelial dysplasia in the biopsy (Brennan et al., 2007; van der Waal, 2009). Histological grading of epithelial dysplasia is the gold standard to predict MT, but it remains somewhat subjective and has a limited value when applied in the assessment of individual patients and lesions.

Management of PMDs is mostly dependent upon both clinical and histological features (Marley et al., 1996). However, at the present time there is no consensus on the treatment of patients with OED and the management plan may vary from initial incisional biopsy to exclude oral cancer to complete excision with an adequate margin resection (Epstein et al., 2007). Therefore, the development of an evidence-based management and surveillance strategy is urgently required.

1.10. Treatment Outcome

Reporting the treatment outcome requires standardisation of parameters as much as possible such as the length of the follow-up time and the type of study population, such as a hospital-based population or a community-based study (van der Waal and Axell, 2002; Holmstrup et al., 2006; Jaber, 2010).

The outcome after treatment is conventionally recorded at the last documented contact with patients and it is either successful in which the patient is noted to be a disease-free with complete resolution or as disease active. Disease active may be recurrence at the same site of primary disease after surgical intervention, or new disease formation at new-sites or indeed cancer development. The former is either MT of the disease at the same site of primary disease or development of cancer at new and distinct oral subsites.

1.10.1. Malignant Transformation

The clinical significance of PMDs is their unpredictable ability to transform to oral cancer. MT may be defined as the appearance of OSCC at the same site of the pre-existing PMD (Amagasa et al., 2006; Lim et al., 2010). The time to MT is the time between the onset of initial diagnosis and the progression to oral cancer confirmed by histological diagnosis (Hsue et al., 2007).

It has been suggested that two important factors should be considered to diagnose a progression of PMD to oral cancer: the same primary site cancer progression and the time to MT (Hsue et al., 2007). The same group suggested at least 6 months latency time for the transformation to be considered to exclude the possibility of concomitant presentations; however, a rapid MT of OL may occur in a shorter time.

The concept of the multistep process of oral cancer development, in which an initial precursor lesion consequently develops into oral cancer, is well-accepted. OL is the best known precursor of OSCC (Reibel, 2003), with progression from hyperplasia, through the increasing grades of dysplasia to CIS and finally OSCC the postulated pathway (Guillaud et al., 2008).

Although cases of oral cancer may derive from clinically identifiable oral PMDs with dysplasia, some cases may also arise from clinically normal appearing mucosa or progress so rapidly they escape the detection of dysplasia (Cowan et al., 2001). It remains uncertain how many OSCCs arise from precursors and how many develop from apparently normal oral mucosa (Reibel, 2003; Hsue et al., 2007). Previous studies have shown that between 16% and 62% of OSCCs are associated with or preceded by clinically detectable PMDs such as OL (Schepman et al., 1998; Lee et al., 2000; Reibel, 2003) providing a rationale for both localization and systemic causes (Lee et al., 2000).

The annual MT rate of OL is between 0.1 to 17% (Johnson et al., 1996; Schepman et al., 1998; Saito et al., 2001; Reibel, 2003; Lodi and Porter, 2008). It is believed that the differences in MT in different parts of the world are probably due to differences in ethnic, environmental factors and lifestyle risk factors, such as different tobacco and dietary habits (Gupta et al., 1989; van der Waal et al., 1997; Shiu et al., 2000; Reibel, 2003). The majority of variations in the assessments of MT rates may well be related to different inclusion criteria used in individual studies, geographical variation, habits, genetic variations and the varied follow-up times (Reibel, 2003) as well as clinical definitions that have been used in different studies (Lee et al., 2000). Therefore, future studies on oral PMDs should state in detail the clinical definition, inclusion criteria, diagnostic criteria, type of biopsy specimen, exclusion of possible etiologic factors, study population and the length of follow-up.

Follow-up studies have shown that between 0.13% and 36.4% of OLs may transform into oral cancer after a follow-up period of 1 to 11 years (Pindborg et al., 1977; Gupta et al., 1980; Silverman et al., 1984; Gupta et al., 1989; Gregg et al., 1992; Bouquot and Whitaker, 1994; Lumerman et al., 1995; Schepman et al., 1998; Shiu et al., 2000; Cowan et al., 2001; Thomson and Wylie, 2002; Reibel, 2003; Shiu and Chen, 2003; Ishii et al., 2004; Chandu and Smith, 2005; Arduino et al., 2009).

MT is thought to be time dependent and the overall risk of MT is dependent on the length of follow-up (Silverman et al., 1984), the longer the follow-up time a higher rate of MT may be detected. A retrospective study on the incidence of MT in OL performed by Lind (1987) showed that carcinoma developed in 11 out of the 157 patients but this 7% incidence increased to 8.9% when the same patients were followed prospectively for 6 more years.

In spite of different treatment modalities, the risk of MT is not completely eliminated and the number of disorders that are prevented from progressing to malignant development is unknown (Scully, 1995; Tradati et al., 1997; Lodi et al., 2002).

Many factors have been found to be associated with an increased risk of MT. These factors were reported in several previous studies, and include the presence and grade of epithelial dysplasia (Banoczy, 1977; Pindborg et al., 1977; Kramer et al., 1978a; Gupta et al., 1980; Silverman et al., 1984; Karabulut et al., 1995; Lumerman et al., 1995; Schepman et al., 1998; Lee et al., 2000). Nevertheless, some dysplastic areas may remain clinically unchanged or may be even regress completely (Gupta et al., 1980) and MT may also take place in non-dysplastic leukoplakia (Holmstrup et al., 2006).

Clinical appearance of PMDs (Mashberg and Meyers, 1976; Banoczy, 1977; Kramer et al., 1978b; Silverman et al., 1984; Lind, 1987; Gupta et al., 1989; van der Waal et al., 1997; Schepman et al., 1998; Reibel, 2003; Holmstrup et al., 2006), location at high-risk anatomical site such as tongue or the FOM (Mashberg and Meyers, 1976; Kramer et al., 1978b; Silverman et al., 1984; Gupta et al., 1989; Lesch et al., 1989; van der Waal et al., 1997; Schepman et al., 1998; Reibel, 2003) and size (Saito et al., 1999; van der Waal and Axell, 2002; Holmstrup et al., 2006) were all found to play a role in MT of PMDs.

Furthermore, female-sex, clinical extent and long standing disease, idiopathic leukoplakia (non-smokers leukoplakia), viral infection, and the presence of candida albicans have also been reported as predisposing factors (Gupta et al., 1980; Silverman et al., 1984; Gupta et al., 1989; Bouquot and Whitaker, 1994; van der Waal et al., 1997; Schepman et al., 1998; Jaber et al., 1999; Reibel, 2003; Napier and Speight, 2008; Jaber, 2010).

Factors that, statistically, carry an increased risk for MT are listed in Table 1.8.

Table 1.8: Factors predisposing to malignant transformation of oral leukoplakias.

Factors	Reference
Dysplastic leukoplakia (mostly high grade dysplasia)	(Schepman et al., 1998; van der Waal, 2009)
Persistent long standing leukoplakia	(van der Waal, 2009)
Idiopathic leukoplakia; non-smoker leukoplakia	(Napier and Speight, 2008; van der Waal, 2009)
Non-homogenous leukoplakia	(van der Waal et al., 1997; van der Waal, 2009)
Leukoplakia in high risk sites; FOM, ventral/lateral tongue and soft palate	(Zhang et al., 2001b; van der Waal, 2009)
Female sex	(Napier and Speight, 2008; van der Waal, 2009)
Leukoplakia size more than 200 mm^2	(Holmstrup et al., 2006)

MT can be explained on the basis of field cancerization or field change theory. This concept was proposed by Slaughter in 1953 based on extensive histologic examination of dysplastic epithelium adjacent to invasive oral cancer. Such dysplasia may account for the relatively high incidence of second primary tumours in patients treated for OSCC (Slaughter et al., 1953) and this has been cited to explain both the occurrence of multiple primary diseases and recurrence after complete surgical excision (Thomson and Wylie, 2002).
The concept of cancerization is supported by various clinical, histopathological and molecular evidences, in which clinically normal control epithelium was compared with adjacent tumour tissue and demonstrated similar subcellular or biochemical changes (Ogden et al., 1993; Thomson, 2002).

Carcinogenesis theories suggest that dysplastic change may occur in any area of mucous membrane exposed to a carcinogen, so patients with oral cancer and PMDs are at risk of developing multiple primary disease within the upper aerodigestive tract from the accumulation of genetic alterations of oncogenes and tumour suppressor genes (Choi and Myers, 2008). While some researchers have suggested that each tumour arises as biologically unrelated and clonally independent genetic mutations, others believe that multiple diseases arise due to widespread migration of clonally related cells (transformed cells) throughout the mucosa (Ogden et al., 1993; Jang et al., 2001; Tabor et al., 2002; Thomas et al., 2003).

The hypothesis of independent cell clones has been supported by data from microsatellite analysis, and p53 mutational analysis (Bedi et al., 1996; Lydiatt et al., 1998; Tabor et al., 2002). However, other genetic analyses have shown that second or multiple cancers even if distant from the abnormal fields can be clonally related and derived from expansion of an original clone (Braakhuis et al., 2002).
Field change can thus explain both recurrence and MT at the same site or new OED and cancer development at new oral subsites based on both clonally related and unrelated mutagenic cells.

Malignant Transformation and Clinical Appearance of PMDs

It has been shown that the clinical appearance of PMDs is an important predictor of MT (McCullough et al., 2010). Non-homogenous leukoplakia, in particular speckled and nodular subtypes showed an increase risk for MT. Studies have shown that 26% of carcinomas developed from sites of specked leukoplakia, while only 2% developed from other types of leukoplakia (Banoczy and Csiba, 1976; Banoczy, 1977). This has been supported by an earlier study conducted by Pindborg (1968) who investigated mouth carcinoma and found 64% of cases arose from areas of speckled leukoplakia. Similarly, the nodular leukoplakia subtype showed a higher incidence of MT (Lind, 1987) and a 16% MT was demonstrated in a series of nodular leukoplakias followed-up over 8 years in India (Gupta et al., 1989).

A study performed by Holmstrup *et al.* (2006) showed that 12% of surgically treated cases developed carcinoma after a mean follow-up period of 7.5 years (range 2.7–15.1 years) in which non-homogenous leukoplakia accounted for the majority compared with homogenous leukoplakias (20% vs. 3%). This finding is consistent with previous results demonstrating a higher potential for malignant development of non-homogenous leukoplakias (Banoczy, 1977; Silverman et al., 1984; Lind, 1987; Gupta et al., 1989; Schepman et al., 1998).

It is well accepted that homogenous leukoplakias carry a lower risk for MT (Axell et al., 1996); however, in a study to assess the clinical usefulness of laser surgery for OL conducted by Ishii *et al.* (2004), all cases of MT (4.12%) were seen in a flat white-spotted type leukoplakias (homogenous type), as they classified leukoplakias into flat type (white-spotted and erythroleukoplakia types) and a bulging type (hillock and verrucous type).

It is generally accepted that the non-homogeneous subtype of OLs, and those located on the tongue and FOM carry an increased risk of MT (Silverman et al., 1984; Gupta et al., 1989; van der Waal et al., 1997; Reibel, 2003).

Malignant Transformation and Site Localization

The lateral tongue and FOM, extending back to the lateral soft palate and tonsillar area combine to form a horseshoe-shaped region of the oral mucosa considered as a high-risk sites with an increased risk of cancer development compared to the remaining low-risk oral subsites (Mashberg and Meyers, 1976; Kramer et al., 1978b; Silverman et al., 1984; Gupta et al., 1989; Boffetta et al., 1992; Zhang et al., 2001b). The possible explanation for higher risk at these subsites may be due to the fact that these sites are lined by thinner non-keratinized epithelial mucosa with a higher permeability as indicated by experimental human studies on oral mucosa (Lesch et al., 1989) and less of a protective barrier against carcinogens pooled in saliva when compared to other areas of the oral cavity.

The lateral tongue and FOM are characterized by a significant proportion of dysplastic changes mainly severe dysplasia compared to the other low-risk subsites (Flynn et al., 1988; Jaber et al., 1999). A reported MT of 13.6% of tongue lesions compared to 1.8% of gingiva has been reported in a hospital-based population study conducted by Ishii *et al.* (2004).

Interestingly, it has been found that dysplasia in high-risk sites for MT, such as the FOM, ventro-lateral tongue and soft palate contain significantly higher frequencies of loss of heterozygosity at chromosomes 3p, 9p, and 17p, compared with other low-risk regions and that this pattern of loss is associated with an increased risk of progression to malignancy (Zhang et al., 2001b).
The use of these markers, together with conventional histopathological examination, may be important for accurate prediction and effective management (Scully et al., 2003).

Malignant Transformation and Risk Factors

It is well documented that tobacco and alcohol are the principal etiological factors in the development of PMDs and oral cancer. However, results of studies have shown that OL

associated with non-smokers are more likely to undergo MT than OL of smokers (Pindborg et al., 1977; Gupta et al., 1980; Silverman et al., 1984; Lind, 1987; Hogewind and van der Waal, 1988; Schepman et al., 1998; Reibel, 2003).

In a study conducted by Silverman *et al.* (1984) of 257 patients with OL, 12% from the 183 smokers developed carcinoma, whereas 32% from 74 non-smokers developed carcinoma. Schepman *et al.* (1998) investigated 166 patients with OL and found a statistically significant increased risk of transformation among female non-smokers compared to smokers. In a recent study performed by Jaber (2010), 4% of tobacco users and 10.8% of non-users transformed to malignancy after a 2-96 month follow-up period.

The reasons for a lower rate of MT of leukoplakias associated with smoking are not clear. Since tobacco as a stimulating factor is absent in non-users, other strong carcinogenic agents may exist (Lee et al., 2006). However, a more likely explanation is that the multistage carcinogenesis pathway factors affecting the development of leukoplakias may be different from those responsible for MT (Lee, 2006). Also, non-tobacco users exhibiting leukoplakias may have intrinsically more unstable mucosa due to pre-existing genetic abnormalities, resulting in higher MT rate.

However, Morse *et al.* (2007) reported that the association between tobacco smoking and dysplasia is at least as strong as the association between smoking and oral cancer, suggesting that smoking may influence oral carcinogenesis in stages prior to the MT of dysplasia to cancer.

Although the role of alcohol in MT of OL has received little attention (Banoczy and Sugar, 1972) a more recent hospital-based case-control study from Taiwan determined that alcohol intake, but not smoking, was strongly associated with the MT of OL to OSCC (Shiu and Chen, 2004). They concluded that smoking and betel quid were two significant risk factors for the occurrence of leukoplakia, whereas alcohol was significantly responsible for MT. In contrast, a study conducted in the Netherlands found no association between alcohol consumption and the MT of OL, but a statistically significant increased risk of transformation among female non-smokers compared to smokers (Schepman et al., 1998).

Malignant Transformation and Oral Epithelia Dysplasia

It is generally accepted that OEDs have a greater tendency for MT (Silverman et al., 1984) and the more severe the dysplasia the greater the risk of progression (Banoczy and Csiba, 1976; Silverman et al., 1984; van der Waal et al., 1997; Reibel, 2003). However, the grade of dysplasia alone is not a reliable predictor of prognosis (Lind, 1987), because not all cases in which dysplastic epithelium are present will progress to malignancy and some may even regress (Pindborg et al., 1977). It has been found that 6% of all OLs may transform over a 10 year period, while histologically confirmed dysplasia may transform in 16–36% of cases (Lumerman et al., 1995). They may also regress at a rate between 10-30% (Hong et al., 1986; Papadimitrakopoulou et al., 2008).

Epithelial dysplasia is an essential factor in determining the malignant potential of OLs (Hsue et al., 2007; Schaaij-Visser et al., 2010). Leukoplakias with dysplasia are more likely to progress to OSCC than those without dysplasia. Studies reported progression of OED to OSCC at rate from 6.6%-36.4% after mean follow-up period of 8.5 years (Pindborg et al., 1977; Silverman et al., 1984; Gregg et al., 1992; Lumerman et al., 1995).

It is generally accepted that the higher grade of OED is associated with higher rate of MT (Hsue et al., 2007). This is supported by a meta-analysis study conducted by Mehanna *et al*. (2009) who found that MT rate for mild to moderate dysplasia was 10.3% compared with 24.1% for severe dysplasia and CIS. According to Lee *et al.* (2000), the risk in moderate or severe dysplasia appears to be at least double (2.3 times) that seen in mild dysplasia or hyperplasia.

The current gold standard of histopathology is reasonably efficient in identifying malignant risk for severe dysplasia and CIS, of which 30 to 40% may undergo recurrence or MT even with aggressive surgical treatment (Vedtofte et al., 1987; Thomson and Wylie, 2002). Furthermore, a hospital-based study conducted by Holmstrup *et al.* (2006) showed that CIS was associated with the highest percentage of MT after surgical intervention (33%) compared to 9% in moderate or severe and 11% in mild dysplasia.

A further study conducted by Kujan *et al.* (2006) demonstrated that high-risk grades of OED tend to have higher rates of MT compared to low-risk OED grades; 84.2% of cases with either severe dysplasia or CIS developed an OSCC, while 15.8% of cases diagnosed with hyperplasia or mild dysplasia showed MT.

According to Guillaud *et al.* (2008), however, the gold standard histopathology may be considered as a poor predictor for the majority of PMDs, particularly hyperplasia or mild-moderate dysplasia in which most do not progress to cancer. Some authors do not attach much significance to mild dysplastic changes (Cruz et al., 1998; Lee et al., 2000); however, mild epithelial dysplasia demonstrated MT rates similar to severe dysplasia in a study conducted by Holmstrup *et al.* (2006) and this may challenge previous assumptions that mild dysplasias can be considered as harmless.

Moderate or severe dysplasia and CIS are accepted to have the highest potential for MT, although, progression to cancer is not predictable (Banoczy and Sugar, 1972; Gupta et al., 1980). This is discordant with the results reported by Holmstrup *et al.* (2006) who did not find a significant relationship between epithelial dysplasia and progression to oral cancer in a cohort of 269 leukoplakias. Similarly, Arduino *et al.* (2009) reported that histological grading had no significant value in predicting malignant development; OSCC may arise without a preceding dysplasia (Schoelch et al., 1999) and no significant tendency to increased MT with increased degree of dysplasia was seen. According to Holmstrup *et al.* (2006) the absence of accurate correlation between histological features of biopsy and the clinical outcome could be related to unrepresentative features of incisional biopsy specimens.

Furthermore, MT rates by grade of dysplasia are difficult to establish (Brennan et al., 2007), because of the inherent subjectivity of the histopathological grading system which is a disadvantage of using existing histological criteria of dysplasia to predict transformation.
Several studies have revealed low-to-moderate inter-observer agreement for the grade of dysplasia diagnosis amongst experienced oral pathologists (Abbey et al., 1995; Karabulut et al., 1995; Brothwell et al., 2003). In a study conducted by Hsue *et al.* (2007), only eight of the 166 patients with OED (4.82%) underwent MT, and none of the severe dysplasias transformed during the follow-up study.

A study with CO_2 laser surgery reported only 1% developing cancer over a mean one year period (van der Hem et al., 2005). The low figure for MT as compared with a similar study in an Irish population which quoted a 14% MT (Cowan et al., 2001) is mainly due to the short follow-up time (1 year) and a higher percentage of low grade dysplasia (83.13%) in the first study which is believed to be at low-risk of MT.

From these data, one can understand that oral carcinogenesis is multifactorial and this may cause treatment failure. In spite of complete removal of clinically evident disease, the altered field may remain and the patient can develop recurrence, second primary disease or undergo MT.

1.10.2. Recurrence

Local recurrence is defined as the reappearance of PMD in the same site of the primary disease which was previously treated and which is confirmed by follow-up biopsy (Lim et al., 2010).

After surgical excision, recurrences are not uncommon irrespective of the time span between the excision and the recurrence event (van der Waal and Axell, 2002). Although there is some difficulty in comparing recurrence rates in different studies, the rate of recurrence is found to vary significantly between different types of surgical treatment, with the highest rate of recurrence associated with cryosurgery 71.4%, followed by laser vaporization 30.1%, excision surgery 25% and laser excision 22.2% (Ishii et al., 2004).

According to Frame (1985), different surgical techniques, patient factors, and different patient follow-up periods may all be responsible for different recurrence rates. Previous reported recurrence rates for OL in laser surgery range from 7.7 to 38.1% (Frame et al., 1984; Vedtofte et al., 1987; Chu et al., 1988; Chiesa et al., 1990; Roodenburg et al., 1991; Chiesa et al., 1993; Schoelch et al., 1999; Thomson and Wylie, 2002; Ishii et al., 2003). This wide range variation of recurrence rates may be attributed to differences in the variety and conditions of the laser surgery, the follow-up time, geographical and racial variations (Ishii et al., 2004).

In one of the earliest studies of the CO_2 laser being used to treat leukoplakia by ablation and excision, Frame *et al.* (1984) reported recurrent or new lesions in 13.6% of cases after 10 months. Reported recurrence rates for PMDs were as high as 34.4% in a study conducted by Silverman *et al.* (1984). Also, one study found an 18% recurrence rate in cases of severe dysplasia or CIS which were all excised with 3-5 mm margins of normal tissue (Vedtofte et al., 1987). Chu *et al.* (1988) reported an initial recurrence rate of 10.8% with a 3-year local control rate of 97% after one to two laser excisions. A later study with CO_2 laser surgery reported good prophylaxis with 10% local recurrence and 1% developing cancer over a mean one year follow-up period (van der Hem et al., 2005).

It has been reported previously by Chiesa *et al.* (1990) that the recurrence rate of leukoplakia increases over time. In a study performed by Chandu and Smith (2005) the mean time to first recurrence/new lesion was 19.1 months, while the mean time to second recurrence was 22.5 months. The median time to recurrence in a study conducted by Guntinas-Lichius *et al.* (2010) was 27 months (range 15–56 months). According to Thomson *et al.* (2008), most episodes of further disease occurred during the first 18 months following treatment.

Removing all local diseased tissue and establishing clear resection margins is the ultimate aim of surgery. This may facilitate disease free recurrence and improve patient survival rates. Regarding resection margins, these are highly dependent on the biological and anatomical situation of the affected region and determined by both functional and aesthetic factors. However, there is no accepted standard for the amount of normal tissue to be removed and the effect of positive margins on local recurrence rates appears to be considerably dependent on the site of the disease.

In spite of complete removal or laser vaporization, adjacent or peripheral dysplastic epithelial tissue may proliferate as a recurrence phenomenon, and such epithelial tissues which may show clinically normal features but consist of highly active cells are probably abundant within the oral mucosa of PMD patients (Ishii et al., 2004). Also, Frame *et al.* (1984) hypothesized that recurrence of leukoplakia might be due to migration of new epithelium from surrounding unstable oral mucosa.

A different explanation of recurrence has been proposed that deep nests of dysplasia may be left *in situ* after any surgical treatment (Lim et al., 2010) and it is therefore important to remove the full thickness of epithelium during ablative laser surgery (Cantarelli Morosolli et al., 2006).

The recurrence of leukoplakia could also be related to the concept of field change or cancerization (Lim et al., 2010) in which the oral epithelial tissue within the area of field change could appear clinically normal. Thus, in such patients, recurrence may be unavoidable, regardless of the type of treatment because all surgical margins might be within an area of field change (Thomson and Wylie, 2002; Chandu and Smith, 2005).

Moreover, the risk of recurrence may be also related to continue exposure to risk factors after treatment (Arduino et al., 2009) and reducing the alcohol intake and cessation of smoking could have the potential to reduce the incidence of recurrence (Poate and Warnakulasuriya, 2006). However, it is estimated that it may take 10-15 years before the risk lessens significantly (Jaber et al., 1999).

Whether recurrence is primarily related to continued exposure to risk factors or to the underlying mechanism that initiated the disease remains unclear, but all patients should be closely monitored for recurrence regardless (Jaber, 2010).

1.11. Follow-up of PMD Patients

Since oral PMDs have a definite but unpredictable tendency for transformation to oral cancer, close and regular follow-up of PMD patients, both treated and untreated cases, is warranted irrespective of clinical or histological characterisations. To date, there are no available universally accepted guidelines in the literature with regard to the intervals and frequency of follow-up examinations (van der Waal and Axell, 2002; Weijers et al., 2008) with follow-up strategies varying from immediate discharge to life-time recall (Epstein et al., 2007). Also, the true advantage of such follow-up appointments remains unproven (Weijers et al., 2008).

Follow-up time is measured from the date of diagnosis to the most recent follow-up or last contact with patients. It is generally accepted that the length and the duration of follow-up for PMD patients is greatly dependent upon both the clinical and histopathology features.

In general, life-long follow-up examination is advised at 6–12 months intervals for both treated and untreated patients; although occasionally a non-treated patient with a dysplastic leukoplakia may need follow-up visits at 3-month intervals (van der Waal and Axell, 2002; Jaber, 2010). The recommended shorter follow-up interval for patients with non-treated dysplastic lesions is mainly due to the potential risk for MT as well as the possibility of developing secondary dysplasia from field change phenomenon.

Different follow-up schedules have been reported by many research groups; Holmstrup *et al.* (2006) reported a follow-up schedule after the immediate postoperative period every 3 months for the first 2 years and thereafter every 6 months during the following years and in case of recurrence, new biopsies were taken. In a study conducted by Arduino *et al.* (2009), patients were followed-up with varying intervals at 3, 6 or 8-months based upon clinical or histopathological features unless death of the patient occurred; new biopsies were taken in case of recurrence or any oncological event.

According to Arduino *et al.* (2009), patients suffering from speckled leukoplakia and/or severe dysplasia were seen every 3 months, whereas those with homogeneous leukoplakia and/or mild-moderate dysplasia every 6 months. Li *et al.* (2011) followed patients for 5 years reviewing postoperatively every month for the first year, every two months during the second year, four-monthly during the third and fourth years and six-monthly thereafter.

Longer follow-up time for patients with PMDs is recommended due to their potential MT and possibility of further disease events (Merkx et al., 2005). This is supported by a study conducted by Chandu and Smith (2005) who observed a fall in disease-free survival after 5 years compared with 3 years post-interventions (33.9% vs. 55.4%). This gives a strong indication that disease active state such as recurrence, new disease or MT may occur 3 years after intervention.

Clearly, the main objective of follow-up is the early detection of recurrence, new disease formation or cancer development to offer patients a rapid, second curative therapy (Merkx et al., 2006). Also, follow-up can provide important information about the efficacy and the scientific value of treatment regimens for the benefit of patients in the future. In addition, psychological support of patients with functional or cosmetic problems as a result of destructive treatments may be facilitated (Merkx et al., 2006).

The effective clinical surveillance of patients with PMDs requires careful oral examination which may be supported by clinical aids and recording of the clinical appearance and size of lesions with photography. However, the duration of follow-up and the frequency of routine visits after treatment are based on anecdotal regions rather than evidence-based practice.

1.12. High risk / Low Risk Patients

It is well accepted that higher-grade OEDs including moderate, severe dysplasia, and CIS have a greater chance of progression to OSCC (Neville and Day, 2002; Rosin et al., 2007), whereas mild dysplasia is a low-risk grade, with a lower risk of progress to cancer. The significance of dysplasia phenotypes as cancer risk prognosticators is well documented (Guillaud et al., 2008) and the strong association of marked dysplasia with increased risk of cancer progression has been observed and confirmed in multiple body sites such as lung, esophagus, breast, cervix, and skin; that is why histopathologic diagnosis of dysplasia is the current gold standard.

PMDs in certain anatomic subsites such as the FOM, ventral and lateral tongue, soft palate and tonsillar pillar have been associated with a greater risk of cancer development (Mashberg and Meyers, 1976; Boffetta et al., 1992; Zhang et al., 2001b) and these locations were classified as high-risk sites whilst the remaining subsites were classified as low-risk (Jayaprakash et al., 2009). A greater proportion of high grade dysplasia was seen on the high-risk anatomical sites (65%) compared with low-risk ones (35%) (Jayaprakash et al., 2009).
In addition to dysplasia grade and localization of PMDs, lifestyle risk factors are also important parameters in the classification of the patient at high-risk or low-risk.

Assessment of the risk factors in PMDs patients may help to identify patients at higher risk of unfavourable clinical outcomes who require more extended care and surveillance. Also, stratification of patients may facilitate treatment planning for each group of patients which may subsequently minimize treatment failure by considering their demography, clinicopathological features and pre-existing or associated risk factors.

Tobacco smoking and alcohol consumption are the two major confirmed risk factors for PMDs. However, according to Ho *et al*. (2007) not all individuals who smoke or drink develop OSCC; individual genetic susceptibility, differences in carcinogen-metabolizing enzyme function, mutagen sensitivity, apoptosis, and chromosomal aberrations either alone or in combination have been hypothesized to modify the risk of OSCC.
Nearly all carcinogens and pro-carcinogens require activation by metabolizing enzymes. Similarly, detoxifying enzymes exist and deactivate carcinogens as well as their intermediate by-products; together these enzymes are termed xenobiotic-metabolizing enzymes. Genetic polymorphisms of these enzymes can modify an individual's response to carcinogens and hence the carcinogenic potential of such exposures (Ho et al., 2007).

One can understand that, patients with PMDs may be classified as either high-risk or low-risk based on the severity, location of dysplasia, the associated risk factors and individual genetic susceptibility. Clinical staging or classification of patients may allow clinicians to design treatment plans, compare results, and assess the likelihood of treatment success or determine the progress of individual disease.

High-risk dysplasia in a high risk individual may be excised with wider surgical margins, while for patients with low-risk dysplasia observational treatment, perhaps with less frequent follow-up would suffice (Schaaij-Visser et al., 2010). The main challenge for early diagnosis of high-risk tissue is the limited ability to differentiate those at risk of progressing into invasive OSCC from those at low-risk (Guillaud et al., 2008). It is as so important to remember that some mild dysplasia cases may transform to oral cancer.

The results of a randomized trial recommended periodic screening with regular surveillance of high-risk patient populations for early identification of PMDs and detection of the early

symptoms of malignancy (Sankaranarayanan et al., 2005). However, early malignant changes are often subtle with late clinical presentation of established tumours remains the norm.

There is thus a real need for the development of a tool with a complementary diagnostic ability, objective, quantitative, and sensitive enough to differentiate between oral tissues at high tendency to progress to malignancy from non-progressing, low-risk ones.

Chapter Two: Study Aims, Objectives and Hypotheses

2.1. Study Aims

The aims of this study were:

1. To determine the socio-demographic and clinicopathological features of a cohort of patients with a single potentially malignant disorder (PMD), diagnosed and treated in the North-East of England in the Oncology/Dysplasia clinic in the Maxillofacial Unit at Newcastle General Hospital.

2. To investigate the risk factors that influence the development and progression of PMDs, that may affect patient treatment outcomes.

3. To determine the outcome of laser treated oral epithelial dysplasia and to identify the significant risk factors for recurrence, development of new-site dysplasia and cancer development of a long term follow-up cohort of patients with oral dysplastic PMDs.

4. To investigate the suitability of the Raman spectroscopy (RS) for distinguishing dysplastic PMDs from morphologically normal tissue and whether RS is able to identify dysplastic tissue changes at early stages.

2.2. Study Objectives

1. To build-up a database of clinical outcomes and histopathological features for patients treated in the North-East of England for PMDs of oral mucosa, using a retrospective patient record.

2. Using archived tissue specimens, assess whether RS is an objective diagnostic tool by trying to detect changes in biochemical tissue structure of diseased tissues and comparing these changes with morphologically normal oral tissue, with the objective of trying to find biochemical tissue markers for early diagnosis of PMDs.

2.3. Study Hypotheses

The null hypotheses behind the present study were:

1. Analysis of clinicopathological findings from PMDs does not aid in the development of "high-risk"/"low-risk" classification systems to predict clinical outcome and direct clinical management protocols.

2. The clinical outcome following PMD treatment is independent of patient demography, PMD clinical type, size, site, grade of oral epithelial dysplasia and risk factor assessment.

3. Residual dysplasia found at resection margins of excisional specimens does not affect clinical outcome following laser excision of individual PMDs.

4. Raman spectroscopy is unable to discriminate morphologically normal tissue from dysplastic tissue.

5. Raman spectroscopy is unable to identify biochemical tissue changes associated with early dysplastic changes seen in PMDs.

Chapter Three: Potentially Malignant Disorders (PMDs): Demographic, clinical and histopathological features of a cohort of 100 patients, who were diagnosed, treated and reviewed at Newcastle General Hospital

Study 1

3.1. Introduction

Oral PMDs are mainly leukoplakias, erythroplakias, erythroleukoplakia, submucous fibrosis, lichen planus and actinic cheilitis as well as inherited cancer disorders (Napier and Speight, 2008; Rapidis et al., 2009; van der Waal, 2009). However, leukoplakia is the most common oral PMD (Gupta et al., 1980; Axell et al., 1996; Pindborg et al., 1997; van der Waal et al., 1997; Lee et al., 2006). PMDs convey that not all the disorders will transform to cancer and reflect a widespread anatomical distribution (Warnakulasuriya et al., 2007). PMD is able to correct the misunderstanding that the disease is associated with either a lesion or a condition; lesions imply cancer is more likely to occur in the same site, whilst conditions correspond to cancer development in any anatomical site (Warnakulasuriya et al., 2007). Moreover, it is unknown precisely which lesions or conditions will undergo MT; therefore, PMD is the best clinical term to describe a group of disorders of morphologically altered tissues that have a higher tendency for MT than apparently normal tissue (Speight and Morgan, 1993; Axell et al., 1996; Warnakulasuriya et al., 2007; van der Waal, 2009).

PMDs may exhibit variable degrees of epithelial dysplasia, which is the most important feature of PMDs (Reibel, 2003). Histologically, it is characterised by cellular atypia and loss of normal maturation and stratification (Pindborg et al., 1997; Gale et al., 2005). The diagnosis and grading of oral epithelial dysplasia is based on a combination of architectural and cytological criteria, but the evaluation of these features is subjective and depends on the pathologist's experience. Thus, there is considerable inter-and intra-observer variations in grading (Abbey et al., 1995; Kujan et al., 2007) with Kappa values showing low to moderate agreement among even experienced oral pathologists (Pindborg et al., 1985; Abbey et al., 1995; Karabulut et al., 1995; Brothwell et al., 2003; Kujan et al., 2007; Arduino et al., 2009).

At present, epithelial dysplasia is considered the most important predictor of MT in PMDs (Lumerman et al., 1995; Cowan et al., 2001; Reibel, 2003; Brennan et al., 2007); however, accurate prediction of clinical outcome for an individual patient is not possible (Napier and Speight, 2008), because not all dysplasia will undergo transformation to malignancy and some may be even regress or disappear. Also, malignancy may develop from non-dysplastic tissue (Pindborg et al., 1977; Silverman et al., 1984; Schepman et al., 1998;

Warnakulasuriya et al., 2008). The presence of dysplasia in oral epithelial tissue is believed to be associated with an expected progression to malignancy and there is a widely accepted concept that the more severe the dysplasia the greater the tendency for MT (Reibel, 2003). This may be related to the accumulation of chromosomal, genomic and molecular alterations within the affected tissues (Warnakulasuriya et al., 2008) associated with exposure to risk factors.

Thus, understanding of the process of carcinogenesis and the associated risk factors is of great benefit to identify high-risk PMDs (Lee et al., 2006) and also to identify patients at increased risk. The carcinogenesis process may be initiated by carcinogens from lifestyle habits. The major risk factors of oral PMDs are generally believed to correspond to those of OSCC such as tobacco and alcohol use (Johnson et al., 1996; van der Waal et al., 1997; Lodi et al., 2002; Neville and Day, 2002).

Tobacco smoking and excessive alcohol consumption are the major risk factors for OL, epithelial dysplasia and OSCC (Blot et al., 1988; Jaber et al., 1999). Approximately 75% of all oral cancers arise in association with tobacco and alcohol use (Llewellyn et al., 2003; La Vecchia et al., 2004) and OLs have been shown to occur up to six times more frequently in smokers than in non-smokers (Hogewind and van der Waal, 1988; Franceschi et al., 1992) in a dose-response relationship; increased risk is associated with an increased number of cigarettes smoked per day in leukoplakia (Roed-Petersen, 1982; Gupta, 1984; Evstifeeva and Zaridze, 1992) and epithelial dysplasia (Kulasegaram et al., 1995; Morse et al., 1996; Jaber et al., 1998; Jaber et al., 1999). Approximately 60% of OLs may disappear if patients stop smoking (Banoczy and Rigo, 1991), whilst continued exposure to risk factors may result in persistent disease (Arduino et al., 2009) and the possibility of developing carcinoma which is 50 to 100 times greater in smokers than in the general population (Cawson, 1975).

The intention of clinical management is early diagnosis and treatment of PMDs which is crucial to prevent MT or recurrence after removal (Dietrich et al., 2004). In addition, it allows effective, less aggressive treatment with lower morbidity and reduced cost (Holmstrup et al., 2006; Epstein et al., 2007) as well as better prognosis and better quality of life (Warnakulasuriya et al., 2008).

Although various treatments modalities have been developed, currently, there is no consensus on the most successful and reliable one (Tradati et al., 1997; Zhang et al., 2001a; Jerjes et al., 2012) and treatment remains controversial, polarized between surgical removal or medical treatment with clinical observation. These modalities include change in patient lifestyle factors, such as tobacco use and alcohol consumption (Silverman et al., 1984; Gupta et al., 1995), medication with retinoids or antifungal agents (Boisnic et al., 1994; Tradati et al., 1994; Scully, 1995), surgical excision (Sako et al., 1972; Vedtofte et al., 1987; Lumerman et al., 1995), cryosurgery (Sako et al., 1972; Tal et al., 1982; Saito et al., 2001), laser vaporisation (Gooris et al., 1999; Ishii et al., 2004; van der Hem et al., 2005) or laser excision (Frame, 1985; Thomson and Wylie, 2002; Ishii et al., 2004). However, the outcome of these interventions appears to vary and long-term follow-up studies are warranted (Holmstrup et al., 2006).

After surgical intervention, recurrences and development of cancer in the same sites have been previously reported in 10-20% and 2-9%, respectively (Vedtofte et al., 1987; Chiesa et al., 1993; Schoelch et al., 1999); however, no randomized clinical trials have been reported (Lodi et al., 2004). Identification of PMDs with high risk of recurrence or MT rate is essential in rationalisation of management of PMDs (Thomson et al., 2008).
However, development of new disease at new sites remains a major problem within oral epithelium and is consistent with the field cancerization theory first described by Slaughter *et al.* (1953). The development of multiple disease sites due to carcinogenic exposure can cause simultaneous genetic defects in the upper aerodigestive tract epithelium putting the whole epithelium at high risk for multicentric disease which may develop synchronously or metachronously, resulting in complicated clinical management situations (Thomson and Wylie, 2002; Thomas et al., 2003; Thomson et al., 2006).

This study investigates the demographic, clinical and histopathological features of a cohort of 100 patients with a single PMD with known clinical outcomes and was carried out to assess which patients are at high risk of further disease or MT.

3.2. Aim

The aim of this study was to develop a new method to assess prognostic factors for patients with oral PMDs in the North-East of England after long term follow-up of laser intervention based upon demographical, clinical and histopathological features and utilizing known clinical outcome data.

3.3. Methods

3.3.1. Study Design

A retrospective cohort study was conducted after obtaining ethical approval from County Durham &Tees Valley 1 Research Ethics Committee on the 9th of July 2009 (Appendix 1-A). Patients with PMDs (leukoplakia, erythroplakia and erythroleukoplakia) and a histological confirmed diagnosis of oral epithelial dysplasia (OED) attending the Oral Oncology/Dysplasia clinics at the Maxillofacial Unit at Newcastle General Hospital were invited to take part in this study. After receiving a patient information sheet (Appendix 1-B) and explaining the study aims, patients signed a consent form (Appendix 1-C) and were enrolled into the study. Patients consented to use their medical records along with biopsy specimens previously used for diagnosis and routinely stored in the Pathology Department at the Royal Victoria Infirmary (RVI). Also, letters have been sent to general practitioners of patients who participated in this study (Appendix 1-D).

All patients in this study underwent a standardised treatment plan by the same clinical team led by Professor Peter Thomson. Patients received an initial clinical oral examination, education about risk factor behaviour; an explanation of the role of MT of unstable mouth lining and all patients underwent laser interventional surgery to excise PMD lesions.

Patients were followed-up post-laser intervention at Newcastle General Hospital, by regular appointments every month, 2 months, 3 months, 4 months, 6 months or every year depending on the need of each individual case.

In this study, all patients at their initial examination and during follow up visits were provided risk factor advice, for example they were encouraged to stop tobacco smoking and reduce alcohol consumption. Candida infections identified by the presence of hyphae or pseudohyphae in periodic acid-Schiff-stained histological sections were treated with antifungal therapy.

3.3.2. Study Criteria

Clinicopathological data were collected from both medical records and pathological reports of patients with PMDs treated and followed-up in the Oral Oncology/Dysplasia clinics at Newcastle General Hospital.

The clinical diagnosis of the PMDs was based on the criteria adopted by the WHO and provided by Pindborg *et al.* (1997); van der Waal and Axell (2002). The histopathological diagnosis of oral epithelial dysplasia of laser excised tissue specimens was made using two classification systems: the WHO system (Gale et al., 2005):

- Mild dysplasia, in which architectural disturbances are limited to the lower third of the epithelium accompanied with cytological atypia.
- Moderate dysplasia, where the architectural disturbance extending into the middle third of the epithelium with a considerable degree of cytological atypia.
- Severe dysplasia, in which architectural disturbance starts with greater than two-thirds of the epithelium associated with significant cytological atypia.
- Carcinoma *in situ,* which comprised full or almost full epithelial thickness architectural abnormalities in the cellular layers accompanied with marked cytological atypia.

And the binary grading system, which comprised the categories of high and low grade dysplasia (Kujan et al., 2006).

Primarily, pathological slides of the selected cases were collected from the pathological archive, reviewed and suitable paraffin blocks were selected. In the pathology department of the RVI, 10-micrometer-thick tissue sections were cut from the formalin fixed paraffin embedded tissue samples using a microtome, mounted onto a glass slide and stained with haematoxylin and eosin (H&E). The histopathological verification of the stained tissue sections were independently assessed by two experienced oral pathologists and then a consensus diagnosis of the grade of epithelial dysplasia was assigned and used in this study.

Selection of Patients

100 patients with PMDs of the oral mucosa were recruited for the current study.

Inclusion Criteria

1) Patients with single oral PMD: leukoplakia, erythroplakia or erythroleukoplakia.
2) Histopathologically confirmed OED.
3) First presentation of oral PMD.
4) No previous treatment for oral PMD.
5) Known clinical outcome.

Exclusion Criteria

1) Patients with multiple PMDs.
2) Patients with non-dysplastic lesions.
3) Patients who did not undergo laser surgery.
4) Previously treated patients.
5) Patients with oral cancer.
6) Patients who had previously undergone head and neck surgery, radiotherapy or chemotherapy.
7) Patients who could not consent for themselves or patients with special communication needs or difficulties in understanding verbal or written information.

3.3.3. Data Collection

Demographical, clinical and histopathological data were collected at various time points starting from the first presentation as a baseline, during treatment, and at each follow-up point.

The data collection, retention and management were all carried out according to Data Protection Act 1998 and Caldicott Principles. Data were stored in a university-based computer secured with password access.

For each patient, data were recorded in a standardised case sheet (Appendix 1-E) which included the following variables:

1. Patient's study number
2. Age
3. Sex
4. Occupation; classified according to the International Standard Classification of Occupations (ISCO-08); Appendix (1-F).
5. Civil status (married, single, divorced and widowed).
6. Patient medical history.
    - *Immunodeficiency*
    - *Diabetes mellitus*
    - *Anaemia*
    - *Candida infection*
    - *Hypertension*
    - *Familial cancer history*
    - *Other medical problems*
7. Clinical types of PMDs (Pindborg et al., 1997).
    - *Leukoplakia*
        - Homogenous leukoplakia
        - Non- Homogenous leukoplakia
            - Speckled
            - Nodular
            - Exophytic
            - Ulcerated
    - *Erythroplakia*
8. Size of PMD in (mm^2) (length x width) depending on excision biopsy report.
9. Anatomical location of the PMDs according to the topographical code (ICD-10).
    1) *Floor of the mouth (C04.0, C04.1)*
    2) *Tongue (C02.0, C02.1, C02.2)*
        - Ventral tongue
        - Lateral tongue
        - Dorsal tongue
    3) *Buccal (cheek) mucosa(C06.0)*
    4) *Soft palate (C05.1)*
    5) *Fauces (C09.1)*
    6) *Hard palate(C05.0)*
    7) *Retromolar area (C06.2)*
    8) *Gum(C03.0, C03.9)*
    9) *Alveolar mucosa*
    10) *Lips (C00.3, C00.4, C00.5, C006)*
        - Lower lip
        - Upper Lip
10. Risk factors assessment
    1) *Tobacco smoking*
        - Non-smoker

        (If the patient never smoked tobacco)

- Ex-smoker
  (If the patient stopped smoking for at least 1 year before initial presentation)
    - Length of smoking history
    - Number of cigarettes smoked per day
- Current smoker (Regular smoker)
    - Length of smoking history
    - Number of cigarettes smoked per day

2) Alcohol consumption

- Non-drinker
  (Never drink alcohol)
- Ex-drinker
  (If the patient stopped drinking for at least 1 year before initial presentation)
    - Length of drinking history
    - Number of units per week
- Current drinker (Regular drinker)
    - Length of drinking history
    - Number of units per week

3) Type of diet

- Processed, prepared food
- Fresh fruit and vegetables.

4) Oral hygiene

- Good
- Bad

5) Mouth wash use

- User
- Non-user

6) Oral prosthesis use

- None
- Full denture
- Crowns and bridges
- Upper or lower denture

11. Initial diagnostic biopsy (incisional biopsy)
    - Pathology reference number
    - Time of biopsy
    - Histopathological diagnosis
      WHO classification system (2005)
        - Mild dysplasia (disorganisation affecting the lower third of the epithelium)
        - Moderate dysplasia (disorganisation affecting up to two- third)
        - Severe dysplasia (disorganisation affecting greater than two- third)
        - Carcinoma in situ (disorganisation affecting the whole epithelial thickness)
12. Observational biopsy (incisional biopsy before any laser excision)
    - Number of biopsies carried out and pathology reference number
    - Date
    - Histopathological diagnosis
13. Laser excision specimen
    - Pathology reference number
    - Date of laser surgery

- Histopathological diagnosis

14. Surgical margin status
    - Free margins
    - Dysplastic margins
        - Mild dysplasia
        - Moderate dysplasia
        - Severe dysplasia
        - Carcinoma *in situ*
15. Follow-up biopsies (incisional biopsies post-laser treatment for patient monitoring)
    - Number of biopsies carried out and pathology reference number
    - Date
    - Histopathological diagnosis
16. Treatment after recurrence
    - Further laser intervention
    - Observation
17. Clinical outcome
    - *Clinical resolution (Disease-free)*
    - *Persistent (same site, same disorder*)
    - *Recurrence after treatment (same site of primary disease)*
        - Time of recurrence
        - Number of recurrences
    - *Development of new disease (new-site)*
        - Time
        - New site
    - *Malignant transformation (same site OSCC)*
        - Time
    - *OSCC development (new-site, new disease)*
        - *Time*
        - location
    - *Malignant events outside the oral cavity*
        - Time
        - Location
18. Follow-up after laser intervention
        - Length of follow-up
        - Frequency

3.3.4. Statistical Analysis

Statistical analysis was performed with SPSS (Statistical Package for Social Sciences) version 17.0 and 19.0 software package (SPSS Inc; Chicago, IL, USA). Descriptive statistical analysis, the Chi-Square, Fisher's Exact, Independent Paired Student T-test, analysis of variance (ANOVA) and bivariate correlation were used to compare the variables and find correlations. Non-parametric tests included the Mann-Whitney U and Kruskal-Wills tests were also used when the data were not assuming a normal distribution. In addition, Kappa statistics to assess inter-observer variability in histopathological diagnosis and Kaplan-Meier analysis for cumulative survival were used. A *p*-value of less than 0.05 was considered to indicate statistical significance.

3.4. Results

A total of 157 medical records were reviewed in this study. However, according to study inclusion criteria, 57 patients were excluded due to the presence of multiple PMDs (n=20), development of oral cancer, or having undergone head and neck surgery, radiotherapy or chemotherapy (n=24), incomplete clinical outcome data (n=3) or lack of dysplasia in pathological reports (n=9) and one observational treatment case, leaving 100 patients available for the clinicopathological dataset analysis.

100 patients with single dysplastic PMD were thus enrolled in this study. The age at first presentation ranged from 30-94 years with an overall mean age of 57.72 years (SD: 12.89). The cohort comprised mostly men, with a male to female ratio of 2: 1. The majority of our study population were retired followed by unemployed, while the remaining was classified according to the International Standard Classification of Occupations (ISCO-08); Appendix (1-F).

Married patients formed the main civil group (62%), followed by single (19%), divorced (10%) and widowed (9%).
The majority of the study population were current smokers (63%) followed by ex-smokers (22%) and non-smokers (15%). The majority of patients in this study were current drinkers (83%), followed by non-drinkers (14%) with the remaining 3% ex-drinkers.

In relation to patients' medical histories in our study population, hypertension occurred in 60% followed by diabetes mellitus 14%, Candida infection 8%, anaemia 4% and immunodeficiency 1%. Also, a variety of other chronic medical conditions were observed in 70% of our PMD patients, such as cardiovascular (28%), respiratory (19%), musculoskeletal (18%), liver (15%), psychological disorders (10%), nervous system disorders (8%), digestive system (7%), endocrine (6%), skin (4%) and renal related problems (3%) which is accounted for the least frequent conditions.

The FOM was the most commonly affected anatomical site (46) followed by lateral (19) and ventral surfaces of the tongue (14), the soft palate (9), buccal mucosa (5), fauces (4), alveolar mucosa (2) and retromolar areas (1).

Clinically, 92% of PMDs presented as leukoplakia, while only 8% manifested as erythroplakia. Homogenous leukoplakia was the main clinical type of PMDs, followed by non-homogenous leukoplakia in which the speckled subtype was the most common.

Histologically, in both incisional and excisional biopsies, the majority of PMDs were diagnosed as mild dysplasia (37%, 43%), followed by moderate (26%, 24%), severe (19%, 10%) and CIS (18%, 10%). Males were more likely to have higher dysplastic features in their PMDs compared with females (55% vs. 48%).

PMDs sizes were classified into 3 categories: minor (< 200 mm^2), intermediate (200-600 mm^2) and major sizes (> 600 mm^2); intermediate (45/97) and minor sized (42/97) were the most common, followed by major size group (10/97).

3.4.1. Sex and Age

Men were the most common in the study population. There were 66 males with a mean age of 56.71 years (SD: 12.01), age range 30-81 years and 34 females with a mean of 59.68 years (SD: 14.45) and age range 33-94 years.

Females tended to be older than male patients at initial presentation, however the difference was not statistically significant (p=0.279; Independent t-test).

Patients were classified into 4 age categories: young, middle aged, old and elderly; (≤ 40), (41-62), (63-84) and (> 85) years, respectively; Figure 3.1. The majority of patients were found within the middle age group 56% (41 males and 15 females), followed by old 33% (19 males and 14 females), young 10% (6 males, 4 females) and only one female was observed in the elderly age group. In this study, 90% of the PMD patients were older than 40 years.

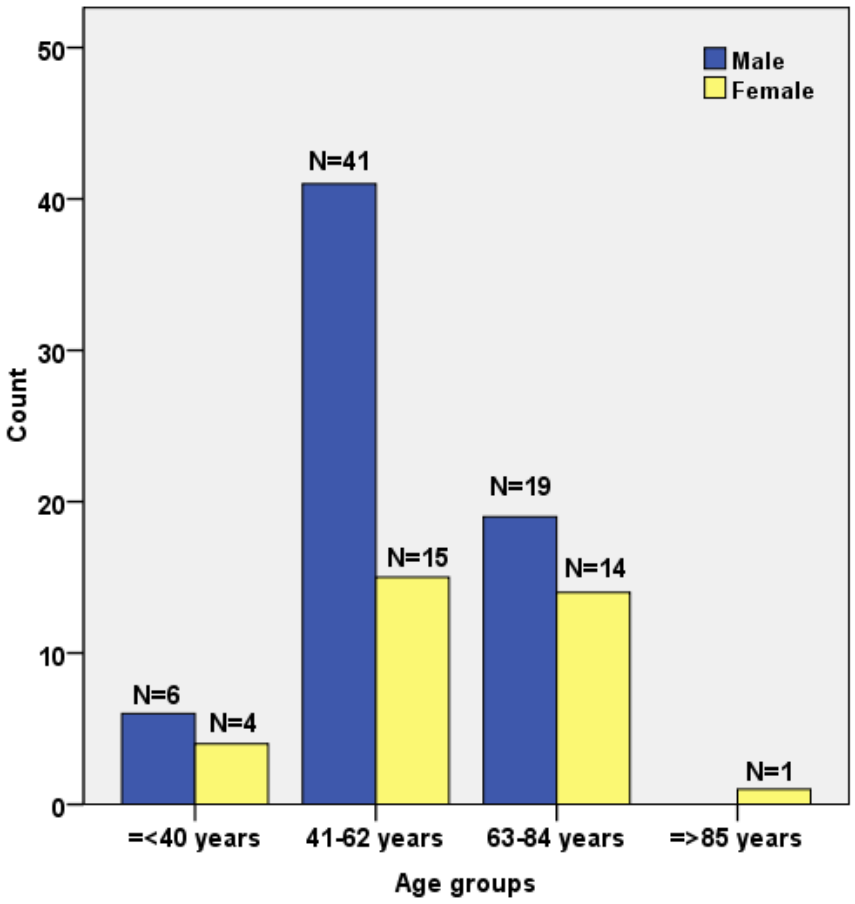

Figure 3.1: Sex distribution according to age group.

3.4.2. Occupation and Marital Status of PMD Patients

Data showed that out of 100 patients, 41 were retired, followed by 25 unemployed, while the remaining 34 of patients were classified according to the International Standard Classification of Occupations (ISCO-08). Service and sale workers represented 13, followed by craft and related trades workers (5); Figure 3.2.

Males were the most common in retired, unemployed, service and sale workers, whilst no females were seen in craft and related trades, machine operators, professional and manager categories; Figure 3.3. An equal distribution of males and females in both clerical support workers and technician/associate professionals was observed. Females were more frequently seen in elementary work compared to males.

Considering civil status, married patients formed the main group (62), followed by single (19), divorced (10) and widowed (9); Figure 3.4.

Males represented the most common in married, single and divorced groups, whilst females showed the higher frequency in widowed group; Figure 3.5.

Using Chi-Square test, a significant relation was found between sex and civil status; 78% (7/9) of widowed were females compared to 22% of males, while 71% (44/62) of married, 70% (7/10) of divorced and 68% (13/19) of single were males compared to 29%, 30% and 32% of females, respectively (p=0.001).

Married patients were the most common in both middle and old age group; Figure 3.6.
A significant relation was found between marital status and age groups; 65% (40/62) of married and 60% (6/10) of divorced were middle age, while 56% (5/9) of widowed were old age (p=0.002; Chi-Square test).

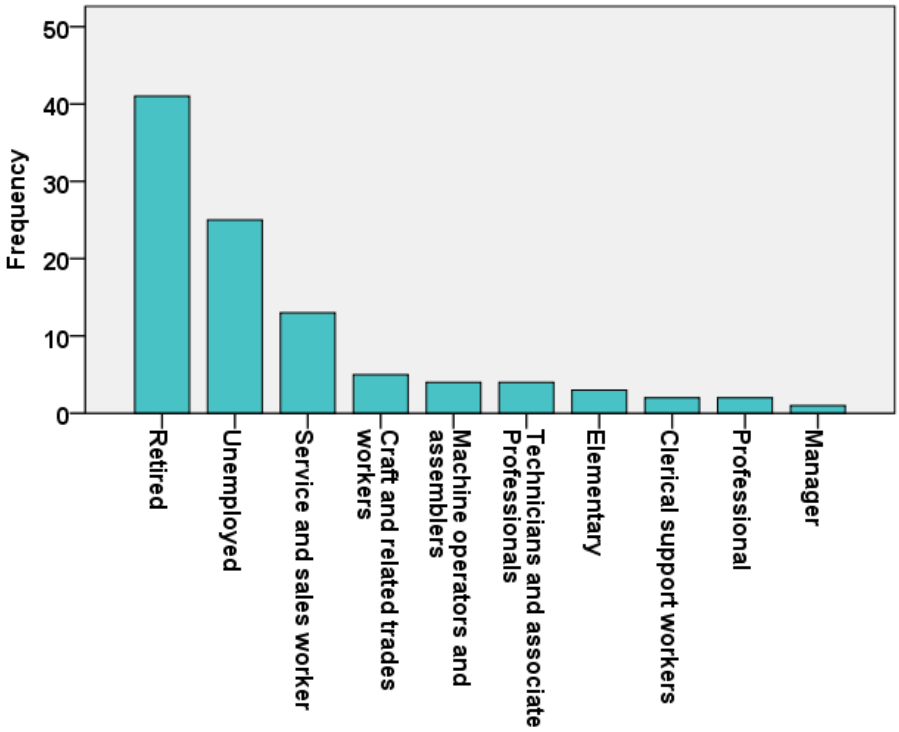

Figure 3.2: Distribution of 100 patients according to occupations.

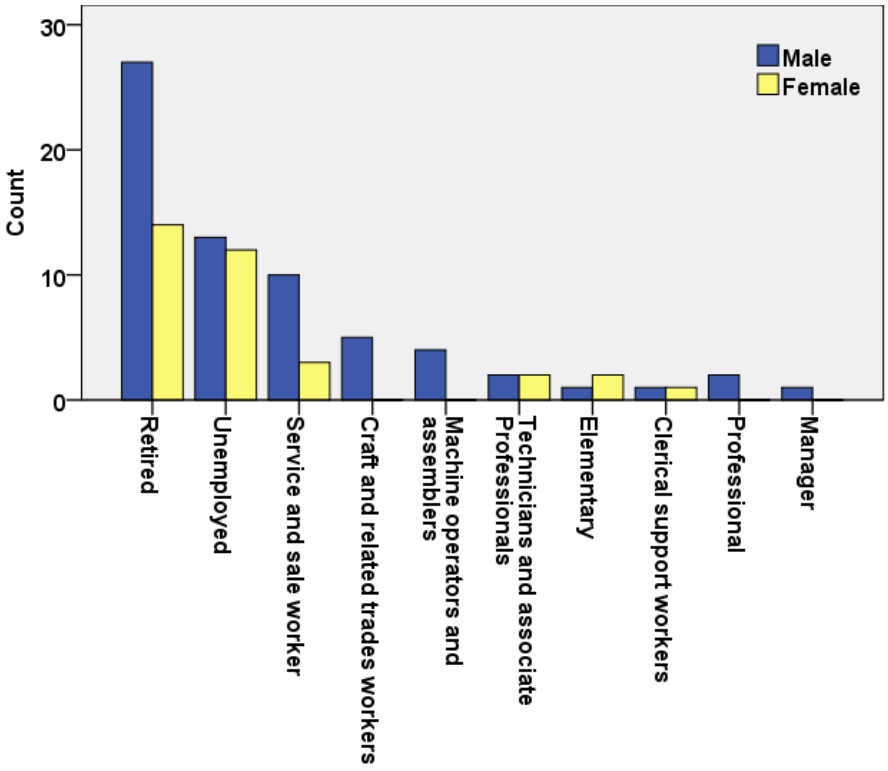

Figure 3.3: Sex distribution according to occupations.

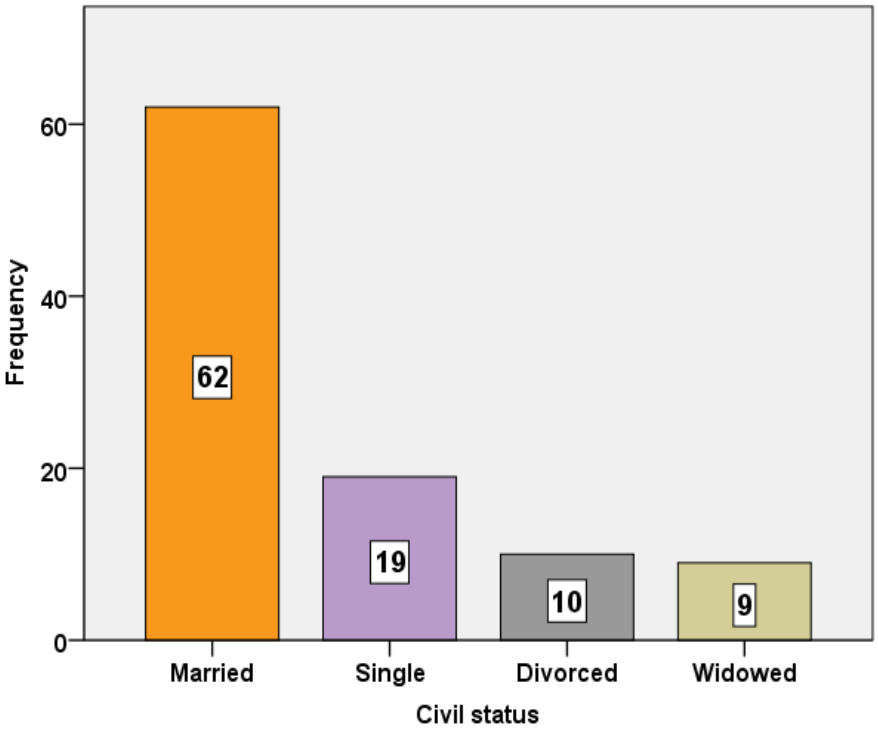

Figure 3.4: Distribution of civil status of 100 PMD patients.

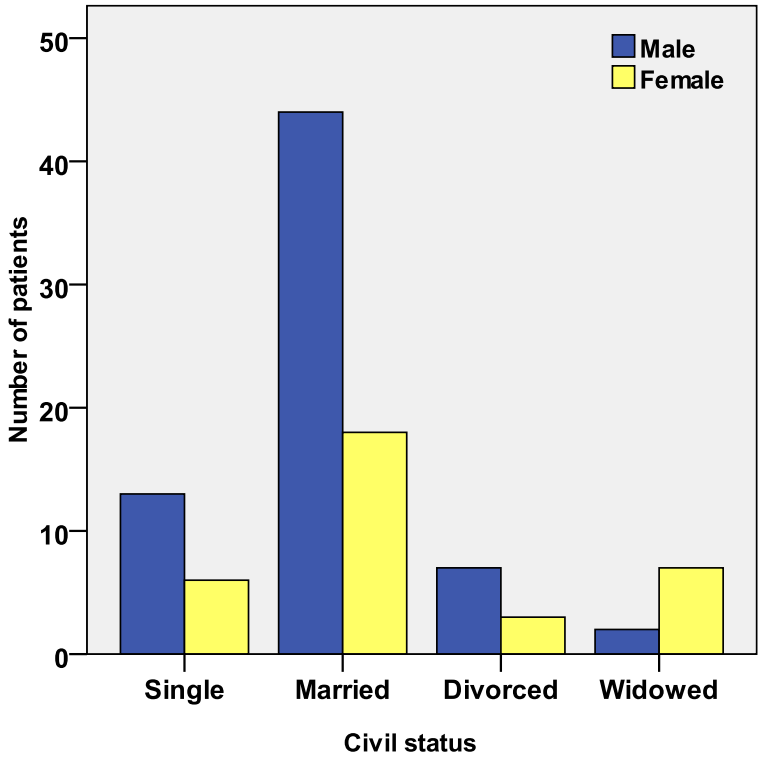

Figure 3.5: Sex distribution according to civil status.

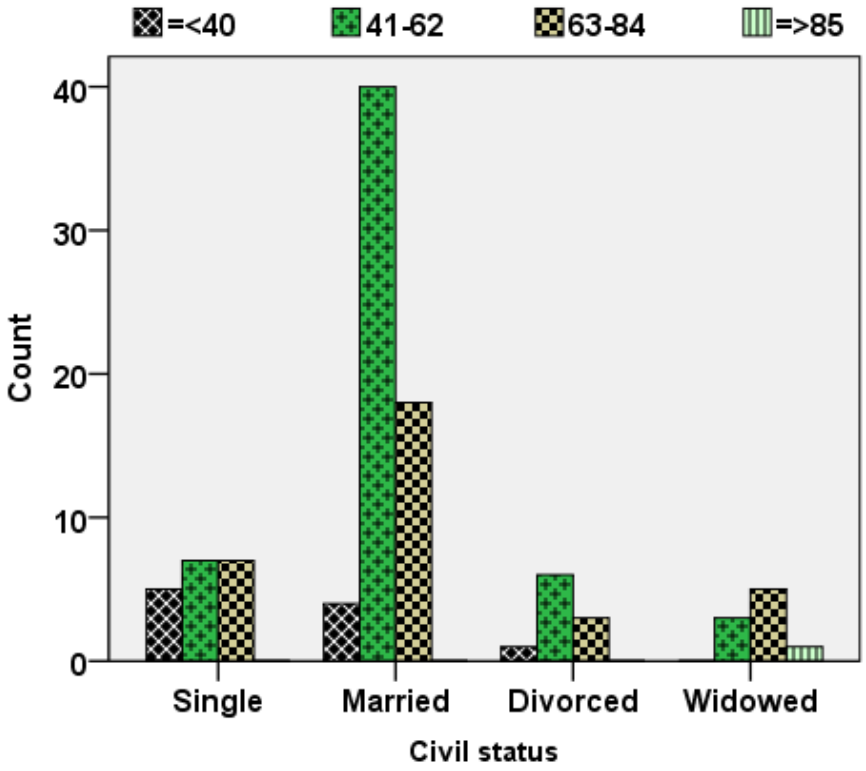

Figure 3.6: Age groups and civil status.

3.4.3. Anatomical Sites of PMDs

In this study, single dysplastic PMDs within the oral cavity were mainly observed in the FOM, lateral tongue, ventral tongue, soft palate, buccal mucosa, fauces, alveolar mucosa and retromolar area.

The data showed that FOM (46) was the most commonly affected site, followed by lateral tongue (19), ventral tongue (14%), soft palate (9), buccal mucosa (5), and fauces (4). Alveolar mucosa (2) and the retromolar area (1) were the least commonly affected oral subsites; Figure 3.7.

For statistical analysis, anatomical subsites were combined to form five groups: FOM, tongue, buccal mucosa, soft palate and other sites (fauces, alveolar mucosa and retromolar area). Accordingly, PMDs were thus most frequently identified in FOM (46), followed by tongue (33), soft palate (9), buccal mucosa (5) and other sites (7).

The sites were then combined to form two groups: high-risk sites (FOM and tongue) and low-risk sites (the remaining oral sites), accordingly the majority of PMDs were distributed among the higher-risk sites (79/100) compared with lower-risk sites (21/100); Figure 3.8.

Anatomical Sites, Sex and Age

Table 3.1 shows sex distribution according to the anatomical oral locations of dysplastic PMDs. FOM, ventral and lateral tongue surface were the main affected sites in both males and females.

Males (66/100) were the predominant in all oral subsites compared with females (34/100).

Ventral tongue and soft palate showed equal distribution in males; whilst buccal mucosa and fauces were equally distributed in females. In female patients, dysplastic PMDs were not observed in the soft palate, retromolar area or alveolar mucosa.

Using Chi-Square test, a significant relation between sex and anatomical oral sites was found (p=0.0001); females showed a higher percentage of dysplastic PMD in FOM and lateral tongue compared to males (53%; 18/34 vs. 42%; 28/66) and (26%; 9/34 vs.15%; 10/66), respectively.

Similarly, a statistical significant association between oral sites classified as high and low-risk groups and sex was observed; dysplastic PMDs were more frequently recorded in males in both high-risk (47 vs. 32) and low-risk sites (19 vs. 2) compared with females (p=0.009; Fisher's Exact test); Figure 3.8.

Figure 3.9 demonstrates the distribution of age groups (young, middle and old age) according to the anatomical sites of single dysplastic PMDs.
The FOM was the main affected anatomical site in all age groups; a significant relation between age and the anatomical sites was found; 80% (8/10) of patients younger than 40 years old were observed with FOM compared to 46% (26/56) of middle age and 36% (12/33) of older age (p=0.010; Chi-Square test).

Figure 3.10 shows the relationship between the anatomical sites of PMDs and age group classified as younger than 40 and older than 41 years.
Patients ≤ 40 years showed mainly FOM dysplastic PMDs (80%), followed by buccal mucosa and soft palate (10% for each). Similarly, patients > 41 years showed mainly FOM dysplastic PMDs (42%), followed by tongue (37%), soft palate 9%, buccal mucosa and fauces equally 4%, alveolar mucosa 2% and retromolar area 1%.

Although FOM was the commonest site of occurrence of PMDs in both ≤ 40 and > 41 years age group, the frequency was higher in younger patients (80% vs. 42%). Chi-Square test showed no significant association between PMD sites and age group (≤ 40 and > 41 years) (p=0.101).

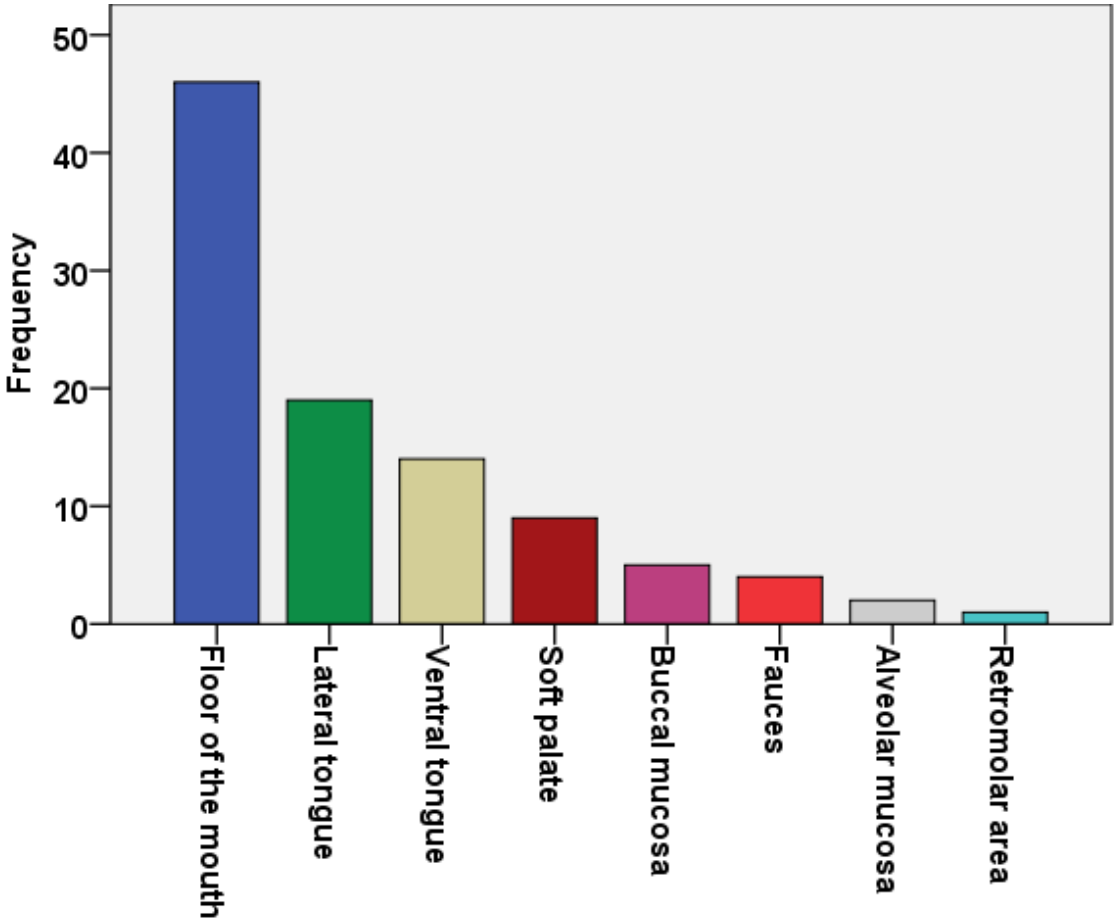

Figure 3.7: Anatomical site distribution of PMDs.

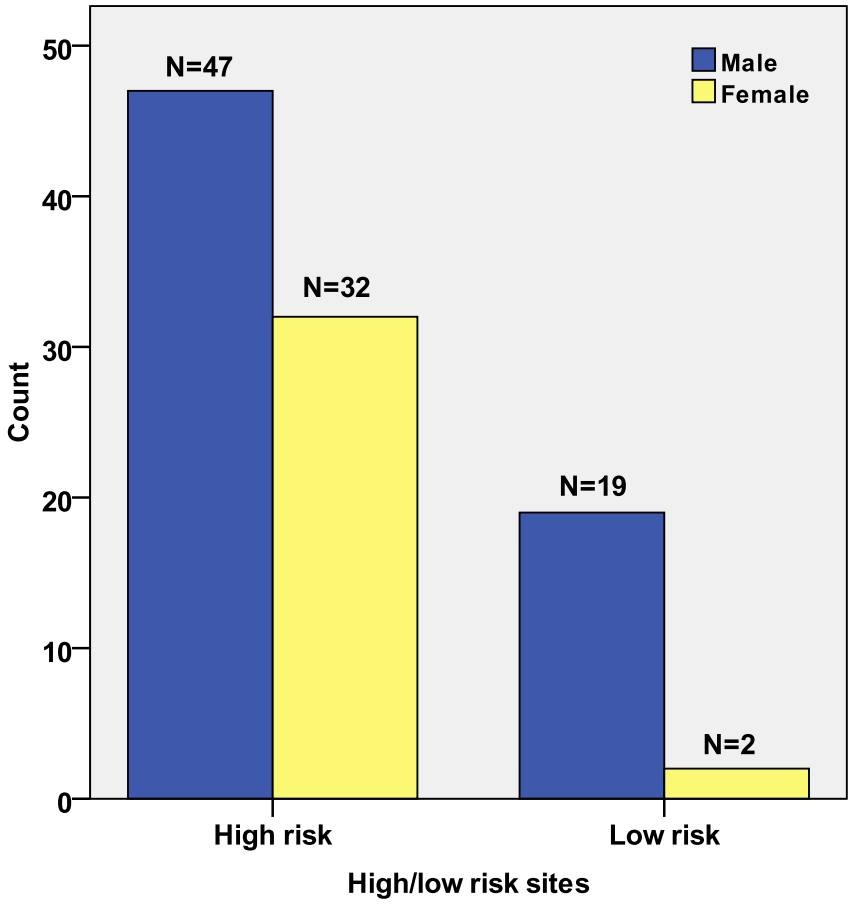

Figure 3.8: Sex distribution according to high/low risk sites.

Table 3.1: Sex distribution according to PMD anatomical site.

PMD anatomical site	Sex		Total
	Male	Female	
FOM	**28** *61%*	**18** *39%*	**46** *100%*
Lateral tongue	**10** *53%*	**9** *47%*	**19** *100%*
Ventral tongue	**9** *64%*	**5** *36%*	**14** *100%*
Soft palate	**9**	**-**	**9** *100%*
Buccal mucosa	**4** *80%*	**1** *20%*	**5** *100%*
Fauces	**3** *75%*	**1** *25%*	**4** *100%*
Alveolar mucosa	**2**	**-**	**2** *100%*
Retromolar area	**1**	**-**	**1** *100%*
Total	**66**	**34**	**100**

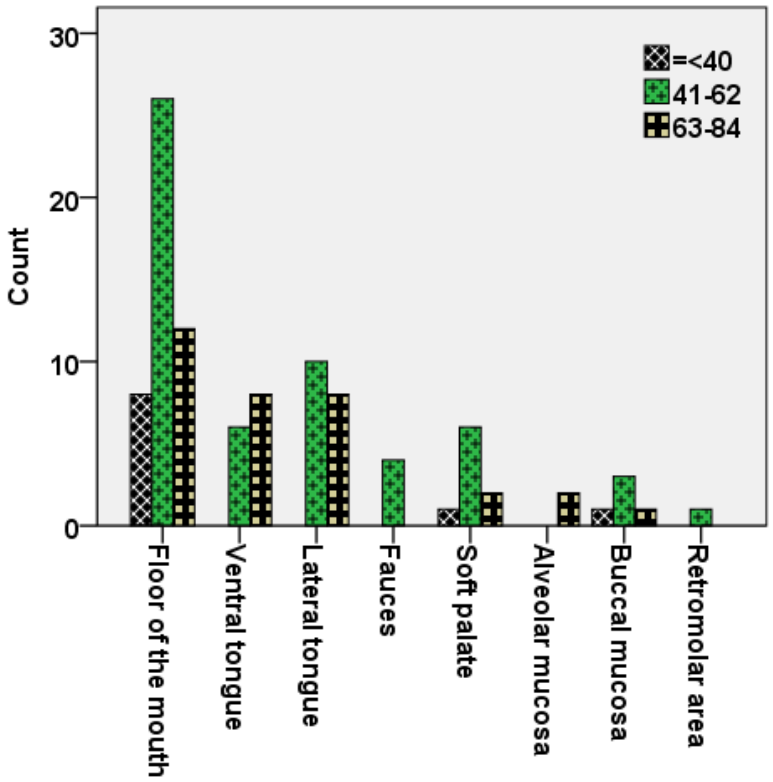

Figure 3.9: Age group according to PMD anatomical sites.

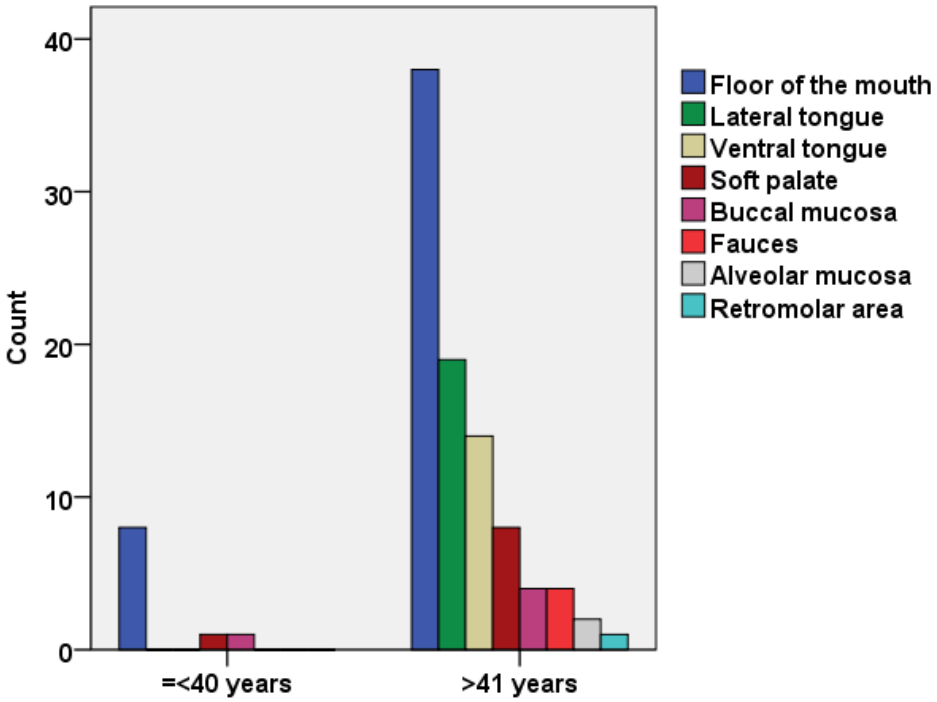

Figure 3.10: Site distribution of PMDs according to two age groups.

3.4.4. Clinical Appearance of PMDs

In this study, as can be seen in Figure 3.11, dysplastic PMDs presented mainly as leukoplakias (92%) with only 8% as erythroplakia. Homogenous leukoplakias formed 67% and non-homogenous 25%, with males showed a higher percentage of non-homogenous leukoplakia compared to females (31%; 18/59 vs. 21%; 7/33), however, Fisher's exact test was not significant (p=0.464); Table 3.2.

Considering non-homogenous leukoplakias, speckled was the most common (16/25), followed by exophytic (5/25), nodular (2/25) and ulcerated (2/25) appearance.
In general, all clinical types of PMDs were seen more frequently in males compared with females; Table 3.3. In females, 97% of PMDs were leukoplakias and only 3% were erythroplakias; whereas, in males, 89% were leukoplakias and 11% were erythroplakias. Comparing males to females, a higher percentage of speckled subtypes were seen in females (18% compared to 15%).
Overall, all erythroplakia cases were observed in patients over 40 years old with 88% (7/8) identified in males compared to only 12% (1/8) in females.

Clinical Appearance and Site Distribution of PMDs

Table 3.4 shows that the FOM represented the most common site for homogenous leukoplakia (37/67), followed by equal distribution in both lateral and ventral surfaces of the tongue (11/67) and soft palate (5/67).

In the FOM, the speckled subtype was the most common amongst non-homogenous subtypes (6/16), followed by pillar of fauces (3/16), whereas an equal distribution was seen in the lateral and ventral surfaces of the tongue and the soft palate (2/16).

The nodular subtype was only seen in the ventral tongue and the buccal mucosa and in an equal distribution. The exophytic subtype was more frequently seen in buccal mucosa (2/5) but was also observed in FOM, lateral tongue and alveolar mucosa (1/4). Ulcerated PMDs were only seen on the lateral surface of the tongue (2/2).

Erythroplakia was seen most on the lateral tongue surface (3/8), followed by the FOM and soft palate (2/8) with retromolar area (1/8) the least affected site.

Regarding high-risk and low-risk oral sites, homogenous leukoplakia was the commonest clinical appearance, followed by speckled leukoplakia and erythroplakia with higher percentage in high-risk sites. Although ulcerated PMDs were only identified in high-risk site (2/2), 60% of exophytic leukoplakias were seen in low-risk oral sites. Nodular lesions were equally seen in both high/low-risk regions; Figure 3.12 and Table 3.5.
Using Chi-square test, a highly significant relation was found between clinical types of dysplastic PMDs and anatomical oral sites (p=0.0001); 55% (37/67) of homogenous leukoplakia and 38% (6/16) of speckled subtype were reported in the FOM. While 38% (3/8) of erythroplakia and all ulcerated cases (2/2) were seen in lateral tongue.

Likewise, a significant association between clinical appearance and anatomical site as high/low-risk sites was found (p=0.002); 88% (59/67) of homogenous leukoplakia, 63% (10/16) of speckled and 63% (5/8) of erythroplakia and all ulcerated cases (2/2) were observed in high-risk sites.

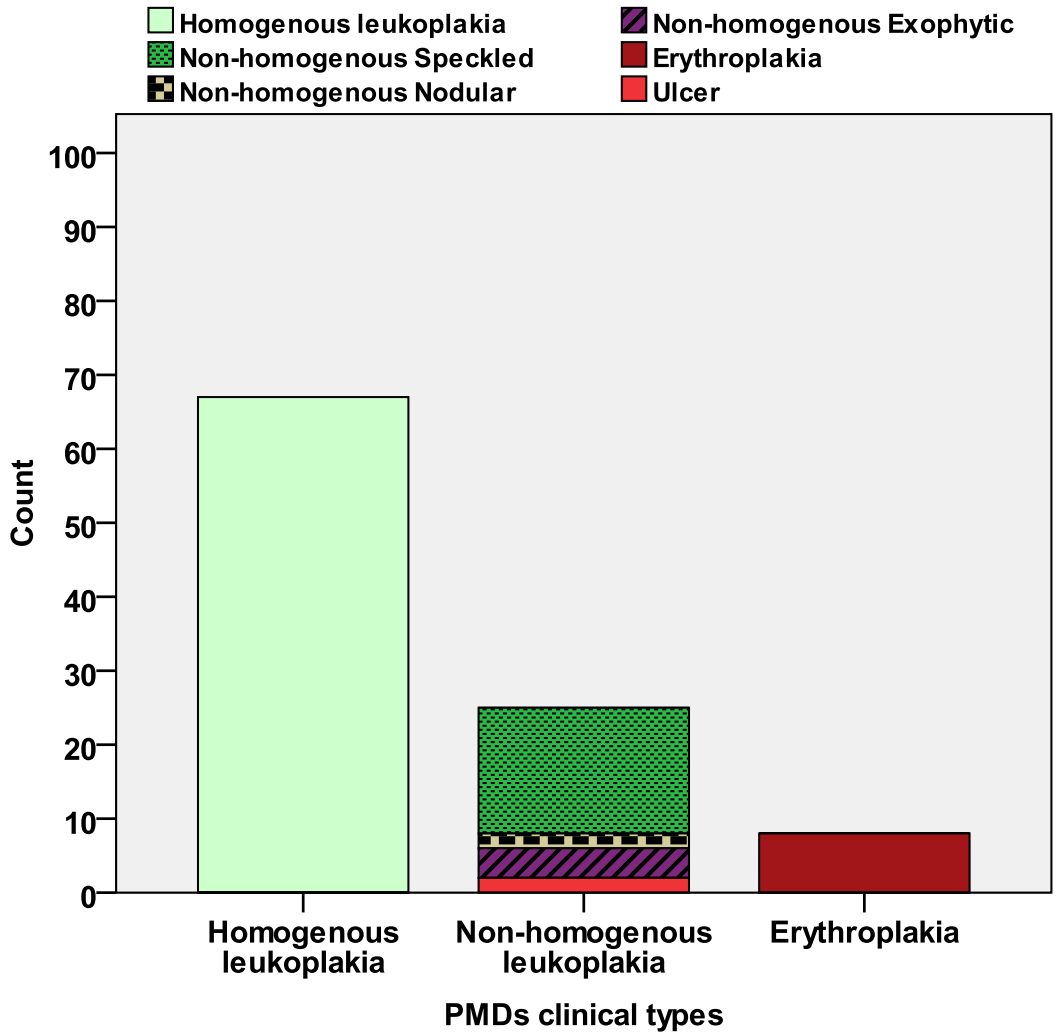

Figure 3.11: Clinical appearance of PMDs at first presentation.

Table 3.2: Sex distribution according to type of leukoplakia.

Types of leukoplakia	Sex		Total
	Male	Female	
Homogenous	41 *69%*	26 *79%*	67 *73%*
Non-homogenous	18 *31%*	7 *21%*	25 *27%*
Total	59 *100%*	33 *100%*	92 *100%*

Table 3.3: Sex distribution according to clinical appearance of PMDs.

Clinical type of PMDs		Sex		Total
		Male	Female	
Homogenous leukoplakia		41	26	67
Non-homogenous leukoplakia	Speckled	10	6	16
	Nodular	2	-	2
	Exophytic	4	1	5
	Ulcerated	2	-	2
Erythroplakia		7	1	8
Total		66	34	100

Table 3.4: Distribution of clinical types of PMDs according to the anatomical sites.

Clinical type of PMDs		Anatomical sites of PMDS								Total
		FOM	Lateral tongue	Ventral tongue	Buccal mucosa	Soft palate	Fauces	Retromolar area	Alveolar mucosa	
Homogenous leukoplakia		37	11	11	1	5	1	-	1	67
Non-homogenous leukoplakia	Speckled	6	2	2	1	2	3	-	-	16
	Nodular	-	-	1	1	-	-	-	-	2
	Exophytic	1	1	-	2	-	-	-	1	5
	Ulcerated	-	2	-	-	-	-	-	-	2
Erythroplakia		2	3	-	-	2	-	1	-	8
Total		46	19	14	5	9	4	1	2	100

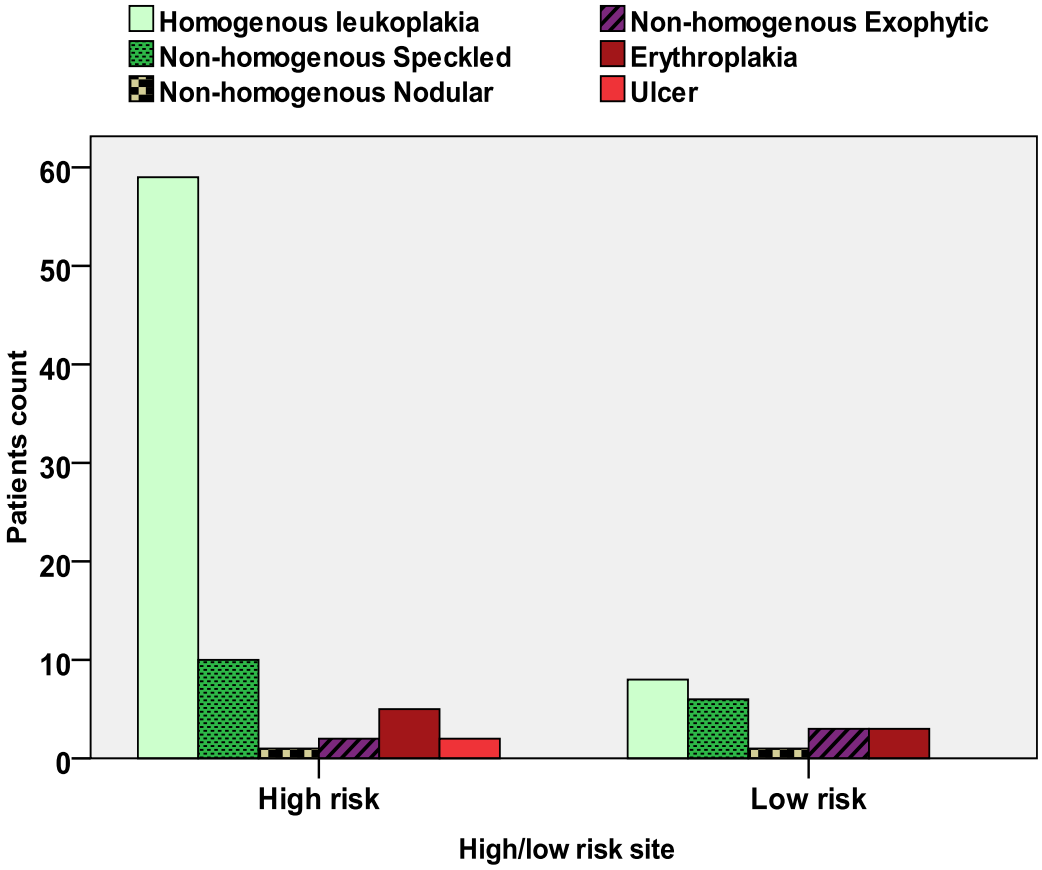

Figure 3.12: Clinical type of PMDs according to high and low risk sites.

Table 3.5: Clinical type of PMDs according to high and low risk sites.

PMD site	Clinical appearance of PMDs						Total
	Homogenous leukoplakia	Non-homogenous leukoplakia				Erythroplakia	
		Speckled	Nodular	Exophytic	Ulcerated		
High risk	**59** *88%*	**10** *63%*	**1** *50%*	**2** *40%*	**2** *100%*	**5** *63%*	**79** *79%*
Low risk	**8** *12%*	**6** *38%*	**1** *50%*	**3** *60%*	**-**	**3** *38%*	**21** *21%*
Total	**67** *100%*	**16** *100%*	**2** *100%*	**5** *100%*	**2** *100%*	**8** *100%*	**100** *100%*

3.4.5. Size of a Single Dysplastic PMD

The size of a single PMD was recorded in mm^2 by multiplying the length by the width, taken from the pathological report of the laser excised specimen. The data were recorded for 97/100 (with 3 cases of laser vaporization).

The size range was 21-1,800 mm^2, with a mean of 299.97 mm^2 (SD: 279.16). PMDs sizes were classified into 3 categories; minor (< 200 mm^2), intermediate (200-600 mm^2) and major sizes (> 600 mm^2) with the intermediate (45/97) and minor size (42/97) were the most common, followed by the major size group (10/97); Figure 3.13.

For statistical analysis, sizes were further classified according to the 75th percentile (third quartile) into 2 groups: < 425 mm^2 and ≥ 425 mm^2; 75% (73/97) of cases were found to be less than 425 mm^2 in size and 25% (24/97) were more than 425 mm^2 in size.

Age and Size

In all age groups, the majority of PMDs were intermediate and minor, followed by major sizes; 46%, 44% and 10%, respectively; Table 3.6.

In the young age group, the majority of PMDs were minor (6/10), followed by intermediate (4/10), while no major sized PMD was observed. The old age groups demonstrated minor and intermediate sized PMDs equally (13/30), followed by major sized (4/30). Whilst in middle age patients, intermediate sized PMDs were the most common (27/56), followed by minor (23/56) and major sized (6/56).

Although the mean size of PMDs was found to increase with increasing age, the correlation was not significant when considering the 4 age groups (r=0.151, n=98, p=0.137; Spearman Correlation); Table 3.7. However, the correlation was found to be significant between PMDs size and age when the 4 groups were compressed into 2 groups: > 41 and ≤ 40 years (r=0.222, n=98, p<0.01); Table 3.8.

Sex and Size

Male patients were most commonly seen in all the size groups, with highest frequency in intermediate, minor and then major size categories; Figure 3.14.

No significant association was found between sex and size groups (p=0.096, Chi-Square test). Consequently, a Mann-Whitney U test was performed which revealed no significant differences between males and females in their average size of PMDs (p=0.089); however, the mean size of PMDs in males (344.50 mm^2, range 21-1,800 mm^2) was greater compared to that of females (216.15 mm^2, range 32-682 mm^2).

Site and Size

Figure 3.15 and Table 3.9 show that the FOM was the main site for minor sized PMDs (23/46), followed by intermediate (22/46) and major sized (1/46).

The lateral surface of the tongue was the main site for major sized PMDs (6/10), followed by the ventral tongue (3/10). The buccal mucosa and fauces showed an equal distribution of minor sized PMDs, similar to ventral tongue and soft palate.

Using Chi-Square test, a significant association was found between size and PMDs oral sites (p=0.0001).

Table 3.10 shows the average sizes of PMDs according to their oral subsites. Investigation the differences in the average size of PMDs among different oral locations showed a highly significant differences (p=0.0001; Kruskal-Wallis test). Subsequently, pair-wise comparisons were performed using Mann-Whitney U test which revealed a significantly smaller mean sized PMDs in the FOM (200.78 mm^2) compared with the lateral tongue (591.63 mm^2) (p=0.001) and to the ventral tongue (369.39 mm^2) (p=0.034).

Table 3.11 shows that major sized PMDs were only seen at high-risk sites (10/10), followed in frequency by intermediate (36/45) and minor sized PMDs (32/42).

Low-risk sites exhibited minor (10/19) and intermediate sized PMDs (9/19), with no major sized PMDs were observed. However, no significant relation was seen between high/low-risk sites and size categories (p=0.301; Chi-Square test).

Using Fisher's Exact test, a significant relation was found between high/low-risk sites and the size of PMDs as smaller than the third quartile (< 425 mm^2), and bigger than the third quartile (≥ 425 mm^2) (p=0.036). Whilst ninety-six percent (23/24) of PMDs larger than the third quartile were seen in the high risk-sites, 95% (18/19) of PMDs located in low-risk sites showed a smaller size than the third quartile (< 425 mm^2); Table 3.12.

Clinical Appearance and Size

As can be seen in Table 3.13 and Figure 3.16, leukoplakia was the most prevalent type of PMD (89/97) in all size categories.

Erythroplakia was only seen in 8 cases mainly in the minor sized group (5/8), followed by intermediate (2/8) and major (1/8).

Considering the non-homogenous subtypes, speckled leukoplakia (13/16) was the most common type identified within the intermediate sized group. An equal distribution of speckled and exophytic subtypes was seen in the minor size category (3/42). Ulcerated PMDs (2) were only seen within the major size category (> 600 mm^2). An equal number of nodular PMD was seen in intermediate and major size categories (1 for each).

Forty-nine percent (31/64) of homogenous leukoplakia and 60% (3/5) of exophytic exhibited minor size PMDs, with 81% (13/16) of speckled intermediate and all ulcerated cases were major size PMDs.

Using Chi-Square test, a significant relation was found between size and clinical appearance of PMDs (p=0.001).

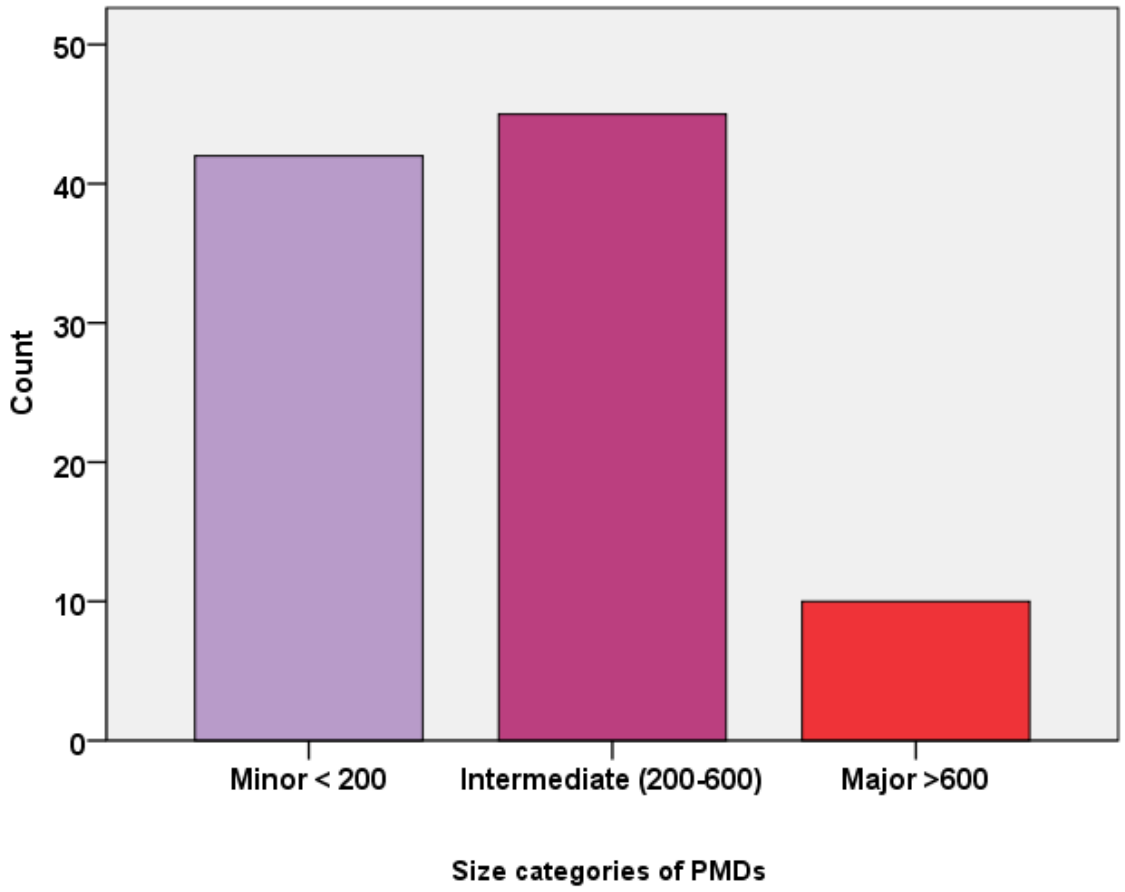

Figure 3.13: Distribution of PMDs according to size categories (mm^2).

Table 3.6: Distribution of PMD size according to age group.

Age group (years)	Size category of PMDs (mm^2)			Total
	Minor < 200	Intermediate (200-600)	Major > 600	
≤ 40	6	4	-	10
41-62	23	27	6	56
63-84	13	13	4	30
≥ 85	-	1	-	1
Total	42 *44%*	45 *46%*	10 *10%*	97 *100%*

Table 3.7: Mean size of PMDs according to four age groups.

Age group (years)	Size of PMDs (mm^2)				
	Mean	N	Minimum	Maximum	SD
≤ 40	160.90	10	32	450	156.425
41-62	298.45	56	21	1,050	234.212
63-84	345.16	30	36	1,800	367.870
≥ 85	375.00	1	375	375	.
Total	299.97	97	21	1,800	279.166

Table 3.8: Mean size of PMDs according to two age groups.

Age group (years)	Mean	N	Maximum	Minimum	SD
≤ 40	160.90	10	450	32	156.425
> 41	315.77	87	1,800	21	286.155
Total	299.97	97	1,800	21	279.166

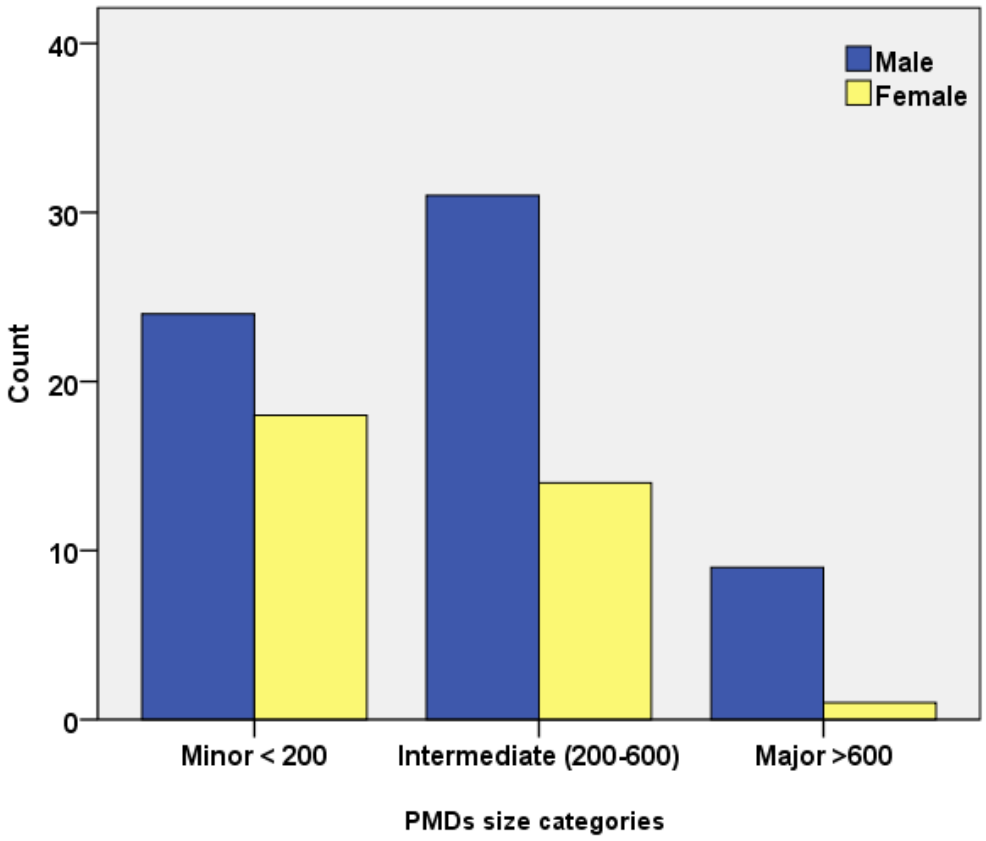

Figure 3.14: Sex distribution according to size category.

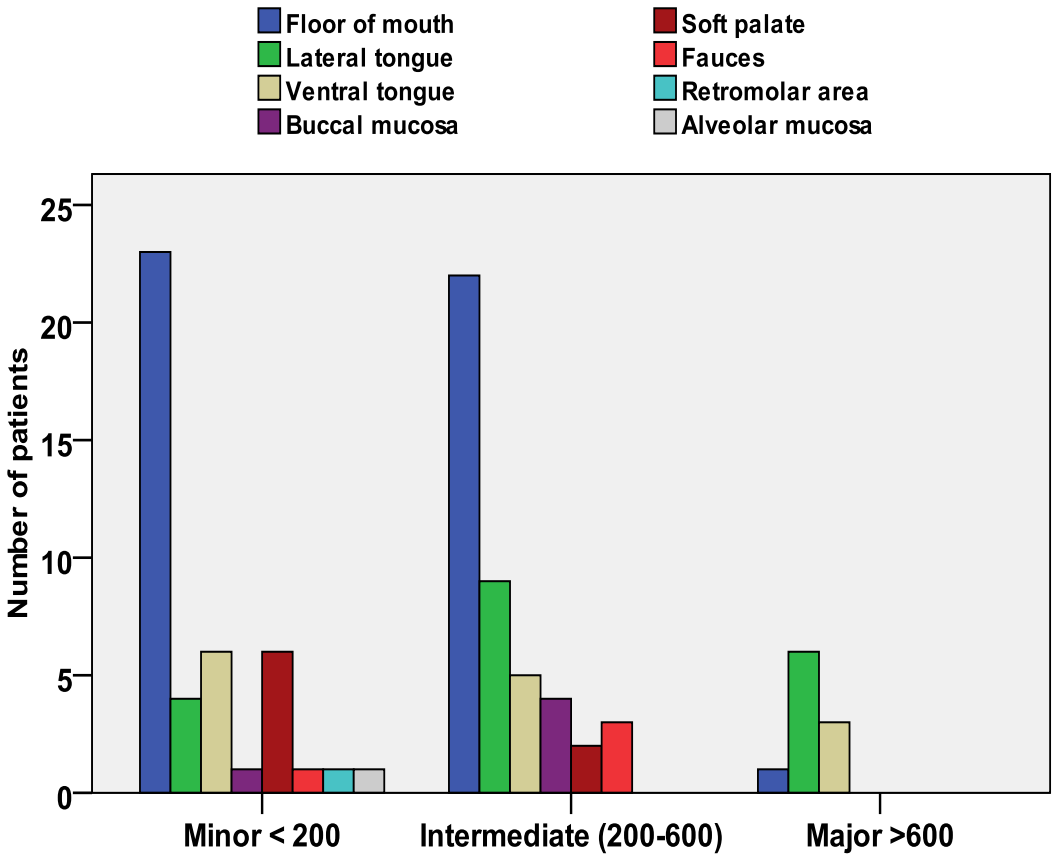

Figure 3.15: PMD anatomical sites according to size (mm^2).

Table 3.9: Size category of PMDs according to anatomical sites.

PMD anatomical sites	Size category (mm^2)			Total
	Minor < 200	Intermediate (200-600)	Major > 600	
FOM	23 *50%*	22 *48%*	1 *2%*	46 *100%*
Lateral tongue	3 *17%*	9 *50%*	6 *33%*	18 *100%*
Ventral tongue	6 *43%*	5 *36%*	3 *21%*	14 *100%*
Buccal mucosa	1 *20%*	4 *80%*	-	5 *100%*
Soft palate	6 *75%*	2 *25%*	-	8 *100%*
Fauces	1 *25%*	3 *75%*	-	4 *100%*
Retromolar area	1 *100%*	-	-	1 *100%*
Alveolar mucosa	1 *100%*	-	-	1 *100%*
Total	42 *44%*	45 *46%*	10 *10%*	97 *100%*

Table 3.10: Mean size of PMDs according to anatomical sites.

PMD anatomical site	Size of PMD (mm^2)				
	N	Mean	Minimum	Maximum	SD
Lateral tongue	18	591.63	105	1,800	403.115
Ventral tongue	14	369.36	66	884	270.436
Buccal mucosa	5	344.20	150	450	128.290
Fauces	4	243.75	160	351	83.348
FOM	46	200.78	32	620	146.324
Soft palate	8	112.62	21	377	115.489
Alveolar mucosa	1	92.00	92	92	-
Retromolar area	1	60.00	60	60	-
Total	97	299.97	21	1,800	279.166

Table 3.11: Distribution of high/low-risk sites according to three PMDs size groups.

Size category (mm^2)	Anatomical site		Total
	High risk	Low risk	
Minor < 200	**32**	**10**	**42**
Intermediate (200-600)	**36**	**9**	**45**
Major > 600	**10**	**-**	**10**
Total	**78** *80%*	**19** *20%*	**97** *100%*

Table 3.12: Distribution of high/low-risk sites according to two PMDs size groups.

Size category (mm^2)	Anatomical sites		Total
	High risk	Low risk	
≥ 425	**23** *96%*	**1**	**24** *100%*
< 425	**55**	**18** 95%	**73**
Total	**79**	**19** *100%*	**97** *100%*

Table 3.13: Clinical appearance of PMDs according to size category.

Clinical appearance of PMDs		PMD size category (mm^2)			Total
		Minor < 200	Intermediate (200-600)	Major > 600	
Homogenous leukoplakia		**31** *49%*	**27** *42%*	**6** *9%*	**64** *100%*
Non-homogenous leukoplakia	Speckled	**3** *19%*	**13** *81%*	**-**	**16** *100%*
	Nodular	**-**	**1** *50%*	**1** *50%*	**2** *100%*
	Exophytic	**3** *60%*	**2** *40%*	**-**	**5** *100%*
	Ulcerated	**-**	**-**	**2** *100%*	**2** *100%*
Erythroplakia		**5** *63%*	**2** *25%*	**1** *13%*	**8** *100%*
Total		**42** *44%*	**45** *46%*	**10** *10%*	**97** *100%*

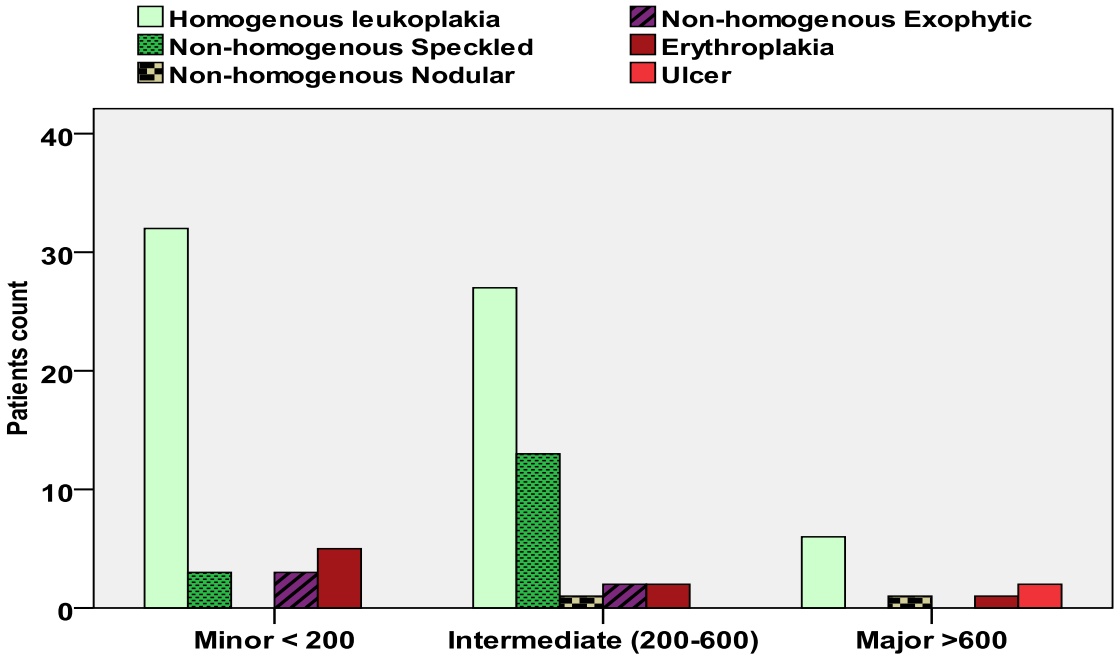

Figure 3.16: Clinical appearance of PMD in relation to size (mm^2).

3.4.6. Histopathological Features of PMDs

Overall, the majority of excised dysplastic PMD specimens were diagnosed histopathologically as mild dysplasia 43% (42), followed by moderate 24% (23), severe 23% (22) and CIS 10% (10). Table 3.14 shows the histopathological diagnosis of both incisional and excisional biopsies according to the WHO classification system.

According to the binary grading system, 54% of the PMDs were diagnosed as high-grade dysplasia and the remaining 46% were diagnosed as low-grade dysplasia as consensus diagnosis of two oral pathologists; Table 3.15.

Table 3.14: Oral epithelial dysplasia: Incisional vs. Excisional.

Oral epithelial dysplasia WHO grading	Incisional Biopsy	Excisional Biopsy
Mild	**37** *37%*	**42** *43%*
Moderate	**26** *26%*	**23** *24%*
Severe	**19** *19%*	**22** *23%*
CIS	**18** *18%*	**10** *10%*
Total	**100** *100%*	**97** *100%*

Table 3.15: Oral epithelial dysplasia: Binary grading system.

Oral epithelial dysplasia			Total
The WHO grading system	Binary grading system		
	Low grade	High grade	
Mild	**42**	**-**	**42**
Moderate	**3**	**20**	**23**
Severe	**-**	**22**	**22**
CIS	**-**	**10**	**10**
Total	**45**	**52**	**97**

Sex, Age and Oral Epithelial Dysplasia

Generally, males were more frequently seen in all dysplastic groups compared with females; however, Chi-Square test showed no significant relation between sex and WHO histopathology consensus diagnosis of excisional specimens (p=0.502); Figure 3.17.

Fifty–two percent of females (17/33) exhibited mild dysplasia compared to 39% (25/64) of males. Females showed higher percentage of severe dysplasia compared to males (24% vs. 21%); whereas higher degree of moderate dysplasia and CIS were seen in males compared with females (27% vs.18%), (13% vs. 6%), respectively; Table 3.16.

Regarding the binary grading system, 55% (35/64) of males were diagnosed with high grade dysplasia, while 52% (17/33) of females were diagnosed with low grade dysplasia; Table 3.17.
Fisher's Exact test showed no significant association between sex and epithelial dysplasia classified as high/low grade dysplasia.

Risk estimate showed that males were 1.13 times more likely to develop higher dysplastic features compared to females (95% CI, 0.744-1.711).

A higher grade of dysplasia was seen in patients within the middle age group (41-62 years), but no significant relation was found between degree of oral epithelial dysplasia and age (p=0.643, Chi-Square test); Figure 3.18.

Table 3.18 shows the mean age of patients with different degrees of oral epithelia dysplasia. Comparing the mean age of patients with different degrees of epithelia dysplasia, higher dysplastic features were observed with advancing age; however ANOVA test was not significant regarding WHO grading (p=0.393). Also, the correlation was not significant between age and oral epithelial dysplasia (r=0.179, n=97, p=0.080, Spearman Correlation); Figure 3.19.

Similarly with low grade and high grade dysplasia, higher grade dysplasia was seen with advancing age, but the differences between the mean age of patients with high and low grade dysplasia was not significant (p=0.323, Independent t-test).
Seventy-percent (7/10) of mild dysplasia was seen in patients less than 40 years compared to only 40% (35/87) in patients older than 41 years; Table 3.19.

Considering the binary grading system, 94% (48/51) of high grade dysplasia was recognized in patients older than 41 years compared to just 6% (3/51) in young age (≤ 40). While 70% (7/10) of patients younger than 40 years were affected with low-grade dysplasia; Table 3.20. However, no significant association between age group and binary grading system (p=0.184; Fisher's Exact test).

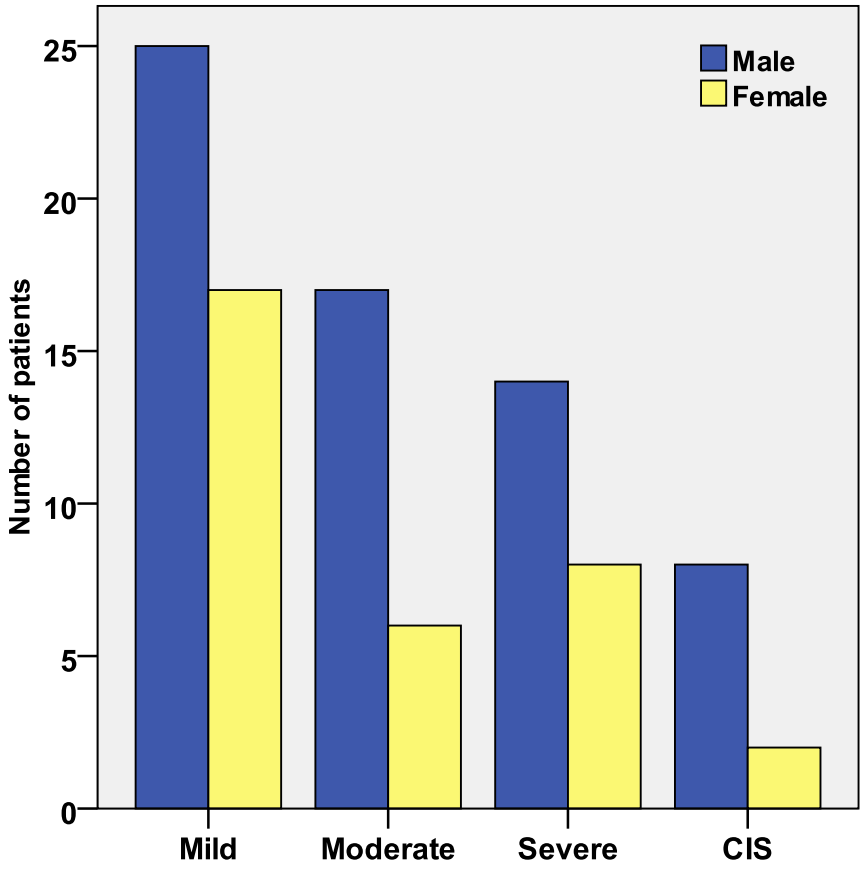

Figure 3.17: Sex distribution according to degree of dysplasia.

Table 3.16: Oral epithelial dysplasia (WHO grading) and sex.

Sex	Oral epithelial dysplasia (consensus grade)				Total
	Mild	Moderate	Severe	CIS	
Male	**25** *39%*	**17** *27%*	**14** *21%*	**8** *13%*	**64** *100%*
Female	**17** *52%*	**6** *18%*	**8** *24%*	**2** *6%*	**33** *100%*
Total	**42** *43%*	**23** *24%*	**22** *23%*	**10** *10%*	**97** *100%*

Table 3.17: Oral epithelial dysplasia (binary grading) and sex.

Sex	Binary grading system		Total
	High grade	Low grade	
Male	**35** *55%*	**29** *45%*	**64** *100%*
Female	**16** *48%*	**17** *52%*	**33** *100%*
Total	**51** *53%*	**46** *47%*	**97** *100%*

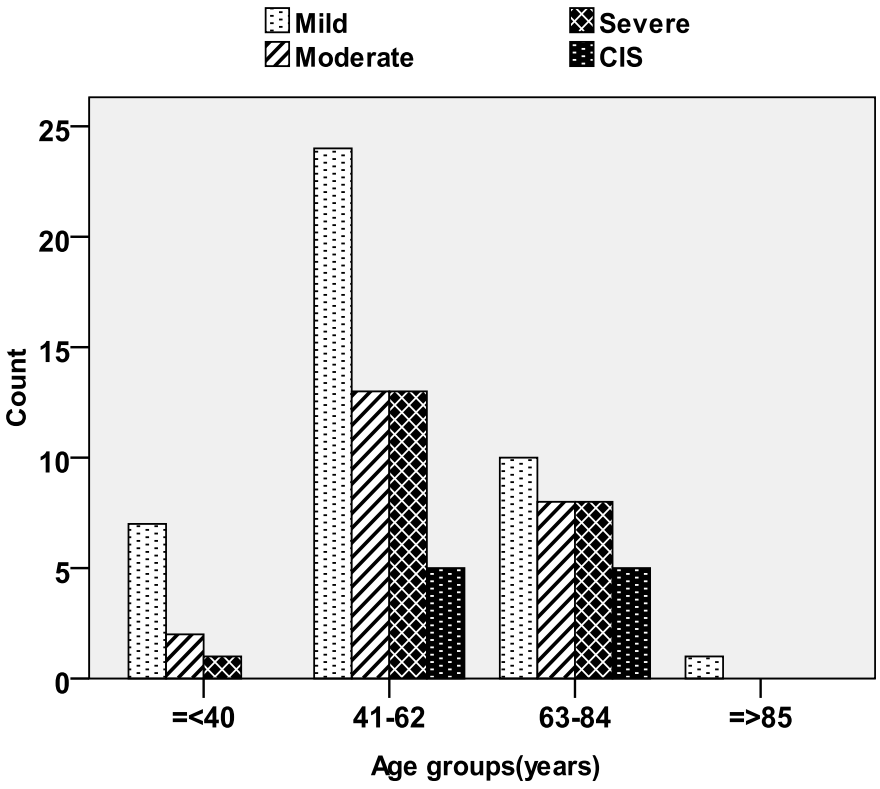

Figure 3.18: Degree of dysplasia in relation to age group.

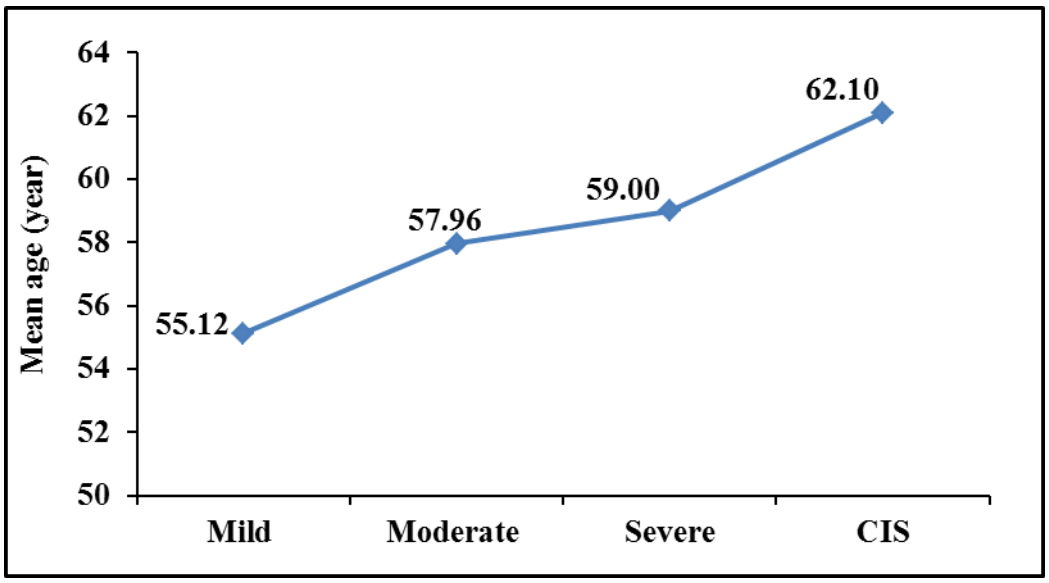

Figure 3.19: Mean age of patients in relation to degree of dysplasia.

Table 3.18: Mean age of patients in relation to degree of dysplasia.

Histopathology (WHO grading)	Mean age (years)	N	Minimum	Maximum	SD
Mild	55.12	42	30	94	13.978
Moderate	57.96	23	39	81	12.568
Severe	59	22	39	81	12.122
CIS	62.1	10	47	76	9.938
Binary grading					
Low grade	56.02	46	30	94	14.177
High grade	58.63	51	39	81	11.622

Table 3.19: The WHO histopathological diagnosis and age group.

Age group (years)	Degree of dysplasia (WHO)				Total
	Mild	Moderate	Severe	CIS	
≤ 40	**7** *70%*	**2** *20%*	**1** *10%*	**-**	**10** *100%*
> 41	**35** *40%*	**21** *24%*	**21** *24%*	**10** *12%*	**87** *100%*
Total	**42** *43%*	**23** *24%*	**22** *23%*	**10** *10%*	**97** *100%*

Table 3.20: Binary grading dysplasia and age group.

Age group (years)	Binary grading system		Total
	Low grade	High grade	
≤ 40	**7** *15%*	**3** *6%*	**10**
> 41	**39** *85%*	**48** *94%*	**87**
Total	**46** *100%*	**51** *100%*	**97** *100%*

Anatomical Site and Oral Epithelial Dysplasia

Table 3.21 shows histopathological diagnosis of dysplastic PMDs according to oral anatomical sites.
Fifty-seven percent (24/42) of mild dysplasia was diagnosed in the FOM, followed by lateral tongue 21% (9/42) and ventral tongue 7% (3/42). Similarly moderate dysplasia was mostly seen in the FOM, followed by lateral and ventral tongue (35%, 22% and 9%, respectively).

Severe dysplasia was seen more frequently in FOM 41% (9/22), followed by ventral tongue 23% (5/22) and lateral tongue 18% (4/22). 50% (5/10) of CIS cases were identified in the FOM, with 30% (3/10) of CIS cases were seen in the ventral tongue.

Severe dysplasia was the main dysplastic feature diagnosed in ventral tongue surface 23% (3/13). Moderate dysplasia was the main dysplastic feature in soft palate 22% (5/23) and mild dysplasia was the most commonly seen dysplasia in the FOM and lateral tongue. Using Chi-Square test, a significant relation was found between degree of epithelial dysplasia and sites of PMDs (p=0.010).

Table 3.22 shows the relation between high/low-grade dysplasia and PMD oral site. A significant association was seen between anatomical site and the binary grading dysplasia (p=0.010; Chi-Square test). The high grade dysplasias were mainly diagnosed in high-risk sites; 41% in FOM, 20% in both lateral and ventral tongue surface, 8% in soft palate and 6% in both buccal mucosa and fauces. All alveolar mucosa cases (2/2) were diagnosed as low-grade dysplasia. High and low-grade dysplasias were equally observed in soft palate.

Similarly, although 80% (41/51) of high-grade dysplasia was diagnosed in the FOM and tongue compared to 20% (10/51) at remaining oral sites, this did not reach statistical significance (p=0.342; Chi-Square test).

Regarding high/low-risk sites in relation to dysplastic features, it is clear that all dysplastic features were more commonly seen in high-risk sites (FOM and tongue) compared to low-risk sites (remaining oral sites), Chi-Square test was significant (p=0.026); Table 3.23.

Table 3.21: Anatomical site of PMD in relation to histopathological diagnosis.

Histopathology (WHO grading)	Anatomical site of PMDs							Total
	FOM	Lateral tongue	Ventral tongue	Buccal mucosa	Soft palate	Fauces	Alveolar mucosa	
Mild	**24** *57%*	**9** *21%*	**3** *7%*	**2** *5%*	**2** *5%*	**1** *2%*	**1** *2%*	**42** *100%*
Moderate	**8** *35%*	**5** *22%*	**2** *9%*	**1** *4%*	**5** *22%*	**1** *4%*	**1** *4%*	**23** *100%*
Severe	**9** *41%*	**4** *18%*	**5** *23%*	**2** *9%*	**1** *5%*	**1** *5%*	**-**	**22** *100%*
CIS	**5** *50%*	**1** *10%*	**3** *30%*	**-**	**-**	**1** *10%*	**-**	**10** *100%*
Total	**46** *47%*	**19** *20%*	**13** *13%*	**5** *5%*	**8** *8%*	**4** *4%*	**2** *2%*	**97** *100%*

Table 3.22: Anatomical site of PMDs according to high/low grade dysplasia.

Histopathology (binary grading)	Anatomical site of PMDs							Total
	FOM	Lateral tongue	Ventral tongue	Buccal mucosa	Soft palate	Fauces	Alveolar mucosa	
Low grade dysplasia	25 *54%*	9 *20%*	3 *7%*	2 *4%*	4 *9%*	1 *2%*	2 *4%*	46 *100%*
High grade dysplasia	21 *41%*	10 *20%*	10 *20%*	3 *6%*	4 *8%*	3 *6%*	- 	51 *100%*
Total	46 *47%*	19 *20%*	13 *13%*	5 *5%*	8 *8%*	4 *4%*	2 *2%*	97 *100%*

Table 3.23: Degree of dysplasia in relation to high/low risk sites.

Anatomical site of PMDs	Histopathological diagnosis				Total
	Mild	Moderate	Severe	CIS	
High risk	36 *86%*	15 *65%*	18 *82%*	9 *90%*	78 *80%*
Low risk	6 *14%*	8 *35%*	4 *18%*	1 *10%*	19 *20%*
Total	42 *100%*	23 *100%*	22 *100%*	10 *100%*	97 *100%*

Clinical Types of PMDs and Oral Epithelial Dysplasia

Overall, 98% (41/42) of mild, 91% (21/23) of moderate, 86% (19/22) of severe dysplasia and 90% (9/10) of CIS appeared as leukoplakia. Erythroplakia manifested mainly in severe dysplasia 43% (3/7) and moderate 29% (2/7) and equally demonstrated in both mild and CIS 14% (1/7); Figure 3.20.
Using Chi-Square test, a significant association was found between the clinical appearance of PMDs (leukoplakia and erythroplakia) and the grade of oral epithelial dysplasia (p=0.035).

As can be seen in Figure 3.21, leukoplakia was equally seen in both low and high grade dysplasia (45/90); whereas 86% (6/7) of erythroplakias were observed within high grade dysplasia compared to 14% (1/7) in low grade dysplasia. However, the association was not significant (p=0.115; Fisher's Exact test).

Considering homogenous and non-homogenous leukoplakia subtypes, the majority of homogenous lesions were diagnosed as mild dysplasia 53% (35/66), whilst the majority of non-homogenous were diagnosed as severe dysplasia 38% (9/24); Table 3.24.
The relation between the grade of dysplasia and types of leukoplakia (homogenous and non-homogenous) approached statistical significance (p=0.051; Chi-Square test).

The majority of homogenous leukoplakias 58% (38/66) were diagnosed as low grade dysplasia, whereas the majority of non-homogenous leukoplakias were diagnosed as high grade dysplasia 71% (17/24); Figure 3.22.

There was a significant association between high/low grade dysplasia and clinical types of leukoplakia (homogenous and non-homogenous) (p=0.031; Fisher's Exact test).
Also, risk estimate showed that non-homogenous leukoplakia was 1.67 times higher risk for high grade dysplasia compared to homogenous leukoplakia.

Considering subtypes of non-homogenous leukoplakia, the speckled subtype showed mainly severe dysplasia 47% (7/15), followed by moderate dysplasia 27% (4/15). Ulcerated cases were equally observed in moderate and severe dysplasia (1/2); Table 3.25. However, no

significant association was found between the non-homogenous subtypes (speckled, exophytic, nodular and ulcerated) and the degree of dysplasia (WHO system) (p=0.792; Chi-Square test).

For the binary grading system, 80% (12/15) of the speckled subtype was diagnosed as high-grade dysplasia. Similarly, ulcerated PMDs were only diagnosed as high-grade dysplasia (2/2); while exophytic non-homogenous leukoplakia was more commonly seen as low-grade dysplasia 60% (3/5). The nodular subtype was equally observed in low and high grade dysplasia (1/2); however, Chi-Square test showed no statistical significance (p=0.304); Figure 3.23.

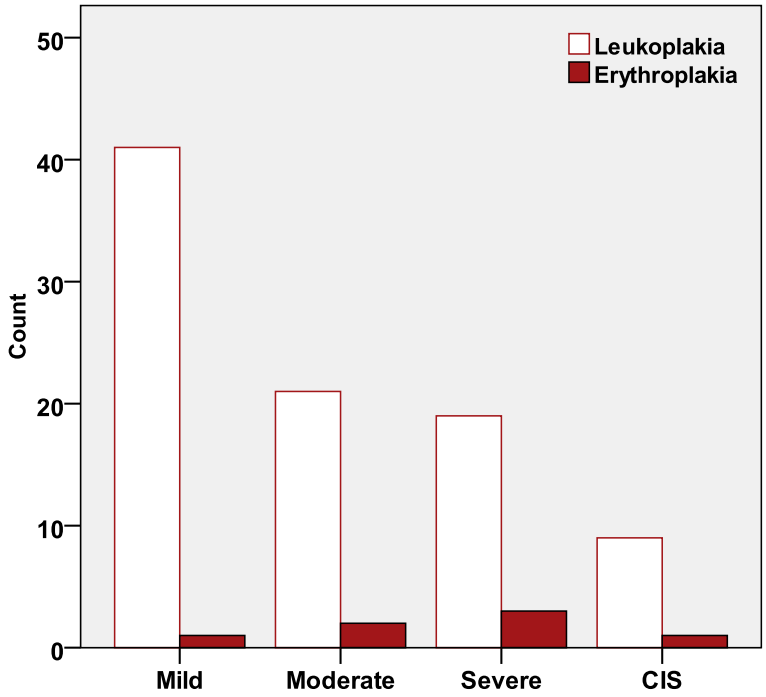

Figure 3.20: Distribution of leukoplakia and erythroplakia according to degree of dysplasia.

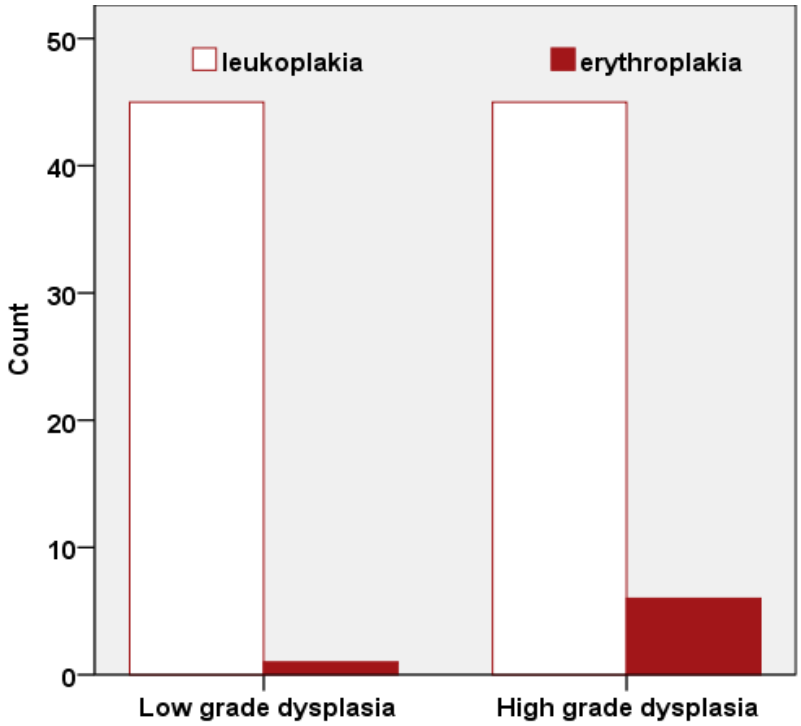

Figure 3.21: Distribution of leukoplakia and erythroplakia according to high/low grade dysplasia.

Table 3.24: Homogenous/non-homogenous leukoplakia in relation to degree of dysplasia.

Histopathology (WHO)	Leukoplakia type		Total
	Homogenous	Non-homogenous	
Mild	**35** *53%*	**6** *25%*	**41**
Moderate	**14** *21%*	**7** *29%*	**21**
Severe	**10** *15%*	**9** *38%*	**19**
CIS	**7** *11%*	**2** *8%*	**9**
Total	**66** *100%*	**24** *100%*	**90** *100%*

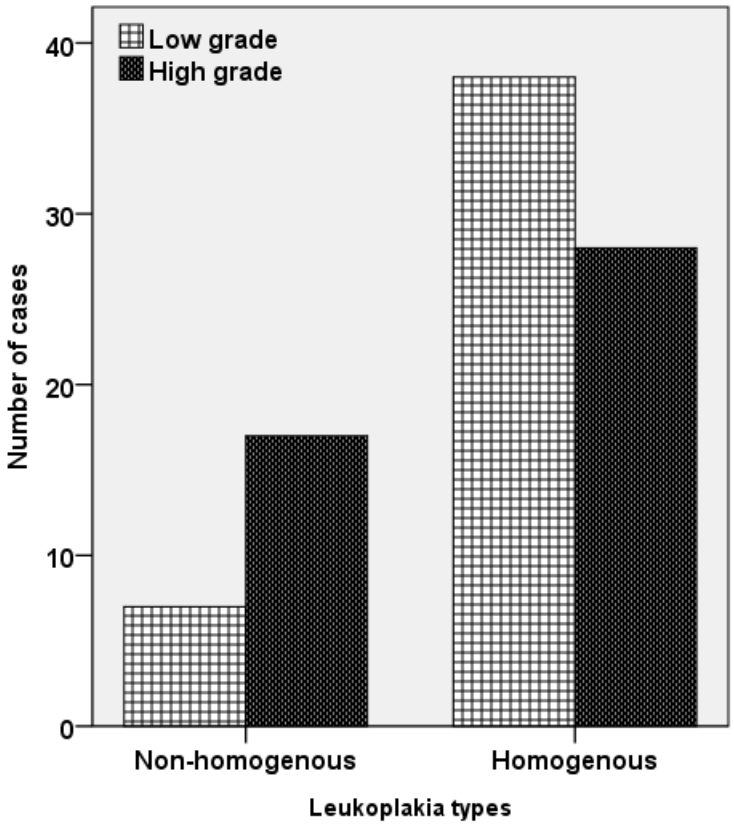

Figure 3.22: High/low grade dysplasia in relation to type of leukoplakia.

Table 3.25: Degree of dysplasia and non-homogenous leukoplakia subtypes.

Histopathology (WHO)	Non-homogenous leukoplakia subtypes				Total
	Speckled	Nodular	Exophytic	Ulcerated	
Mild	3 *20%*	1 *50%*	2 *40%*	-	6 *25%*
Moderate	4 *27%*	-	2 *40%*	1 *50%*	7 *29%*
Severe	7 *47%*	1 *50%*	-	1 *50%*	9 *38%*
CIS	1 *7%*	-	1 *20%*	-	2 *8%*
Total	15 *100%*	2 *100%*	5 *100%*	2 *100%*	24 *100%*

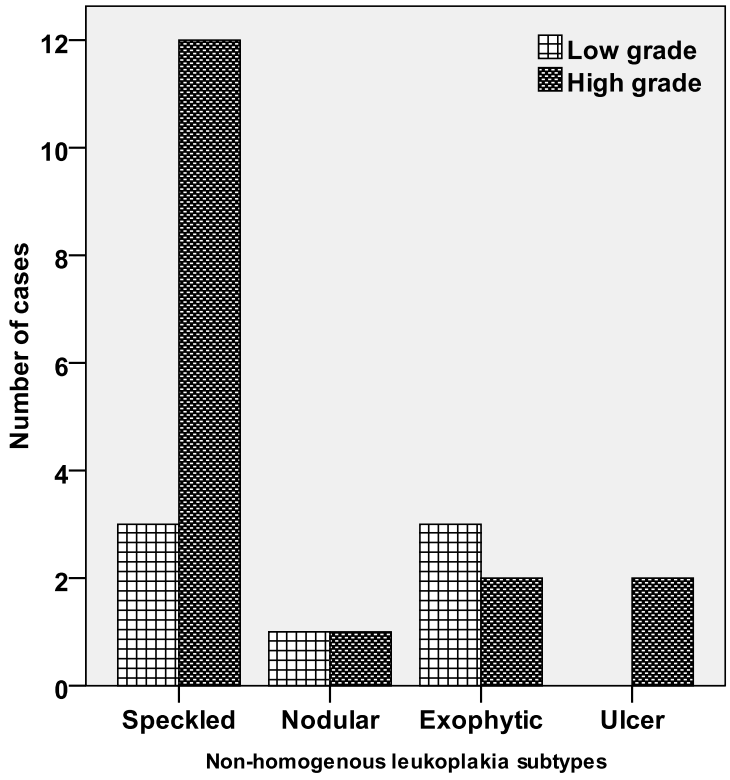

Figure 3.23: High/low-grade dysplasia and non-homogenous leukoplakia subtypes.

Sex, Clinical Types and Degree of Oral Epithelial Dysplasia

Generally, a higher proportion of leukoplakia was found in both males (59/66) and females (33/34). However, erythroplakia presented as high as 88% (7/8) in males compared to 12% (1/8) in females; Table 3.26.

However, the relation was not significant between sex and clinical appearance of PMDs (leukoplakia and erythroplakia) (p=0.259; Fisher's Exact test).

Homogenous and non- homogenous leukoplakias were more predominant in males compared to females. 72% of non-homogenous and 61% of homogenous leukoplakia was observed in males, whilst 28% of non-homogenous and 39% of homogenous leukoplakia was seen in females; Table 3.27.

In female patients, homogenous and non-homogenous PMDs showed mainly mild dysplasia; 54% of homogenous leukoplakia showed mild dysplasia, 8% CIS and the remaining 38% was divided equally between moderate and severe dysplasia. Similarly, 50% of non-homogenous leukoplakia was diagnosed as mild dysplasia, followed by 33% severe and 17% moderate dysplasia; Table 3.28.

Using Chi-Square test, no significant relation was seen between homogenous/non-homogenous leukoplakia and the degree of epithelial dysplasia in females (p=0.439).

In males, 53% (21/40) of homogenous leukoplakia showed mild dysplasia; followed by 23% (9/15) moderate dysplasia, while severe dysplasia and CIS were equally diagnosed (13%; 5/40 for each). Non-homogenous leukoplakia mainly exhibited severe (39%; 7/18) and moderate dysplasia (33%; 6/18); Table 3.29.

A significant association was seen between homogenous/non-homogenous leukoplakia and the degree of epithelial dysplasia in males (p=0.003; Chi-Square test).

Both males and females presented with higher proportion of speckled leukoplakia; 56% (10/18) of male patients and 86% (6/7) of females affected by speckled leukoplakia. While exophytic was more commonly seen in males 22% (4/18) compared to 14% (1/7) in females; nodular and ulcerated subtypes were only seen in males and in equal frequency (2/18); Table 3.30.

Taking into consideration the binary grading system in male patients, as can be seen in Figure 3.24 and Table 3.31; 83% (5/6) of erythroplakias in men were diagnosed as high grade dysplasia. Also, the majority of leukoplakia (52%) was identified as high grade dysplasia, although the relation was not significant (p=0.209; Fisher's Exact test).

Furthermore, in males 78% (14/18) of non-homogenous leukoplakias were diagnosed with high grade dysplasia, whilst the majority of homogenous was low grade 60% (24/40); Figure 3.25 and Table 3.32.

Using Fisher's Exact test, a significant association between leukoplakia types (homogenous and non-homogenous) and binary grading dysplasia was found in males (p=0.011; Chi-Square test). All ulcerated and 90% of speckled non-homogenous leukoplakia in males was identified as high-grade dysplasia; Figure 3.26 and Table 3.33.

However, the relation between binary dysplasia and non-homogenous subtypes was not significant (p=0.078; Chi-Square test).

In females, the majority of leukoplakias 53% were low grade dysplasia with one case of erythroplakia diagnosed as high-grade dysplasia; Figure 3.27 and Table 3.34.

Fisher's Exact test showed no significant relation between clinical type of PMD (leukoplakia/erythroplakia) and binary grading diagnosis in females (p=0.485).

Female non-homogenous leukoplakia was equally distributed in both high and low-grade dysplasia, but the majority of homogenous leukoplakia (54%) was low grade dysplasia; Figure 3.28 and Table 3.35. Statistically, Fisher's Exact test was not significant (p=1.000).

Regarding the non-homogenous subtypes, 60% of speckled subtypes in females exhibited high grade dysplasia with only one exophytic subtype which showed low-grade dysplasia; Figure 3.29 and Table 3.36. Statistically, Fisher's Exact test was not significant (p=1.000).

Table 3.26: Sex and clinical appearance of PMDs.

Sex	Leukoplakia	Erythroplakia	Total
Male	**59** *64%*	**7** *88%*	**66** *66%*
Female	**33** *36%*	**1** *12%*	**34** *34%*
Total	**92** *100%*	**8** *100%*	**100** *100%*

Table 3.27: Sex and type of leukoplakia.

Sex	Leukoplakia clinical type		Total
	Non-homogenous	Homogenous	
Male	**18** *72%*	**41** *61%*	**59** *64%*
Female	**7** *28%*	**26** *39%*	**33** *36%*
Total	**25** *100%*	**67** *100%*	**92** *100%*

Table 3.28: Clinical type of leukoplakia and degree of dysplasia in females.

Degree of dysplasia	Leukoplakia clinical type		Total
	Homogenous	Non-homogenous	
Mild	**14** *54%*	**3** *50%*	**17** *53%*
Moderate	**5** *19%*	**1** *17%*	**6** *19%*
Severe	**5** *19%*	**2** *33%*	**7** *22%*
CIS	**2** *8%*	**-**	**2** *6%*
Total	**26** *100%*	**6** *100%*	**32** *100%*

Table 3.29: Clinical type of leukoplakia and degree of dysplasia in males.

Degree of dysplasia	Leukoplakia clinical type		Total
	Homogenous	Non-homogenous	
Mild	**21** *53%*	**3** *17%*	**24** *41%*
Moderate	**9** *23%*	**6** *33%*	**15** *26%*
Severe	**5** *13%*	**7** *39%*	**12** *21%*
CIS	**5** *13%*	**2** *11%*	**7** *12%*
Total	**40** *100%*	**18** *100%*	**58** *100%*

Table 3.30: Sex and non-homogenous leukoplakia subtypes.

Sex	Non-homogenous leukoplakia subtype				Total
	Speckled	Nodular	Exophytic	Ulcerated	
Male	**10** *56%*	**2** *11%*	**4** *22%*	**2** *11%*	**18** *100%*
Female	**6** *86%*	-	**1** *14%*	-	**7** *100%*
Total	**16** *64%*	**2** *8%*	**5** *20%*	**2** *8%*	**25** *100%*

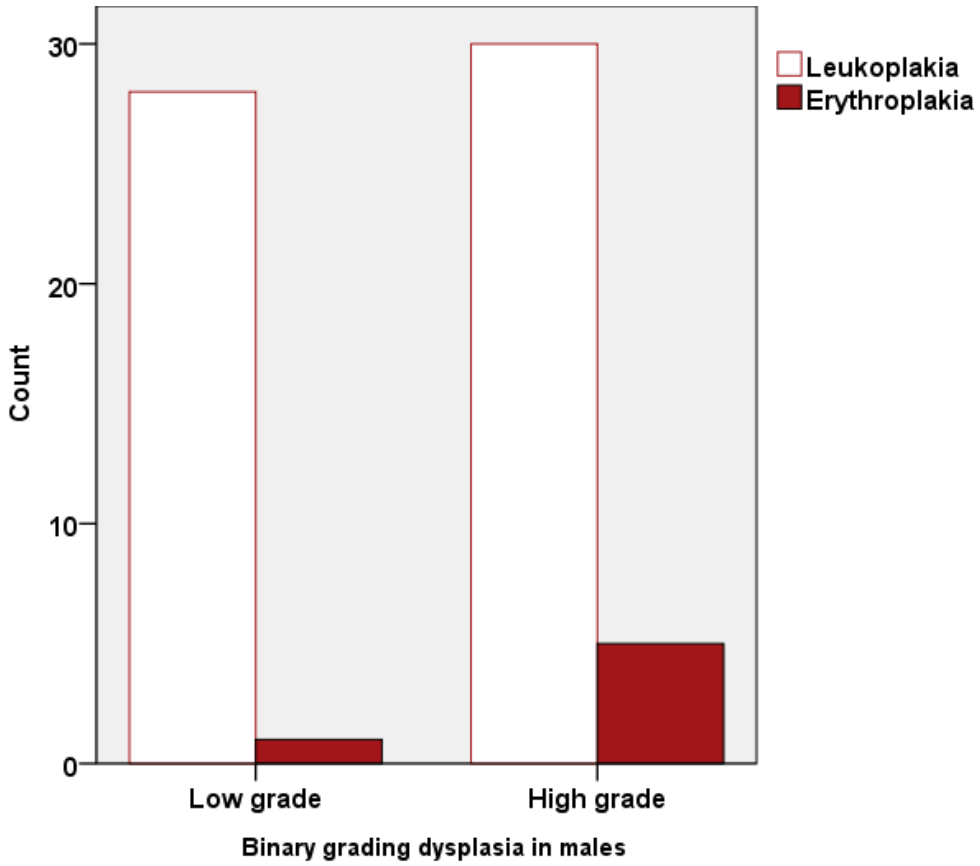

Figure 3.24: Leukoplakia and erythroplakia in relation to high/low grade dysplasia in males.

Table 3.31: Leukoplakia and erythroplakia in relation to high/low grade dysplasia in males.

Binary grading dysplasia	Leukoplakia	Erythroplakia	Total
Low grade	**28** *48%*	**1** *17%*	**29** *45%*
High grade	**30** *52%*	**5** *83%*	**35** *55%*
Total	**58** *100%*	**6** *100%*	**64** *100%*

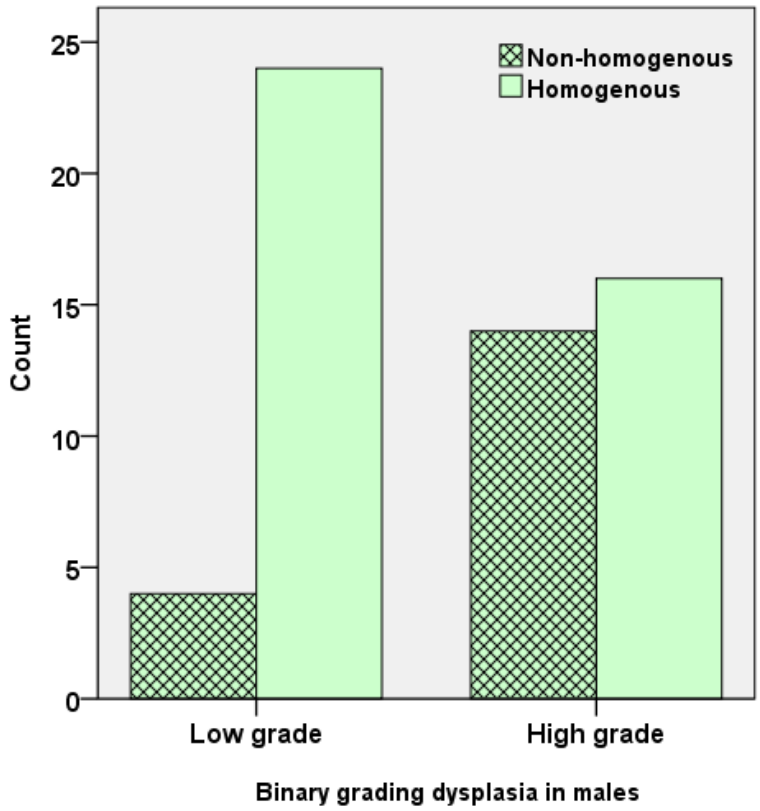

Figure 3.25: Types of leukoplakia and high/low grade dysplasia in males.

Table 3.32: Types of leukoplakia and high/low grade dysplasia in males.

Binary grading	Leukoplakia clinical type		Total
	Non-homogenous	Homogenous	
Low grade	**4** *22%*	**24** *60%*	**28** *48%*
High grade	**14** *78%*	**16** *40%*	**30** *52%*
Total	**18** *100%*	**40** *100%*	**58** *100%*

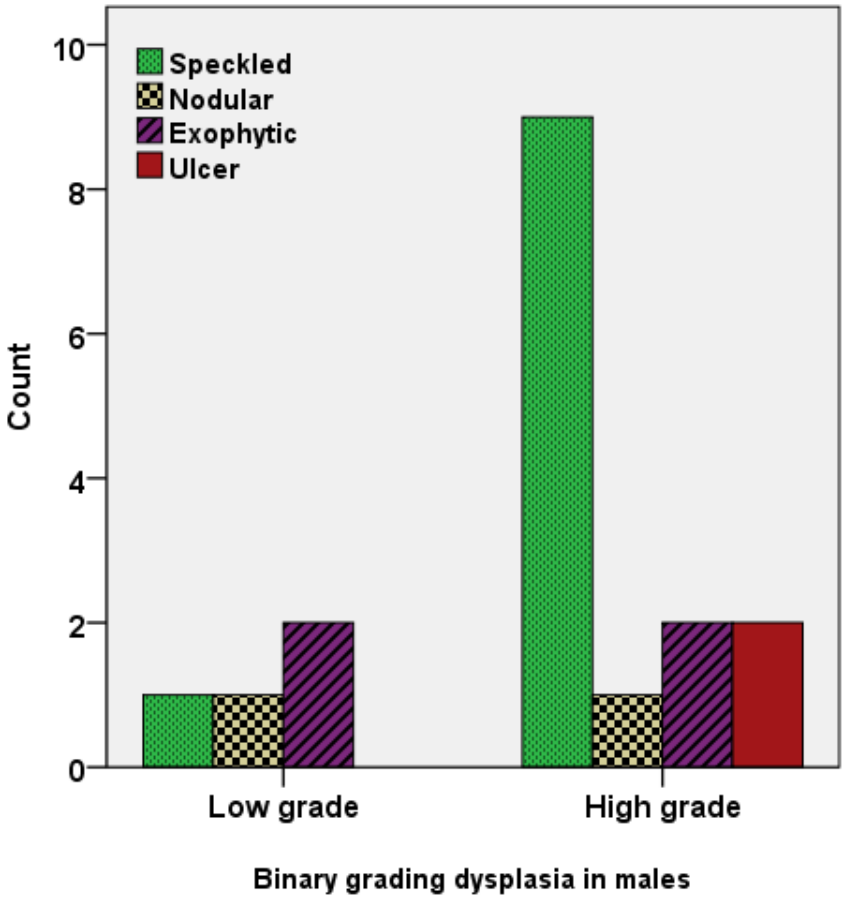

Figure 3.26: Non-homogenous leukoplakia subtypes according to high/low grade dysplasia in males.

Table 3.33: Non-homogenous leukoplakia according to high/low grade dysplasia in males.

Binary grading dysplasia	Non-homogenous leukoplakia				Total
	Speckled	Nodular	Exophytic	Ulcerated	
Low grade	**1** *10%*	**1** *50%*	**2** *50%*	**-**	**4** *22%*
High grade	**9** *90%*	**1** *50%*	**2** *50%*	**2** *100%*	**14** *78%*
Total	**10** *100%*	**2** *100%*	**4** *100%*	**2** *100%*	**18** *100%*

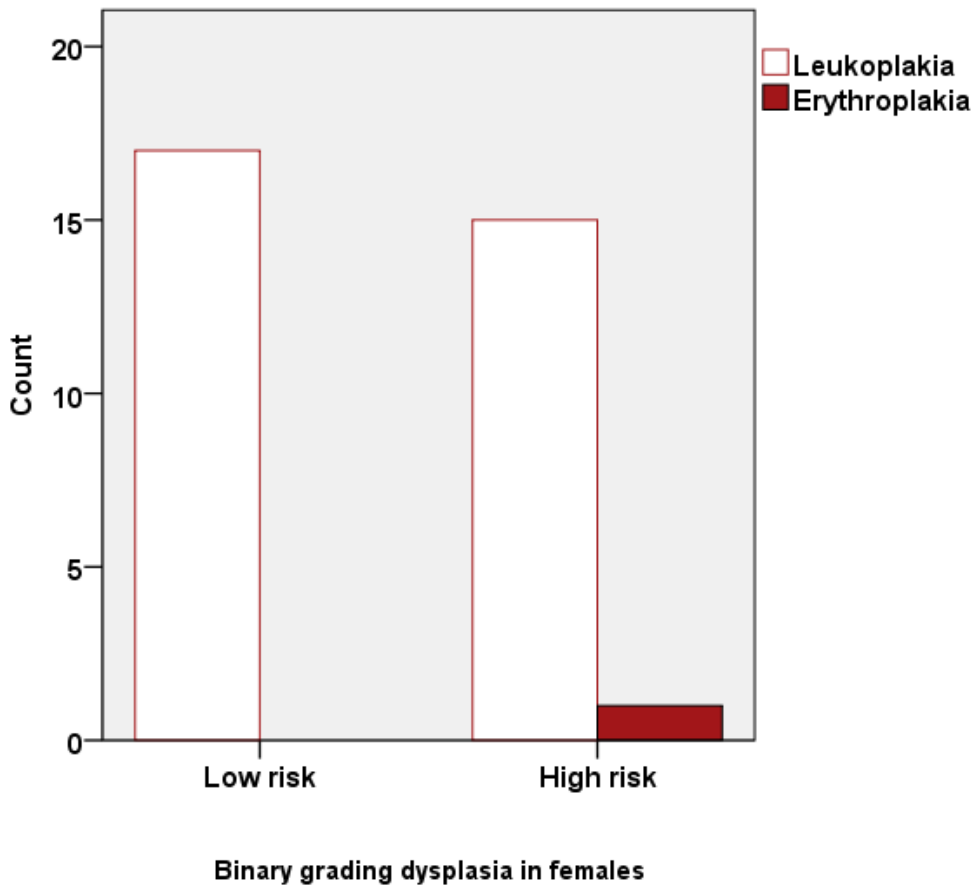

Figure 3.27: Leukoplakia and eythroplakia according to high/low grade dysplasia in females.

Table 3.34: Leukoplakia and eythroplakia according to high/low grade dysplasia in females.

Binary grading dysplasia	Leukoplakia	Erythroplakia	Total
Low grade	**17** *53%*	**-**	**17** *52%*
High grade	**15** *47%*	**1** *100%*	**16** *48%*
Total	**32** *100%*	**1** *100%*	**33** *100%*

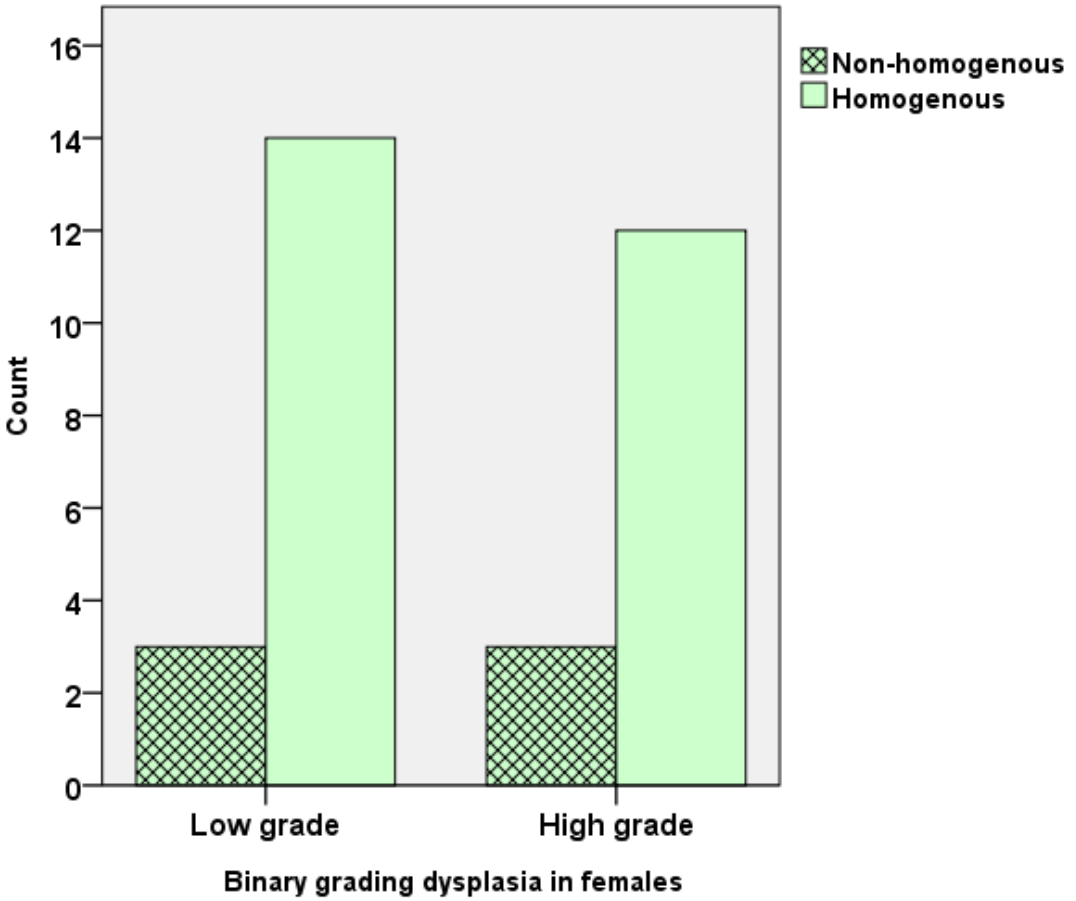

Figure 3.28: Leukoplakia types according to high/low grade dysplasia in females.

Table 3.35: Leukoplakia types according to high/low grade dysplasia in females.

Binary grading dysplasia	Leukoplakia clinical type		Total
	Non-homogenous	Homogenous	
Low grade	**3** *50%*	**14** *54%*	**17** *53%*
High grade	**3** *50%*	**12** *46%*	**15** *47%*
Total	**6** *100%*	**26** *100%*	**32** *100%*

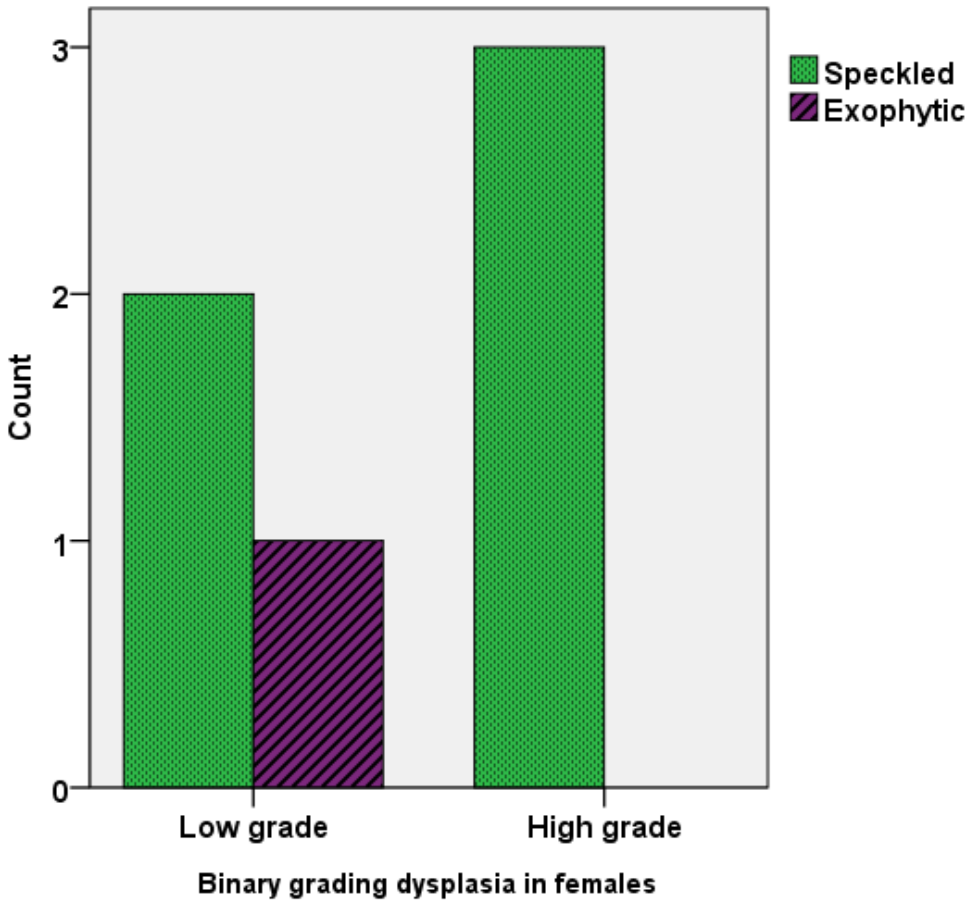

Figure 3.29: Non-homogenous leukoplakia subtypes according to high/low grade dysplasia in females.

Table 3.36: Non-homogenous leukoplakia subtypes according to high/low grade dysplasia in females.

Binary grading dysplasia	Non-homogenous subtypes		Total
	Speckled	Exophytic	
Low grade	**2** *40%*	**1** *100%*	**3** *50%*
High grade	**3** *60%*	**-**	**3** *50%*
Total	**5** *100%*	**1** *100%*	**6** *100%*

Size of PMDs and Oral Epithelial Dysplasia

Figure 3.30 shows that the majority of minor sized PMDs 49% (20/41) and 44% (20/45) of intermediate sized PMDs were diagnosed with mild dysplasia. 50% (5/10) of major sized PMDs were diagnosed with severe dysplasia with only one case of mild dysplasia seen in the major size group.

Chi-Square test indicated a significant relation between oral epithelial dysplasia (mild, moderate, severe and CIS) and size (minor, moderate and major) (p=0.029).
Subsequently, Spearman correlation revealed a positive significant correlation between the degree of dysplasia and size of PMDs (r=0.272; n=96; p< 0.01), whereby increased size of PMD was associated with increased severity of epithelial dysplasia; Figure 3.31.

Considering the mean size of PMDs, significant differences were observed among oral dysplasia groups (p=0.044; Kruskal-Wallis). Subsequently, Mann-Whitney U test was performed for pair-wise comparison and showed a significant difference between mild and severe dysplasia (239.44 vs. 375.70 mm^2) (p=0.026) and between mild and CIS (239.44 vs. 481.50 mm^2) (p=0.027).

Although there were clear differences in the mean size between mild/moderate, moderate /CIS, severe/CIS and moderate/severe, statistically Mann-Whitney U test showed no significance differences (p=0.796) (p=0.092) (p=0.477) (p=0.137), respectively.

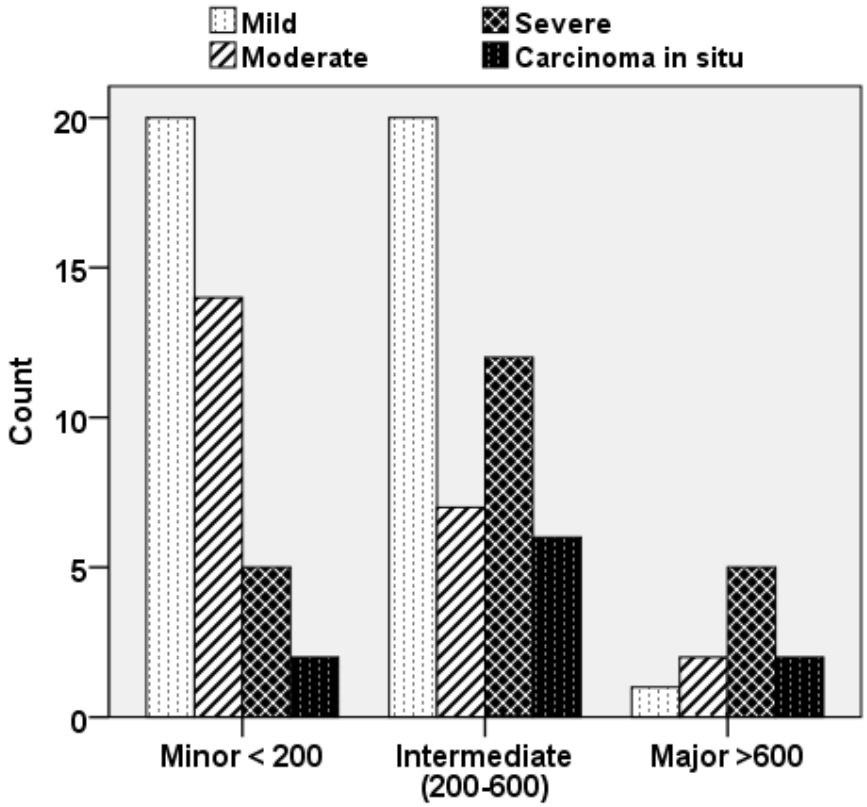

Figure 3.30: Degree of dysplasia in relation to PMD size category (mm^2).

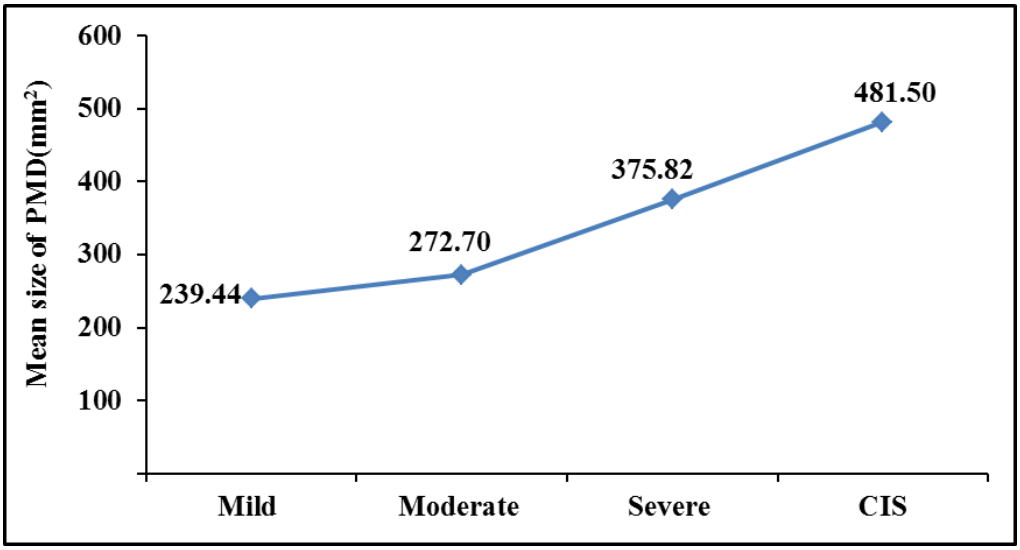

Figure 3.31: Mean size of PMDs in relation to degree of dysplasia.

3.4.7. Resection Margins

The status of resection margins was recorded for 87 patients, whilst no data were reported for the remaining 13 patients of the study population.

Surgical margins were clear in 44% (38). Residual dysplasia was reported in 56% (49) of the margins, distributed as 23% (20) mild, 15% (13) moderate and severe equally, and 3% (3) CIS; Figure 3.32.

As shown in Figure 3.33, clear margins were commonly seen in mildly dysplastic PMDs, while margins with residual dysplasia were more frequently seen in PMDs with higher dysplastic features (moderate, severe and CIS).

Figure 3.34 shows that mild dysplasia at the surgical margins was mainly seen in cases of mild dysplasia 60% (12/20), moderate dysplasia at the margin was mainly seen in moderate dysplasia cases 54% (7/13), severe dysplasia at the margin was usually seen in severe dysplasia cases 54% (7/13) and CIS at the resection margins was mainly seen in CIS cases 68% (2/3).

A highly significant association was found between histopathology of surgical margins and histopathology of laser excised tissue specimens (p=0.0001; Chi-Square test). Clear margins were mainly observed in mild dysplasia cases and decreased in frequency with increased dysplasia severity, whilst dysplasia at the resection margins was mostly the same grade of the excised lesion.

Furthermore, 58% (21/36) of free-margins and 65% (13/20) of mildly dysplastic margins were seen in low grade dysplasia cases, while 77% (10/13) of moderately dysplastic margins, 83% (10/12) of severely dysplastic margins and all margins with CIS (3/3) were observed in high grade dysplasia cases; Figure 3.35.

Using Kaplan-Meier analysis, a clear fall in the overall disease-free survival rates of patients with dysplastic margins can be seen in the Kaplan-Meier curve showing in Figure 3.36. The disease-free survival rates 1-year, 2-years, 3-years and 5-years post-laser surgery for patients with dysplastic margins were 87%, 70%, 59% and 43%, respectively. Comparing the disease-free survival rates of patients with free and dysplastic margins, Figure 3.37 shows lower rates

in cases with dysplastic margins than free-margins, 1-year (83% vs. 94%), 2-years (75% vs. 81%) and 4-years (55% vs. 61%) postoperatively; however, Log-Rank test was not significant (p=0.337).

A significant relationship was seen between the clinical extent of dysplasia and resection margin status (p=0.010; Chi-Square test); Table 3.37. Clear margins (23/40; 57%) were mainly observed in minor sized dysplasias compared with dysplastic margins (17/40; 43%). The majority of margins with residual dysplasia (6/9; 67%) were seen in major sized dysplasia compared to 33% (3/9) cases with clear margins. Similarly, for cases of intermediate size, 72% (26/36) showed dysplasia in the margins compared to 28% (10/36) with clear margins.

Table 3.38 shows the relationship between the histopathology of resection margins and the size of the excised dysplasia; a highly significant association was found (p=0.005; Chi-Square test). The majority of residual dysplasia in margins of minor sized cases (10/46) were mild dysplasia, followed by moderate and then severe dysplasia–CIS. Major sized cases were associated with higher grades dysplasia in the margins; 33% (3/9) severe dysplasia-CIS, followed by 22% (2/9) moderate dysplasia, whilst mild dysplasia was least common (11%; 1/9).

Four groups were defined according to the number of margins involved by residual dysplasia: one, two, three or four. The majority of cases only showed one margin involvement (61%; 30/49), followed by four margins (17%; 8/49), three (10%; 5/49) and then two (12%; 6/49); Figure 3.38.

Out of the 49 cases with dysplasia in the margins, 65% were male (32/49) and 35% (17/49) female. A significant relation was found between sex and degree of dysplasia in the margins (p=0.012; Chi-Square test); higher grades of dysplasia in the margins were mainly associated with male patients, whilst residual mild dysplasia was most commonly seen in females; Figure 3.39.

The extent of resection margin involvement was higher in males compared to females; Figure 3.40. All three margin involvement cases, 75% of four margin, 67% of two margin and 57% of one margin involvement were all observed in males. However, the relation between sex and extent of dysplasia in the margins (number of margins involved) was only marginally significant (p=0.051; Chi-Square test).

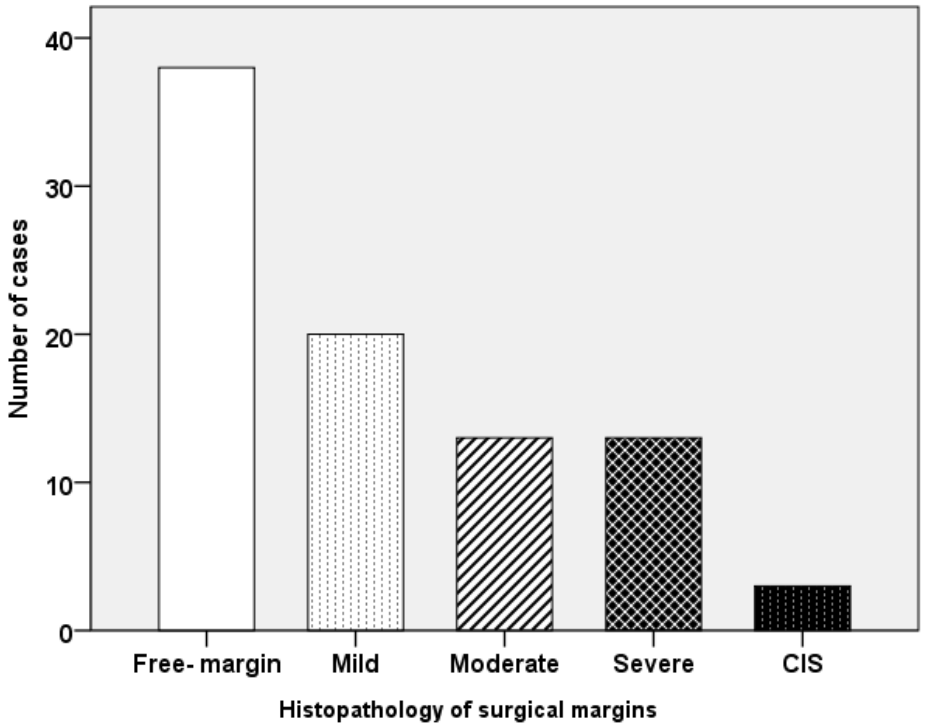

Figure 3.32: Histopathological diagnosis of surgical margins of PMD patients.

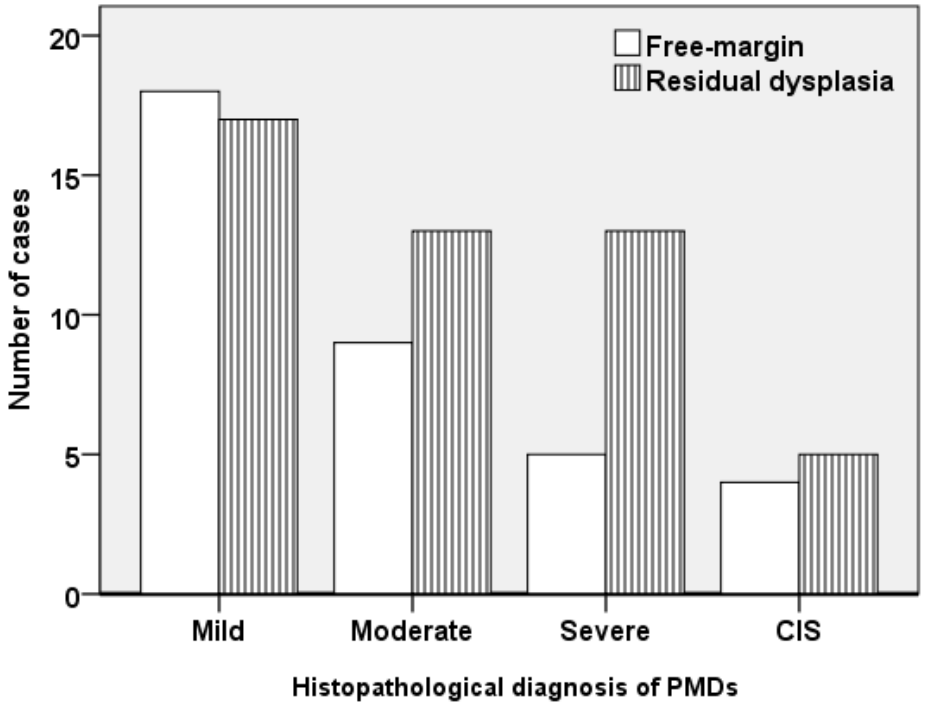

Figure 3.33: Resection margin status and PMD histopathology diagnosis.

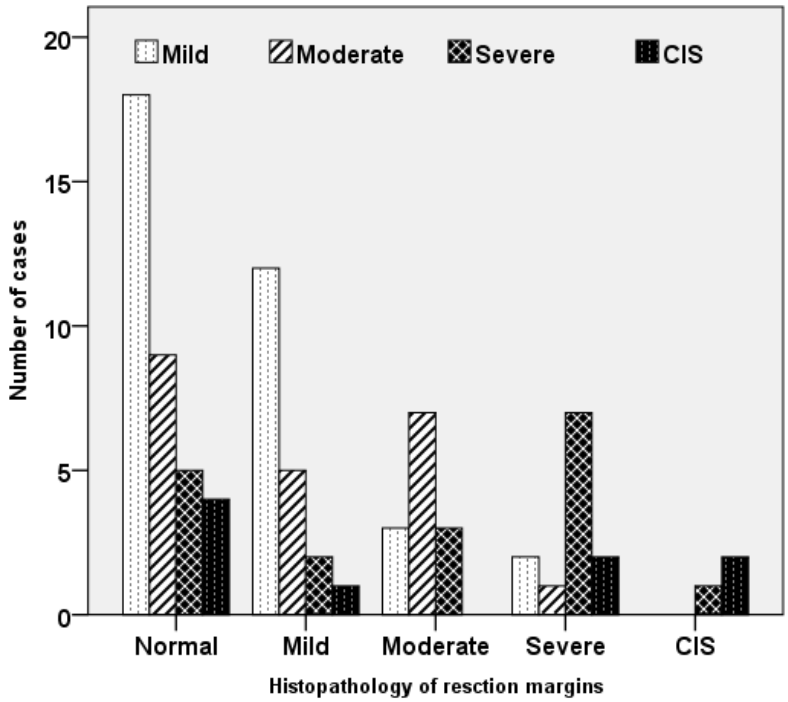

Figure 3.34: Histopathological diagnosis of PMDs in relation to histopathological diagnosis of resection margins.

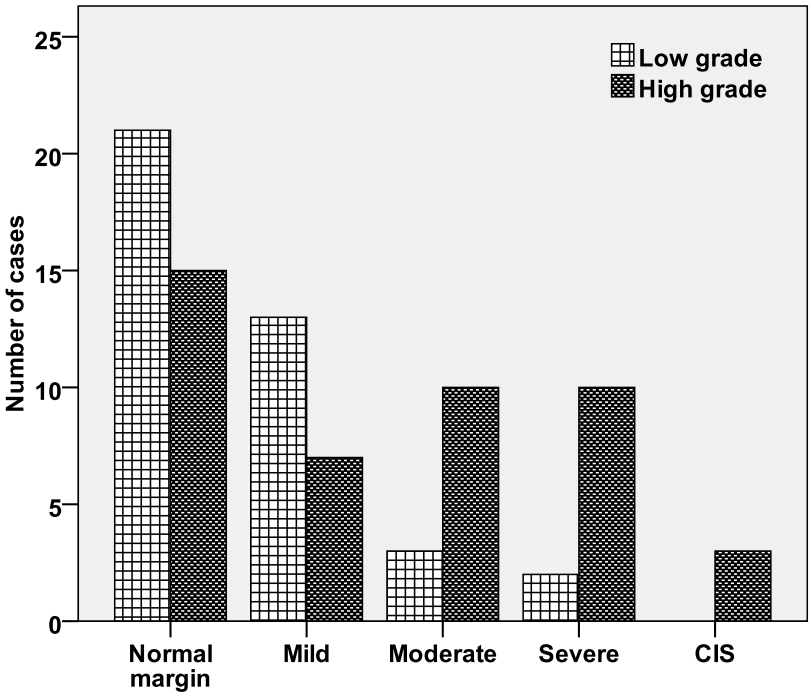

Figure 3.35: Distribution of high/low grade dysplasia according to histopathology of resection margins.

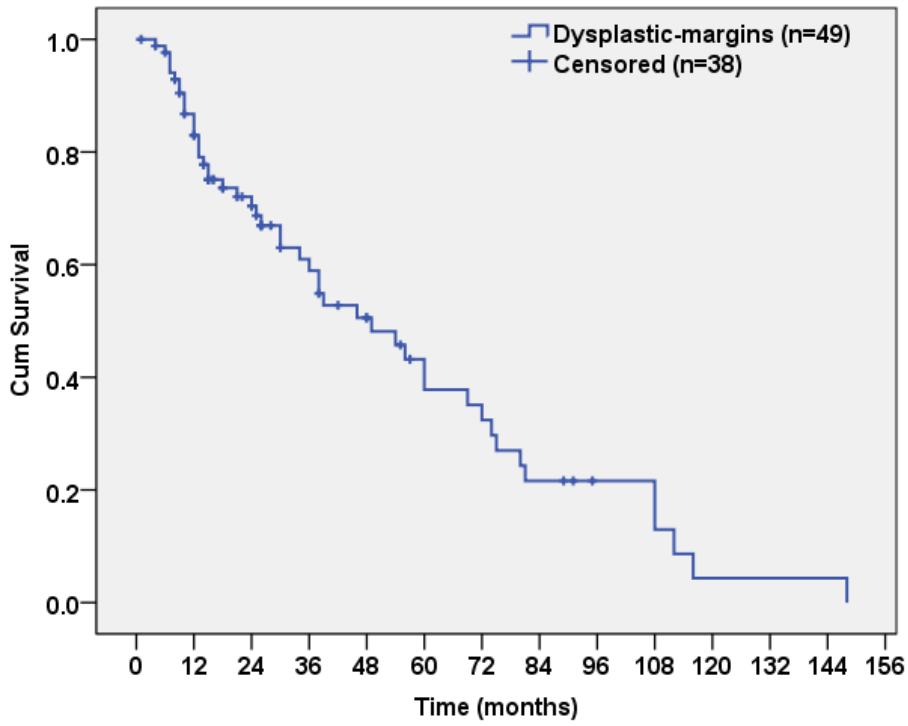

Figure 3.36: Overall disease-free survival rate of dysplastic margins by Kaplan-Meier analysis.

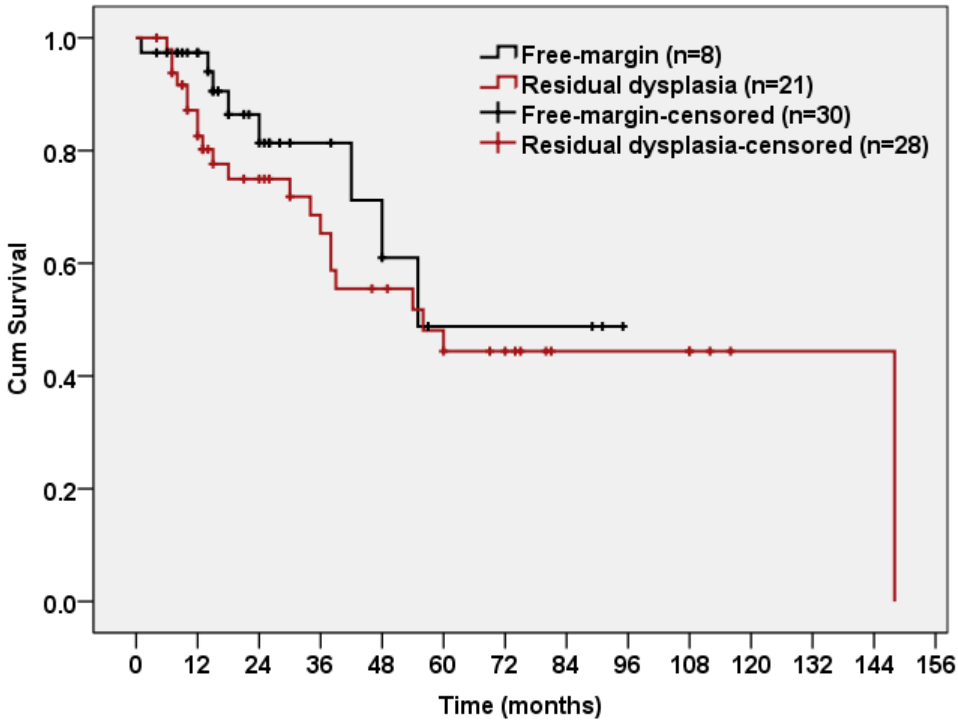

Figure 3.37: Kaplan-Meier analysis of patients with free and dysplastic margins.

Table 3.37: Resection margin status in relation to PMD size category.

Size of PMD (mm^2)	Resection margin status		Total
	Free-margin	Dysplastic	
Minor < 200	**23** *57%*	**17** *43%*	**40** *100%*
Intermediate (200-600)	**10** *28%*	**26** *72%*	**36** *100%*
Major > 600	**3** *33%*	**6** *67%*	**9** *100%*
Total	**36** *42%*	**49** *58%*	**85** *100%*

Table 3.38: Histopathology of resection margins in relation to PMD size category.

Size of PMD (mm^2)	Histopathology of resection margins				Total
	Free-margin	Mild	Moderate	Severe	
Minor < 200	**23** *57%*	**10** *25%*	**4** *10%*	**3** *8%*	**40** *100%*
Intermediate (200-600)	**10** *28%*	**9** *25%*	**7** *19%*	**10** *28%*	**36** *100%*
Major > 600	**3** *33%*	**1** *11%*	**2** *22%*	**3** *33%*	**9** *100%*
Total	**36** *42%*	**20** *24%*	**13** *15%*	**16** *19%*	**85** *100%*

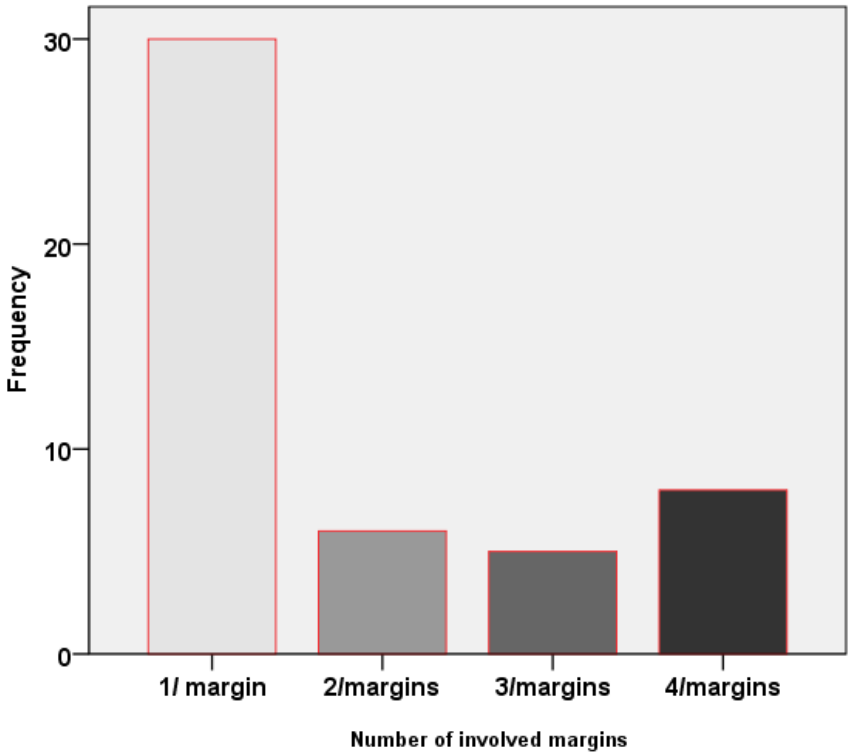

Figure 3.38: Distribution of margin involvement in dysplastic resection margins.

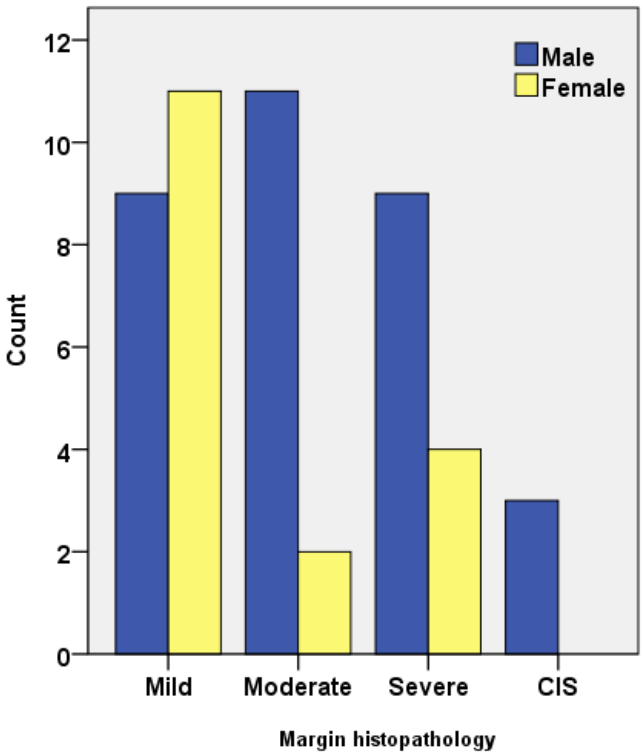

Figure 3.39: Sex and resection margin histopathology.

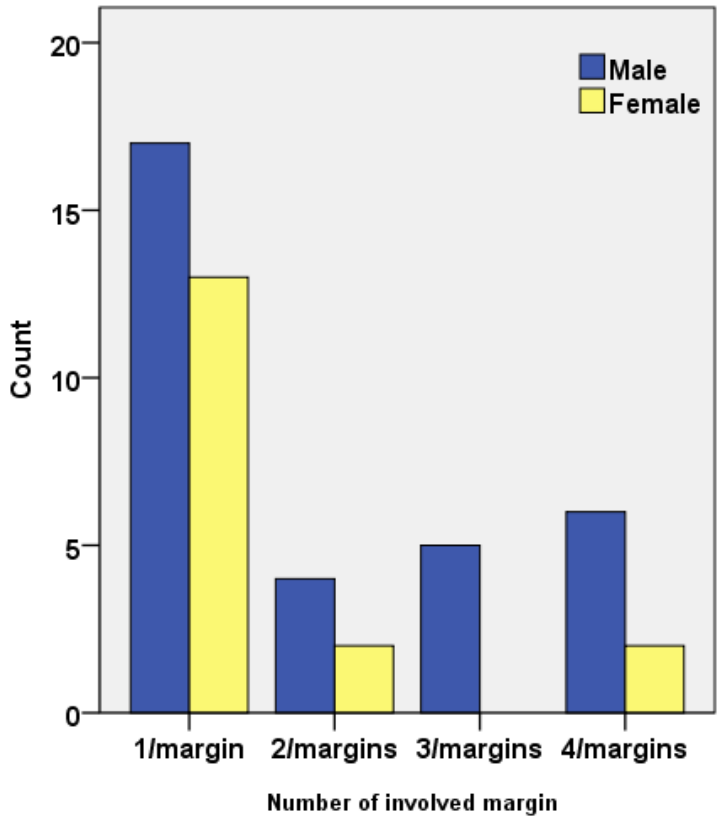

Figure 3.40: Sex and the number of involved margins.

3.4.8. Inter-observer Variation between Pathologists

To assess the variability between pathologists in grading oral epithelial dysplasia, a total of 106 post-laser specimens were independently scored by 2 oral pathologists using both the WHO scoring system of mild, moderate, severe dysplasia, CIS and the binary grading system as high/low-grade dysplasia.

The degree of agreement between the pathologists was characterized by Kappa statistics.
For the WHO scoring, Kappa statistics showed a substantial agreement between the two pathologists (Kappa value=0.644, $p< 0.001$).
As shown in Table 3.39, although a complete agreement between the 2 pathologists in grading CIS, the disagreement between the two oral pathologists was mainly seen in 10 cases; 5 moderate, 4 severe cases with only 1 mild dysplasia.

Considering the binary grading system, Kappa analysis showed also a substantial agreement between the two pathologists (Kappa value=0.756, $p< 0.001$); however, the Kappa value was higher compared with the WHO scoring system which indicates a higher agreement between pathologists using the two grading system.
Table 3.40 shows that the 2 pathologists disagreed in 9 cases in high grade dysplasia which were scored as low grade dysplasia with similar number of cases in low grade dysplasia which were graded as high grade dysplasia.

Table 3.39: Crosstabulation of the WHO scoring of oral epithelial dysplasia of two oral pathologists.

1st pathologist (WHO scoring)	2nd pathologist (WHO scoring)				Total
	Mild	Moderate	Severe	CIS	
Mild	42 *89%*	5 *11%*	-	-	47 *100%*
Moderate	4 *16%*	17 *68%*	4 *16%*	-	25 *100%*
Severe	-	7 *29%*	14 *58%*	3 *13%*	24 *100%*
CIS	-	1 *10%*	2 *20%*	7 *70%*	10 *100%*
Total	46 *43%*	30 *28%*	20 *19%*	10 *9%*	106 *100%*

Table 3.40: Crosstabulation of binary scoring system of two oral pathologists.

1st pathologist (binary scoring)	2nd pathologist (binary scoring)		Total
	Low grade	High grade	
Low grade	47 *81%*	11 *19%*	58 *100%*
High grade	2 ***4%***	46 ***96%***	48 ***100%***
Total	49 *46%*	57 *54%*	106 *100%*

3.5. Discussion

Establishing an accurate and accessible dataset for oral PMD patients in the North East of England was an important aim of this study as it is vital for patients' management by providing a baseline for comparison at follow-up appointments and comprises a unique data set for further investigations.

The patient cohort in this study comprised more males (66%) than females (34%) (ratio 2: 1) which is in line with most previous studies (Silverman et al., 1976; Gupta et al., 1980; Axell, 1987; Jaber et al., 2003; Jaber, 2010).
The International Standard Classification of Occupations (ISCO-08) was used to classify the occupation of the study population, although the majority of patients were either retired (41%) or unemployed (25%). As expected, females were predominant in elementary work with males and females equally seen in clerical and technical work. Assessing the influence of different occupations on the clinicopathological features of PMDs was not feasible because of the small number of patients in each group.

The study demonstrated that 90% of patients with dysplastic PMDs were over the age of 40 years and 56% of patients were between 41-62 years, regardless of sex, which agrees with the results of other studies (Waldron and Shafer, 1975; Schepman et al., 1998; Jaber et al., 2003). This also supports a view that leukoplakia, the most common PMD, is not usually diagnosed before the age of 30-years, with it's highest prevalence in the fifth decade (van der Waal et al., 1997).

Excisional specimens are able to provide a much more representative tissue sample for the assessment of the degree of dysplasia compared to incisional specimens, which may be inadequate for definitive diagnostic purposes due to sampling errors (Goodson and Thomson, 2011). Thus, in the present study, excisional specimens were used, with the highest dysplasia score recorded.
Overall, mild dysplasia was the predominant dysplastic feature seen in our study sample (43/100) which is quite similar to the findings recognized in a study of Western European residents conducted by Jaber *et al*. (2003) (47%; 297/630). However, a lower proportion of

mild dysplasia 37% (167/456) was reported in a study of 456 patient hospital records with oral epithelial dysplasia (Jaber, 2010). Whereas a higher percentage of mild dysplasia (65%; 135/207), was reported in a retrospective hospital-based study conducted by Arduino *et al.* (2009). Using incisional biopsies instead of excisional specimens in Arduino *et al.'s* study may explain the higher percentage of mild dysplasia compared to other studies.

In this study, a higher grade dysplasia was observed with advancing age, but ANOVA test was not significant; 70% of mild dysplasias were identified in patients less than 40 years, whilst 60% of severe dysplastic features were recognized in patient older than 40 years. Likewise, 94% of high grade dysplasia was reported in patient older than 40 years and 70% of young patients were associated with low grade dysplasia. This is consistent with previous studies that showed higher dysplastic features affecting patients in their 50s and 60s (Lumerman et al., 1995; Arduino et al., 2009).

Mild and severe dysplasia was more predominant in females, whilst moderate dysplasia and CIS was more commonly seen in males; however, the relation was not significant between sex, age and the degree of epithelial dysplasia which agrees with other studies (Jaber et al., 2003; Arduino et al., 2009). The non-significant relation may be due to the small sample size in some dysplastic groups.

In the current study, the majority of high grade dysplasia (81%) was identified at high-risk sites (FOM and tongue) compared to 19% in low-risk sites (all other remaining oral sites).

In the present study, the FOM and lateral tongue surfaces were the most frequent sites for oral dysplastic PMDs. This is similar to the findings of Mincer *et al.* (1972), Thomson and Wylie (2002), and Hamadah (2007). Others have reported that buccal mucosa, FOM (Jaber et al., 2003); gingiva, tongue and palatal mucosa (Ishii et al., 2004); FOM, lip, tongue, and buccal mucosa (Lim et al., 2010) and lateral border of the tongue followed by the buccal mucosae (Arduino et al., 2009) are the most common sites for PMDs. The differences in the most common oral subsites may be related to geographical differences, race, and social habits (Ishii et al., 2004).

Male patients were common in all affected oral sites, although females showed a higher percentage of FOM and lateral tongue sites compared to males, whilst the soft palate, retromolar area and alveolar mucosa were not affected in female patients. In this study, a statistically significant relation between patient sex and site of dysplastic PMDs was found which is inconsistent with a study conducted by Jaber *et al.* (2003) who investigated the clinical characteristics of patients with oral epithelial dysplasia attended the oral medicine clinics in London and Bristol between 1972 and 1996.

Considering site-distribution and the age of patients, 80% (8/10) of FOM cases were reported in patients ≤ 40 years compared to 42% (38/90) in patients > 41 years. This may support the view that all patients younger than 40 years were tobacco users, whilst patients > 40 years were a mixture of user and non-users. Similar to what has been reported by Schepman *et al.* (2001), that FOM leukoplakia was the most common lesion in tobacco smokers.

In this study, the majority of cases presented as leukoplakias (92%) which agrees with Chadu and Smith's study (2005). Most leukoplakias were homogenous (67%) with a smaller proportion of non-homogenous leukoplakias (25%) and erythroplakias (8%) which is in agreement with other studies (Jaber et al., 2003; Amagasa et al., 2006). However, Jaber *et al.* (2003) showed a higher incidence of non-homogenous leukoplakias and lower erythroplakias compared to this study, at 45% and 2% respectively. Holmstrup *et al.* (2006) reported a higher percentage of non-homogenous leukoplakias (49%) than homogenous type, with erythroplakia (9%) in accordance with this present study.

Clinical appearance and localisation of PMDs was significantly associated in the present study. Ulcerated PMDs were only seen in the lateral tongue as a high-risk site, speckled leukoplakias and erythroplakias were more frequently reported in the FOM and lateral tongue as a high risk site, and 60% of exophytic subtypes were seen in low-risk sites, such as buccal mucosa, whilst nodular subtypes were only seen in ventral tongue and buccal mucosa. There is no clear explanation for the site predilection for specific clinical appearance of PMDs, but this may be due to the biology of each particular clinical type which favours a specific anatomical site.

In the present study, all erythroplakia cases (8/8) were reported in patients over 41 years old, with 88% (7/8) reported in males, 83% of which exhibited severe dysplasia (high grade dysplasia) compared to 12% (1/8) in female patients. Similar findings were reported by Scully (2004), although other studies have demonstrated no sex predilection (Shafer and Waldron, 1975; Hashibe et al., 2000a). There is no obvious reason why erythroplakia is more predominant in males and in patients over 41 years.

Also, 72% of non-homogenous leukoplakia and 61% of homogenous leukoplakia were reported in male patients with 39% of non-homogenous leukoplakia exhibiting severe dysplasia (high grade dysplasia), whereas 53% of homogenous showed mild dysplasia (low grade dysplasia).

Regarding the clinical appearance of PMDs and their degree of dysplasia, erythroplakia primarily showed severe dysplasia, with 86% of it observed in high-risk sites.
In the current study, the relation between the degree of dysplasia and leukoplakia types was significant (p=0.031; Fisher's Exact test). The majority of homogenous leukoplakia (58%) was identified as a low grade dysplasia in which mild dysplasia was most frequent (53%). The majority of non-homogenous leukoplakia (71%) was recognized as high grade dysplasia of which severe dysplasia was the most common (38%).

This study has demonstrated that non-homogenous leukoplakia displayed a 1.7 times increased risk for high grade dysplasia compared to homogenous lesions. 80% of speckled non-homogenous subtypes were mainly diagnosed as high grade dysplasia, in which severe dysplasia formed the most frequent grade (47%). All ulcerated cases showed high grade dysplasia and 60% of exophytic subtypes were low grade dysplasia. These associations in our study were statistically significant and agree with a recent study conducted by Vazquez-Alvarez *et al.* (2010) on the correlation between clinical and pathological diagnoses for OL in 54 patients.

Considering non-homogenous subtypes and sex, this study showed that all ulcerated and nodular cases, 80% of exophytic and 63% of speckled subtypes were seen in male patients, with all ulcerated and 90% of speckled showing high grade dysplasia. In females, no nodular

or ulcerated subtypes were seen, with the majority of speckled subtypes (60%) exhibiting high grade dysplasia.

In the current study, the size of dysplastic PMDs was recorded by multiplying the length by the width of the laser excised specimens obtained from the pathological reports, which is similar to Holmstrup *et al.* (2006). It is worth noting that obtaining a precise measurement of microscopic specimens is problematic and shrinkage by 1 mm of specimens after formalin fixation is a general consensus amongst surgeons and pathologists (Huang et al., 2010). This shrinkage will affect all samples equally. However, taking the measurement from the histopathological report remains the most accurate method because recorded measurements in the medical records of patients are not always appropriately standardised.

A positive correlation between lesion size and patient age was found in this study ($r=0.222$, $n=98$, $p<0.01$; Pearson correlation). The mean size increased with increasing age of patients; however, this correlation was is not significant when considering the four age groups, but was significant when the 4 age groups were compressed into two. Larger sized PMDs may have been present for a longer time compared to small size lesions (Napier et al., 2003) and this may explain the chronicity of the disorder with age. Male patients presented with greater mean sized PMDs compared to female patients (344.5 vs. 216.2 mm^2), but this did not reach statistical significance. The bigger size of PMDs in males and with increased age has no obvious cause, but may reflect a general neglect of oral health by males compared to females.

In the present study, the FOM was the main site for minor sized PMDs (50%), whilst lateral tongue was the principal site for major sized PMDs (55%). While no major sized PMDs were seen in low-risk sites, major sized PMDs were observed only at high-risk sites (10/10).
A lower statistically significant mean size of PMDs in the FOM was seen compared to lateral and ventral tongue surfaces (200.8 vs. 591.6, 369.4 mm^2), respectively. There is no clear explanation for the differences in the size of dysplasia affecting the tongue and the FOM.

Considering the relation between clinical appearance and size, whilst 63% of erythroplakias were minor in size, a significant relation was found between size and non-homogenous subtypes, with 60% of exophytic minor sized, 81% of speckled subtypes intermediate in size,

and all ulcerated PMDs major sized. There is no clear reason for the differences in the sizes among the non-homogenous subtypes.

The current study showed a positive significant correlation between the size of PMDs and the degree of oral epithelial dysplasia present, with an increased severity of dysplasia associated with larger sized PMDs. Histopathologically, 49% of minor sized and 44% of intermediate sized PMDs were associated with mild dysplasia, whilst 50% of major sized lesions showed severe dysplasia. A significant difference in the mean size of mild dysplasia from both severe dysplasia and CIS was observed; mild dysplasia was significantly smaller in size than severe dysplasia and CIS. However, differences in the mean size between mild-moderate, moderate-severe, moderate-CIS and severe-CIS did not reach level of significance. Dysplasia severity may be related to a longer time of presentation and larger sized PMDs may be related to the chronicity of the PMD, perhaps explaining the observed relation between higher grade of dysplasia and larger sized PMDs.

In this study, both the WHO (2005) and binary systems were used for grading oral epithelial dysplasia by two expert oral pathologists using a consensus diagnosis for both grading systems. A substantial agreement using Kappa statistics for both WHO and binary grading systems was seen; with an improvement in the pathologists' agreement using the binary scoring system compared to the WHO system. This is clearly reflected by the higher kappa value for the binary system compared to the WHO (Kappa=0.756 compared to 0.644).

The improvement in the pathologists' agreement for the binary scoring system is consistent with a previous study (Kujan et al., 2006). The two-scoring scheme combines severe dysplasia, CIS and the majority of moderate dysplasia into high grade dysplasia, with mild dysplasia categorised as low grade dysplasia, providing an easier, less subjective system with better discriminatory powers than the WHO scoring system (Kujan et al., 2006).
However, it is worth noting that poor to moderate inter-observer agreement, which can be related to subjective judgments and varied individual pathologist experience and which has been reported in several previous studies (Pindborg et al., 1985; Karabulut et al., 1995; Kujan et al., 2007), was not found in the current study and this is probably due to the highly expert

oral pathology team who assessed the grade of oral epithelial dysplasia in our study specimens.

Microscopically, the resection margin was defined as the distance between the outer edge of the dysplastic tissue and the cut edge of the tissue specimen. In this study the resection margin status of 87 excisional specimens were obtained from histopathological reports but no data on margins were available for the rest of the cases. This was either due to the thermal damage of margins due to heating effect from laser intervention making the pathological reporting difficult (Jerjes et al., 2012) or the margin status was not recorded in the pathology report.

The study showed that clear margins were reported in 44% of cases (38/87), while the remaining 56% (49/87) showed different grades of residual dysplasia. A significant association was found between margin status and the degree of dysplasia, with clear margins primarily seen in mild dysplasia cases. Interestingly, margins showing mild dysplasia were more frequently observed in mild dysplasia cases which were similar for moderate, severe and CIS margins which were all more commonly reported in lesions with the same grade of dysplasia.

The clinical extent of dysplastic PMDs was significantly associated with resection margin status; clear resection margins were mainly seen in minor sized lesions whilst dysplastic margins were more frequently observed in major and intermediate sized PMDs. Similarly, a significant relation was found between the size of PMDs and the degree of dysplasia in the margin; major sized dysplasias primarily showed higher grades of dysplasias in their margins, whereas minor sized dysplasia frequently exhibited only mild dysplasia in the margins. These observations clearly reflect an association between the clinical extent of dysplasia and the risk of residual foci of dysplasia occurring in surgical margins. Since large size dysplasias may have been present for a long time, with subsequently more extensive dysplastic changes arising in the mucosa, removal of the entire disease becomes more difficult with an increased likelihood of incomplete surgical excision due to biological and anatomical restriction.

In this study, the extent of dysplasia in margins was also investigated; one margin involvement was the most common, followed by 4 margins, three margins and 2 margins involvement. Males were more frequently affected with dysplastic margins and more margin involvement with significantly higher grade dysplasia in their resection margins compared to females. This may be related to the fact that male patients had larger sized PMDs (344.5 vs. 216 mm^2) and exhibited more severely dysplastic features in their PMDs compared to females; however, this was not significant.

In the present study, Kaplan-Meier analysis showed lower cumulative disease-free survival rates for patients with dysplastic margins compared to patients with free resection margins; the disease-free survival rate of patients with dysplastic margins dropped from 87% 1-year after laser intervention to 75% at 2-years, 65% at 3-years and 44% 5-years post-laser treatment. While disease-free survival rates of patients with non-dysplastic margins 1-year, 2-years, 3-years and 5-years post-laser surgery, were 97%, 81%, 81% and 49%, respectively.
This may suggest that the status of the resection margins is an important prognostication parameter and any dysplastic margin may require re-intervention or very close follow-up taking in consideration other clinicopathological and associated risk factors.

3.6. Conclusions

1. Males were most common in our cohort, with a male to female ratio was 2: 1.
2. Ninety-percent of our PMDs patients were over the age of 41 years.
3. The majority of this study population were retired and married.
4. The most common PMD sites in these patients were the FOM and lateral tongue surface.
5. Females had the highest percentage of FOM PMDs (53%) and lateral tongue (26%) compared to males, at 42% and 15%, respectively.
6. The soft palate, retromolar area and alveolar mucosa were not affected in female patients.
7. Patients younger than 40 years exhibited the highest percentage of FOM PMDs compared to older age groups (80% vs. 42%).
8. Patients younger than 40 years were mainly diagnosed with low grade dysplasia (70%).
9. Patients older than 41 years were mainly affected by high grade dysplasia (94%).
10. Higher dysplastic features were associated with advancing age.
11. Erythroplakia is more predominant in males (88%; 7/8) and in patients over the age of 41 years.
12. Homogenous leukoplakias were the most common clinical presentation (67%).
13. The majority of homogenous leukoplakias were identified as low grade dysplasias (58%).
14. The majority of non-homogenous leukoplakias were diagnosed as high grade dysplasias (71%).
15. Ulcerated PMDs were only seen in the lateral tongue (2/2), the site which was previously classified as a high-risk site.
16. Erythroplakias and speckled leukoplakias were more frequently reported in the FOM and lateral tongue, the sites which were previously classified as high-risk sites.
17. Exophytic subtypes were mainly seen in buccal mucosa (2/5), the site which was previously classified as a low-risk site.
18. Nodular subtypes were only seen in ventral tongue and buccal mucosae.
19. No nodular or ulcerated subtypes were seen in female patients.
20. All ulcerated and nodular cases were seen in male patients.
21. The size of PMDs increased with increasing age of patients.
22. The severity of dysplasia is associated with larger sized PMDs.
23. Clear surgical margins were primarily seen in mildly dysplastic PMDs (60%).

24. Dysplastic margins were more frequently seen in PMDs with higher dysplastic features.
25. Clear surgical margins were significantly observed in minor sized lesions.
26. Major sized PMDs were significantly associated with higher dysplastic features in the margins.
27. Mild dysplasia in the margins was mainly seen in minor sized PMDs.
28. Males exhibited a higher number of margin involvement compared to females.
29. Significantly, males showed higher dysplastic features in their dysplastic margins.

Chapter Four: Risk Factors of Patients with PMD in the North-East of England

Study 2

4.1. Introduction

Oral squamous cell carcinomas may be preceded by PMDs which can be detected morphologically as leukoplakia, erythroplakia or erythroleukoplakia, reflecting the multi-step process of oral cancer development (Melrose, 2001; Brinkman and Wong, 2006; Williams et al., 2008).
It has been estimated that one-third of oral PMDs progress to cancer (Saran et al., 2008). Thus, it is important to identify patients at risk of developing PMDs and to detect these disorders as early as possible. Current research efforts are aimed towards early identification of risk factor(s) associated with oral carcinogenesis and to recognize those patients at increased risk (Bloching et al., 2007).

A risk factor is a variable that might be associated with an increased risk of a disease and it either acts as a disease initiator or promoter (Binnie, 1991). Risk factors for PMDs generally correspond to those of OSCCs (Dietrich et al., 2004). Assessment of potential risk factors is essential for early diagnosis and treatment of oral cancer and PMDs (Mawardi et al., 2011), and to help identify patients at risk of unfavourable clinical outcome and treatment failures such as recurrence or malignant transformation and who require extended surveillance.

Several risk factors have been associated with the aetiology of PMDs and OSCCs, but tobacco smoking and alcohol use (Reichart, 2001) are the most important. They are independently and synergistically associated with high risk in a dose-dependent pattern (Blot et al., 1988; Castellsague et al., 2004).

It has been suggested that the association between tobacco smoking and oral epithelial dysplasia is at least as strong as the association between smoking and oral cancer (Morse et al., 2007). However, the risk association for these preventable aetiological factors is not necessarily constant over the multi-step pathway of oral carcinogenesis (Morse et al., 2007).

In the current study, the exposure profile of smoking and drinking behaviour including number of cigarettes smoked per day and the units of alcohol consumed per week, history of use and total lifetime accumulation (pack-year score) for tobacco smoking were all

considered. These exposure parameters were studied in relation to patient demography and clinicopathological features of PMDs such as oral anatomical site, clinical appearance, size and the presence of epithelial dysplasia. In addition, local factors (intraoral dental prosthesis wear) and the presence of systemic disease such as immunodeficiency, anaemia, diabetes mellitus, hypertension, a familial cancer history and oral candida infection were also investigated.

4.2. Aims

The aim of this study was to identify the associated risk factors in patients presenting with new, single dysplastic PMDs and to investigate the possible relation and correlation with their clinicopathological behaviour in order to classify patients into high and low-risk groups to aid future management protocols.

4.3. Methods

Utilising the records of the 100 PMD patients, the presence of aetiological risk factors such as tobacco smoking, alcohol consumption, history of systemic disease and oral health status were identified. Data regarding risk factor assessment were collected from patients' medical records at initial presentation and at each follow-up appointment up to the most recent clinic review.

Patients were classified as non-smokers (if they had never smoked tobacco), ex-smokers (if the patient stopped tobacco smoking for at least 1 year before initial presentation) and current smokers (regular smokers). Data collected included the number of cigarettes or grams of tobacco smoked per day and the length of smoking history in terms of years.

Regarding alcohol behaviour, patients were classified as non-drinkers (if they had never drunk alcohol), ex-drinkers (if the patient stopped drinking for at least 1 year before initial presentation) and current drinkers (regular drinkers). Data including the number of alcohol units consumed per week and the length of drinking history were recorded.

For each patient in this study, the history of any concurrent medical problems, oral health status such as mouthwash use, the presence of dental prostheses (dentures, crowns and bridges) and a family cancer history were all recorded.

4.3.1. Statistical Analysis

Statistical analysis was performed with SPSS (Statistical Package for Social Sciences) version 17.0 and 19.0 software package (SPSS Inc; Chicago, IL, USA). Descriptive statistical analysis, the Chi-Square, Fisher's Exact, Independent/Paired Student T-test, ANOVA and bivariate correlation were used to compare the variables and find correlations. Non-parametric tests including Mann-Whitney U and Kruskal-Wills tests were also used when data did not assume a normal distribution. In addition, logistic regression analysis with the -2 log likelihood ratio test statistic was performed to identify the significant predictor(s) for non-homogenous leukoplakia and for high grade dysplasia to develop. A *p*-value of less than 0.05 was considered to indicate statistical significance.

4.4. Results

4.4.1. Tobacco Smoking Behaviour in PMD patients

At initial presentation, 63 out of the 100 patients (46 males and 17 females) were current smokers, followed by 22 ex-smokers (15 males and 7 females); only 15 were non-smokers (5 males and 10 females); Figure 4.1.

Current smokers were divided into 3 groups according to the number of cigarettes smoked per day: light (< 10), intermediate (10-20) and heavy smokers (> 20). Overall, intermediate smokers were most common (60%; 38/63), followed by heavy smokers (37%; 23/63) and light smokers (3%; 2/63); Figure 4.2.

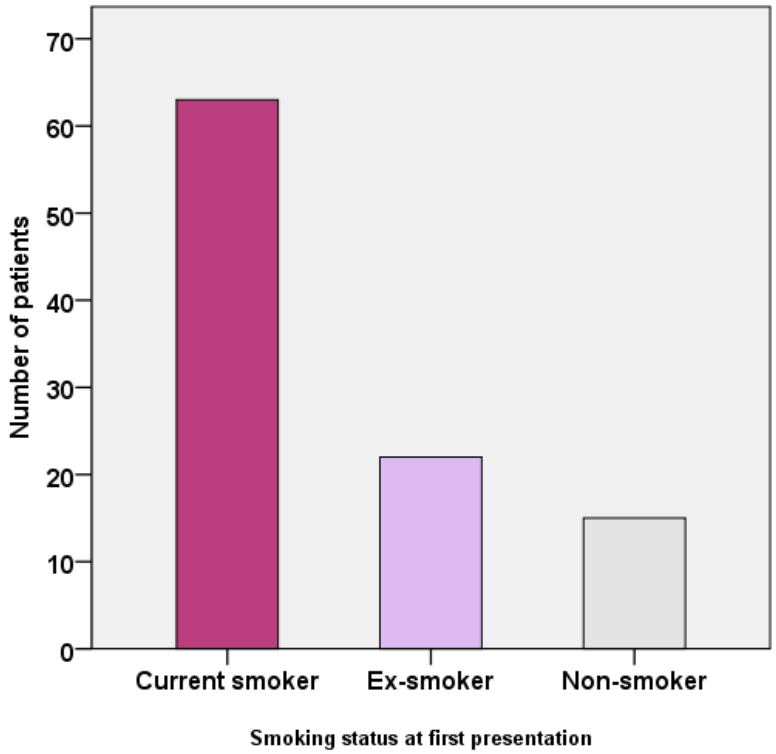

Figure 4.1: Distribution of 100 PMD patients according to smoking status.

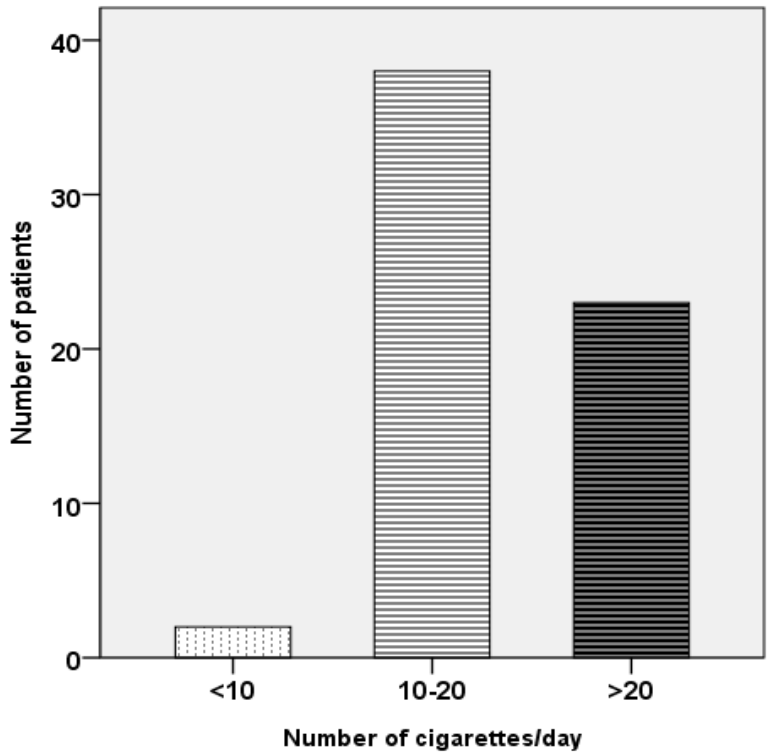

Figure 4.2: Current smoker categories according to the number of cigarettes.

Age, Sex, Occupation and Smoking Status

Overall, significant differences were found in mean age between current smokers and both non-smokers and ex-smokers (p=0.0001, One way ANOVA), whilst no significant differences were found between non-smokers and ex-smokers (p=0.998). Current smokers (mean age, 52.27 years; SD: 9.73) were younger than both non-smokers (66.87 years; SD: 15.53) and ex-smokers (67.09 years; SD: 10.14).

All patients ≤ 40 years were tobacco users, with 90% current smokers and 10% ex-smokers. In middle age (41-62 years), the majority of patients were current smokers (80%), followed by 11% ex-smokers and 9% non-smokers. In old age (63-85 years), ex-smokers were most common (46%), followed equally by current and non-smokers (27% for each). In this study, there was only one elderly patient (> 85 years) who was a non-smoker; Figure 4.3.

A significant relation was also found between smoking status and sex (p=0.014; Chi-Square test). Current smokers were predominantly males 73% (46/63) with the remaining 27% (17/63) females. Similarly, the majority of ex-smokers 68% (15/22) were males compared to 32% (7/22) females, whilst non-smokers were mainly females 67% (10/15) compared to males (33%; 5/15); Figure 4.4.

Female current smokers were significantly younger than both non-smokers and ex-smokers (mean age 52.47 years vs. 68.20 years, 65 years), (p=0.004, p=0.021, respectively). No significant differences were observed in mean age between non-smokers and ex-smokers (p=0.740; Independent t-test).
Similarly, male current smokers had a significantly lower mean age compared to ex-smokers (52.20 years vs. 68.07 years) (p=0.0001), whereas the differences between current smokers and non-smokers (52.20 years vs. 64.20 years) (p=1.000) and between non-smokers and ex-smokers were not significant (64.20 years vs. 68.07 years) (p=0.624).

A highly significant association was found between smoking status and employment status (employed, unemployed or retired) (p=0.0001; Chi-Square test). The majority of employed and unemployed patients were current smokers, whilst the majority of retired patients were

ex-smokers. Eighty-eight percent (22/25) of unemployed patients were current smokers, compared to 74% (25/34) of employed and 44% (18/41) of retired patients; Figure 4.5.

Table 4.1 shows the relation between occupation status and number of cigarettes smoked per day. Using Chi-Square test, a significant relation was found between occupational status and the intensity of smoking (p=0.032). Employed patients were mainly heavy smokers 44% (11/25), unemployed often intermediate smokers (68%; 15/22), and retired patients only light smokers (2/2). Retired patients smoked the least number of cigarettes per day (mean 18, range 7-30) compared to both unemployed (mean 25, range 10-60) and employed patients (mean 25, range 10-50).

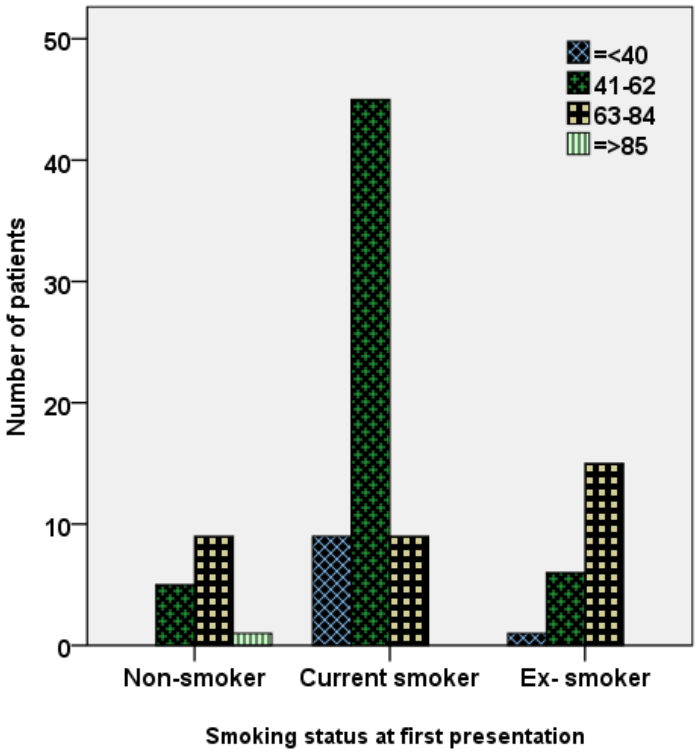

Figure 4.3: Age group according to smoking status.

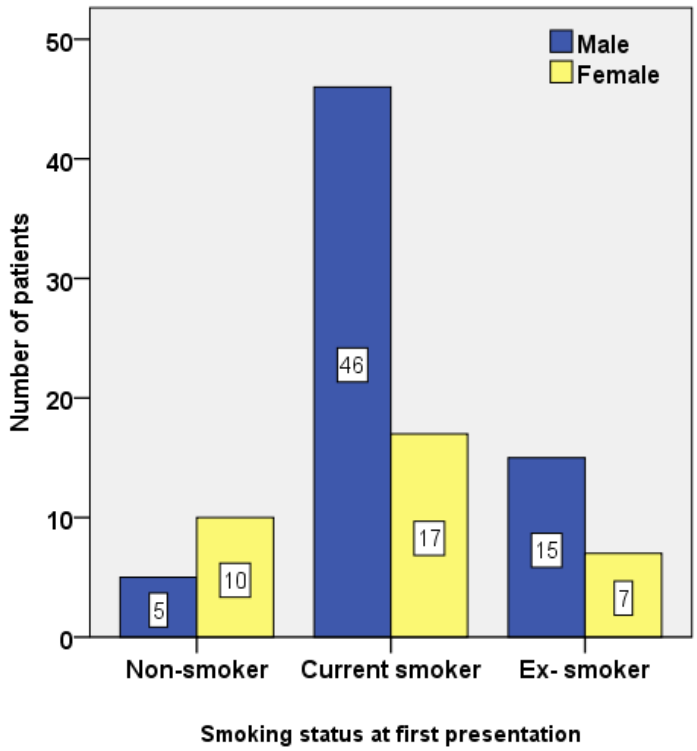

Figure 4.4: Sex distribution according to smoking status.

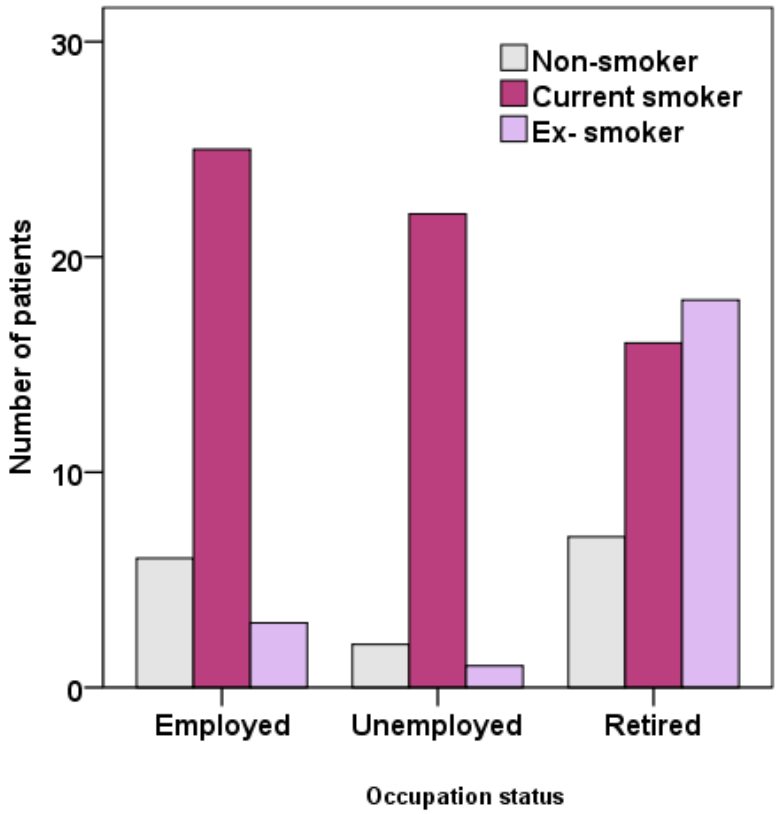

Figure 4.5: Smoking status according to occupation of PMD patients.

Table 4.1: Occupational status according to the number of cigarettes.

Tobacco smoking (cigarettes/day)	Occupational status			Total
	Employed	Unemployed	Retired	
< 10	-	-	**2** *100%*	**2** *100%*
10-20	**14** *37%*	**15** *39%*	**9** *24%*	**38** *100%*
> 20	**11** *48%*	**7** *30%*	**5** *22%*	**23** *100%*
Total	**25** *40%*	**22** *35%*	**16** *25%*	**63** *100%*

Smoking Status and Anatomical Site

Figure 4.6 and Table 4.2 summarise the relation between smoking status and PMD anatomical sites. Using Chi-Square testing, a significant relationship was found between smoking status and anatomical site (p=0.0001).
Out of 63 current smokers, 38 (60%) displayed FOM PMDs, followed by soft palate (7/63) and ventral tongue (6/63); whereas an equal distribution of PMDs were observed in lateral tongue, buccal mucosa and faucial pillars (4/63 for each).

In non-smokers, 93% (14/15) of PMDs were seen in the tongue (9 lateral and 5 ventral surfaces); with the remaining one non-smoker case seen in buccal mucosa.
PMDs in ex-smokers were mainly observed in the tongue (9/22; 6 lateral and 3 ventral surfaces), followed by the FOM (8/22). No PMDs were seen in the buccal mucosa and fauces; PMDs in the retromolar area and alveolar mucosa were only seen in ex-smokers.
PMDs in the soft palate were seen in tobacco users: current smokers (78%; 7/9), followed by ex-smokers (22%; 2/9), but not in non-smokers.

Considering high-risk (FOM and tongue) versus low-risk sites (all remaining other sites), there was no significant difference (p=0.104; Chi-Square test), although high-risk sites were predominant in all smoking groups; Figure 4.7.

Different site predilections in smokers and non-smokers were seen, with FOM most frequently affected in current smokers, and tongue more common in non-smokers. However, both FOM and tongue were similarly affected in ex-smokers (8/22) and (9/22), respectively; Table 4.3.

Figure 4.8 and Figure 4.9 demonstrate sex-site distribution in relation to smoking status. A significant association was found between smoking status and sex (p=0.014; Chi-Square test). Both male and female non-smokers showed no FOM PMDs, with the tongue the only affected site in non-smoker females and 80% of non-smoking males. The FOM was mainly affected in current smokers in both females and males (82%; 14/17 and 52%; 24/46,

respectively). The tongue was the main affected site in male ex-smokers (6/15), whereas FOM was the most commonly affected site in female ex-smokers (4/7).

Current smokers younger than 40 years more frequently developed FOM PMDs (7/9), but with no tongue lesions, whilst patients older than 41 years showed PMDs in both FOM (31/54) and tongue (10/54).

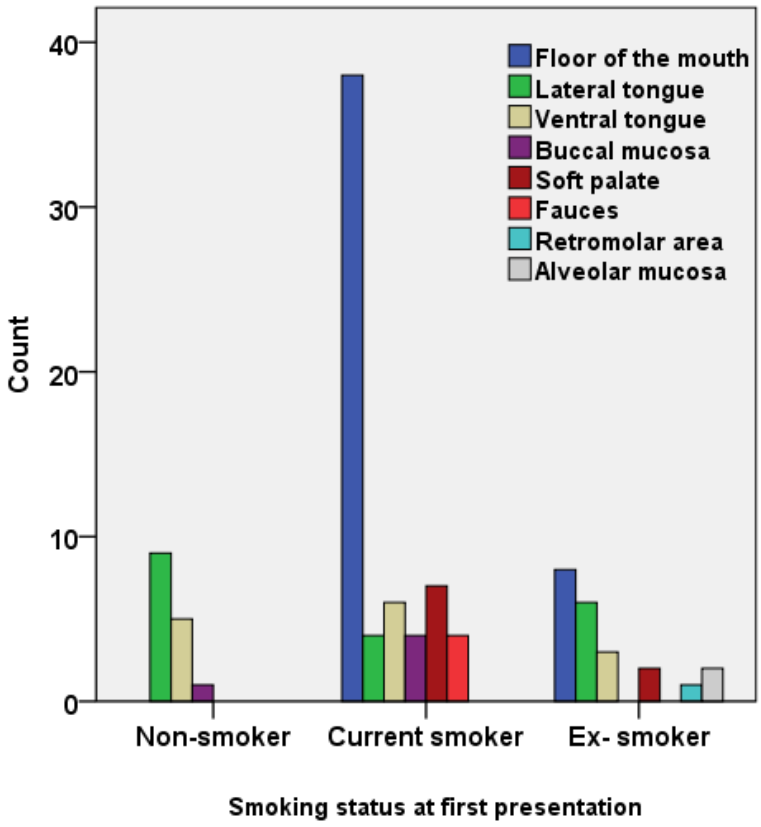

Figure 4.6: PMD anatomical site in relation to smoking status.

Table 4.2: PMD anatomical site in relation to smoking status.

PMD anatomical sites	Smoking status at first presentation			Total
	Non-smoker	Current smoker	Ex-smoker	
FOM	-	38	8	46
Lateral tongue	9	4	6	19
Ventral tongue	5	6	3	14
Buccal mucosa	1	4	-	5
Soft palate	-	7	2	9
Fauces	-	4	-	4
Retromolar area	-	-	1	1
Alveolar mucosa	-	-	2	2
Total	15	63	22	100

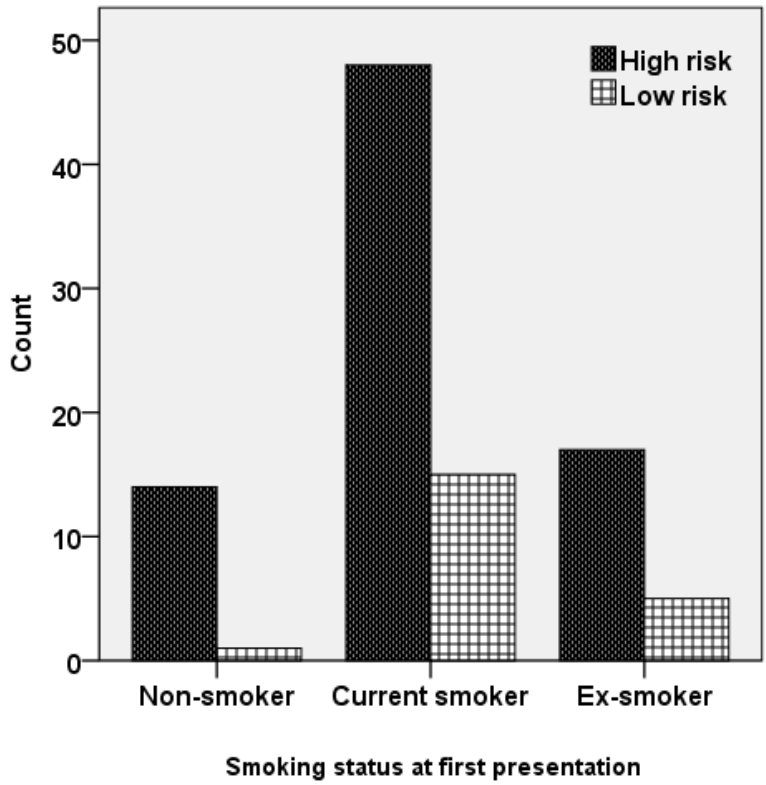

Figure 4.7: Smoking status in relation to high/low-risk anatomical sites.

Table 4.3: Smoking status in relation to five anatomical sites.

Smoking status	PMD anatomical sites					Total
	FOM	Tongue	Soft palate	Buccal mucosa	The remaining sites	
Non-smoker	-	14	-	1	-	15
Current smoker	38	10	7	4	4	63
Ex-smoker	8	9	2	-	3	22
Total	46	33	9	5	7	100

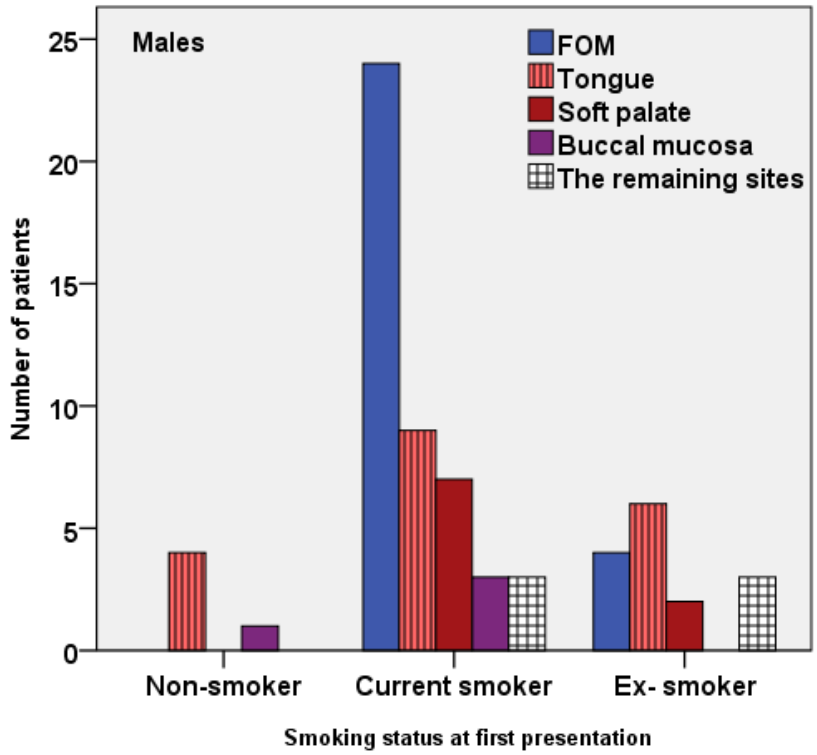

Figure 4.8: Smoking status and PMD oral sites in males.

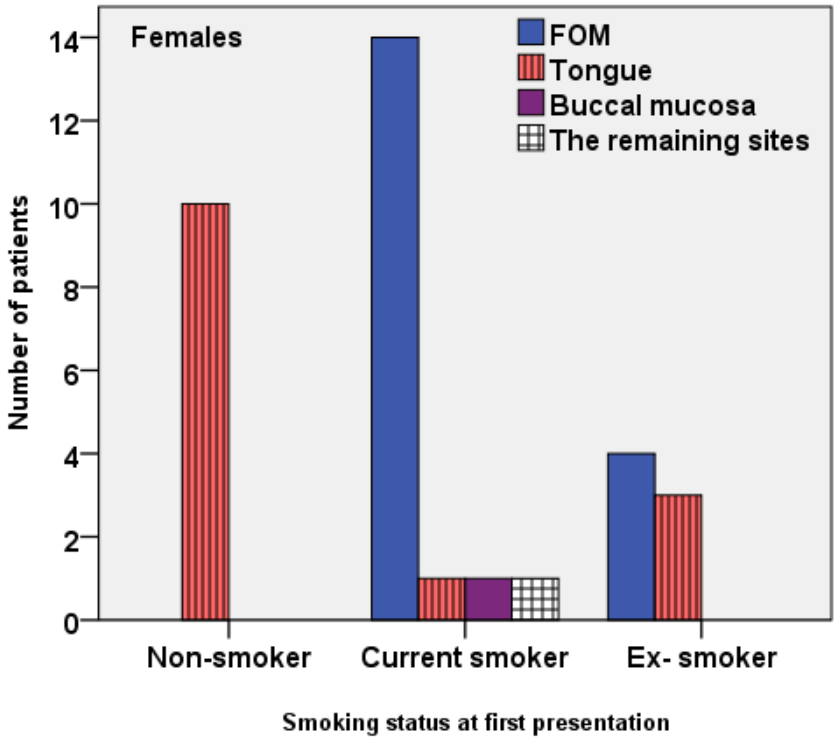

Figure 4.9: Smoking status and PMD oral sites in females.

Smoking Status and Clinical Appearance

In all groups (Figure 4.10), homogenous leukoplakia was the main clinical type (67/100), followed by speckled non-homogenous leukoplakia (16/100), erythroplakia (8/100) and exophytic non-homogenous subtypes (5/100). Ulcerated PMDs were observed equally in current and ex-smokers (1/2), but were not seen in non-smokers. Nodular and exophytic subtypes were seen equally in non-smokers (1/15 for each), with nodular also seen equally in current and non-smokers (1/2), but not in ex-smokers.

In current smokers, higher frequencies of exophytic lesions (3/5) were seen with an equal distribution between non-smokers and ex-smokers (1/5). No significant relation was found between smoking status and PMD clinical appearance (p=0.068; Chi-Square test).

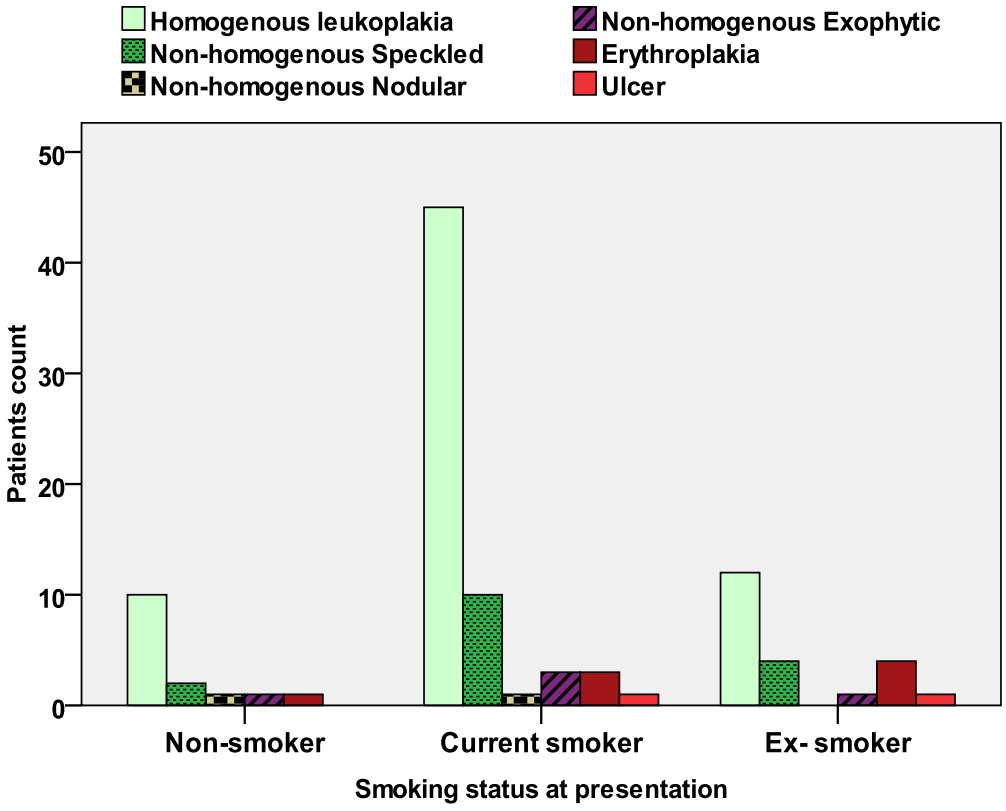

Figure 4.10: PMD clinical appearance according to smoking status.

Smoking Status and Size of PMD

Three size categories were defined in this study: minor < 200 mm^2, intermediate 200-600 mm^2 and major > 600 mm^2.

In current smokers, the majority of PMDs were intermediate (49%; 31/63), followed by minor (44%; 28/63) and major (6%; 4/63). In ex-smokers, minor size PMDs were most common (10/20), followed by intermediate (8/20) and major (2/20).

Non-smokers exhibited mainly intermediate sized PMDs (6/15), followed by minor (5/15) and major (4/15); Figure 4.11.

Kruskal-Wallis testing showed significant differences in mean size between smoking groups (p=0.026). Subsequently, a Mann-Whitney U test was performed which revealed a highly significant difference between current and non-smokers (p=0.006). Whereas the differences between ex-smokers and both current and non-smokers were non-significant (p=0.594 and p=0.093, respectively).

Figure 4.12 shows the association between PMDs mean size and smoking status. Non-smokers showed the highest mean size (473.20 mm^2), followed by ex-smokers (354.25 mm^2) with current smokers showing smallest mean size (241.49 mm^2).

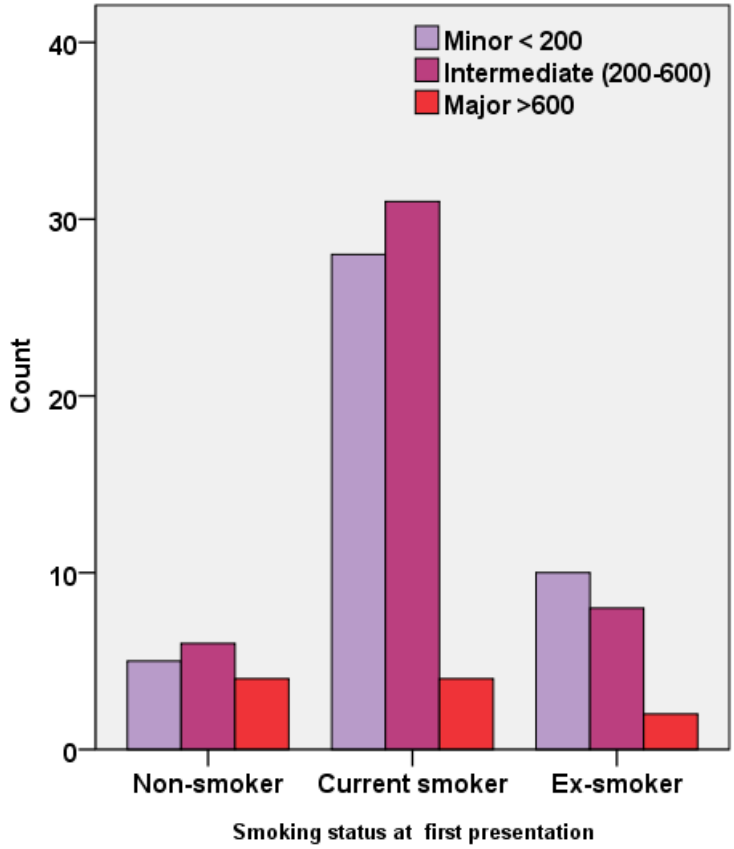

Figure 4.11: Distribution of PMD size (mm^2) according to smoking status.

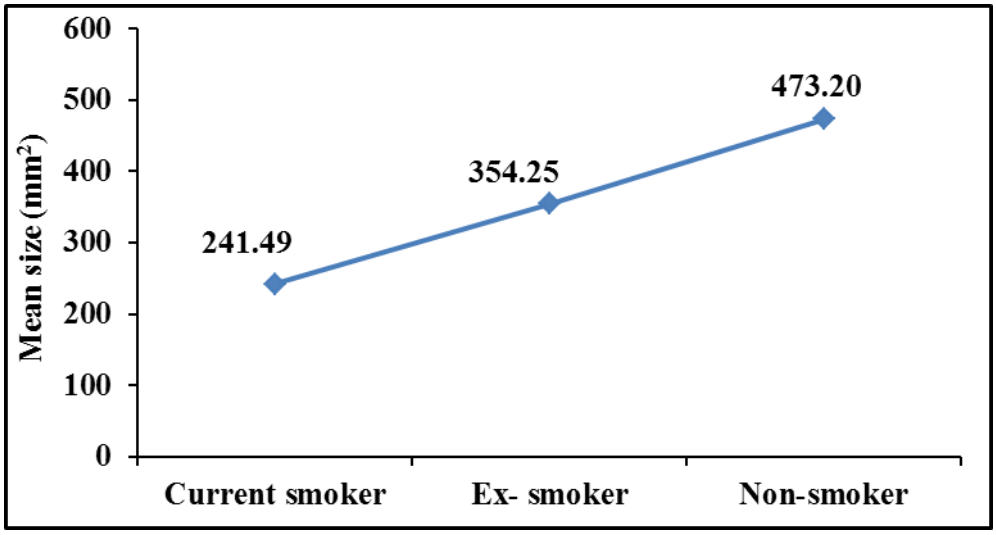

Figure 4.12: Mean size of PMD (mm^2) according to smoking status.

Smoking Status and Dysplasia Grading

Generally, a higher presentation of dysplastic features were seen in current smokers (63/97), compared to 19/97 for ex-smokers and 15/97 in non-smokers; Table 4.4.

No significant relation was found between smoking status and grade of epithelial dysplasia, however (p=0.105; Chi-Square test).

Table 4.4: Degree of epithelial dysplasia in relation to smoking status.

Smoking status (at first presentation)	Oral epithelial dysplasia (consensus diagnosis WHO system)				Total
	Mild	Moderate	Severe	CIS	
Non-smoker	6 *40%*	2 *13%*	5 *33%*	2 *13%*	15 *100%*
Current smoker	27 *43%*	16 *25%*	15 *24%*	5 *8%*	63 *100%*
Ex-smoker	9 *47%*	5 *26%*	2 *11%*	3 *16%*	19 *100%*
Total	42 *43%*	23 *24%*	22 *23%*	10 *10%*	97 *100%*

Number of Cigarettes per day, Tobacco Grams per week and Sex

Overall, the average number of cigarettes smoked per day was 23, and the range 7 to 60. No significant differences were found between males and females in mean number of cigarettes smoked per day (p=0.345; Independent t- test); males showed a slightly higher mean number (24) compared to females (21).

The number of cigarettes smoked/day was converted into grams/week (1 cigarette contains approximately 1 gram of tobacco), providing a range of 49-420 grams/week with a mean of 164 (SD: 73.67).

To assess the influence of smoking intensity, smokers were classified according to the number of cigarettes smoked per day into 3 groups: light (< 10), intermediate (10-20) and heavy smokers (> 20). Males consumed more tobacco than females, 78% (18/23) of heavy smokers were males compared to only 22% (5/23) of females. Similarly, 68% (26/38) of intermediate smokers were males compared to 32% (12/38) females; Figure 4.13.

No significant relationship was found between sex and intensity of smoking in terms of cigarettes/day (p=0.160; Chi-Square test).

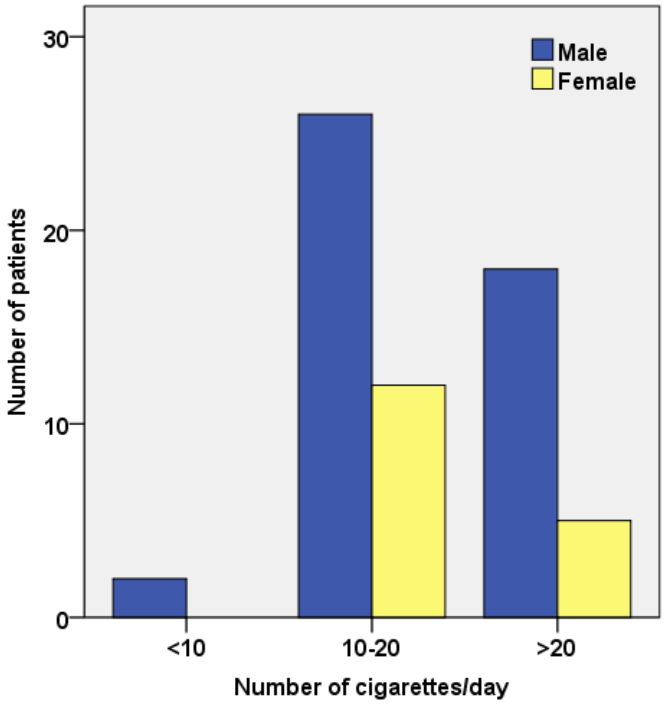

Figure 4.13: Number of cigarettes smoked per day in males and females.

Number of Cigarettes and Age of Patient

Figure 4.14 and Table 4.5 summarise the number of cigarettes smoked per day in relation to age. Higher frequencies of intermediate, followed by heavy smoking were seen in all age groups. Eighty-two percent (19/23) of heavy and 66% (25/38) of intermediate smokers were middle age; 16% (6/38) of intermediate and 9% (2/23) of heavy smokers were old age. Whilst Chi-Square test showed no significant relation between number of cigarettes smoked per day (light, intermediate, heavy) and age groups (p=0.137), Kruskal-Wallis testing showed a significant difference in average tobacco intake (cigarettes/day) between age groups (p=0.026). Subsequently, a Mann-Whitney U test was performed and revealed a significant difference in mean tobacco intake between middle and old age (p=0.009), whilst no significant differences were observed regarding other age groups. Middle age patients consumed the highest average number of cigarettes per day (25), followed by young (22) and then old (16); Figure 4.15.

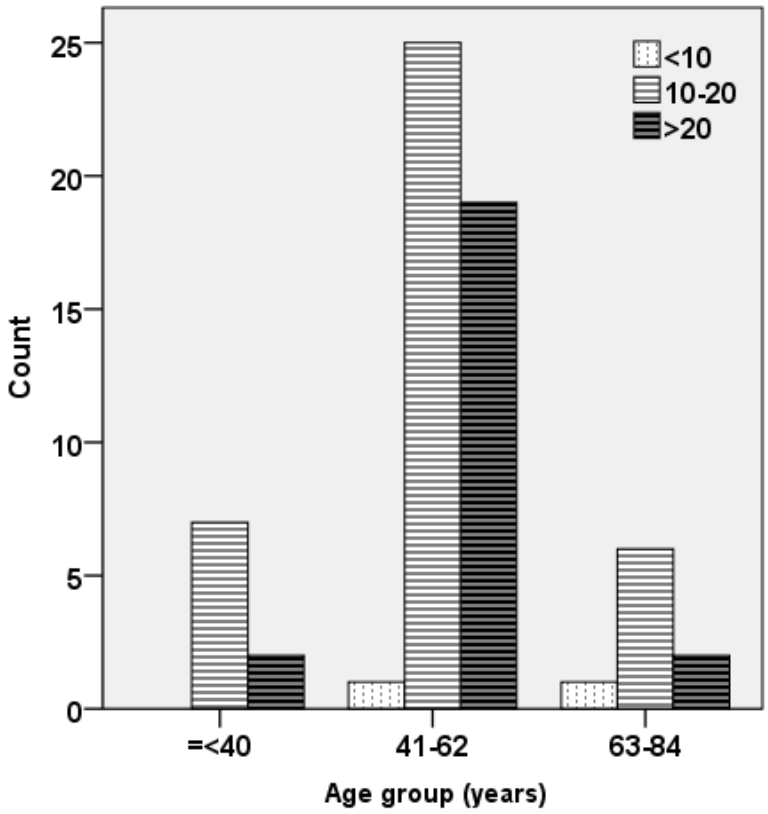

Figure 4.14: Number of cigarettes smoked per day according to age group.

Table 4.5: Number of cigarettes smoked per day according to age group.

Smoking intensity (cigarettes/day)	Age group (years)			Total
	≤ 40	41-62	63-84	
< 10	-	1 *50%*	1 *50%*	2 *100%*
10-20	7 *18%*	25 *66%*	6 *16%*	38 *100%*
> 20	2 *9%*	19 *82%*	2 *9%*	23 *100%*
Total	9 *14%*	45 *72%*	9 *14%*	63 *100%*

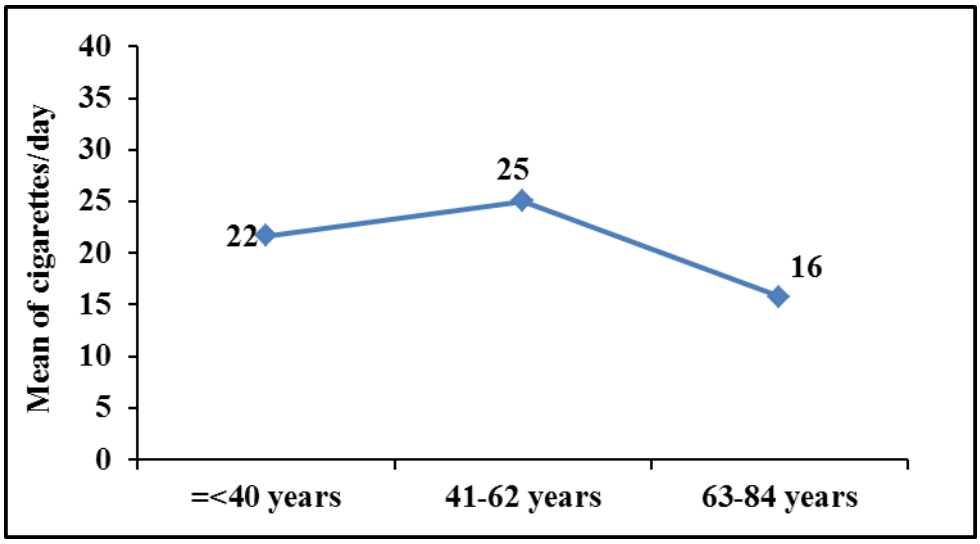

Figure 4.15: Mean cigarettes smoked per day in relation to age group.

Number of Cigarettes per day and PMD Anatomical Site

The FOM was the main site affected by PMDs in intermediate smokers 61% (23/38) and heavy smokers 37% (14/38); Figure 4.16.

The intensity of smoking in term of cigarettes smoked per day did not influence PMD site distribution; Chi-Square test was not significant considering all sites (p=0.085), 5 anatomical sites (FOM, tongue, soft palate, buccal mucosa and other sites) (p=0.086) or high/low-risk sites (p=0.144).

Mann-Whitney U test also showed no significant relation between amount of tobacco smoked per day and PMDs in high or low-risk oral sites (p=0.960).

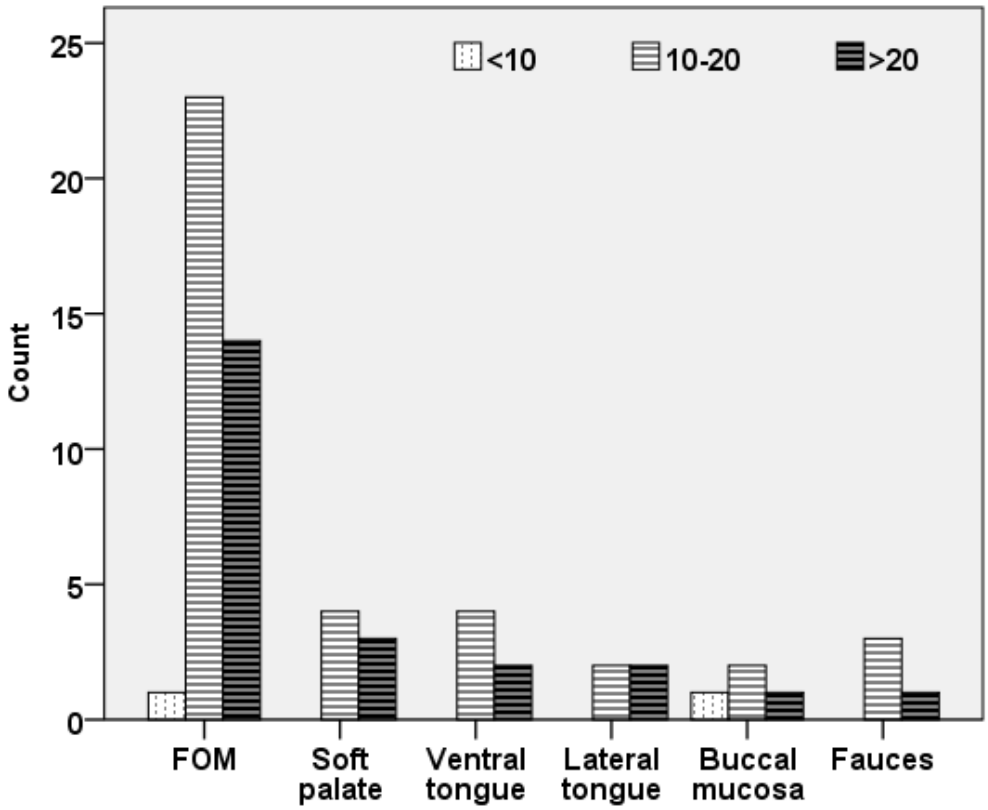

Figure 4.16: Number of cigarettes smoked per day in relation to PMD anatomical site.

Number of Cigarettes per day and Clinical Appearance of PMD

Figure 4.17 shows the distribution of clinical type of PMD in relation to number of cigarettes smoked per day.

Erythroplakia was only seen in heavy smokers (3/3), whilst light smokers exhibited only exophytic non-homogenous leukoplakia (2/2). Homogenous and specked non-homogenous leukoplakias were both more frequently seen in heavy and intermediate smokers.

Whilst Chi-Square test was significant (p=0.001); Kruskal-Wallis testing showed no significant difference in the average number of cigarettes smoked per day and clinical type of PMD (p=0.274).

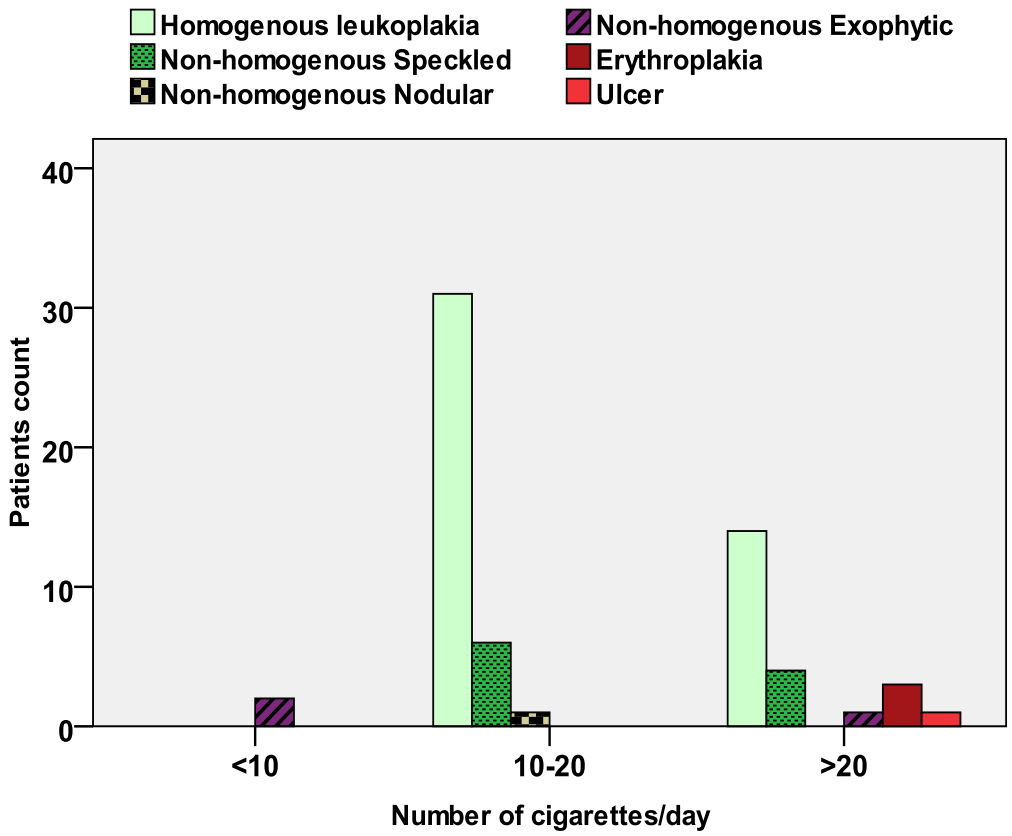

Figure 4.17: Clinical appearance of PMDs according to number of cigarettes/day.

Number of Cigarettes and Size of Dysplastic PMD

Patients who smoked more than 20 cigarettes/day demonstrated the highest frequency of minor sized PMDs (48%; 11/23), followed by intermediate (39%; 9/23) and major size (13%; 3/23).

Fifty-percent (19/38) of intermediate smokers showed intermediate sized PMDs (200-600 mm^2), followed by minor (45%; 17/38) and major size (5%; 2/38). Light smokers showed only intermediate size PMDs (2/2); Table 4.6.
Chi-Square testing showed no significant association between intensity of smoking and size of PMD (p=0.392).

A weak negative, albeit non-significant, correlation was observed between the number of cigarettes smoked per day and PMD size (r=-0.50, n=63, p>0.01). Heavy smokers showed the smallest mean size compared with light smokers (247.48 mm^2 vs. 316 mm^2).

Table 4.6: Association between size of PMDs and the number of cigarettes/day.

Number of cigarettes/day	PMD size (mm^2)			Total
	Minor < 200	Intermediate (200-600)	Major > 600	
< 10 Light smoker	**-**	**2** *100%*	**-**	**2** *100%*
10-20 Intermediate	**17** *45%*	**19** *50%*	**2** *5%*	**38** *100%*
> 20 Heavy smoker	**11** *48%*	**9** *39%*	**3** *13%*	**23** *100%*
Total	**28** *44%*	**30** *48%*	**5** *8%*	**63** *100%*

Number of Cigarettes and Dysplasia Grading

Patients smoking 10-20 cigarettes per day, which was the largest group in this study, exhibited a higher number of dysplasia cases (38/63), compared to heavy smokers (23/63). Severe dysplasia (9/23) was the main dysplastic feature in heavy smokers (> 20), followed by moderate (7/23) and mild dysplasia (6/23). Light smokers (< 10) showed the least dysplastic features (1 mild and 1 CIS), whereas intermediate smokers were more frequently diagnosed with mild dysplasia (20/38); Table 4.7 and Figure 4.18.

Using Chi-Square testing, a significant association was found between number of cigarettes smoked per day and degree of dysplasia (p=0.005). However, Kruskal-Wallis test showed no significant difference in the average amount of tobacco smoked per day and different grades of dysplasia (p=0.183).

Patients with mild and moderate dysplasia smoked the same average cigarettes per day (23), whilst severe dysplasia patients smoked a higher average number (26). Patients with CIS reported the least tobacco intake (17).
No significant correlation was found between number of cigarettes smoked per day and degree of dysplasia (r=0.116; n=63; p>0.01).

Table 4.7: Number of cigarettes smoked per day and degree of dysplasia.

Smoking intensity (cigarettes /day)	Oral epithelia dysplasia (consensus diagnosis WHO system)				Total
	Mild	Moderate	Severe	CIS	
< 10	1	-	-	1	2
10-20	20	9	6	3	38
> 20	6	7	9	1	23
Total	27 *43%*	16 *25%*	15 *24%*	5 *8%*	63 *100%*

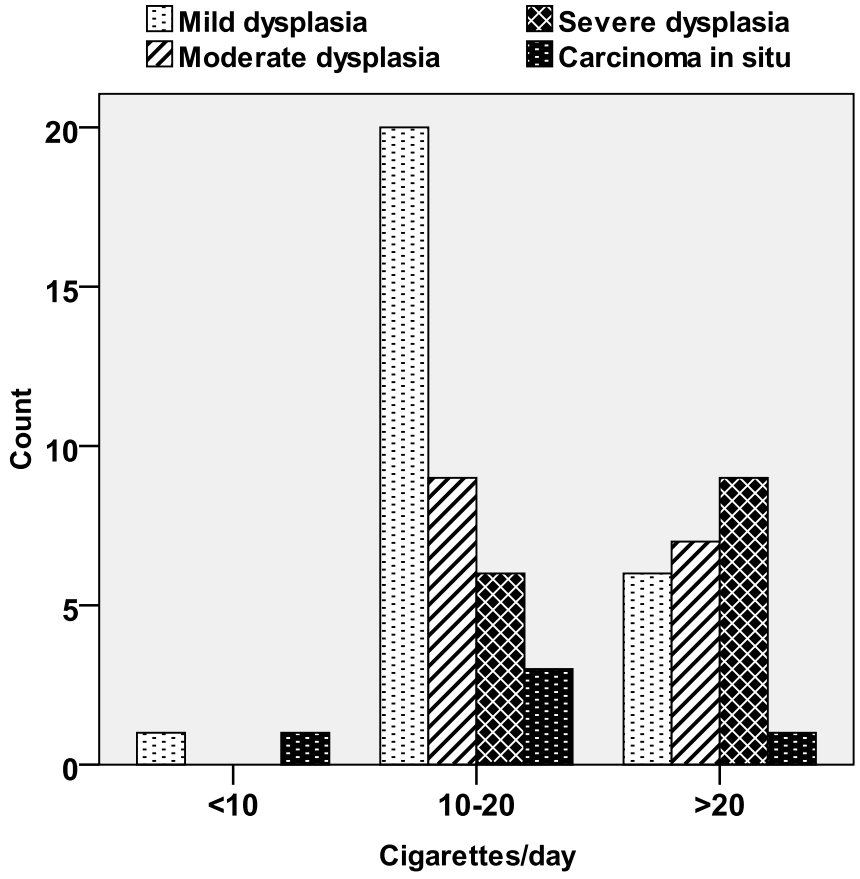

Figure 4.18: Association between numbers of cigarettes smoked per day and degree of dysplasia.

Smoking History and Sex

Smoking history was recorded for 36 patients (28 males and 8 females), whilst no reliable data were found for the remaining 27 current smokers.

Duration of smoking was divided into 3 categories: 10-30 years, 31-50 years and > 50 years; Figure 4.19.

Overall, 58% (20/36) of current smokers reported a long history (31-50 years), followed by 39% (14/36) of relatively long (10-30 years), with only one patient more than 50 years smoking history.

Male smokers had longer smoking histories (31-50 years) compared to females (64%; 17/28 vs. 38%; 3/8). Females mainly had relatively long histories (10-30 years) compared to males (63%; 5/8 vs. 32%; 9/28). No significant relation was found between the length of smoking history and sex (p=0.310; Chi-Square test).

A significant difference was found between males and females in mean smoking history (p=0.034; Mann-Whitney U test), with males showing a longer mean history (37.11 vs. 28.13 years).

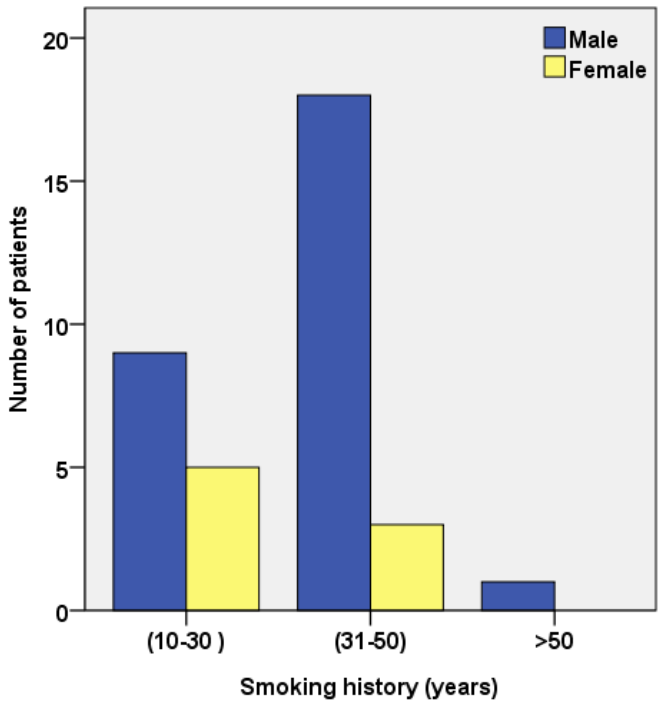

Figure 4.19: Sex distribution in relation to the length of smoking history.

Smoking History and Number of Cigarettes

A negative correlation was found between the number of cigarettes smoked per day and length of smoking history (years), although it did not reach statistical significance (r=-0.317, n=36, p>0.01).

Patients with a history of 10-30 years consumed a higher number of cigarettes/day compared to those who had smoked for 31-50 years (28 vs. 22).

Patients who smoked < 10 cigarettes/day had the highest average smoking history (46.4 years), followed by patients smoking 10 to 20 (35.05 years) and those smoking > 20/day (33.57 years); Figure 4.20.

The majority of heavy 57% (8/14) and intermediate smokers 55% (11/20) had a long smoking history (31-50 years), although Chi-Square test was not significant (p=0.254); Figure 4.21.

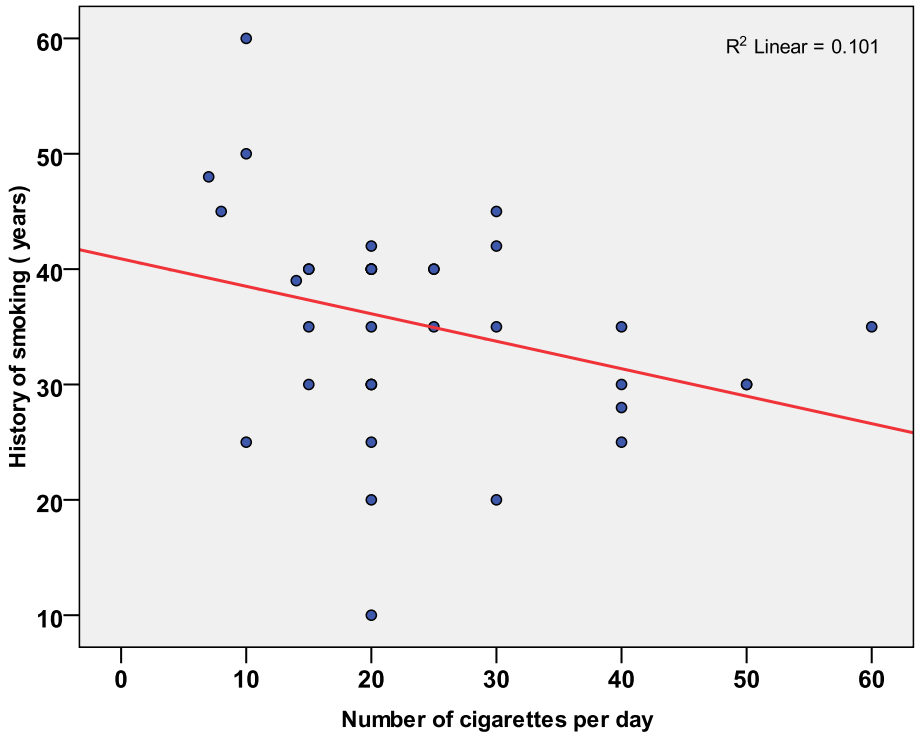

Figure 4.20: Correlation between number of cigarettes and history of smoking.

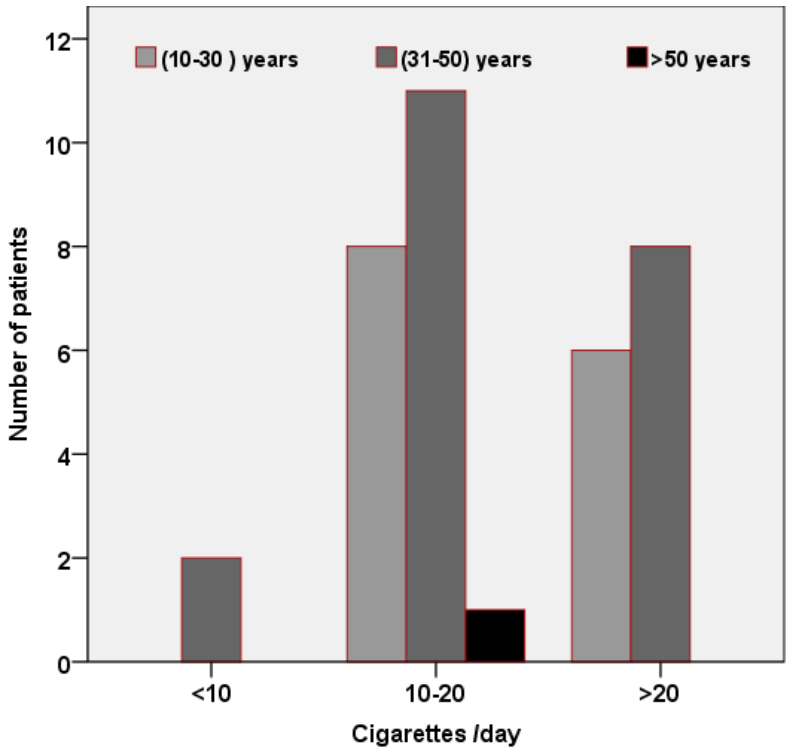

Figure 4.21: Length of smoking history according to the number of cigarettes.

Smoking History and Anatomical Site of PMDs

Overall, patients with long smoking histories (31-50 years) showed higher numbers of PMDs (21/36) compared to those with shorter histories (10-30 years) (14/36); Table 4.8.

A significant relationship was found between smoking history and PMD site (p=0.043; Chi-Square test). The FOM was the main site for PMDs with long histories (11/19), followed by ventral tongue and soft palate equally (3/4), whilst buccal mucosa and fauces were mainly affected in patients with shorter histories.

High-risk sites showed higher frequencies of PMDs compared to low-risk sites for both long and relatively long smoking histories, although patients with a longer history developed more PMDs in high risk sites than those with a shorter duration of smoking (16/26 vs. 9/26); Table 4.9. Chi-Square testing, however, showed no significant relation between smoking history and high/low-risk sites (p=0.438).

Table 4.8: Length of smoking history in relation to PMD anatomical site.

PMD anatomical sites	Smoking history (years)			Total
	10-30	31-50	> 50	
FOM	8	11	-	19 *53%*
Lateral tongue	1	2	-	3 *8%*
Ventral tongue	-	3	1	4 *11%*
Buccal mucosa	2	1	-	3 *8%*
Soft palate	1	3	-	4 *11%*
Fauces	2	1	-	3 *8%*
Total	14	21	1	36 *100%*

Table 4.9: Length of smoking history in relation to high/low risk sites.

PMD anatomical sites	History of smoking (years)			Total
	10-30	31-50	> 50	
High risk	9	16	1	26
Low risk	5	5	-	10
Total	14	21	1	36

Smoking History and Clinical Appearance of PMD

As shown in Figure 4.22, homogenous leukoplakia was the main clinical appearance in patients with a long smoking history, followed by those with a shorter history (67% vs. 57%). The one patient with a history > 50 years also had homogenous leukoplakia.

Chi-Square test showed no significant relationship between smoking history and the clinical type of PMDs: leukoplakia and erythroplakia (p=0.522), homogenous and non-homogenous leukoplakia (p=0.498) or non-homogenous leukoplakia subtypes (p=0.455). However, the relation was significant when all clinical types were combined (p=0.049).

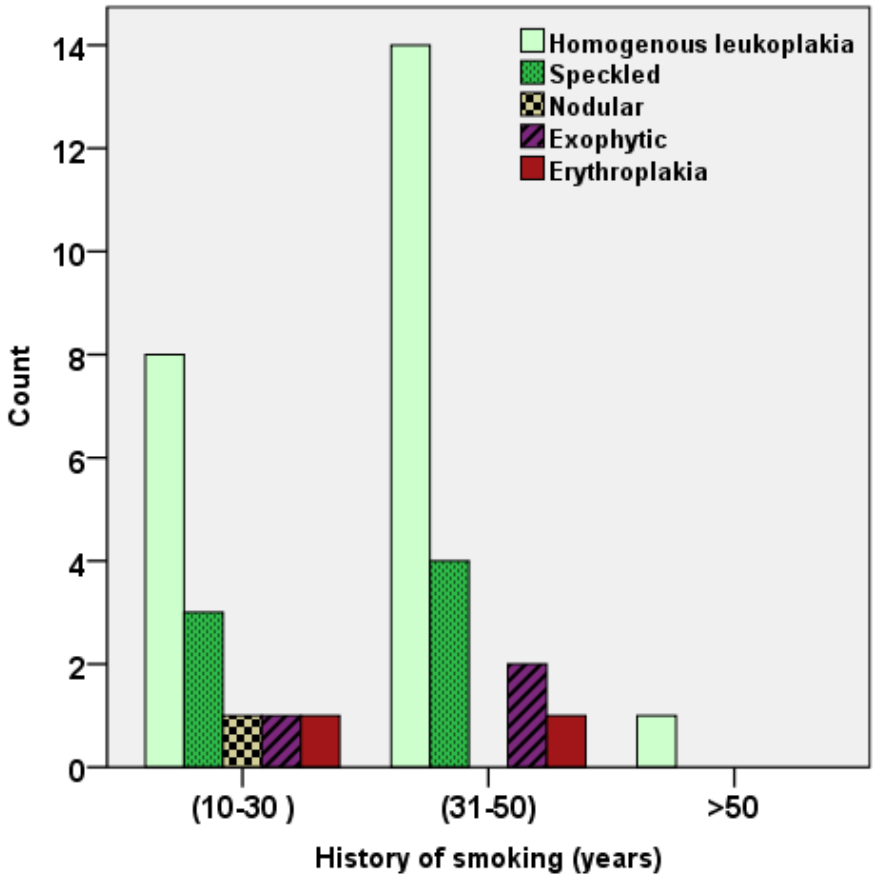

Figure 4.22: Clinical type of PMDs according to smoking history.

Smoking History and Size of Dysplastic PMDs

All major sized (2/2), 60% (9/15) of minor and 53% (10/19) of intermediate sized PMDs were observed in patients with a long smoking history (31-50 years); Figure 4.23.

Patients with a short history (10-30 years) showed 47% (9/19) intermediate, followed by 33% (5/15) minor size. However, no significant relation was found between smoking history and size of PMDs (p=0.233; Chi-Square test).

A negative, albeit, non-significant, correlation was found between PMD size and smoking history (r=-0.050; n=98; p>0.01); patients with a longer history were liable to develop smaller sized PMDs.

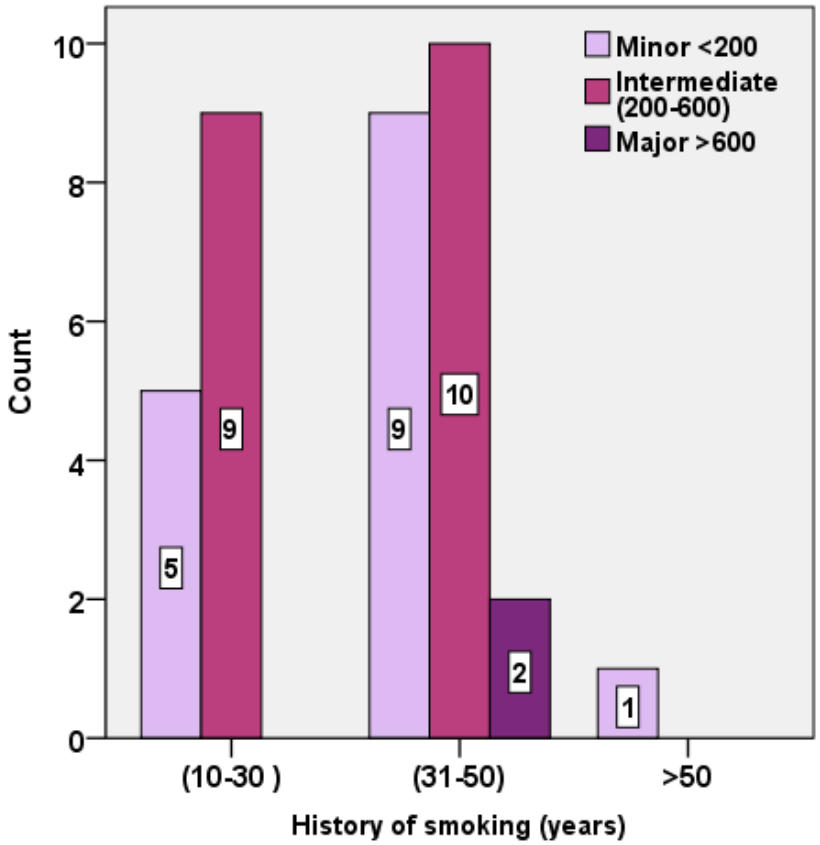

Figure 4.23: PMD size (mm^2) in relation to smoking history.

Smoking History and Dysplasia Grading

Overall, patients with long smoking histories were likely to develop more dysplastic lesions (21/36) compared to those with a short history (14/36).

In this study, the one patient with a history > 50 years developed moderate dysplasia; Table 4.10.

No significant relationship was found between smoking history and degree of dysplasia (p=0.141; Chi-Square test).

Table 4.11 shows mean smoking history in relation to degree of dysplasia. A positive, though non-significant, correlation was found between length of smoking history and degree of dysplasia (r=0.117, n=48, p>0.01); a longer smoking history was associated with increased severity of dysplasia. Patients with mild dysplasia showed the shortest duration of smoking (32.7 years), whilst patients with CIS exhibited the longest history of smoking (41 years).

Table 4.10: Oral epithelial dysplasia in relation to smoking history.

Smoking history (years)	Oral epithelial dysplasia (consensus diagnosis WHO system)				Total
	Mild	Moderate	Severe	CIS	
10-30	7 *50%*	4 *29%*	3 *21%*	-	14 *100%*
31-50	9 *43%*	6 *29%*	3 *14%*	3 *14%*	21 *100%*
> 50	-	1 *100%*	-	-	1 *100%*
Total	16 *44%*	11 *31%*	6 *17%*	3 *8%*	36 *100%*

Table 4.11: Mean smoking history in relation to degree of dysplasia.

Oral epithelial dysplasia	N	Mean smoking history (years)	SD
Mild	23	32.65	9.708
Moderate	14	32.93	13.170
Severe	8	33.75	8.763
CIS	3	41.00	6.557
Total	48	33.44	10.459

Pack-years Score

In this analysis, the effect of both intensity and duration of tobacco smoking was investigated. The lifetime (cumulative tobacco smoking) or pack-years score was calculated by multiplying the number of cigarettes smoked per day by history of smoking, divided by 20 cigarettes (one pack) (Prignot, 1987). The mean pack-year score was 40.78 with a range of 10 to 150 (SD: 20.61).

Males showed a higher mean pack-years score than females (43.23 vs. 32.18), however, the differences were not significant (p=0.200; Mann-Whitney U test). A negative, non-significant, correlation was found between pack-years score and both age of patients (r=-0.210, n=36, p>0.01) and size of dysplasia (r=-0.0127, n=36, p>0.01).

Smoking Trends in PMD Patients

Figure 4.24 shows the percentage of smokers versus the percentage stopping smoking during their clinical management time. Higher percentages of smokers were seen up until 108 months.

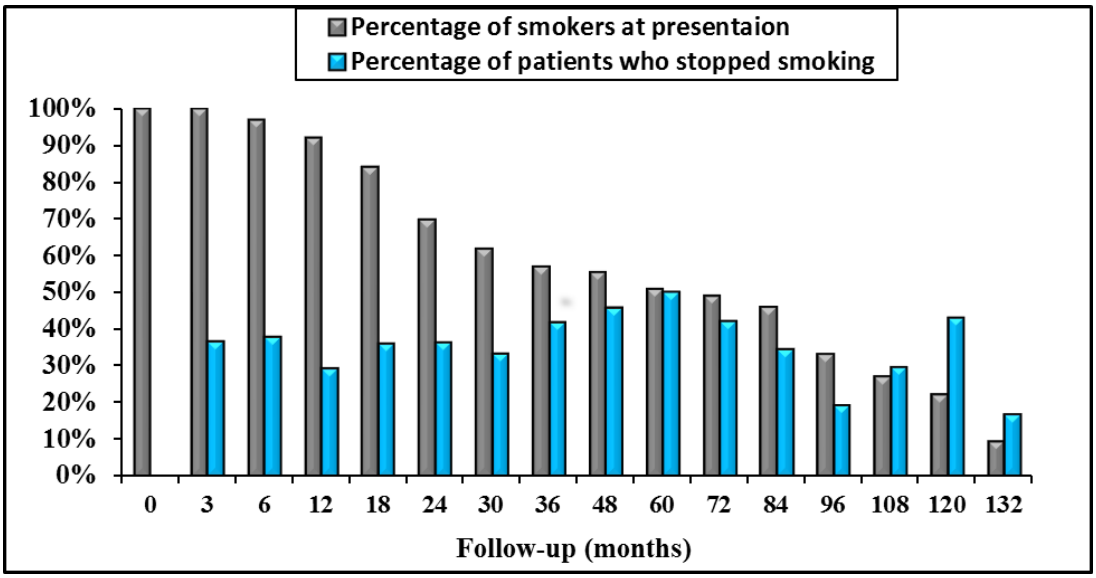

Figure 4.24: Percentage of smokers and quitters during the clinical management time.

Figure 4.25 compares smoking status at initial presentation and at most recent follow-up. A highly significant difference was found between initial presentation and most recent clinic follow-up in the number of patients in different smoking status groups (p=0.0001, Sign test). Sixty-three current smokers at first presentation decreased to 36 at the most recent follow-up, whilst 22 ex-smokers increased to 49 at most recent clinic review.

A significant decrease in the number of cigarettes smoked per day was seen at the most recent clinical follow-up compared to first presentation (p=0.0001, Sign test). The number of light smokers increased from 2 to 20, intermediate smokers decreased from 38 to 14, and heavy smokers reduced from 23 to 2; Figure 4.26.

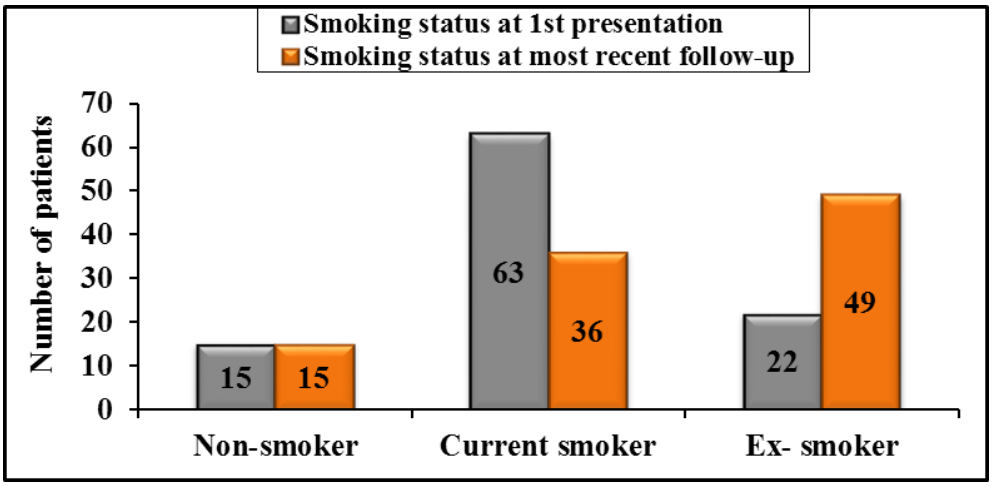

Figure 4.25: Smoking status at first and most recent follow-up time.

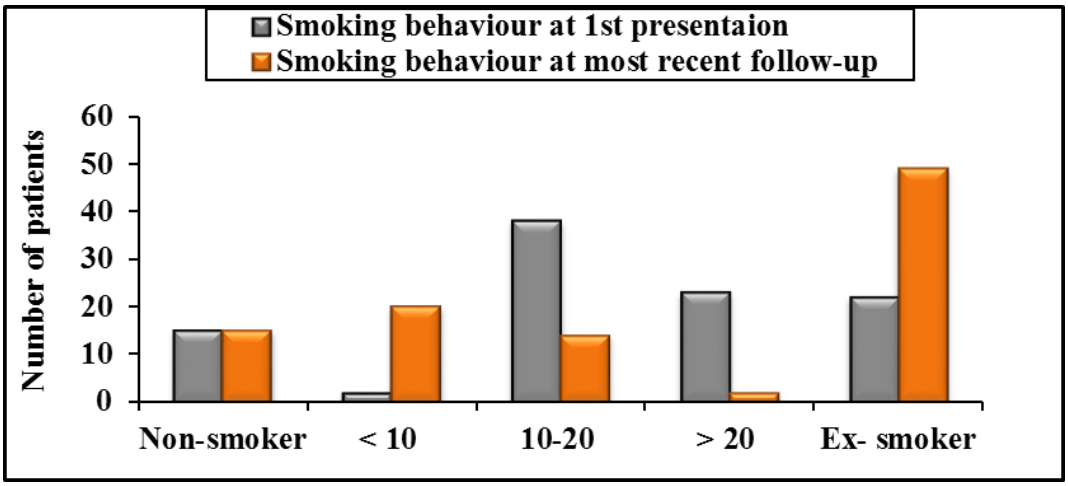

Figure 4.26: Smoking behaviour (cigarettes/day) at first and most recent follow-up.

Table 4.12 compares smoking behaviour between males and females at first presentation and at most recent clinic follow-up.

No heavy smoking female patients were seen at most recent clinic follow-up, whilst intermediate female smokers decreased from 12 to 2. In males, heavy smokers decreased from 18 to 2, whilst the 2 light smokers increased to 17.

Table 4.12: Smoking behaviour at first and most recent follow-up according to sex.

Smoking status		1st presentation			Most recent time		
		Male	Female	Total	Male	Female	Total
Non-smoker		5	10	15	5	10	15
Current smoker (cigarettes/day)	< 10 Light	2	-	2	17	3	20
	10-20 Intermediate	26	12	38	12	2	14
	> 20 Heavy	18	5	23	2	-	2
Ex-smoker		15	7	22	30	19	49
Total		66	34	100	66	34	100

4.4.2. Alcohol Consumption amongst PMD Patients

The majority of patients were current drinkers (83/100), followed by non-drinkers (14/100), with the remaining 3 ex-drinkers; Figure 4.27.

Current drinkers consumed alcohol to an average of 30 units/week (SD: 30.59) and a range of 1-140 units/week. Alcohol consumption was divided into 3 categories: 39 light drinkers (1-14 units/week), 10 intermediate (15-28 units/week) and 34 heavy drinkers (> 28 units/week); Figure 4.28.

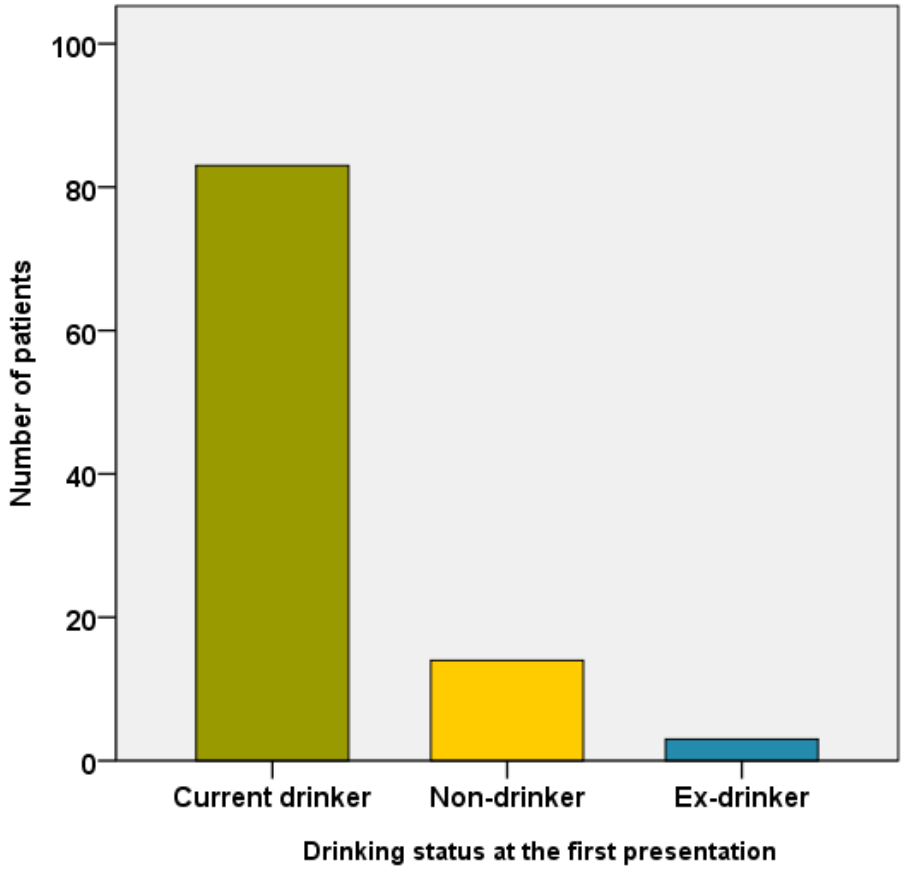

Figure 4.27: Drinking status of 100 PMD patients at first presentation.

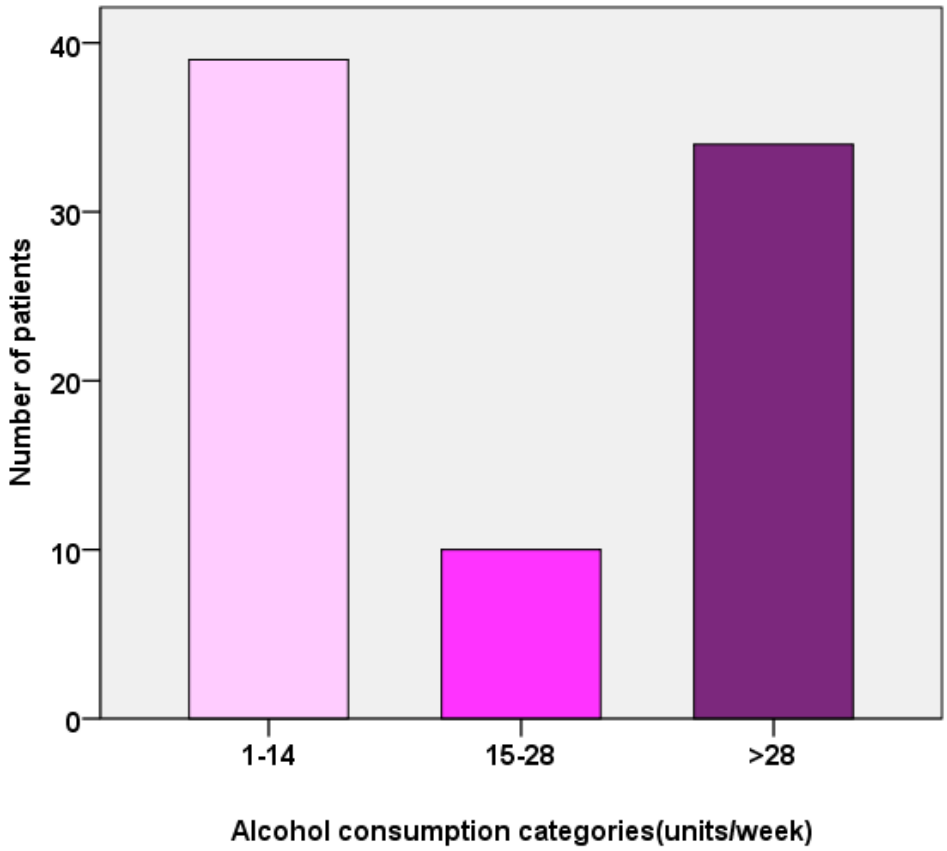

Figure 4.28: Alcohol consumption (units per week) at first presentation.

Age, Sex and Drinking Status

Figure 4.29 shows that the majority of current drinkers were middle aged (57%; 47/83), followed by old (31%; 26/83) and young patients (12%; 10/83).
Chi-Square testing showed a significant association between drinking status and age (p=0.031). Young patients were all current drinkers, with ex-drinkers only seen in middle age. One elderly patient (> 85 years) was a non-drinker.

A significant relationship was found between drinking status at first presentation and sex (p=0.001; Chi-Square test), with the majority of current drinkers male 71% (59/83), and most non-drinkers female 71% (10/14). No females were observed in the ex-drinker category which comprised 3 males; Figure 4.30.

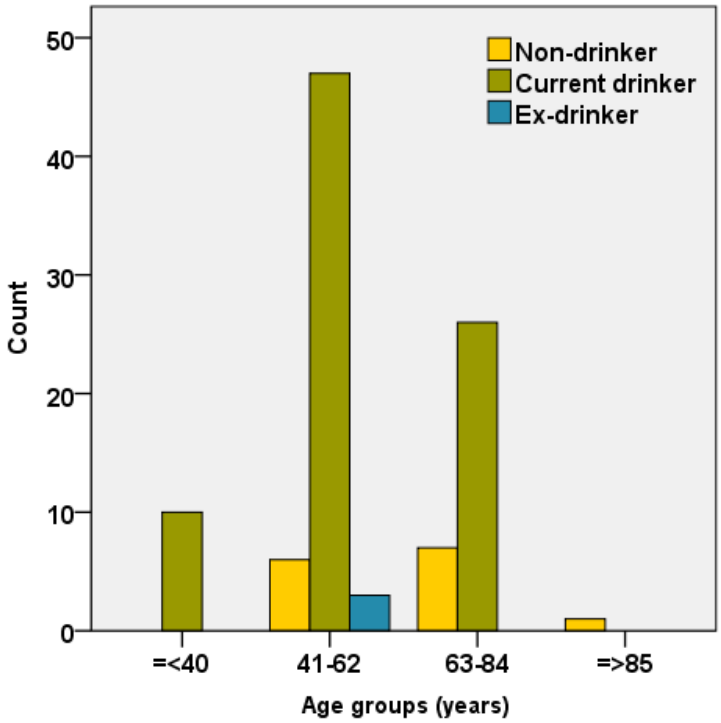

Figure 4.29: Drinking status according to age.

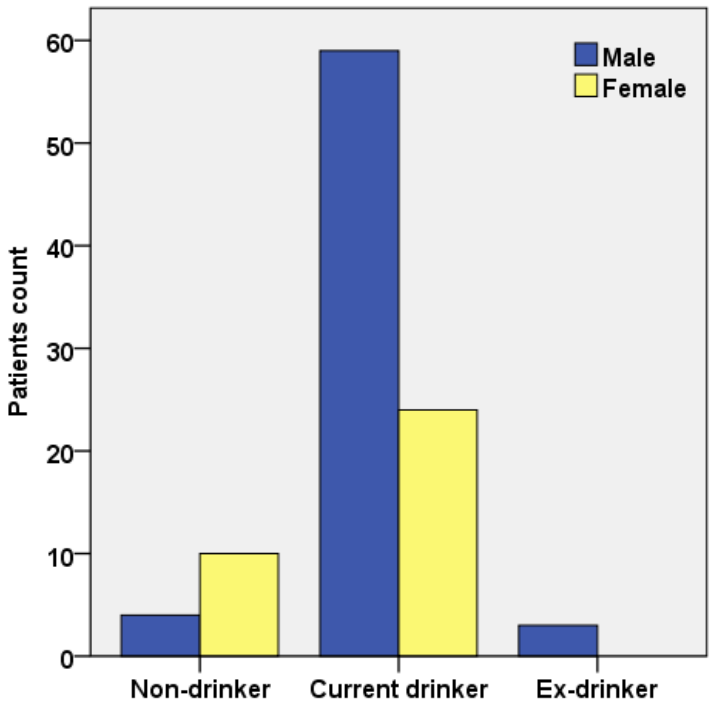

Figure 4.30: Sex distribution and drinking status at first presentation.

Age, Sex and Number of Alcohol units/week

A highly significant relation was found between age and alcohol intake (units/week) (p=0.002; Chi-Square test). Fifty-percent (5/10) of young and 53% (25/47) of middle age patients were heavy drinkers, whilst the majority of old age 77% (20//26) were light drinkers; Table 4.13 and Figure 4.31.

Table 4.14 shows mean alcohol intake (units/week) in relation to age. Using Kruskal-Wallis testing, a significant difference was found between age groups and the amount of alcohol consumed per week (p=0.003). Subsequently, a Mann-Whitney U test was performed and showed a highly significant difference between middle age and old age (p=0.001), with old age patients consuming the lowest amount of alcohol compared to both young and middle age patients (15.9 vs. 34.7 and 36.87 units/week).

A negative, significant correlation was found between amount of alcohol intake (units/week) and age (r=-0.240, n=83, p=0.029), with increasing age associated with a decreased alcohol intake.

Out of 83 current drinkers (59 males and 24 females), males were more frequently heavy drinkers 97% (33/34), followed by 92% (9/10) intermediate and 44% (17/39) light drinkers. The majority of light drinkers were females 56% (22/39), followed by 10% (1/10) intermediate and 3% (1/34) heavy drinkers; Figure 4.32.

Females drank a range of 1-70 units/week with a mean of 9.50 (SD: 13.58), whilst males ranged from 4-140 units/week with a mean of 38.4 (SD: 31.692).
Chi-Square test showed a highly significant relation between sex and amount of alcohol consumed (p=0.001). Subsequently, a Mann-Whitney U test confirmed that males consumed a higher average amount of alcohol per week compared to females (p=0.0001).

Current drinkers were further classified into two groups: those who consumed > 28 and < 28 units/week. Males formed the majority consuming > 28 units/week (97%; 33/34), whilst females mainly drank < 28 units/week (96%; 23/24).
In this study, 58% (38/66) of males and 9% (3/34) of females exceeded the recommended UK department of health alcohol intake guidelines.

Figure 4.33 and Table 4.15 summarise drinking behaviour at first presentation and most recent clinic follow-up.
Significant changes in the amount of alcohol intake by both males and females were seen at the most recent clinical follow-up, with a Mann-Whitney U test confirming a lower mean alcohol intake in females compared to males (p=0.0001). Females consumed alcohol between 1-20 units/week with a mean of 7.42 (SD: 5.055), whilst males drank between 1-147 units/week with a mean of 35.86 (SD: 35.340).

Table 4.13: Amount of alcohol consumed (units/week) according to age.

Age groups (years)	Alcohol intake (units/week)			Total
	1-14	15-28	> 28	
≤ 40	**4** *40%*	**1** *10%*	**5** *50%*	**10** *100%*
41-62	**15** *32%*	**7** *15%*	**25** *53%*	**47** *100%*
63-84	**20** *77%*	**2** *8%*	**4** *15%*	**26** *100%*
Total	**39** *47%*	**10** *12%*	**34** *41%*	**83** *100%*

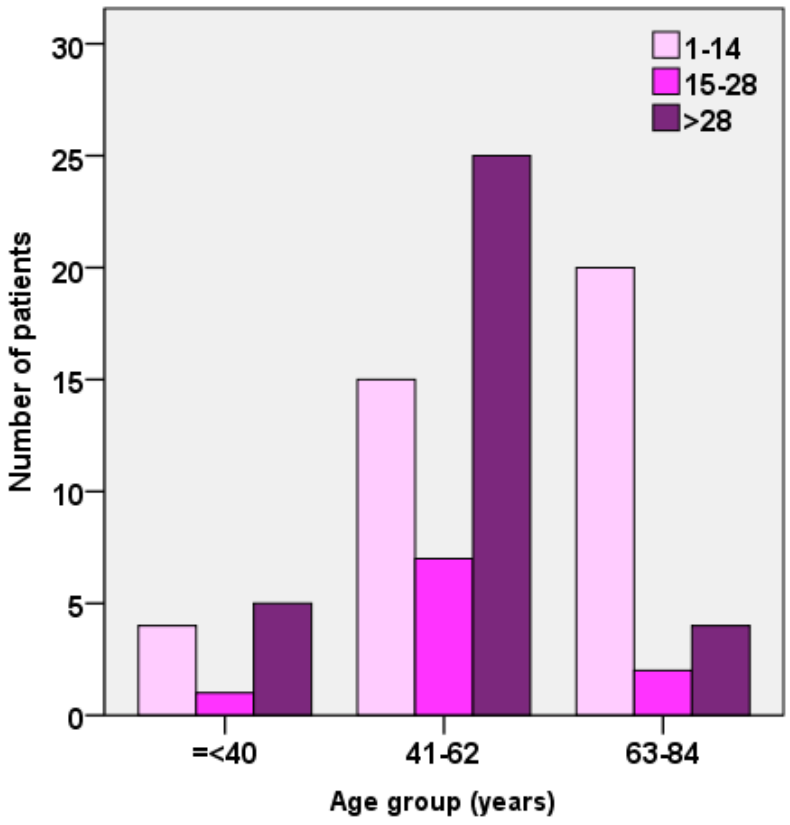

Figure 4.31: Amount of alcohol consumed (units/week) according to age.

Table 4.14: Mean alcohol consumption in relation to age.

Age groups (years)	Mean alcohol intake (units/week)	N	Maximum	Minimum	SD
≤ 40	34.70	10	84	2	29.341
41-62	36.87	47	140	1	33.438
63-84	15.92	26	80	4	19.978
Total	30.40	83	140	1	30.584

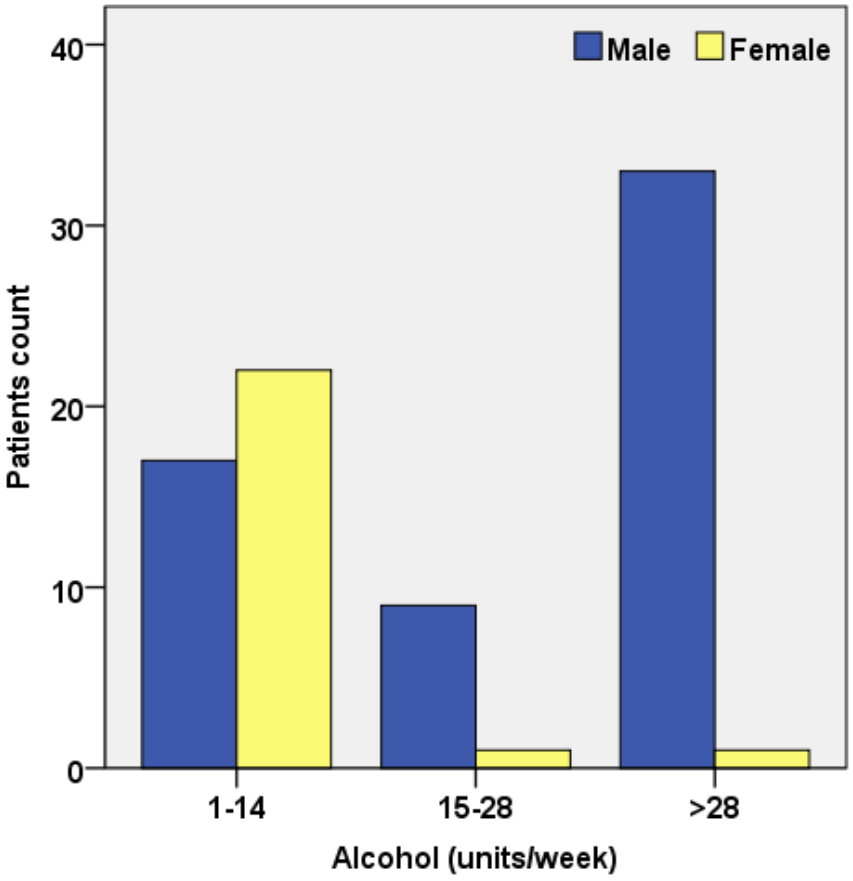

Figure 4.32: Sex distribution and alcohol intake.

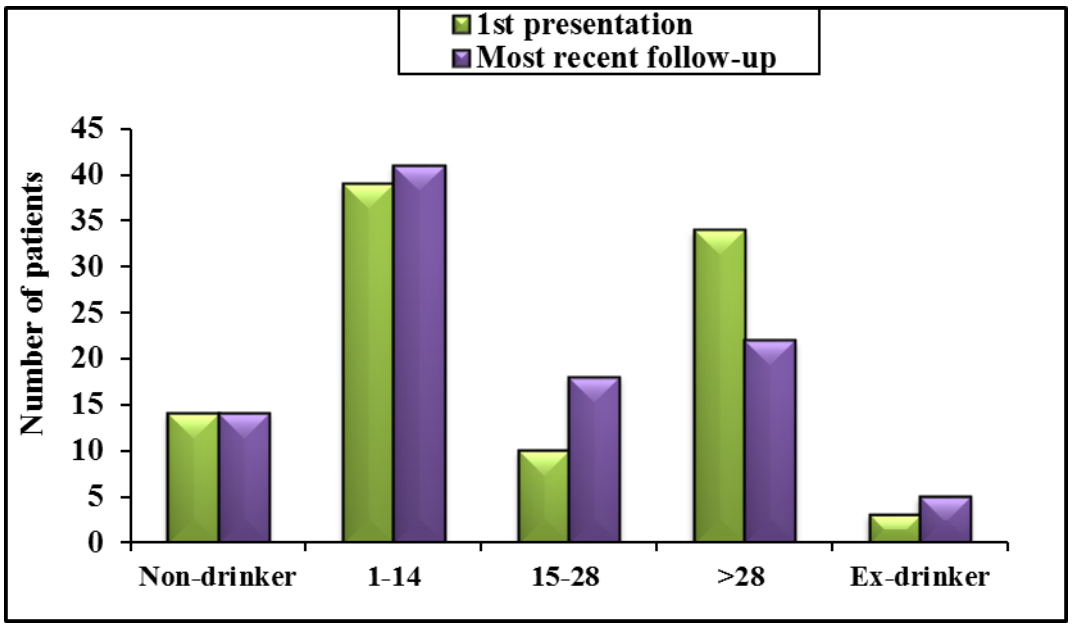

Figure 4.33: Drinking behaviour at initial and most recent follow-up.

Table 4.15: Drinking behaviour at initial and most recent follow-up in relation to sex.

Drinking status		1st presentation			Most recent time		
		Male	Female	Total	Male	Female	Total
Non-drinker		4	10	14	4	10	14
Current drinker (units/week)	1-14	17	22	39	19	22	41
	15-28	9	1	10	16	2	18
	>28	33	1	34	22	0	22
Ex-drinker		3	0	3	5	0	5
Total		66	34	100	66	34	100

Drinking Status, Amount of Alcohol (units/week) and Anatomical Site of PMDs

The relationship between drinking status and anatomical site of PMD can be seen in Figure 4.34.

Current drinkers were more frequently affected with FOM PMDs 46% (38/83), followed by lateral 19% (16) and ventral tongue 15% (12).

Similarly, non-drinkers mainly showed FOM PMDs (43%), followed by lateral (21%) and ventral tongue (14%); buccal mucosa, fauces and alveolar mucosa were not affected in non-drinkers. The FOM (67%; 2/3) and soft palate (33%; 1/3) were the only affected anatomical sites in ex-drinkers. Chi-Square testing showed a significant relation between drinking status and anatomical site (p=0.030).

Eighty-percent (66/83) of current drinkers had PMDs at high-risk sites, compared to 20% (17/83) at low-risk sites, which was similar for non-drinkers and ex-drinkers, although non-significant (p=0.709; Chi-Square test); Table 4.16.

Table 4.17 demonstrates PMD site in relation to alcohol intake. The FOM was the principal site for PMDs in heavy drinkers 56% (20/36), followed by light 41% (14/34) and intermediate drinkers 31% (4/13). All faucial pillar lesions (4/4) and the majority of soft palate PMDs (4/5) were seen in heavy drinkers.

Using Chi-Square test, a highly significant relation was found between amount of alcohol intake (units/week) and all anatomical sites (p=0.0001), 5 sites (p=0.001) or high/low-risk sites (p=0.025). All heavy, light and intermediate drinkers developed PMDs more frequently in high-risk compared to low-risk sites; Table 4.18.

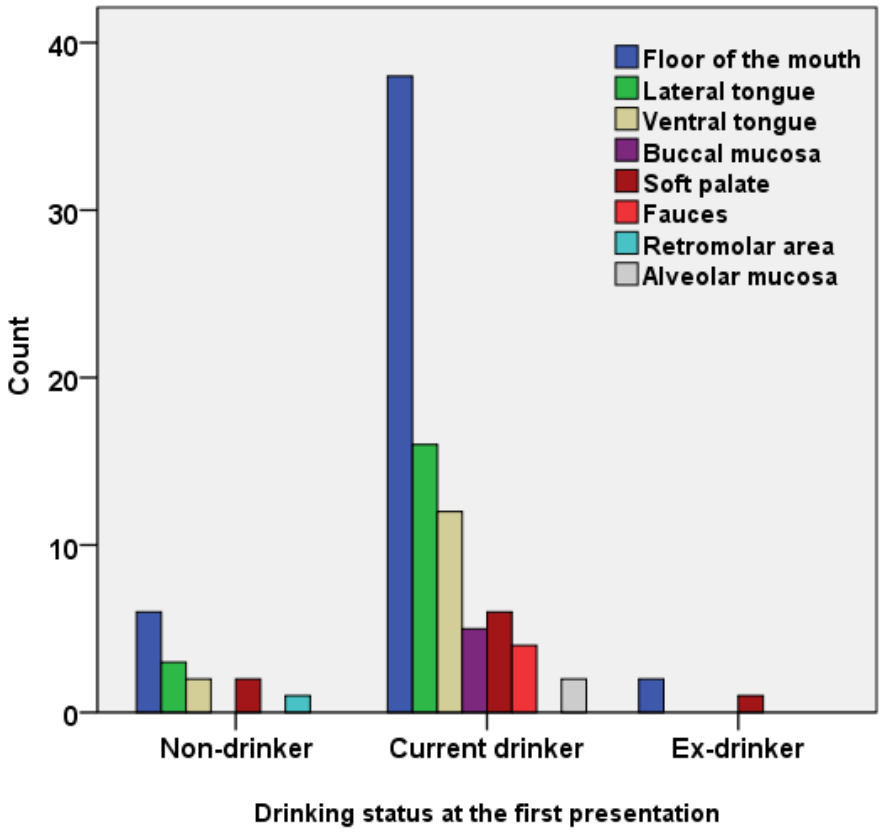

Figure 4.34: PMD anatomical site in relation to drinking status.

Table 4.16: PMD high and low-risk sites according to alcohol status.

Alcohol status	PMDs anatomical site		Total
	High risk	Low risk	
Non-drinker	**11** *79%*	**3** *21%*	**14** *100%*
Current drinker	**66** *80%*	**17** *20%*	**83** *100%*
Ex-drinker	**2** *67%*	**1** *33%*	**3** *100%*
Total	**79** *79%*	**21** *21%*	**100** *100%*

Table 4.17: Anatomical site of PMD according to the amount of alcohol drinking.

PMD anatomical sites	Alcohol intake (units/week)			Total
	1-14	15-28	> 28	
FOM	**14**	**4**	**20**	**38** *46%*
Lateral tongue	**11**	**3**	**2**	**16** *19%*
Ventral tongue	**7**	**2**	**3**	**12** *15%*
Buccal mucosa	**2**	**1**	**2**	**5** *6%*
Soft palate	**-**	**1**	**5**	**6** *7%*
Fauces	**-**	**-**	**4**	**4** *3%*
Alveolar mucosa	**-**	**2**	**-**	**2** *2%*
Total	**34**	**13**	**36**	**83** *100%*

Table 4.18: Alcohol intake in relation to high/low-risk sites.

Alcohol intake (units/week)	PMD anatomical sites		Total
	High risk	Low risk	
1-14	**32** *94%*	**2** *6%*	**34** *100%*
15-28	**9** 69%	**4** 31%	**13** *100%*
> 28	**25** *69%*	**11** *31%*	**36** *100%*
Total	**66** *80%*	**17** *20%*	**83** *100%*

Drinking Status, Amount of Alcohol (units/week) and Clinical Appearance of PMD

Table 4.19 shows the relationship between drinking status and clinical appearance of PMDs. Homogenous leukoplakias and specked non-homogenous leukoplakias were the most common clinical types in all groups.

In current drinkers, homogenous leukoplakia, followed by the specked subtype, erythroplakia, exophytic subtypes and an equal distribution of ulcerated and nodular subtypes were seen.
Out of 8 erythroplakias, 7 were reported in current drinkers with the remaining a non-drinker.
All ulcerated PMDs were observed in current drinkers.
Using Chi-Square testing, no significant relation was found between clinical type of PMD and drinking status (p=0.318).

Table 4.20 summarises the relationship between amount of alcohol intake and type of leukoplakia; most homogenous leukoplakias were seen in light drinkers (52%; 28/54) compared to 37% (20/54) in heavy drinkers. Fifty-percent (11/22) of non-homogenous leukoplakias were reported in heavy drinkers compared to 36% (8/22) in light drinkers.

Figure 4.35 shows the distribution of alcohol intake (units/week) according to the clinical appearance of PMDs, which showed a significant association (p=0.013; Chi-Square test).
Erythroplakias were observed equally in heavy and light drinkers (43% each), specked non-homogenous subtypes were most common in heavy drinkers compared to light drinkers (62% vs. 30%). Similarly, exophytic subtypes were more frequently seen in heavy drinkers compared to light drinkers (60% vs. 40%).

Table 4.19: Clinical type of PMD in relation to drinking status.

Clinical appearance of PMDs		Drinking status			Total
		Non-drinker	Current drinker	Ex-drinker	
Homogenous leukoplakia		11	54	2	67
Non-homogenous leukoplakia	Speckled	2	13	1	16
	Nodular	-	2	-	2
	Exophytic	-	5	-	5
	Ulcerated	-	2	-	2
Erythroplakia		1	7	-	8
Total		14	83	3	100

Table 4.20: Alcohol intake in relation to type of leukoplakia.

Types of leukoplakia	Amount of alcohol intake (units/week)			Total
	1-14	15-28	> 28	
Homogenous	**28** *52%*	**6** *11%*	**20** *37%*	**54** *100%*
Non-homogenous	**8** *36%*	**3** *14%*	**11** *50%*	**22** *100%*
Total	**36** *47%*	**9** *12%*	**31** *41%*	**76** *100%*

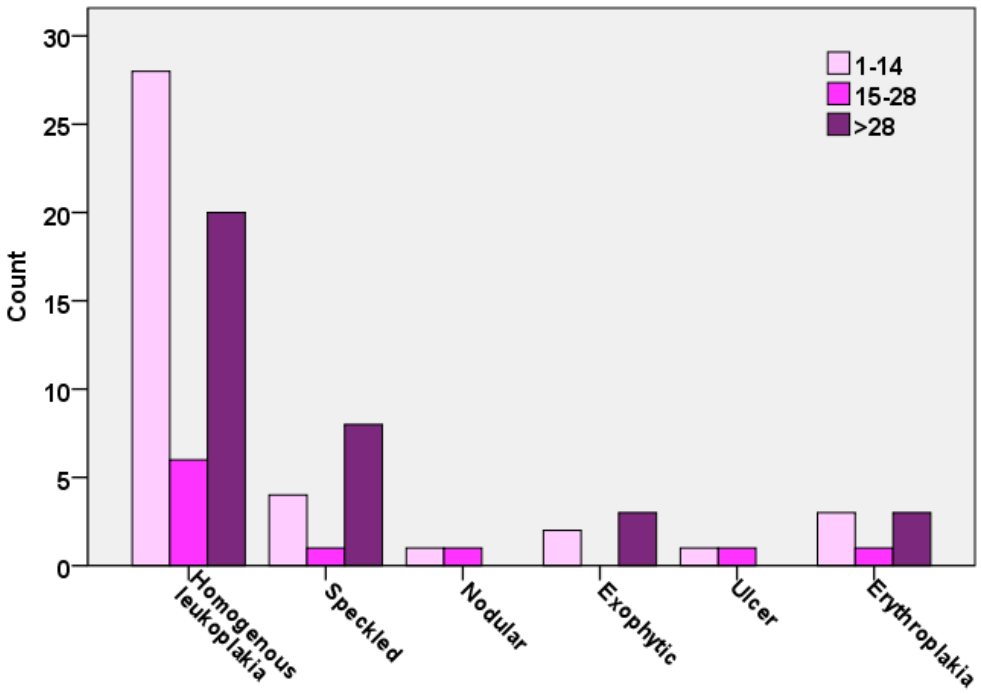

Figure 4.35: Alcohol intake (units/week) in relation to clinical appearance of PMDs.

Drinking Status, Amount of Alcohol (units/week) and Dysplasia Grading

Table 4.21 and Figure 4.36 show the relationship between drinking status and oral epithelial dysplasia. In current drinkers, mild dysplasia (40%; 33/83) was most common, followed by moderate (25%; 21/83), severe dysplasia (23%; 19/83) and CIS (12% (10/83). Similarly, non-drinker patients were mainly diagnosed with mild dysplasia 64% (7/11), followed by moderate and severe (18%; 2/11) equally; no CIS was diagnosed in this group. Ex-drinkers were mainly affected with mild (2/3), followed by severe (1/3), whilst no moderate dysplasia or CIS were seen. Using Chi-Square testing, no significant relation was found between drinking status and degree of dysplasia (p=0.059).

A lower mean alcohol intake was seen in patients with mild dysplasia (22) compared to moderate (37), severe (29) and CIS (43); however, the differences were not significant (p=0.449; Kruskal-Wallis test); Table 4.22. No significant correlation was found between alcohol intake and degree of dysplasia (r=0.097, n=83, p>0.01).

Table 4.23 shows alcohol intake in relation to the grade of dysplasia. The majority of mild and severe dysplasias were observed in light drinkers, whilst the majority of moderate dysplasia and CIS were seen in heavy drinkers. However, no significant relation was found between alcohol intake and degree of dysplasia (p=0.067; Chi-Square test).

Overall, at the most recent follow-up, average alcohol intake was lower than at first presentation (27.5 vs. 30 units/week), albeit non-significant (p=0.541; Sign test). Ex-drinkers increased from 3% to 5%, intermediate drinkers increased from 12% to 21%, whilst heavy drinkers decreased from 34% to 23%; Figure 4.37.

Chi-Square test showed a highly significant relation between sex and both drinking status (p=0.001) and number of alcohol units consumed per week (p=0.0001) at most recent follow-up. Although there were no clear changes in female drinking behaviour, males showed noticeable changes at most recent follow-up, with heavy drinkers decreasing from 33 to 22, intermediate drinkers increasing from 9 to 16, and light drinkers increasing from 17 to 19; Table 4.24.

Table 4.21: Drinking status and epithelial dysplasia grading.

Drinking status	Oral epithelial dysplasia (consensus WHO scoring)				Total
	Mild	Moderate	Severe	CIS	
Non-drinker	**7**	**2**	**2**	**-**	**11**
Current drinker	**33**	**21**	**19**	**10**	**83**
Ex-drinker	**2**	**-**	**1**	**-**	**3**
Total	**42** *43%*	**23** *24%*	**22** *23%*	**10** *10%*	**97** *100%*

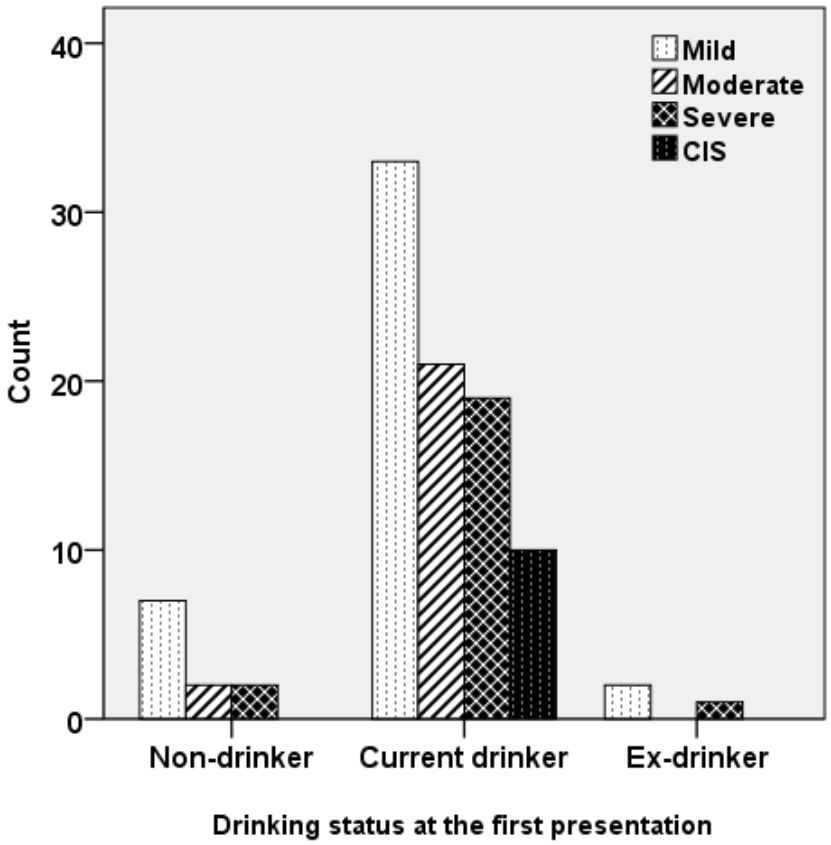

Figure 4.36: Drinking status and epithelial dysplasia grading.

Table 4.22: Average alcohol intake in relation to the degree of epithelial dysplasia.

Dysplasia grade	Mean (units/week)	N	Minimum	Maximum	SD
Mild	**22.06**	**33**	**1**	**60**	**17.960**
Moderate	**37.00**	**21**	**2**	**120**	**37.199**
Severe	**29.37**	**19**	**4**	**140**	**35.603**
CIS	**43.10**	**10**	**4**	**112**	**35.127**
Total	**30.05**	**83**	**1**	**140**	**30.594**

Table 4.23: Alcohol intake in relation to degree of dysplasia.

Alcohol (units/week)	Oral epithelial dysplasia				Total
	Mild	Moderate	Severe	CIS	
1-14	**16**	**8**	**10**	**3**	**37**
15-28	**6**	**4**	**2**	**-**	**12**
> 28	**11**	**9**	**7**	**7**	**34**
Total	**33** *40%*	**21** *25%*	**19** *23%*	**10** *12%*	**83** *100%*

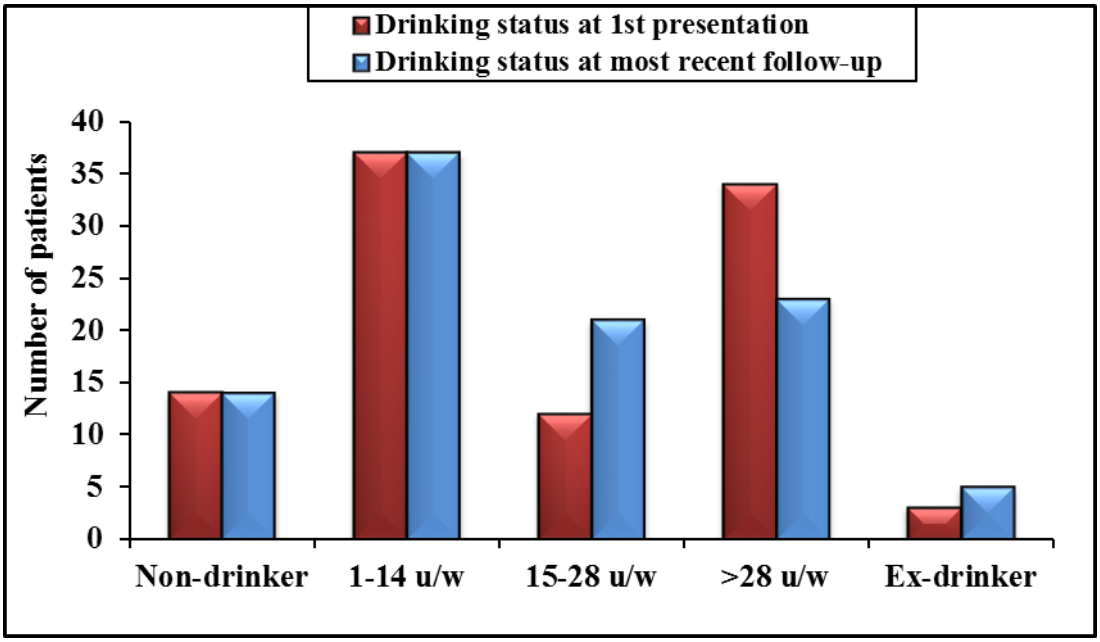

Figure 4.37: Drinking status at first presentation and most recent follow-up.
(u/w=units/week)

Table 4.24: Drinking status at first presentation and most recent follow-up, according to sex.

Drinking status		1st presentation			Most recent follow-up time		
		Male	Female	Total	Male	Female	Total
Non-drinker		4	10	14	4	10	14
Current drinker (units/week)	1-14	17	22	39	19	22	41
	15-28	9	1	10	16	2	18
	> 28	33	1	34	22	-	22
Ex- drinker		3	-	3	5	-	5
Total		66	34	100	66	34	100

The Association between Tobacco Smoking and Alcohol Drinking

Overall, Chi-Square testing showed a significant relationship between smoking and alcohol behaviour in the study population both at first presentation (p=0.003) and most recent follow-up (p=0.039); Figure 4.38.

At initial presentation, current drinkers were mainly also current smokers (92%), followed by non-smokers (73%) and ex-smokers (64%). Fifty-percent (7/14) of non-drinkers were also ex-smokers, followed by non-smokers (29%; 4/14) and current smokers (21%; 3/14).
In this study, there were only 3 ex-drinkers, 2 (67%) were current smokers and the remaining one (33%) an ex-smoker.

No significant correlation was found between the number of cigarettes smoked per day or tobacco grams per week and the amount of alcohol intake at the time of presentation (r=0.053, n=63 p>0.01), (r=0.045, n=63, p>0.01), respectively.

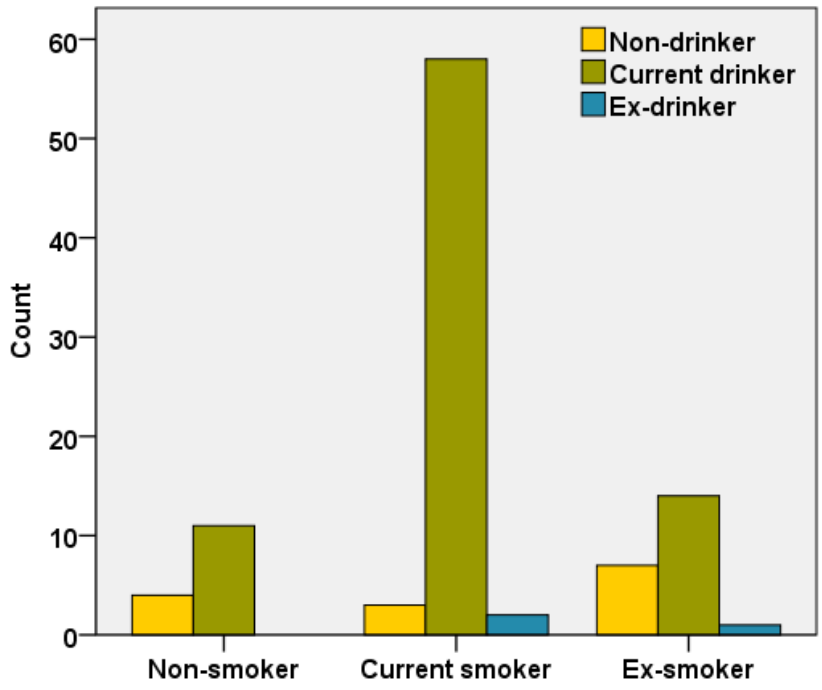

Figure 4.38: The relation between drinking and smoking status at first presentation.

4.4.3. Medical History of Patients with PMDs

In this study, five medical problems were investigated: immunodeficiency, anaemia, oral candida infection, diabetes mellitus and hypertension.

Eighty-eight patients reported a positive medical history with one or more active, systemic diseases. Hypertension (60%) was the most common, followed by diabetes mellitus (15%), candida infection (8%), anaemia (4%) and immunodeficiency (1%); Figure 4.39.

Figure 4.40 shows the distribution of systemic disease in relation to male and female patients; 55% of males and 33% of females were affected. Males were primarily affected by hypertension, candida infection and anaemia, whilst females more frequently had diabetes mellitus.

Using Fisher's Exact testing, no significant association was found between systemic health status and sex (p=0.536). Seventy-percent of PMD patients also reported other medical conditions such as those related to the cardiovascular (28%), respiratory (19% and musculoskeletal systems (18%), liver disease (15%), psychological disorders (10%), nervous system (8%), digestive system (7%), endocrine (6%), skin (4%) and renal problems (3%); Figure 4.41 and Table 4.25.

No significant association was found between the occurrence of other medical problems and sex, although males were more likely to be affected than females (46/70 vs. 24/70) (p=1.000; Fisher's Exact test).

A significant association was found between age group and systemic health status (p=0.045; Chi-Square test). Patients with a positive medical history were mainly in middle age (53%; 47/88), followed by old (38%; 33/88) and young age groups (8%; 7/88).
All patients in old age (33/33), 84% (47/56) of middle age and 70% (7/10) of young age had a positive medical history; Table 4.26 and Figure 4.42.

Table 4.27 shows a significant relationship between systemic health and degree of dysplasia (p=0.021; Chi-Square test). In general, higher dysplastic features were seen in patients with a positive medical history: 40% (34/85) mild, 26% (22/85) severe, 22% (19/85) moderate dysplasia and 12% (10/85) CIS. Patients with negative medical histories had no severe dysplasia or CIS, with mild the main dysplastic feature (67%; 8/12), followed by moderate (33%; 4/12).

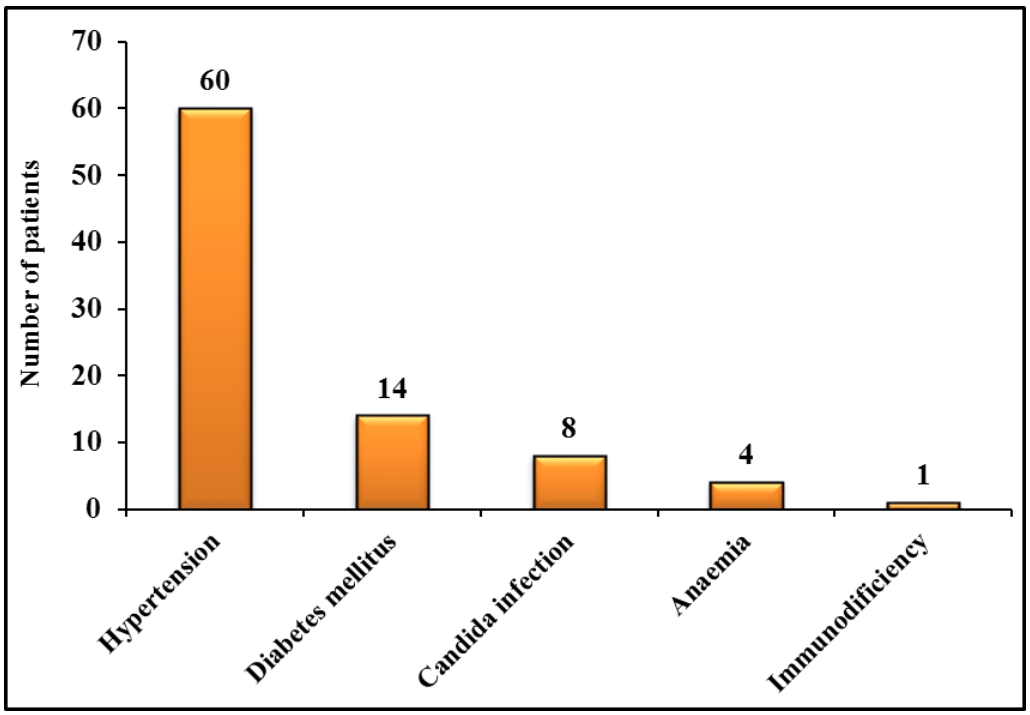

Figure 4.39: Distribution of systemic disease amongst 100 PMD patients.

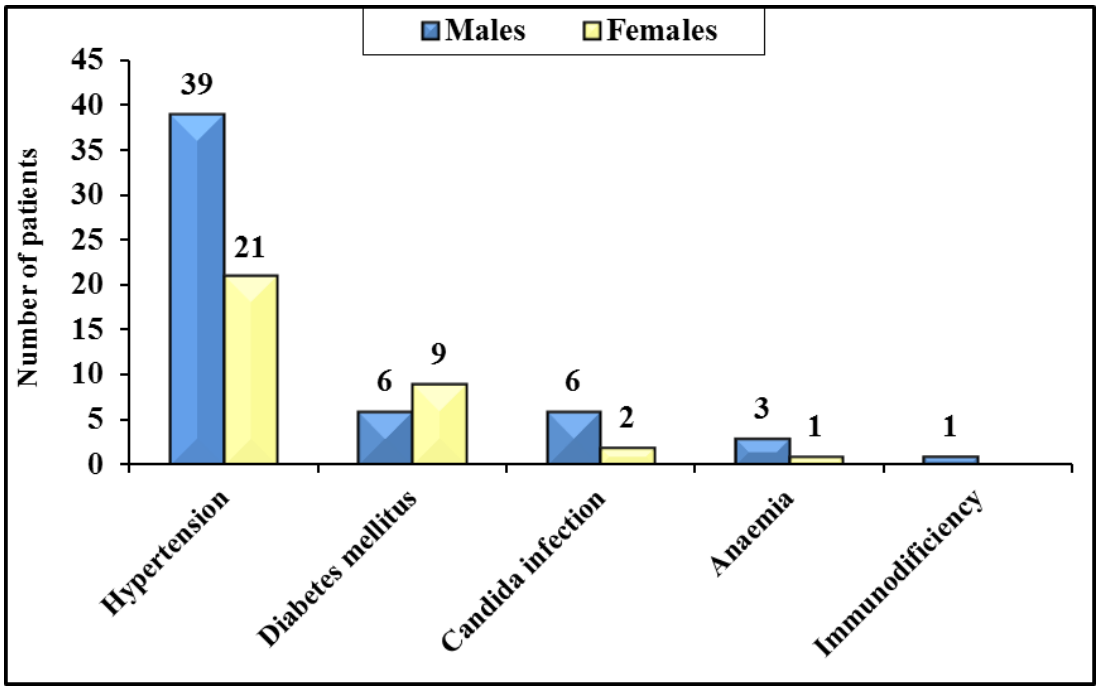

Figure 4.40: Distribution of systemic disease according to sex.

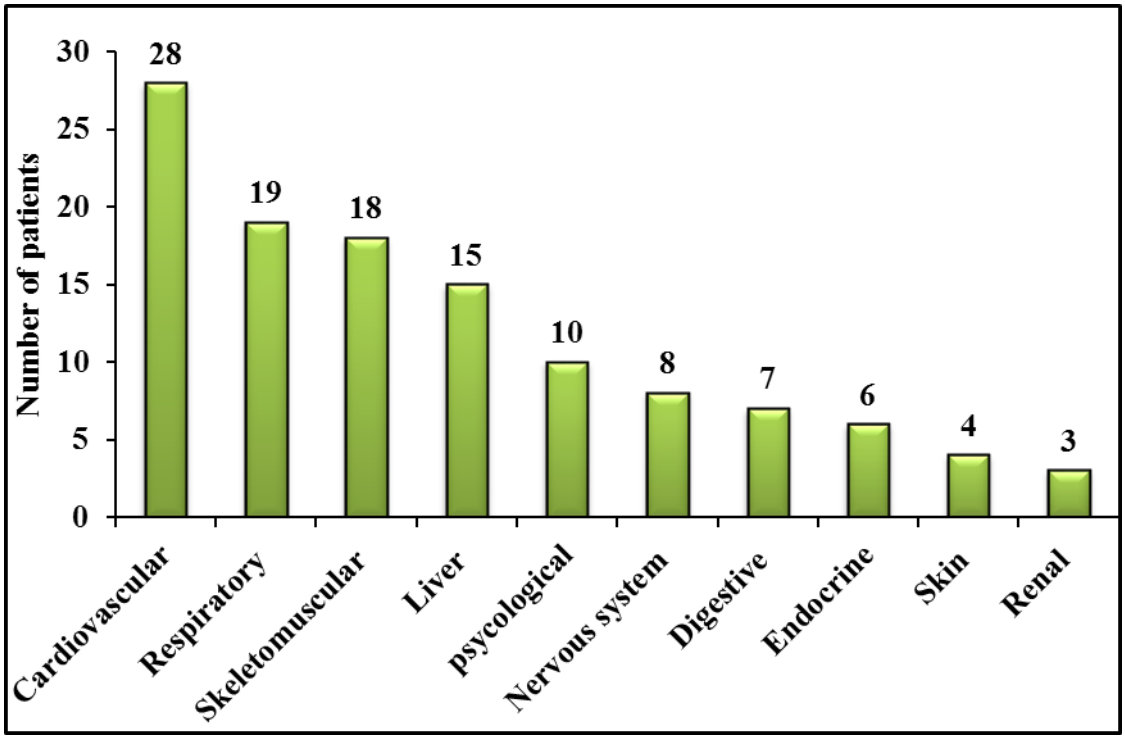

Figure 4.41: Other medical conditions recorded in 100 PMD patients.

Table 4.25: Details of the other associated medical conditions.

System	Associated medical problems
Cardiovascular	Myocardial Infarction, Cerebrovascular accident (CVA) Cardiac failure, Ischemic heart disease, Rheumatic fever
Respiratory	Asthma, Chronic cough, Recurrent chest infection, TB, Partially collapsed lung
Skeleton-muscular	Asymmetric cervical myelopathy, Osteoarthritis, Rheumatoid arthritis, Spondylosis, Autoimmune dermatomyositis
Liver	Hepatitis, Alcoholic related jaundice, Liver cirrhosis
Psychological	Anxiety, Depression
Nervous system	Multiple sclerosis, Peripheral neuropathy, Epilepsy
Digestive	Esophagitis, Stomach and duodenal ulcer, Celiac disease
Endocrine	Hyperthyroidism, Hypothyroidism
Skin	Psoriasis, Eczema
Renal	Kidney problem, Prostate

Table 4.26: Systemic health status in relation to age.

Age group (years)	Systemic health status		Total
	No systemic disease	Positive systemic disease	
≤ 40	**3** *25%*	**7** *8%*	**10** *10%*
41-62	**9** *75%*	**47** *53%*	**56** *56%*
63-84	**-**	**33** *38%*	**33** *33%*
≥ 85	**-**	**1** *1%*	**1** *1%*
Total	**12** *100%*	**88** *100%*	**100** *100%*

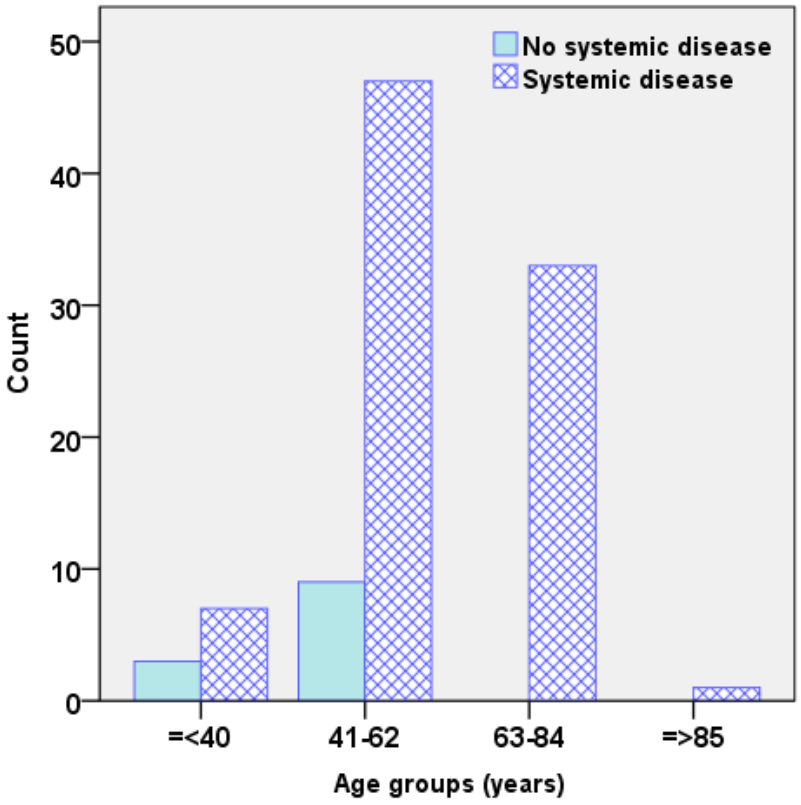

Figure 4.42: Systemic health status in relation to age.

Table 4.27: Systemic health status in relation to the degree of dysplasia.

Degree of dysplasia	Systemic health status		Total
	No systemic disease	Positive systemic disease	
Mild	**8** *67%*	**34** *40%*	**42** *43%*
Moderate	**4** *33%*	**19** *22%*	**23** *24%*
Severe	**-**	**22** *26%*	**22** *23%*
CIS	**-**	**10** *12%*	**10** *10%*
Total	**12** *100%*	**85** *100%*	**97** *100%*

Immunodeficiency

Out of the 100 patients, one 48 years old male was immune-suppressed resulting from cyclosporine and prednisolone use for autoimmune dermatomyositis. This patient had severe dysplasia and had significant comorbidity at the time of diagnosis, including thyroid problems, epilepsy, psoriasis and celiac disease.

Anaemia

Four patients reported anaemia diagnosed 1-2 years before initial PMD diagnosis; three males (42, 65 and 71 years) and one female (55 years). Two patients had moderate, one severe and one mild dysplasia.

Oral Candida Infection

Eight cases of oral candida infection were reported, two in females and six in males. Four cases were seen in tongue, three in buccal mucosa and one in soft palate. Two cases exhibited homogenous leukoplakia, five with non-homogenous leukoplakia and only one with erythroplakia. Four patients had mild dysplasia, two moderate and similar numbers with severe dysplasia.

The majority of patients in this group were current drinkers (88%) and smokers (75%).
Candida infection was diagnosed at initial presentation in six patients, whilst the remaining two were identified after laser intervention during follow-up.
Five patients with candida infection used dental prostheses, (2 crowns and bridges and 3 dentures).

Diabetes Mellitus (DM)

In this study, 15 patients had DM; ten type II and five type I. A significant relation was found between DM status and sex (p=0.036; Fisher's Exact test); 60% (9/15) were reported in females compared to 40% (6/15) in males.

An equal distribution of diabetic patients was seen in middle and old age groups (7/15), whilst only one patient was in the young age group (≤ 40 years); Chi-Square test was non-significant (p=0.483); Figure 4.43.

Figure 4.44 shows the relation between PMD clinical appearance and DM. No significant association was found between DM and leukoplakia or erythroplakia (p=0.602; Fisher's Exact test), homogenous or non-homogenous lesions (p=1.000; Fisher's Exact test) or between non-homogenous subtypes (p=0.521; Chi-Square test).
A significant association was found between DM and clinical appearance, however, when all clinical types were considered together (0.035; Chi-Square test).

Chi-Square testing showed no significant relationship between PMD anatomical site and DM status, considering all sites (p=0.151) or high/low-risk sites (p=0.731; Fisher's Exact test). However, PMDs were more frequently seen in high-risk sites in DM patients (87%; 13/15) compared to low-risk sites (13%; 2/15).

Figure 4.45 shows no significant relationship between DM and degree of dysplasia (p=0.219; Chi-Square test). In diabetic patients, mild (5/15) was most common, followed by severe (4/15) and moderate dysplasia and CIS equally (3/15 for each).

The majority of diabetics were diagnosed with high grade dysplasia compared with non-diabetics (9/15; 60% vs. 44/85; 52%), although no significant association was found (p=0.589; Fisher's Exact test).

Most diabetic patients were current smokers (8/15), followed by ex-smokers (4/15) and non-smoker (3/15); Figure 4.46. Amongst current smoker, 5 were intermediate, whilst 3 were

heavy smokers. No significant relation was found between diabetic status and intensity of smoking (cigarettes/day) (p=0.469; Chi-Square test).

Similarly, Chi-Square testing was non-significant between DM and drinking status (p=0.579), although most diabetic patients were current drinkers (13/15).

Light drinkers (77%; 10/13) were most common, followed by heavy drinkers (3/13) in diabetic patients, whilst the majority of non-diabetic patients were heavy drinkers (44%; 31/70); Figure 4.47.

A borderline significant association was seen between DM and intensity of alcohol intake (units/week) (p=0.050; Chi-square test).

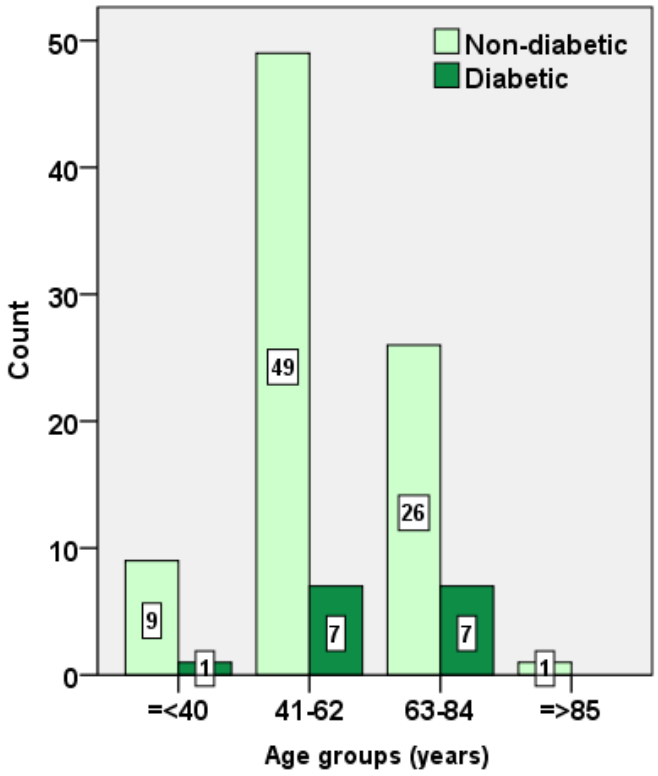

Figure 4.43: Diabetes mellitus in relation to age.

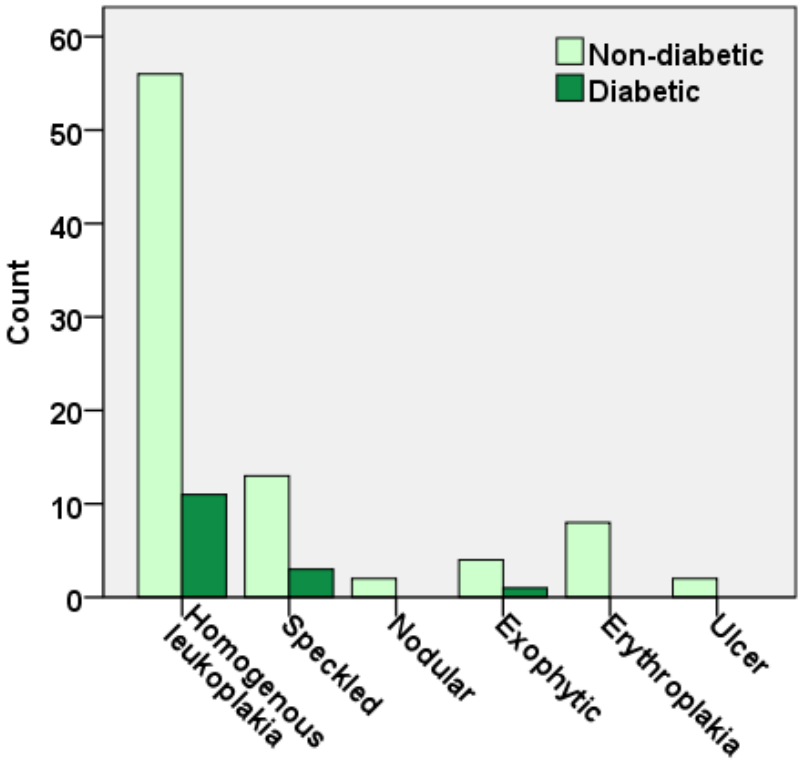

Figure 4.44: Diabetes mellitus in relation to PMD clinical appearance.

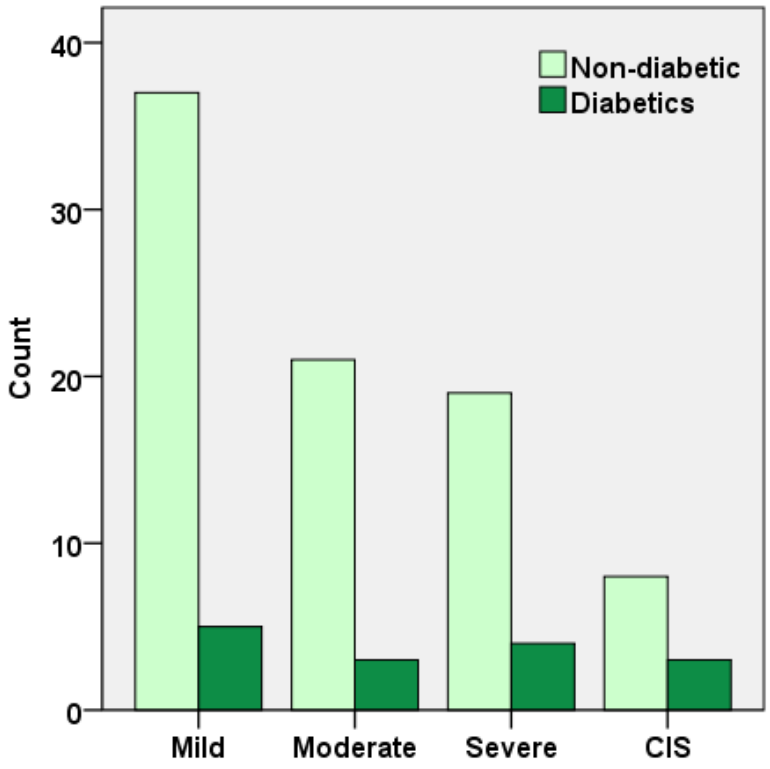

Figure 4.45: Diabetes mellitus in relation to degree of dysplasia.

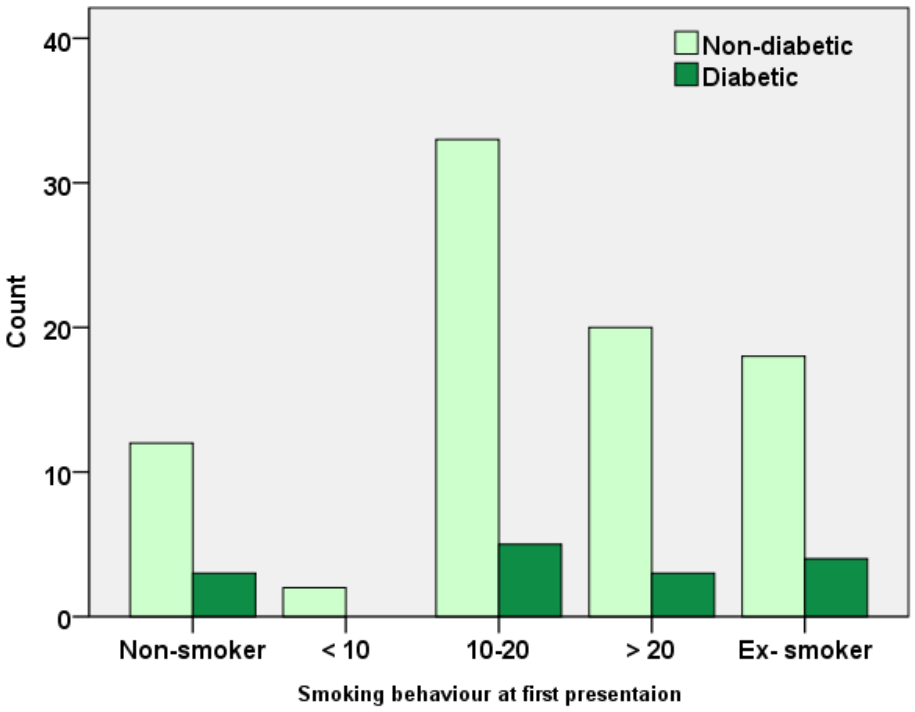

Figure 4.46: Diabetes mellitus in relation to smoking behaviour of PMD patients.

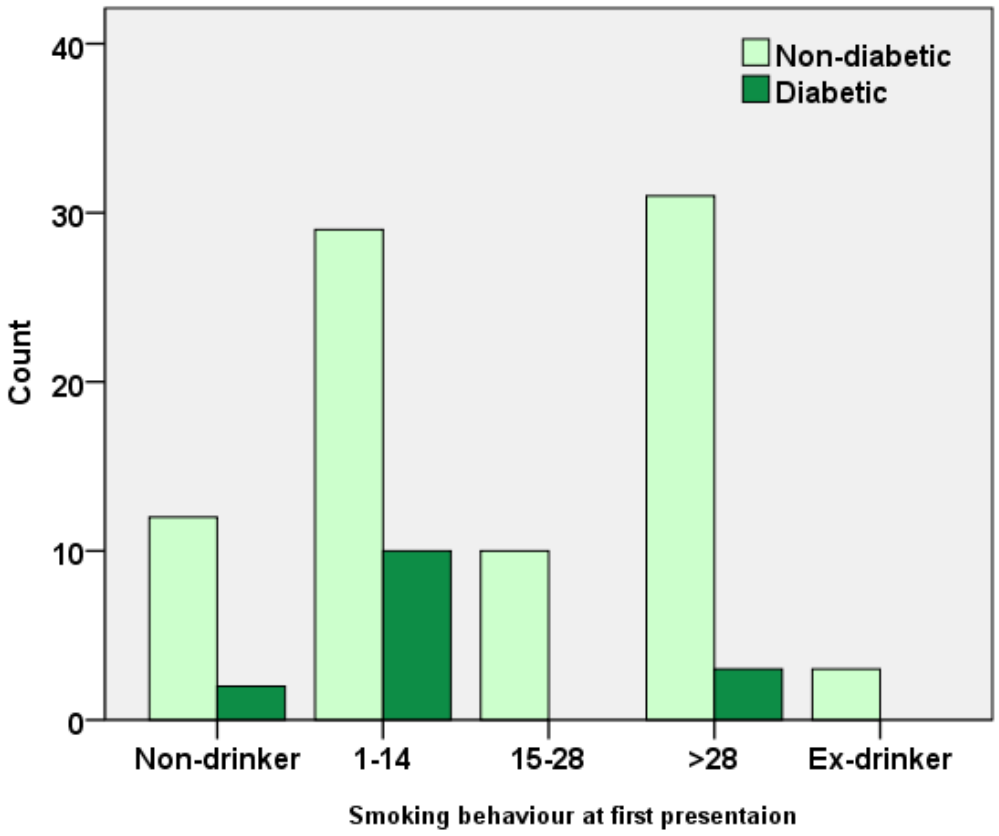

Figure 4.47: Diabetes mellitus in relation to drinking behaviour of PMD patients.

Hypertension

Hypertension was the most common systemic disease, affecting 60% of PMD patients, who used a variety of medical treatments.

Although males (39/60) were more common than females (21/60), no significant relationship was found between hypertension status and sex (p=0.832; Fisher's Exact test); Figure 4.48.

A significant difference was found in mean age between hypertensive and normotensive patients (p=0.02; Independent t-test), with hypertensive patients (60.05 years) older than normotensive ones (54.23 years).

Females with hypertension showed a higher mean age than hypertensive males (63.33 years vs. 58.38 years), although this was not significant (p=0.14; Independent t-test).

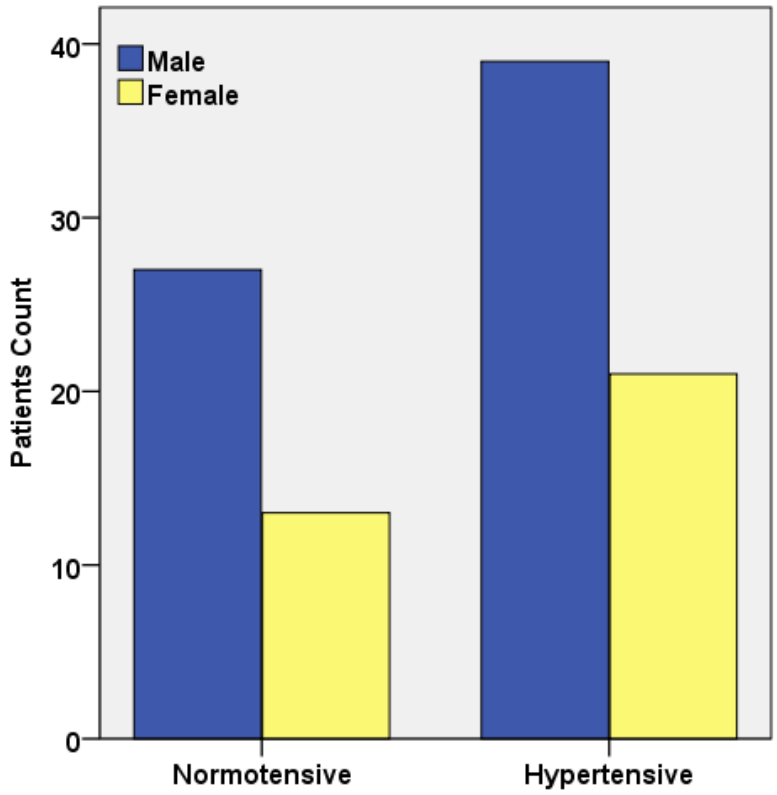

Figure 4.48: Sex distribution according to hypertension status.

Hypertension and PMD Clinical Appearance

Table 4.28 shows that out of 60 patients with hypertension, 54 had leukoplakia and 6 erythroplakia.

Homogenous leukoplakia was the main clinical type in both normotensive (70%; 28/40) and hypertensive patients (65% (39/60).

Erythroplakia was more frequently seen in hypertensive patients (6/8) compared with normotensive patients (2/8), although no significant relation was found between hypertension status and leukoplakia or erythroplakia (p=0.471; Fisher's Exact test).

Non-homogenous leukoplakia was more often seen in hypertensive patients (60%; 15/25) compared to normotensive (40%; 10/25), albeit non-significant (p=1.000, Fisher's Exact test). A significant association was found between hypertension and non-homogenous leukoplakia subtypes (p=0.028; Chi-Square test). Speckled, non-homogenous leukoplakias were predominantly seen in hypertensive patients (75%; 12/16) compared to normotensive (25%; 4/16). All nodular and 60% (3/5) of exophytic were more likely to appear in normotensive patients, whilst ulcerated was seen equally in hypertensive and normotensive patients.

Table 4.28: Association between hypertension status and PMD clinical appearance.

Clinical appearance of PMDs		Hypertension status		Total
		Normotensive	Hypertensive	
Homogenous leukoplakia		28	39	67
Non-homogenous leukoplakia	Speckled	4	12	16
	Nodular	2	-	2
	Exophytic	3	2	5
	Ulcerated	1	1	2
Erythroplakia		2	6	8
Total		40	60	100

Hypertension and PMD Anatomical Site

Figure 4.49 displays PMD anatomical site in relation to hypertension status. A significant relation was found (p=0.0001; Chi-Square test), with FOM, lateral and ventral tongue, and soft palate the main sites for PMDs in patients with hypertension. The faucial pillars were equally seen in normotensive and hypertensive patients, and buccal mucosa was most common in normotensive patients.

In hypertensive patients most PMDs were seen at high-risk sites (63%; 50/79), compared to normotensive patients (37%; 29/79); Fisher's Exact test was not significant, however (p=0.217); Figure 4.50.

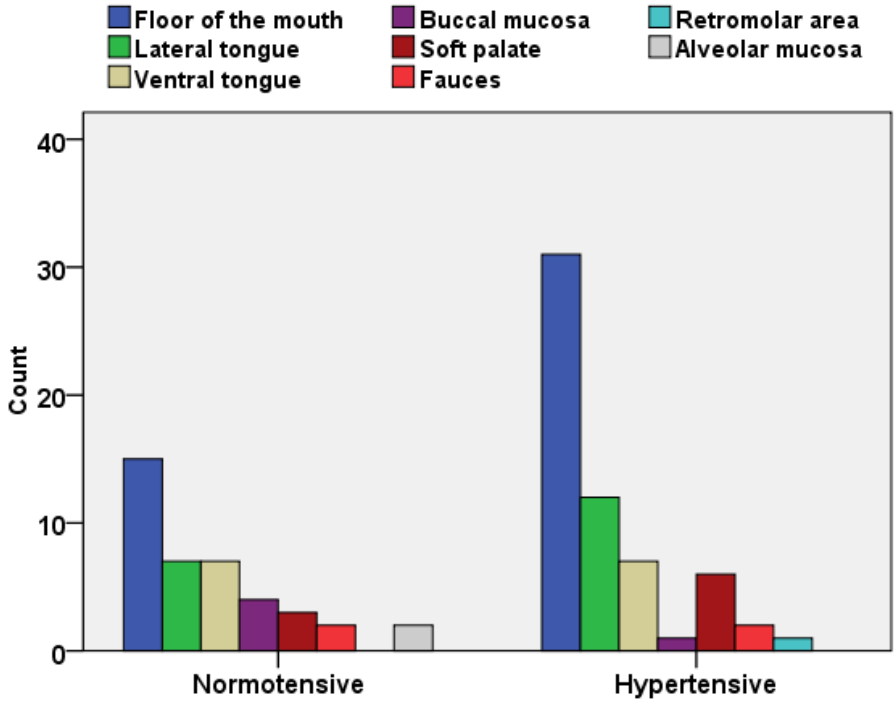

Figure 4.49: PMD anatomical site in relation to hypertension status.

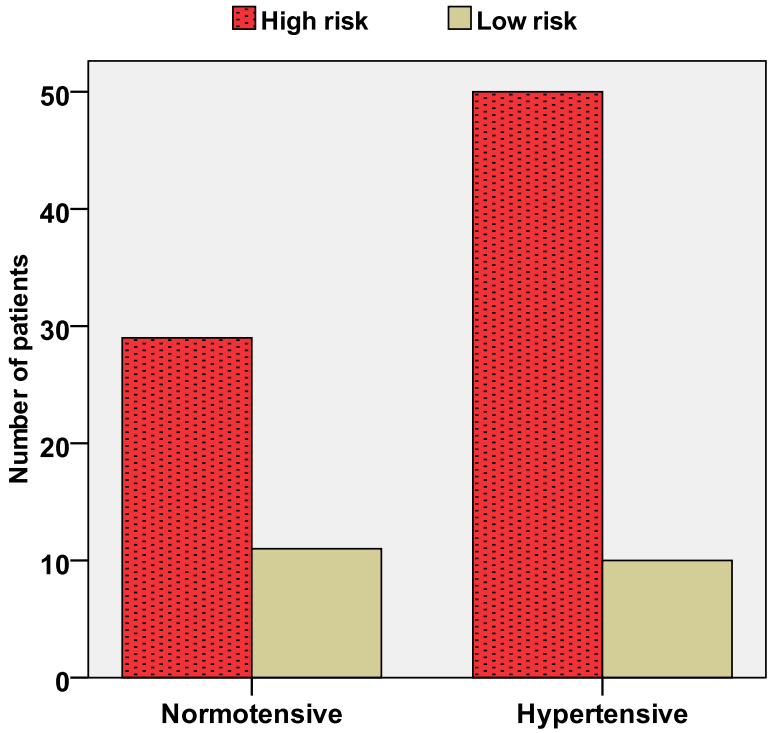

Figure 4.50: High and low-risk sites in relation to hypertension status.

Hypertension and PMD Size

Patients with hypertension showed predominantly minor sized PMDs, followed by intermediate and major size, whilst normotensive patients mainly showed intermediate sized lesions; Figure 4.51.

Although a lower mean size was seen in hypertensive compared to normotensive patients (291.93 vs. 312.13 mm^2), a Mann-Whitney U test was not significant (p=0.523), and

Chi-Square testing showed no significant relationship between hypertension and PMD size (p=0.629).

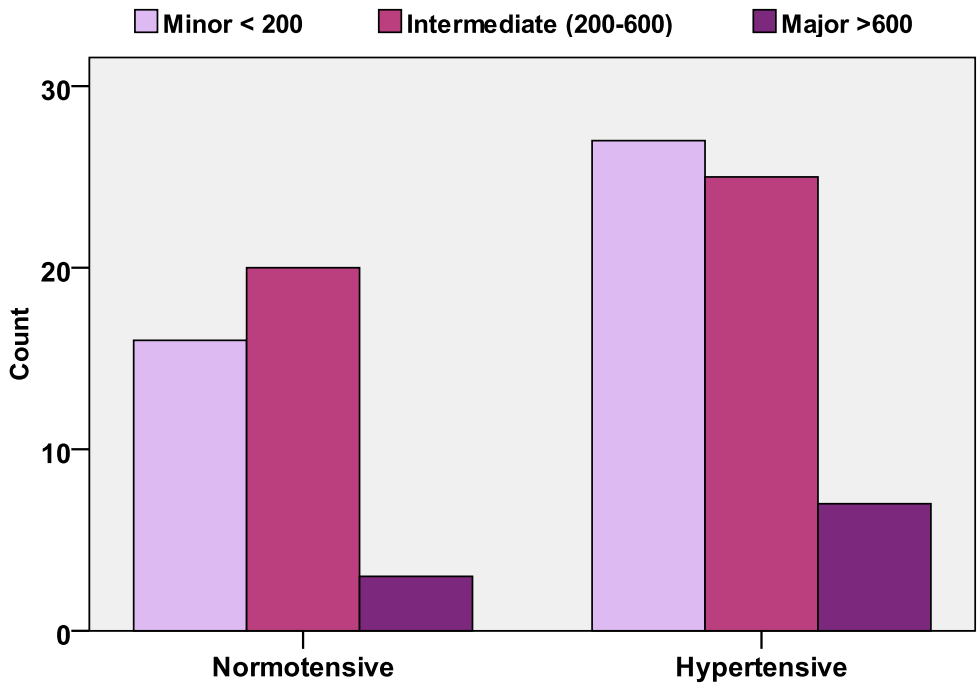

Figure 4.51: Size of PMD (mm^2) according to hypertension status.

Hypertension Status and Dysplasia Grading

Table 4.29 and Figure 4.52 summarise a significant association between hypertension status and degree of dysplasia (p=0.001; Chi-Square test).

Mild dysplasia was seen equally in both hypertensive and normotensive patients, whereas 90% of CIS, 73% of severe and 48% of moderate dysplasia were reported in patients with hypertension.

Table 4.29: Oral epithelial dysplasia in relation to hypertension status.

Hypertension status	Degree of dysplasia				Total
	Mild	Moderate	Severe	CIS	
Normotensive	**21** *50%*	**12** *52%*	**6** *27%*	**1** *10%*	**40** *41%*
Hypertensive	**21** *50%*	**11** *48%*	**16** *73%*	**9** *90%*	**57** *59%*
Total	**42** *100%*	**23** *100%*	**22** *100%*	**10** *100%*	**97** *100%*

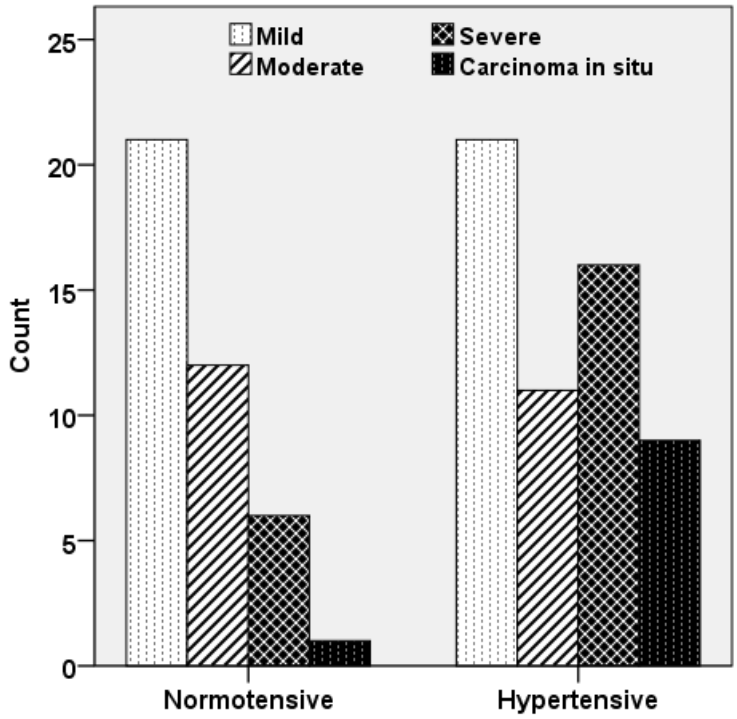

Figure 4.52: Oral epithelial dysplasia according to hypertension status.

Hypertension Status, Tobacco and Alcohol Use

Using Chi-Square testing, no significant relationship was found between hypertension and smoking status (p=0.921) or alcohol drinking status (p=0.061), although all smoking and drinking status subgroups were more frequently seen in hypertensive patients.

Family Cancer History

Full and detailed information related to family cancer history in medical records was limited to 15 patients in this study; 10 had a negative family history and 5 were positive. In the 5 positive histories, a first degree relative was involved; 4 fathers (prostate, throat, bowel and oral cancer) and one maternal history of breast and stomach cancer.

Patients with a positive familial history were all alcohol drinkers and tobacco smokers; 3 males (1< 40 years and 2 between 41-62 years old) and 2 females younger than 40 years.
Four smoked less than 20 cigarettes per day and one was an ex-smoker; 2 males drank more than the recommended units of alcohol (> 21 units) per week.

Out of the ten patients with a negative family cancer history, seven were male (two < 40 years and five between 41-62 years) and 3 female (41-62 years); five drank more than the recommended units, 3 were non-smokers, 4 smoked < 20 cigarettes/day, 2 smoked > 20 and one was an ex-smoker.

Dental Prosthesis Wear

The majority (53%) of PMD patients wore dental prostheses; 25% complete dentures, 17% crowns and bridges and 11% either upper or lower dentures; Figure 4.53.

No significant relation was found between PMD size and dental prosthesis wear (p=0.684; Chi-Square test), although patients wearing prostheses were more likely to have intermediate sized dysplasia, followed by minor and major lesions. Non-wearers showed a higher prevalence of minor sized lesions; Table 4.30 and Table 4.31.

Table 4.32 shows the average size of PMDs in relation to intraoral dental prostheses types. Overall, patients with prostheses exhibited larger mean sizes than non-wearers (332.5 vs. 269.5 mm^2). Patients with crowns and bridges had larger sized PMDs compared to denture wearers (345 vs. 330.5 mm^2), although in both cases Mann-Whitney U tests were not significant (p=0.557, p=0.647, respectively).

In dental prostheses wearers, the majority of PMDs were seen at high-risk sites; 77% (41/53) compared with 23% (12/53) low-risk sites.

Patients with FOM dysplasia were mainly dentures wearers 40% (14/35) compared to 31% for tongue. Whilst crowns and bridges were more frequently associated with tongue lesions (47%) compared to FOM (41%), the relation was non-significant (p=0.338; Chi-Square test).

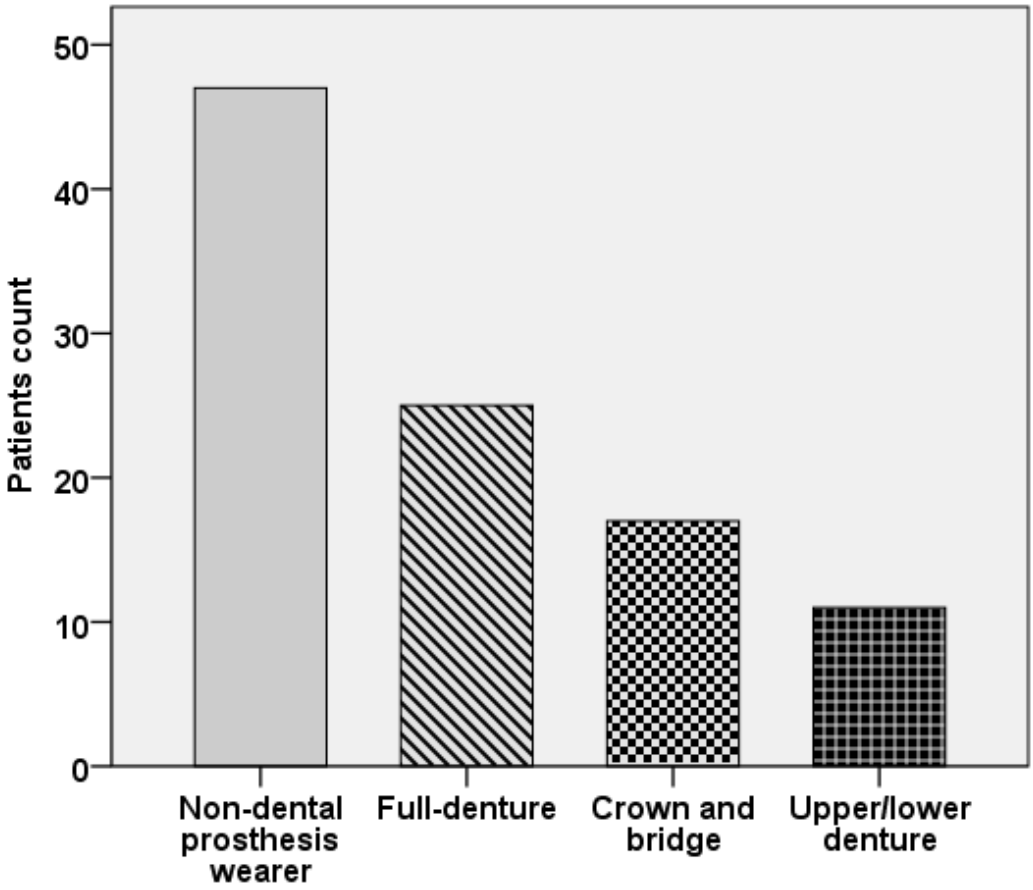

Figure 4.53: Dental prostheses in PMD patients.

Table 4.30: Dental prostheses wear and PMD size.

PMD size categories (mm^2)	Dental prosthesis wear		Total
	Yes	No	
Minor < 200	19	23	42
Intermediate (200-600)	26	19	45
Major > 600	5	5	10
Total	50 *52%*	47 *48%*	97 *100%*

Table 4.31: Types of dental prosthesis according to PMD size.

PMD size categories (mm^2)	Types of dental prosthesis				Total
	Full denture	Upper/lower denture	Crowns and bridges	Non-wearer	
Minor	9	4	6	23	42
Intermediate	12	6	8	19	45
Major	2	1	2	5	10
Total	23 *24%*	11 *11%*	16 *17%*	47 *48%*	97 *100%*

Table 4.32: Mean PMD size according to dental prosthesis wear.

Types of dental prosthesis	Mean	N	Maximum	Minimum	SD
Full denture	330.5	23	1,800	36	377.566
Upper/lower denture	319	11	1,050	40	307.349
Crowns and bridges	345	16	945	21	267.198
Non-wearer	269.5	47	884	36	217.439
Total	301.98	97	1,800	21	279.166

Oral Health Status and Mouthwash Use

In this study, a deficiency in recording oral hygiene status and mouthwash use was noted in PMD patient files.

Out of 100 patients, data retrieved from the medical records showed information for only 12 patients; 9 were mouthwash users, although no detailed information was available. Also, no specific information was recorded in relation to oral hygiene status.

4.4.4. Logistic Regression Analysis

Non-homogenous Leukoplakia

In order to investigate predictors of developing non-homogenous leukoplakia, logistic regression analysis was performed. Several independent risk factors were initially considered separately, and then -2 log likelihood ratios were applied to assess the statistical significance of these variables. Multivariable logistic regression analysis was then used to explore the possible effects of these risk factors.

Table 4.33 presents Odds-Ratios (OR) and 95% confidence intervals (CI) of the univariable and multivariable predictor model for non-homogenous leukoplakia development.

Regression analysis showed that patient age had no effect (OR=1.012, 95% CI, 0.977-1.048) (p=0.516), whilst the risk for non-homogenous leukoplakia to occur in males was 1.631 times higher than females (95% CI, 0.599-4.441), although this was not significant (p=0.339).

The analysis supported that the majority of homogenous leukoplakias were associated with smoking. A reduction in risk of non-homogenous leukoplakia development by 65% was seen in ex-smokers, 80% for smoking > 20 cigarettes/day and 71% for smoking ≤ 20, compared to non-smokers, however these were non-significant (p=0.773, p=0.641, p=0.478, respectively). This observation indicates that non-homogeneous leukoplakias were more common in non-smokers or in patients with lower smoking activity, emphasising the inherent high-risk in these patients.

Drinking > 28 units/week increased the risk for non-homogenous leukoplakia by 3-times compared to non-drinkers (95% CI, 0.566- 16.177), whilst drinking ≤ 28 units/week caused a 1.78-times higher risk compared to non-drinkers (95% CI, 0.341-9.293), although both were non-significant (p=0.196, p=0.494, respectively).

Patients with a history of systemic disease had an increased risk of non-homogenous leukoplakia 4.2-times higher than those without (95% CI, 0.510- 34.737), although this was not significant (*p*=0.182).

In the final regression model, when smoking and drinking were entered together, the reduction in risk for non-homogenous leukoplakia development was also seen among current smokers, with a 38% and 47% reduction risk was observed in patients who smoked ≤ 20 and > 20 cigarettes/day, respectively.

Drinkers exceeding 28 units/week exhibited a 6.27 times increased risk of non-homogenous leukoplakia development compared to non-drinkers, however, this did not reach statistical significance (95% CI, 0.717- 42.796) (*p*=0.061).

Table 4.33: Logistic regression models for non-homogenous leukoplakia.

Clinical appearance	Risk Factors	Uni-variable analysis		Multi-variable analysis	
		Odds (95% CI)	p-value	Odds (95% CI)	p-value
Non-homogenous leukoplakia	Age	1.012 (0.977-1.048)	0.516		
	Sex				
	Females	Reference category			
	Males	1.631 (0.599-4.441)	0.339		
	Smoking status				
	Non-smokers	Reference category			
	Smoking ≤ 20 cig/day	0.800 (0.175-3.651)	0.773	0.381 (0.080-1.811)	0.225
	Smoking > 20 cig/day	0.645 (0.192-2.167)	0.478	0.467 (0.080-2.716)	0.396
	Ex-smokers	0.714 (0.173-2.941)	0.641	1.232 (0.0250-6.083)	0.798
	Drinking status				
	Non-drinker	Reference category			
	≤ 28 units/week	1.779 (0.341-9.293)	0.494	2.652 (0.469-14.977)	0.270
	> 28 units/week	3.025 (0.566-16.177)	0.196	6.265 (0.917-42.769)	0.061
	Medical history				
	Negative	Reference category			
	positive	4.211 (0.510-34.737)	0.182		

High Grade Dysplasia

Age of patient, sex, clinical types of leukoplakia, anatomical site and size of dysplasia, medical history, smoking and drinking behaviours were entered as independent variables to try to predict the most important factor (s) associated with high grade dysplasia.

Table 4.34 summarises logistic regression analysis of univariable and multivariable predictor models for high grade dysplasia.

The analysis showed that patient age did not significantly influence the development of high grade dysplasia (OR=1.017, 95% CI, 0.985-1.049) (p=0.312).
Males had a 1.338 times higher risk than females for developing high grade dysplasia (95% CI, 0.572-3.128), although this was not significant (p=0.502).

Non-homogenous leukoplakia was a significant predictor of high grade dysplasia development (p=0.020), increasing the risk 3.3-times compared to homogenous lesions (95% CI, 1.205-9.018).

Lesion size > 600 mm^2 was a significant predictor of high grade dysplasia development (p=0.026), with an 11.5-times higher risk than minor sized lesions (< 200 mm^2) (95% CI, 1.331-99.329).

Alcohol drinking > 28 units/week was a significant predictor for high grade dysplasia (p=0.038), with a 4.89-times higher risk compared to non-drinkers (95% CI, 1.089-21.950). Drinking ≤ 28 showed 2.78-times the risk of non-drinkers (95% CI, 0.658-11.727), although non-significant (p=0.164).

A history of systemic disease was found to be a significant predictor of high grade dysplasia development (p=0.047), with a 4-times increased risk compared to patients with no systemic disease (95% CI, 1.016-15.981).

The multivariate logistic regression final model utilized the goodness of fit statistic -2 log likelihood ratio, including leukoplakia type, PMD size, medical history, smoking and drinking behaviours.

A positive history of systemic disease remained the only significant predictor in the multivariable model for high grade dysplasia occurrence (p=0.026), with an increased risk of 7.45-times compared to patients with no systemic disease (95% CI, 1.269- 43.721).

In the final model, lesion size > 600 mm^2 showed a 7.635-times higher risk for high grade dysplasia development compared to small lesions, but this was non-significant (p=0.090).

Drinking > 28 units/week showed a 4.760-times higher risk for high grade dysplasia compared to non-drinker, however, this was not significant (p=0.105).

Table 4.34: Logistic regression analysis for high grade dysplasia.

Histopathology	Risk Factors	Uni-variable analysis		Multi-variable analysis	
		Odds (95% CI)	p-value	Odds(95% CI)	p-value
Binary grading system **High grade dysplasia**	Age	1.017 (0.985-1.049)	0.312		
	Sex Females Males	 reference category 1.381 (0.572-3.128)	 0.502		
	Leukoplakia types Homogenous **Non- homogenous**	 Reference category **3.296 (1.205-9.018**)	 **0.020**	 2.481 (0.745-8.261)	 0.139
	PMDs site FOM Tongue Remaining sites	 Reference category 1.825 (0.721-4.620) 1.095 (0.366-3.281)	 0.204 0.871		
	PMDs size (mm^2) Minor < 200 Intermediate 200-600 **Major > 600**	 Reference category 1.460 (0.624-3.418) **11.500 (1.331-99.329)**	 0.383 **0.026**	 0.756 (0.257-2.225) 7.635 (0.727-80.179)	 0.612 0.090
	Medical history No **Yes**	 Reference category 4.029 (1.016--15.981)	 **0.047**	 7.449 (1.269-43.721)	 **0.026**
	Smoking status Non-smokers Smoking ≤ 20 cig/day Smoking > 20 cig/day Ex-smokers	 Reference category 0.576 (0.173-1.915) 1.429 (0.364-6.6127) 0.485 (0.122-1.922)	 0.368 0.609 0.303	 0.427 (0.085-2.156) 1.638 (0.253-10.588) 0.212 (0.033-1.354)	 0.303 0.604 0.101
	Drinking status Non- drinker ≤ 28 u/w **> 28 u/w**	 Reference category 2.778 (0.658-11.727) **4.889 (1.089-21.950)**	 0.164 **0.038**	 2.329 (0.425-12.747) 4.760 (0.721-31.414)	 0.330 0.105

4.5. Discussion

Assessment of risk factors in PMD patients may facilitate treatment planning and identify patients at higher risk of developing OSCC and/or further disease who require extended follow-up.

Patients in this study formed a homogenous cohort, since all were diagnosed with dysplastic PMD, all underwent standardised laser intervention and were then followed-up by the same clinical team.

The current study investigated possible effects from the two most widely recognised risk factors of tobacco and alcohol use, in addition to systemic disease and dental factors, on demographic, clinical and histopatholgical features of dysplastic PMDs.

4.5.1. Tobacco Smoking Behaviour in PMD Patients

Three measurements of tobacco exposure were used: number of cigarettes smoked per day, duration of smoking (years) and pack-years score which captures both dose and duration of exposure (Dietrich et al., 2004). For each smoker, the status and intensity of smoking were reported at each clinic follow-up, in addition to first presentation and most recent follow-up, because smoking behaviour changes from one point to another during the clinical management time.

At initial presentation, the majority of patients were active smokers (63%), with 22% ex-smokers and only 15% non-smokers. This is in accordance with other studies: Hamadah (2007) found that 70% of leukoplakias were associated with current smokers, 20% with ex-smokers and 10% with non-smokers, Freitas *et al.* (2006) reported a higher proportion of leukoplakias in current smokers (79%) and Hogewind and van der Waal (1988) found that 64% of leukoplakias occurred in smokers compared to 36% idiopathic or smoking-unrelated leukoplakia.

In this study, amongst current smokers, a higher percentage of males (73%) compared to females (27%) was seen and the proportion of male smokers was higher than non-smokers

(73% vs. 33%). Female patients were more likely to be non-smokers than smokers (67% vs. 27%). This sex difference is in agreement with most of the previous studies (Freitas et al., 2006; Hamadah, 2007) and may be explained by social habits, more responsibility and stress on males who need a coping mechanism for the stress or may reflect women's low economic resources with more health awareness.

Current smokers were significantly younger than both non-smokers and ex-smokers for both male (52 vs. 64, 68 years) and female patients (52 vs. 68, 65 years), which agrees with Freitas *et al.* (2006) who found a significantly lower mean age of smokers compared to non-smokers with PMDs (49 vs. 59 years).

Males smoked for a significantly longer time (mean 37 years) compared to females (28 years) although, interestingly, male and female current smokers were of similar mean age (52.20 vs. 52.47 years). Males presumably started smoking earlier than females with increased risk arising from long-standing exposure.

Patients consuming < 10 cigarettes per day had the highest mean smoking history (46 years), compared to those smoking 10-20 cigarettes per day (35 years) and those smoked > 20 per day (33.6 years); however, the correlation was not significant. It is clear that both the number of cigarettes smoked per day and the length of smoking history needs to be recorded, therefore, for every PMD patient.

Females were more often non-smokers than males (67% compared to 33%), which is similar to the findings of other studies (Muscat et al., 1996; Schepman et al., 1998; Freitas et al., 2006).
Similar to Muscat *et al.* (1996), who found a greater percentage of non-smoking females over the age of 50 years, our non-smoker females had a mean age of 68 years.

Although males consumed a higher amount of tobacco than females, no significant differences were seen between males and females in the average number of cigarettes smoked per day.

The study showed that patients in middle age (41-62 years) smoked a significantly higher average number of cigarettes per day compared to those older than 63 years (25 vs. 16 cigarettes/day); 88% of heavy smokers and 66% of intermediate smokers were middle-aged.
One reason for the lower average number of cigarettes intake in older ages may be the health problems caused by smoking, which are more likely to appear later in life, compared to younger age who have not yet developed many secondary diseases. As smokers get older, many forced by health problems to either reduce or quite smoking.

It has been reported that unemployment individuals smoke more (Lin, 2010), which may be related to the stress from low income (Greenwood et al., 2003). Smoking in this group of patients has been described as a coping mechanism for the stress associated with deprivation and low income (Marmot, 1997; Stead et al., 2001).
The current study supports this view: 88% of unemployed patients smoked tobacco in the range 10 to 60 cigarettes/day, compared to 74% employed and 44% retired who smoked 10-50 and 7-30 cigarettes/day, respectively. Smoking fall in retired individuals may be one of the substantial changes in lifestyle accompanied occupational retirement (Nooyens et al., 2005). Individuals who undergo the transition into retirement are more likely to reduce or quit smoking than those who do not (Lang et al., 2007), which may be explained by the fact that retired patients are usually old age with systemic diseases compared to younger patients. In addition, a change in economic resources after retirement may influence the smoking habit.

The role of tobacco as a principal risk factor for oral leukoplakia development is well documented (Axell, 1987; Schepman et al., 2001). Baric *et al.* (1982) found that leukoplakia was 6-times more likely to affect smokers compared to non-smokers.

It is known that the type, quality and quantity of tobacco smoking affects localization of leukoplakia in the oral cavity (Schepman et al., 2001). In this study, compared to all other oral subsites, FOM leukoplakias were significantly more common in current smokers than non-smokers. In contrast, leukoplakias on lateral and ventral tongue surfaces were significantly more frequent in non-smokers. The preferential localization of FOM PMDs in current smokers may relate to the accumulation of tobacco-related toxins in saliva which act as a reservoire for carcinogens in tobacco products (Lederman, 1964). In addition, a lower

degree of keratinization and higher permeability of oral mucosa at this location may also influence carcinogenesis of tobacco-related toxins (Mashberg and Meyers, 1976; Lesch et al., 1989).

This is similar to Freitas *et al.'s* (2006) study, which found that FOM and buccal mucosa were more frequent sites amongst smokers, whilst tongue leukoplakia was the main location amongst non-smokers.

Furthermore, a correlation between cigarette smoking and cell proliferative activity was reported in oral anatomic sites exposed to smoking (Cancado et al., 2001; Paiva et al., 2004). This correlation was investigated using cytological analysis conducted by Cancado *et al*. (2004), who found a significant larger number of argyrophilic nucleolar organizer regions (AgNORs) per nucleus among FOM smokers compared to lateral tongue smears with a significant correlation between number of cigarettes smoked per day in FOM cases but not lateral tongue. This suggests that oral mucosa is susceptible to alterations in the genetic patterns of the cellular regulation in cigarette smokers and in less keratinized regions (Cancado et al., 2001), reflected by a cell proliferative activity and may explain the more frequent FOM PMDs in smokers and lateral tongue in non-smokers.

Patients younger than 40 years more frequently presented with FOM PMDs, with no tongue lesions, whilst patients older than 41 years showed both FOM and tongue PMDs, but with more FOM cases. This further supports FOM as the preferential localization in smokers, because all young patients were tobacco users (90% current and 10% ex-smokers) compared to patients > 41 years who were a mixture of current, non- and ex-smokers.

In both male and female patients, FOM was the main affected subsite in current smokers, 52% and 82%, respectively. However, this finding disagrees with Schepman *et al.* (2001) who found buccal mucosa significantly more common in male smokers and FOM more frequent in female smokers. They suggested that different ways of placing cigarettes during smoking, more centred between the lips in females and to the side in males, may account for the difference.

In non-smokers, the tongue was the only affected oral site in females compared to 80% in non-smoker males, which is in agreement with other studies (Schepman et al., 2001; Freitas

et al., 2006). There is no clear reason why lateral tongue is the only affected site in non-smoking females and was the predominant site in non-smoker males. However, this indicates that non-smokers who develop PMDs in lateral tongue surface may be subjected to other more potent carcinogenic factors such as nutritional deficiency, viral infection and chronic irritation, but this did not detract from the well-established role of tobacco in oral carcinogenesis (Neville and Day, 2002) in the most dependent part of oral cavity (FOM). Further studies are needed to confirm this finding and investigate the potential associated factors.

The soft palate was not affected in non-smokers and was more involved in current than ex-smokers (78% vs. 22%). To the best of our knowledge, this finding is reported for the first time and suggests that the soft palate is a specific, smoking-related site, although further studies with larger sample sizes are required to confirm and investigate this finding. This finding supports the hypothesis of carcinogenic effect of tobacco smoke on the oral epithelium through a direct contact to this high-risk site with a thin unprotected epithelium that devoid of keratin (Schwartz, 2008). This is supported by a study conducted by Boffetta *et al*. (1992) who found that tobacco smoking was more strongly associated with soft palate cancer than with lesions in more anterior sites. In addition, soft palate is a less accessible site for a mechanical cleansing action with prolonged tobacco-related carcinogens contact, compared to anterior parts of the oral cavity.

FOM, ventral tongue and soft palate were the most significantly affected sites in patients with long smoking histories. This study has shown that the duration of smoking was more significant than its intensity in relation to site distribution. This suggests that long-term continuous exposure is more harmful than the amount of tobacco smoked at any one time-point. This may reflect intensity-duration molecular mechanisms in the FOM, tongue and soft palate from prolonged and continuous contact to tobacco-related carcinogens. This is supported by a study on lung cancer conducted by Lubin *et al*. (2008) who indicated that smoking duration is much more important than the intensity in causing lung cancer and they indicated that for fixed pack-years, smoking at a lower intensity for a longer duration was more harmful compared to a higher intensity for a shorter duration. This was explained by the behavioural factors, with heavy smokers inhaling less deeply while maintaining addiction-

sufficient nicotine levels, decreasing the carcinogenic effect per cigarette and decreasing risks at higher intensities (Lubin et al., 2008) with saturation activation pathways (Lutz, 1998).

In this study the joint effect of intensity and duration of tobacco smoking was investigated using lifetime tobacco exposure (pack-years). The negative correlation between tobacco cumulative effect and age of patients did not reach significance. When smokers get older, they were found to consume a lower number of cigarettes per day, which may be due to the health problems that are more likely to appear at older ages, forcing them to reduce smoking intensity. Although the mean pack-years score for males was higher than females (43 vs. 32), which was expected due to longer smoking history and higher mean number of cigarettes smoked per day, the difference was not significant. This may be due to the small number of females relative to males (8 vs. 28), which may be inadequate to detect statistical significance.

This study showed a significant relation between numbers of cigarettes smoked per day and the clinical type of PMDs. Erythroplakias were only seen in heavy smokers, with the highest number of cigarettes smoked per day (38). Exophytic non-homogenous leukoplakias were more frequently observed in light-smokers (18).

Homogenous leukoplakias in this study were seen in patients with a long smoking history (mean 34.8 years), but with no significant relation, which may be due to the small number of cases in each clinical type.
To the best of our knowledge, this is the first study to consider the relation between smoking status, intensity of smoking and PMD clinical appearance.

Significantly, current smokers exhibited the smallest mean size of PMD compared to non-smokers (241.5 vs. 473 mm^2). Minor sized PMDs were most common in heavy smokers who showed the smallest mean size compared to light smokers (247.5 vs. 316 mm^2). A negative correlation, albeit non-significant, was found between smoking intensity (cigarettes/day) and PMD size; the higher the intensity, the smaller the size. A longer smoking history was also associated with smaller sized PMDs. While there is no clear biological explanation for this phenomenon, it may be that non-smokers have intrinsically wider field changes within the oral cavity as opposed to more localized tobacco initiated lesions.

Freitas *et al.* (2006) found no significant difference between lesion size in smokers and non-smokers. These authors measured lesion size clinically at the time of diagnosis, however, whilst this study used accurate measurements from pathology reports following lesion excision. Freitas *et al.* (2006) did show a higher percentage of smaller sized lesions in smokers compared to non-smokers (66% vs. 55%), with only 8% of larger size lesions found in smokers.

This study showed a higher presentation of dysplastic lesions in current smokers compared to both ex-smokers and non-smokers (65% vs. 20% and 15%, respectively).
A significant relationship was found between smoking intensity and degree of dysplasia; heavy smokers showed primarily severe dysplasia, whilst light smokers showed the least dysplastic features (1 mild dysplasia and 1 CIS).

A positive correlation was found between smoking history and degree of dysplasia, with a longer history associated with higher dysplasia severity. These findings suggest that increasing dysplasia is associated with longer exposure to risk factors (Napier and Speight, 2008; Warnakulasuriya et al., 2008). Previous studies on oral cancer and epithelia dysplasia have reported increased risk with higher levels of smoking which may decrease following smoking cessation (Blot et al., 1988; Morse et al., 1996; Morse et al., 2007).

4.5.2. Alcohol Consumption in PMD Patients

Existing studies show controversial data on the role of alcohol as an independent risk factor for oral PMDs. Recently, more evidence to support alcohol as an independent risk factor for leukoplakia and erythroplakia has been found.

In a hospital-based, cross-sectional study of 2017 patients, oral leukoplakias were seen in those who were drinking but not smoking (Saraswathi et al., 2006). This is supported by a prospective cohort study in US males, which found that alcohol consumption was an independent risk factor for the incidence of PMDs (Maserejian et al., 2006a). In an Indian

study conducted by Hashibe *et al.* (2000a), alcohol was a significant factor for oral leukoplakia even after adjusting for tobacco habits.

The relationship between drinking alcohol and erythroplakia was also demonstrated in a case-control study of 100 erythroplakia patients and 47,773 controls with a dose-response relationship for both frequency and duration after adjusting for age, sex and smoking habits (Hashibe et al., 2000b).

Case control studies on the risk of oral epithelial dysplasia in Great Britain have considered alcohol to be important only in conjunction with tobacco, suggesting a synergistic effect (Jaber et al., 1998; Jaber et al., 1999). A more recent study in Taiwan supports the notion that alcohol intake was not a risk factor for the development of oral leukoplakia (Lee et al., 2003).

The current study found 83% of patients were current drinkers at initial presentation. Light drinkers (1-14 units/week) were the most common, followed by heavy drinkers (> 28), whilst intermediate drinkers (15-28) were least common. A significant association was found between drinking status and patients' sex and age: males were predominantly current drinkers, whilst females were often non-drinkers. All young patients were current drinkers whereas middle age patients were usually ex-drinkers. Common reasons for non-drinking in females may be due to cultural or family traditions, pregnancy or maternal responsibility, whilst males drink due to social pressures and may have greater disposable income. Middle aged patients with more health problems may quit drinking compared to younger patients who have not yet developed drinking-associated diseases.

Higher levels of alcohol intake in males are reported in studies from all over the world. The present study found that males formed 97% of heavy drinkers with an average alcohol intake of 38 units/week (compared to females 9.5 units/week). Data from the Office for National Statistics in 2006 showed men to be drinking twice as much alcohol as women, with an average 18.7 units a week, compared to 9.0 for women. Males in our PMD cohort consumed twice the National Statistics average, emphasising the difference between general population and high risk hospital-based studies.

In the United Kingdom, safe levels for alcohol drinking are less than 21 units per week for men and 14 units per week for women, with a high risk of developing several health problems associated with > 50 units/week for men and 35 for women. In this study, 58% of males consumed > 21 units/week and 9% of women consumed > 14 units per week, both exceeding the guidelines established by the Department of Health, and emphasising that our patients were at higher risk of alcohol misuse.

Furthermore, the study showed that 29% of males reported drinking over 50 units/week and 3% of females over 35 units/week. In England, in 2008, 7% of men reported drinking over 50 units per week with 5% of women drinking over 35 units. This study showed a higher percentage of male drinkers at higher risk than the general population (29% vs. 7%), whilst female drinkers reported a lower percentage (3% vs. 5%). This suggests underreporting of alcohol intake in female patients and emphasises the need for more objective bioassays for alcohol risk assessment in addition to self-reporting. The reasons for inaccuracy of self-reporting may be to deny problem drinking, avoid personal criticism or the inability to remember drinking patterns because of alcohol intoxication itself (Sommers et al., 2002). This may reflect complex physiological, psychological, and social differences between males and females (Sommers et al., 2002).

All exposures were measured by self-reporting and some degree of exposure misclassification may occur as a result of underreporting. Unreliability of self-reporting of alcohol consumption has been reported previously by Babor *et al*. (1987). The validity of reporting alcohol intake in heavy drinkers in females who tend to underreport heavy drinking of alcohol may be problematic and also this may occur among males (Muscat et al., 1996). However, since relatively few heavy drinker females in this study, the misclassification of heavy drinkers as intermediate may result in slightly spurious effects associated with intermediate drinkers, but also may lead to substantial underestimation of risk in heavy drinkers group.

Similarly, it is difficult to obtain a reliable information about the amount of tobacco intake and type of the product (van der Waal and Axell, 2002); thus study limitation should be considered when interpretate the findings related to both alcohol and tobacco. This

emphasises the need for more objective and reliable way of measurement, in addition to self-reporting. Laboratory test modalities for more accurate and useful risk assessment have been used; cotinine which is a nicotine metabolite with a long half-life in the circulation has been used to confirm the dose and duration of tobacco exposure (Atkin et al., 2002; Sinha et al., 2012) and mean corpuscular volume (MCV) or other biomarkers for alcohol use (Neumann and Spies, 2003; Goodson et al., 2010).

This study showed a significant correlation between patient age and number of alcohol units consumed per week: old age drinkers consumed the lowest level of alcohol compared to both young and middle aged. This agrees with the Office for National Statistics (2008), which reported lower alcohol consumption in the oldest age groups in both males and females. Alcohol consumption declining with age may be connected to changes in life circumstances and attitudes such as ill health, less ability to process alcohol, increased interaction with medications and a reduced income in retirement.

Whilst no significant relationship was found between drinking status and PMD site, the FOM was the most frequent site in heavy drinkers (> 28 units/week). This supports the hypothesis of the carcinogenic effect of alcoholic beverages on the oral mucosa through direct contact (Boffetta et al., 1992).

Alcohol may be an independent risk factor for oral PMDs regardless of frequency, drinking pattern, type of beverage or smoking history, and the association has been seen in non-smokers (Maserejian et al., 2006a). Alcohol intake of more than 30 g/day increased the risk of developing oral PMDs by 2.5-times, with one additional drink per day increasing risk by 22% (Maserejian et al., 2006a).

The relation between amount of alcohol intake and PMD clinical appearance was significant. Homogenous leukoplakias were more commonly seen in light drinkers, with a higher presentation of speckled and exophytic subtypes in heavy drinkers. Seven out of eight patients with erythroplakia were current drinkers. Heavy exposure to alcohol-related carcinogens, associated with genetic profile factors, may be responsible for certain clinical types of PMDs development.

Patients with mild dysplasia had the lowest average alcohol intake (22), whilst those with CIS had the highest average alcohol intake (43), albeit non-significant.

This study demonstrated a significant relationship between the two major risk factors, tobacco and alcohol at first presentation and most recent follow-up. Whilst 92% of current drinkers at initial presentation were also current smokers, half of non-drinkers were ex-smokers. This is similar to Bien and Burge (1990) who reported that over 90% of alcoholic inpatients were smokers. Smoking and alcohol consumption can be seen as complementary behaviours, occurring at social, psychological and physiological levels, with a factor which increases or decreases one behaviour will also tend to increase or decrease the other (Room, 2004). Nicotine appears to facilitate ethanol consumption by reducing the intoxicating effects of alcohol, enhancing more drinking (Johnson et al., 1991; Chen et al., 2001).

4.5.3. Medical History of PMD patients

Medical problems such as hypertension, diabetes mellitus, oral candida infection, anaemia and immunodeficiency were seen in 88% of the study population (55% males and 33% females).

In this study, a significant relationship was found between patient age and medical history. All PMD patients older than 63 years, 84% of middle aged and 70% < 40 years, had one or more systemic disorders. This agrees with a considerable body of opinion that suggests as human lifespan increases, there is an increased prevalence of disease. Elderly patients often suffer from medical problems which may be due to complex physiological, biochemical and molecular changes (Malaguarnera et al., 2010). In addition, there is a decreased efficiency of both host defense mechanisms and healing (Soames and Southam, 2005).

Regular medication intake was reported by PMD patients with systemic disease in this study; however, investigating the interaction of these medications and the behaviour of PMDs was unachievable because most patients took multiple medications of varying dose and duration.

Investigating systemic disease in this study cohort revealed that hypertension was most common, followed by diabetes mellitus; this distribution agrees with a study on the prevalence of oral mucosal lesions in alcohol misusers in India, conducted by Rooban *et al.* (2009). In this study, 60% of PMD patients were hypertensive and usually older than normotensive ones, in particular females were older than males, but differences did not reach significance.

Associations between systemic diseases and oral PMDs have not been examined or reported widely. In this study, non-homogenous leukoplakias were more frequently seen in hypertensive patients, albeit non-significant. A significant association was found between hypertension and non-homogenous subtypes; 75% of speckled lesions were seen in hypertensive patients, whilst all nodular and 60% of exophytic subtypes were seen in normotensive patients. The higher prevalence of non-homogenous leukoplakia in hypertensive patients and the significant association between certain clinical non-homogenous subtypes and hypertension may be due to an effect from anti-hypertensive medications which may influence the clinical appearance of PMDs.

FOM, lateral and ventral tongue surfaces as well as soft palate were the most common affected sites in hypertensive patients, whereas buccal mucosa was most frequent in normotensive. In this study, 70% of major sized lesions were seen in hypertensive patients.

A higher frequency of CIS and severe dysplasia were seen in hypertensive patients and it is possible that anti-hypertensive drugs may play an enhancing role. According to Tangjarturonrasme *et al.* (2007), medications such as Atenolol cause oral lichenoid reactions which can undergo dysplastic change, and malignant transformation (Fatahzadeh et al., 2004), although the pathogenesis of this adverse reaction is still unclear.

To the best of our knowledge, this is the first study to investigate the relation between oral PMD behaviour and systemic hypertension, but it is clear further research is warranted in this area.

Similar to Saini *et al.* (2010) diabetic patients in this study were mainly affected with type II diabetes mellitus, with females significantly more commonly affected. This is consistent with an Indian study conducted by Dikshit *et al.* (2006) who found that diabetes mellitus was mainly associated with PMDs in females.

Leukoplakia was the only clinical type of PMD seen in our diabetic patients. It has been reported that diabetes mellitus is a risk factor for oral cancer and oral leukoplakia, with diabetic patients 3-times more likely to develop oral leukoplakias than non-diabetics (Dietrich et al., 2004; Ujpal et al., 2004). A similar association was found by Albrecht *et al.* (1992) who noted a 6.2% prevalence of oral leukoplakias amongst diabetic patients, compared with 2.2% in controls. However, Saini *et al.* (2010) found no association between diabetes mellitus and oral PMDs and suggested that adjustment for confounders such as tobacco and alcohol may be an important factor to consider.
Erythroplakias and speckled leukoplakias were only seen in non-diabetic patients. Dikshit *et al.* (2006), however, reported diabetes as an independent risk factor for oral leukoplakia and also erythroplakia in women.

The majority of diabetic patients in this study were current smokers and drinkers, with intermediate smokers and light drinkers most common. Oral PMDs in diabetic patients were often seen in high-risk sites (FOM and tongue). However, no relationship between diabetic mellitus and PMD behaviour was significant, probably because of the small number of patients.

Diabetes mellitus may lead to metabolic and immunological changes along with inflammatory and atrophic changes affecting oral mucosa (Ponte et al., 2001). The atrophic changes may promote development of oral PMDs (Albrecht et al., 1992; Ujpal et al., 2004).

The precise role of candida infection in oral carcinogenesis remains unclear. Cawson (1966) suggested that fungal infection may be superimposed upon pre-existing disorders rather than initiating them. This is supported by Holmstrup and Bessermann (1983) who found that a non-homogenous candidal leukoplakia converted into homogenous leukoplakia following candida infection control.

Barrett *et al.* (1998), however, found a significant correlation between dysplasia severity and infection with candida; 22% of dysplasias infected with fungi showed more severe dysplastic features compared with 8% of non-infected ones. The same group concluded that, following histological confirmation of dysplasia, anti-fungal therapy should be part of clinical management.

Evidence supports the role of candida species in oral carcinogenesis based on the fact that certain species have a higher production of nitrosamine compounds which play a role in initiation of carcinogenesis (Sanjaya et al., 2011).

In this study, eight cases were diagnosed with oral candida infection; six were seen at initial presentation and affected equally with mild, moderate and severe dysplasia, whilst two with mild dysplasia were diagnosed after laser intervention during follow-up.
Candida infection was diagnosed in five non-homogenous leukoplakias which agrees with Krogh *et al*. (1987) who evaluated various strains of candida in leukoplakia and found that rarely occurring candida albicans were increasingly isolated from non-homogenous leukoplakia. All cases with candida albicans in this study were tobacco users, with the majority heavy smokers. This agrees with the hypothesis of epithelial alteration caused by cigarette smoking (increased keratinization) allowing candida colonization and enhanced adherence (Sanjaya et al., 2011). In this study, it is unclear whether candida infection diagnosed at first presentation was secondary to dysplastic changes (co-incidental) or had led uncontrolled epithelial growth initiating change (Sanjaya et al., 2011). Further understanding of the biological effects of candida and oral epithelial dysplasia is warranted.

In this study, detailed information relating to family cancer history in medical records was limited, leading to a reduced ability to evaluate the importance of this factor for PMD patients. The medical background of our patients with a positive family history of cancer did not reveal any predisposing factors and the number of young patients was not large enough to provide conclusive information in respect of familial antecedents as a risk factor for this group. Three out of five patients with a familial cancer history were < 40 years. A familial cancer history may be an increased risk for young patients, since tobacco and alcohol use

require relatively long durations of exposure and these factors may be less implicated in OSCC development in younger patients (Hirota et al., 2008).

4.5.4. Dental Prosthesis Wear

More than half of PMD patients in this study wore dental prostheses, mainly removable appliances, which were either complete or upper/lower dentures. The need for dentures is not surprising since more than 90% of patients were older than 40 years and 35% older than 62 years.

Overall, patients wearing dental prosthesis had larger sized PMDs, with FOM lesions more commonly seen in denture wearers. PMDs affecting the tongue were more frequently seen in patients with crowns and bridges, although this was not significant. It has been said that denture wear is not associated with an increased risk of oral cancer (Zheng et al., 1990; Marshall et al., 1992; Fisher et al., 2005); however, large sized PMDs in wearers may be associated with chronic physical irritation, particularly to FOM and tongue epithelium (Piemonte et al., 2010). Further, well-designed studies in this respect with more information about dental prosthesis wear in relation to oral anatomical sites are required.

Since the current study is a retrospective investigation using data recorded in patients' medical records for diagnosis and follow-up, information concerning mouthwash use was limited to 12 patients, with nine who did not use mouth wash and only 3 mouthwash users, which did not allow for statistical analysis.

In many available mouthwashes, the presence of ethanol has raised concern regarding safety. It has been suggested that a heavy alcoholic-mouthwash may expose oral mucosa to the level of alcoholic beverages that induce oral cancer (Winn et al., 2001). Therefore, it has been recommended that the use of alcohol-containing mouth rinses in high-risk populations should be restricted (Reidy et al., 2011).

Although some substantial evidence may support the relationship between ethanol and oral cancer, the assumed relation is inconsistent and lacks scientific evidence (Badran et al., 2010). Well-designed studies are needed in future to determine any causal relationships between alcohol-containing mouthwashes and oral cancer and require larger sample sizes, detailed parameters and assessment of residual confounders such as alcohol and tobacco use.

Similarly, limited information about oral hygiene status prevented statistical evaluation in this respect and our study was not specifically designed to address the relationship between PMD behaviour and oral health. It has been reported that a poor periodontal condition was associated with a small but statistically significant increase in risk of cancer in non-smokers (Michaud et al., 2008). The periodontal status in our patients was not formally assessed but further studies are warranted to investigate possible associations between dysplastic PMDs and oral hygiene parameters such as chronic periodontitis. Routine dental visits as a predictor for favourable oral hygiene status are recognised and may be associated with a decreased risk of oral cancer and early diagnosis of PMDs.

Likewise, limited information about dietary habits in this study did not allow statistical analysis. Associations between nutritional deficiencies and SCC remain possible and a number of case-control studies have consistently shown patients with oral cancer have poor dietary habits (Rossing et al., 1989). Also, it has been reported that increased consumption of citrus fruits reduce the risk of oral PMDs by 30-40% using multivariate-adjusted relative risks, whilst no consistent associations were apparent for vegetable intake (Maserejian et al., 2006a).

The relationship between diet and the risk of oral cancer and PMDs is not fully understood and may be related to the fact that not all chemical constituents of food are identifiable and the role of different micronutrients is not entirely clear (Petti, 2009). However, the protective effects of diet may be explained by mechanical cleansing effects, a dilution action and anti-oxidant and anti-carcinogenic properties (Potter and Steinmetz, 1996; Lucenteforte et al., 2009).

By analysing hospital records and finding omissions in data collection for information such as diet, familial cancer history, oral health status and mouthwash usage, we recommend the use of a standardized case sheet to ensure both consistency and reliability in data collection (Appendix 1-E).

4.5.5. Logistic Regression Analysis

This analysis revealed that various factors previously believed to be important predictors of non-homogenous leukoplakia development, such as patient age, smoking and drinking intensity as well as a history of systemic disease were non-significant. However, males were at increased risk for non-homogenous leukoplakia 1.6-times compared to females. It may be the high prevalence of tobacco and alcohol habits in males that explain this, although further studies on a biomolecular basis are required.

Compared to non-drinkers, drinking alcohol > 28 units/week increased the risk for non-homogenous leukoplakia 3-times whilst drinking < 28 (units/week) increased the risk 1.8-times. The higher risk of developing non-homogenous leukoplakia in such patients was reported for the first time in this study, although in general, alcohol has been suggested as an independent risk factor for oral leukoplakia (Hashibe et al., 2000a; Maserejian et al., 2006a; Saraswathi et al., 2006).

The final regression model showed that simultaneous exposure to tobacco and alcohol increased the risk for non-homogenous leukoplakia to develop by 6.3-times compared to non-drinkers. This reflects the synergistic effects of both risk factors (Castellsague et al., 2004) and is supported by previous studies (Franco et al., 1989; Garrote et al., 2001) which suggested that the combination of smoking and drinking has supra-multiplicative effects.

Homogenous leukoplakia appears to be more common in smokers, whilst non-homogenous lesions are less closely related to tobacco use. In this study, current and past tobacco smoking reduced the risk of non-homogenous leukoplakia development by 65% for smoking > 20 cigarettes/day, 80% for < 20 and 71% in ex-smokers. The reduction in the risk of non-

homogenous leukoplakia was further supported by final regression model, which showed a further reduction in the risk of smoking > 20 and < 20 cigarettes/day (47% and 38%, respectively). This finding clearly supports the well-known relationship between tobacco smoking and the development of homogenous leukoplakia. Previous studies showed that tobacco smoking was the strongest independent risk factor for oral leukoplakia (Gupta, 1984; Morse et al., 1996; Reichart, 2001) and since homogenous leukoplakia is the most common clinical type and consistent with these findings, smoking may be associated with a reduction in the risk of non-homogenous leukoplakia, however, further studies are required to investigate this finding.

Regression analysis showed that non-homogenous leukoplakia was a significant predictor for high grade dysplasia, with a 3.3 times increased risk than homogenous lesions. This finding is consistent with a previous study by Lee *et al.* (2006) who reported a 5.7-times higher potential for dysplasia in non-homogenous leukoplakias. This is further supported by earlier studies which showed an association between non-homogenous leukoplakia and epithelial dysplasia (Mincer et al., 1972; Banoczy and Csiba, 1976). This may be explained by accumulated of more genetic changes with subsequent more severe dysplastic features in non-homogenous leukoplakia compared to homogenous due to a longer presentation time and different biomolecular conformations.

The size of dysplastic PMD was a significant predictor for high grade dysplasia, with sizes exceeding 600 mm^2 showing an 11.5-times increased risk compared to sizes < 200 mm^2. This is supported by the final regression model which confirmed sizes > 600 mm^2 increased the risk for high grade dysplasia by 7.6-times, albeit non-significant. This may be due to a longer presentation time of larger lesions with accumulation of more genetic alteration and resultant higher dysplastic grading (Napier and Speight, 2008; Warnakulasuriya et al., 2008).

Regression analysis showed that alcohol drinking was a significant predictor of high grade dysplasia, with > 28 units/week showing 4.9-times the risk of non-drinkers. This is consistent with multivariate data analysis of Lee *et al.*(2006) which showed a 1.48 fold risk of dysplasia development in drinkers compared to no-drinkers, and is supported by other studies which found increased alcohol consumption associated with an increased risk of dysplasia,

particularly in heavy drinkers (Jokelainen et al., 1996a; Morse et al., 1996; Howie et al., 2001).

In the present study, a positive history of systemic disease was identified as a significant predictor of high grade dysplasia development and showed a 4-times higher risk than patients with clear medical histories. This is supported by the significant relationship between the degree of dysplasia and systemic disease found in this study, whereby no severe dysplasia or CIS was seen in patients without systemic disease, whilst high grade dysplasia was seen in those with systemic disorders. This may be due to PMD patients with systemic disorders being of an older age and taking a variety of medications which may play an enhancing role.

In the final multivariate regression model, the only significant predictor of high grade dysplasia development was a positive medical history, which increased the risk by 7.5-times. This was a higher risk than that identified by the univariable regression model (4-times), reflecting the enhancing effects of other factors incorporated into the multivariate analysis such as PMD size, type of leukoplakia and smoking and drinking behaviour. This finding further emphasises the importance of a systemic history of patients with PMDs as an important prognostic factor for high grade dysplasia and unfavourable prognosis.

4.6. Conclusions

The results of the present study provide a solid ground for additional research and emphasise the importance of risk factor assessment in association with clinical and pathological features to identify patients at high risk.

4.6.1. Tobacco Smoking

1. The majority of PMD patients were active smokers.
2. Male smokers (73%) were more common than non-smoking males (33%).
3. Males were significantly more common in the current smokers group (73%), compared to females (27%).
4. Males were most often heavy and intermediate smokers, compared to females.
5. Female non-smokers (67%) were more predominant than male non-smokers (33%).
6. Significantly, females formed the majority of non-smokers (67%).
7. In both male and female patients, current smokers were significantly younger than non-smokers or ex-smokers.
8. Males smoked for a significantly longer duration of time compared to females (37.11 vs. 28.13 years).
9. The majority of heavy and intermediate smokers were middle aged.
10. Light smokers showed the longest smoking history compared with both intermediate and heavy smokers.
11. Males started smoking earlier than females, subjecting themselves to increased risk from long-standing exposure to tobacco smoke.
12. Unemployed patients consumed the largest amounts of tobacco compared to employed or retired patients.
13. Significantly, in both males and females, FOM leukoplakias (60%) arose more frequently in current smokers than non-smokers.
14. Leukoplakias in the lateral and ventral tongue surfaces (93%) were significantly more common in non-smokers compared to current smokers.
15. All patients younger than 40 years were tobacco users and mainly presented with FOM lesions.

16. Patients older than 41 years comprised tobacco users and non-users and developed both FOM and tongue PMDs.
17. In non-smokers, the tongue was the only affected site in females and in 80% of males.
18. The soft palate only developed PMDs in tobacco users, most commonly in current rather than ex-smokers.
19. Patients with FOM leukoplakias were mainly intermediate and heavy smokers.
20. The joint effect of intensity and duration of tobacco smoking was negatively correlated with patient age and PMD size.
21. The mean pack-years score was higher in males (43.23) than females (32.18).
22. Erythroplakias only affected heavy smokers.
23. Exophytic subtypes were more commonly seen in light-smokers.
24. Patients with homogenous leukoplakias exhibited long smoking histories.
25. Current smokers showed smaller mean PMD sizes compared to non-smokers (241.5 vs. 473 mm^2); heavy smokers exhibited smallest mean size compared with light smokers (247.5 vs. 316 mm^2).
26. Patients with longer smoking histories more often presented with smaller sized PMDs.
27. A higher presentation of all dysplastic features was seen in current smokers compared to both ex-smokers and non-smokers.
28. Heavy smokers were significantly more likely to exhibit severe dysplasia compared with light smokers.
29. The longer the smoking history, the higher the dysplasia severity seen.

4.6.2. Alcohol Use

1. The majority of the study population were current drinkers, light drinkers were most common, followed by heavy and intermediate drinkers.
2. All patients younger than 40 years were current drinkers.
3. Males were predominantly current drinkers (71%), with females mainly non-drinkers (71%).
4. Significant sex differences in alcohol consumption were seen, with 97% of males consuming > 28 units/week compared with 96% of females consuming < 28 units/week.
5. Males showed twice the average UK recommended alcohol consumption.

6. PMD patients are a high-risk group, with 58% of males and 9% of women routinely exceeding recommended alcohol intake.
7. In both males and females, a lower level of alcohol consumption was seen in elderly patients.
8. The FOM was the most common site for PMDs in heavy drinkers (56%).
9. A higher number of homogenous leukoplakias occurred in light drinkers compared to heavy drinkers (52% vs. 37%).
10. A higher number of non-homogenous leukoplakias was observed in heavy drinkers compared to light drinkers (50% vs. 36%).
11. A higher presentation of speckled (62%) and exophytic subtypes (60%) were seen in heavy drinkers compared to light drinkers (30% and 40%).
12. Almost all erythroplakias were seen in current drinkers.
13. The majority of patients with mild dysplasia were light drinkers, whereas the majority of CIS cases were heavy drinkers.
14. Half of non-drinkers were ex-smokers.
15. 92% of current drinkers were also current smokers.

4.6.3. Systemic Health Disorders

1. 88% of the study populations had a positive medical history.
2. Hypertension followed by diabetes mellitus was the most common systemic diseases.
3. Hypertensive patients mainly presented with speckled subtypes.
4. Higher grade dysplasias were more frequently seen in hypertensive patients compared to normotensive; CIS (90% vs. 10%), severe dysplasia (73% vs. 27%) and moderate dysplasia (52% vs. 48%).
5. Females were more commonly affected with diabetes mellitus than males (60% vs. 40%).
6. All PMDs in diabetic patients were leukoplakias.
7. The majority of diabetic patients were intermediate smokers and light drinkers.
8. PMDs in diabetic patients were seen mainly in high-risk sites (87%).

4.6.4. Dental Factors

1. Dental prosthesis wearers mainly presented with large sized PMDs.
2. Patients wearing dentures commonly presented with FOM PMDs, whilst those with crowns and bridges mainly developed tongue lesions.

4.6.5. Logistic Regression Analysis

1. Logistic regression analysis emphasised the inherent high-risk of non-smokers and showed that non-homogeneous leukoplakias were more frequently seen in these patients or in patients with lower tobacco intake.
2. Heavy drinking (> 28 units/week) had 3-times greater risk for developing non-homogenous leukoplakia than non-drinkers, whilst drinking ≤ 28 (units/week) had 1.78-times higher risk.
3. Patients with systemic health disorders showed 4.2-times higher risk for developing non-homogenous leukoplakia than those without.
4. Males showed a 1.338-time greater risk than females for developing high grade dysplasia.
5. Non-homogenous leukoplakia was a significant predictor of high grade dysplasia, increasing the risk by 3.3-times compared to homogenous lesions.
6. Lesion size exceeding 600 mm^2 was a significant predictor of high grade dysplasia, increasing the risk 11.5-times.
7. Heavy drinking (> 28 units/week) was a significant predictor for developing high grade dysplasia, increasing the risk 4.89-times greater than non-drinkers.
8. A history of systemic health disorders was a significant predictor of high grade dysplasia occurrence, increasing the risk 4-times.

Chapter Five: Clinical Outcome

Study 3

5.1. Aims

The aim was to investigate the long-term follow-up outcome of a cohort of patients with single dysplastic oral PMDs who had been treated with laser surgery and to identify significant risk factors for recurrent or new PMD disease formation and for the development of OSCC and malignant transformation.

5.2. Methods

Clinical outcome for the 100 PMD patients was determined as either disease-free (clinical resolution) or disease-active. Disease active (DA) included: persistent disease (same site), recurrent disease (same site), further disease (new-site), malignant transformation (MT) (same site) and development of oral squamous cell carcinoma (OSCC) at sites distant from the primary dysplasia and proven histologically.

For each patient, times were calculated from the first presentation of the patient to the time of incisional biopsy, excisional biopsy (laser surgery) and the most recent clinical contact with patients.

After laser intervention, time to event and number of each event such as recurrences, further disease formation, MT and OSCC were recorded for each patient and at each clinical review time, as proven histologically.

Also, the number of observational biopsies (prior to laser surgery) and follow-up biopsies (after laser surgery), the number of laser interventions, the length of follow-up period and the frequency of clinical review were also recorded.

All patients in this study attended regular follow-up appointments in dedicated oral dysplasia clinics.

5.2.1. Statistical Analysis

Statistical calculations were performed using SPSS, version 17.0 and 19.0 (Statistical Package for the Social Sciences, Chicago, IL, USA).

The outcome of the categorical variables for the whole group of oral PMDs such as recurrence, malignant development and new disease formation were summarized as frequencies and percentages. The Chi-Square test or Fisher's Exact test were used to evaluate the relationship between the categorical variables. Continuous variables were expressed as mean ± SD and were compared using Independent student t-test or Mann-Whitney U test for pair-wise comparison. ANOVA or Kruskal-Wills tests were used for group comparison.

Spearman's and Pearson's correlations were used to find and evaluate the correlation between variables.

The Kaplan-Meier survival analysis method with Log-Rank test was used to assess the differences between the outcome groups and to calculate the overall cumulative survival rate for the total cohort and stratified by baseline tobacco smoking, alcohol consumption and consensus histopathological diagnosis for both the binary and the WHO grading systems.

Further statistical analysis was performed using univariate and multivariate logistic regression analysis to determine the independent predictors of recurrent, further disease, MT and cancer development, using the factors primarily found to be significant by univariate analysis. Odds ratios (OR) and corresponding confidence intervals (CI) were calculated at an ordinary level of significance (p=0.05).

The factors were incorporated as independent variables in the analysis if they had an association with the outcome variables at a p-value < 0.15.

Patient age, size of dysplasia, number of cigarettes/day, length of smoking history, pack score, tobacco grams/week and the number of alcohol units/week were used as covariate whilst sex, clinical appearance, anatomical site of PMD, oral prosthesis wear, smoking status, drinking status and histopathological diagnosis (WHO and binary grading system) were entered as independent variables to predict the most important factors for clinical outcome.

For single factor analysis, age was entered as a continuous variable to perform a trend test.

Considering the localization of PMDs, oral subsites were stratified into 3 locations to facilitate the regression analysis (FOM, tongue and other remaining sites).

Logistic regression model selection utilised the goodness of fit statistic -2 log likelihood ratio. Firstly, all one predictor and all combinations of two predictor models were used. The two predictor model with the smallest value of the log likelihood ratio was used as a basis for fitting three predictor models; each of the remaining predictors was added, one at a time, to the best two predictor model and the log likelihoods assessed. The three predictor model with the smallest value of-2 log likelihood was used as a basis for fitting 4 predictor models; each of the remaining predictors was added to that model to see if fit was significantly increased.
If the criterion of the first analysis was not fulfilled for any factor, the factor was excluded from the regression analysis.

For all statistical tests, p-values ≤ 0.05 were considered statistically significant.

5.3. Results

5.3.1. Analysis by Clinical Outcome

In this study, at the most recent patient follow-up appointment, 5 groups of treatment outcomes were defined and subsequently studied:

1. Disease-free (DF) (clinical resolution).
2. Recurrent disease (recurrence of dysplasia at the same site as the primary dysplasia).
3. Further disease (development of new-site dysplasia).
4. Malignant transformation (MT) (development of OSSC in the same site as the primary dysplasia).
5. OSCC Development in which OSCC was diagnosed arising at a site distant from the primary dysplasia.

The latter 4 categories were considered to be disease active (DA).

Following laser intervention and at the most recent follow-up time, 62 out of the 100 patients (39 males and 23 females) of the study population remained DF, whilst 38 were DA, developing recurrent, further disease, MT and OSCC development.

Out of the 38 DA patients (27 males and 11 females), same site recurrence was reported in 17 patients, further (new-site) disease in 14, 5 underwent MT (same site) and 2 developed OSSC at new sites distant from the primary dysplasia; Figure 5.1.

Figure 5.2 shows the relation between age groups and the clinical outcome. Middle age patients were predominant in all outcome groups, although patients younger than 40 years exhibited no cancer development; they were equally affected by recurrent and further dysplasia formation.

Using Chi-Square test, no significant relation was found between patient age and clinical outcome (p=0.118).

Males were usually the most common in all clinical outcome groups; Figure 5.3. However, no significant association was found between sex and treatment outcome (p=0.811; Chi-Square test).

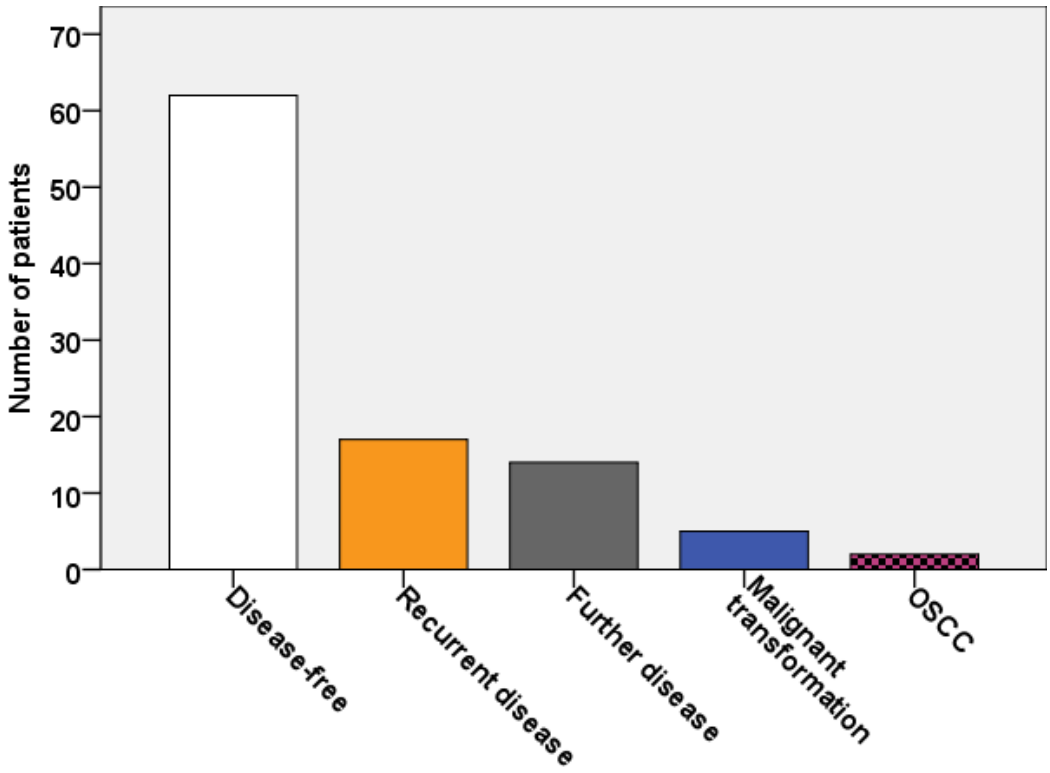

Figure 5.1: Clinical outcome of 100 patients with dysplastic PMDs treated with laser surgery.

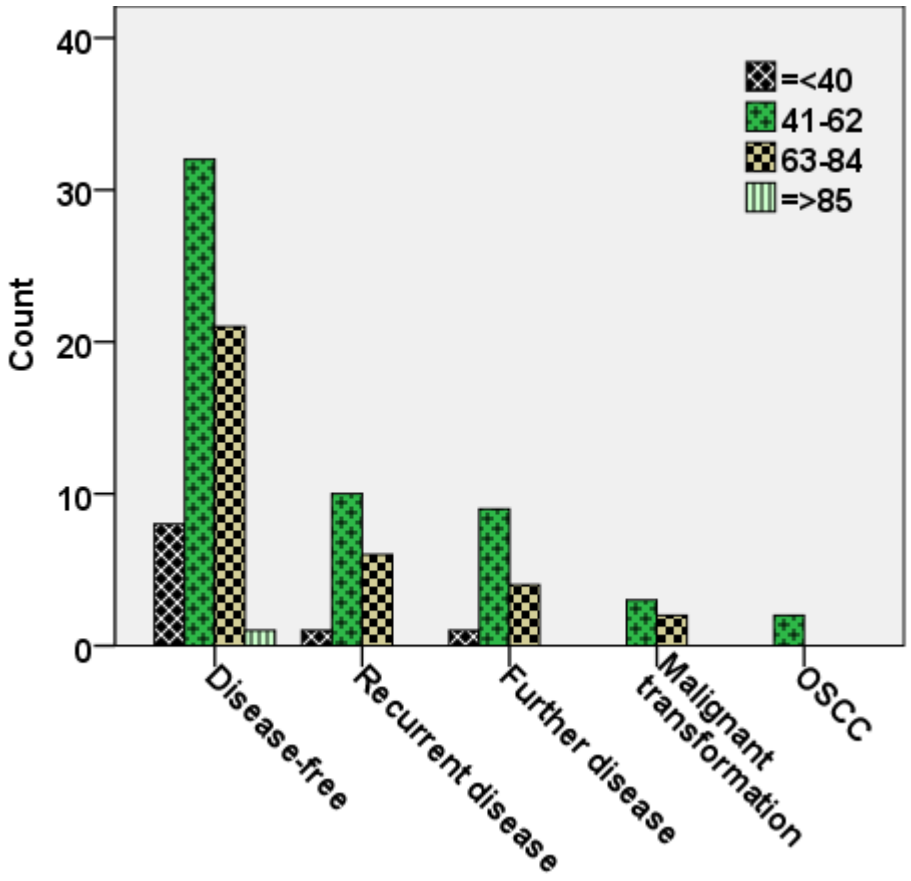

Figure 5.2: Distribution of age group according to clinical outcomes.

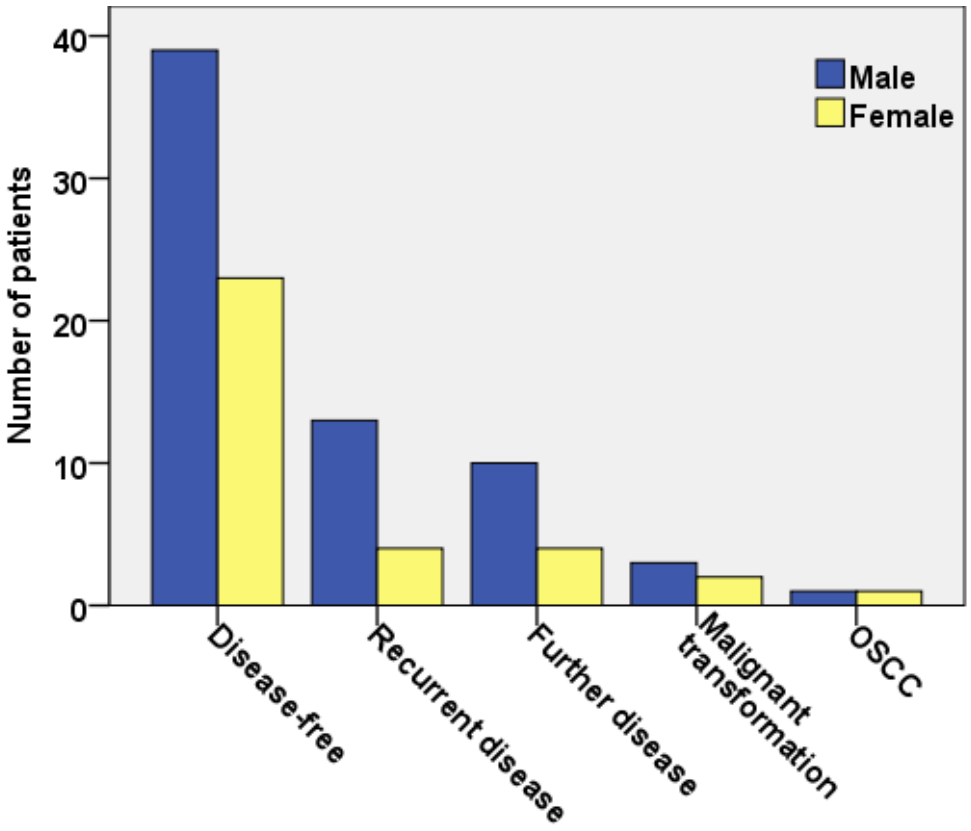

Figure 5.3: Sex distribution in relation to clinical outcomes.

Time to Treatment Considerations

To investigate the influence of time on the diagnosis and management of patients with oral epithelial dysplasia, time intervals were calculated from the point of presentation to the clinical event of interest.

Figure 5.4 illustrates a flowchart of defined time points during sequential diagnosis and management of patients with PMDs.

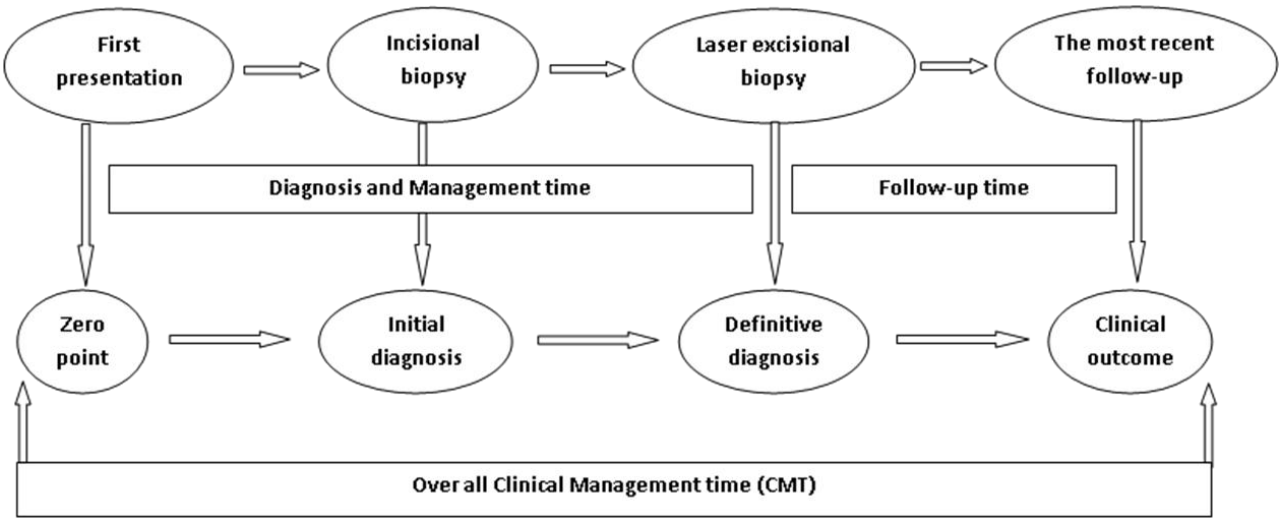

Figure 5.4: Different time points in relation to management of PMD patients.

In general, the time between the first presentation of patients (zero point) and the most recent follow-up appointment was defined as the *total clinical management time*, which ranged from 5-189 months in this study with a mean of 71.86 months (SD: 45.077).

Prior to laser intervention, the number of observational biopsies taken from patients ranged between 0 to 7, whilst the number of follow-up biopsies following laser surgery varied between 0-13. Patients in this study underwent between 1 to 6 laser interventions and 5-78 follow-up appointments depending on the clinical course of the patient.

A negative significant correlation was found between number of observational biopsies and the degree of dysplasia (r=-0.291, n=100, *p*=0.003), with higher number of observational biopsies were reported in patients with mild dysplasia (73%; 16/22) compared to 27% (6/22) in higher grades of dysplasia.

The time between the first presentation and initial diagnostic biopsy (incisional biopsy) was defined as the *provisional diagnostic time (PDT).*

In this study, the provisional diagnostic time ranged from 0-24 months with a mean of 1.74 months (SD: 4.089). Incisional biopsies were taken on the same day as first presentation in 52% of patients, from 39% of patients within the first 3 months of initial presentation and from 6% between 4-12 months. A delay of more than 12 months in taking the initial diagnostic biopsies was reported for only 3 cases due to patients' delays; Figure 5.5.

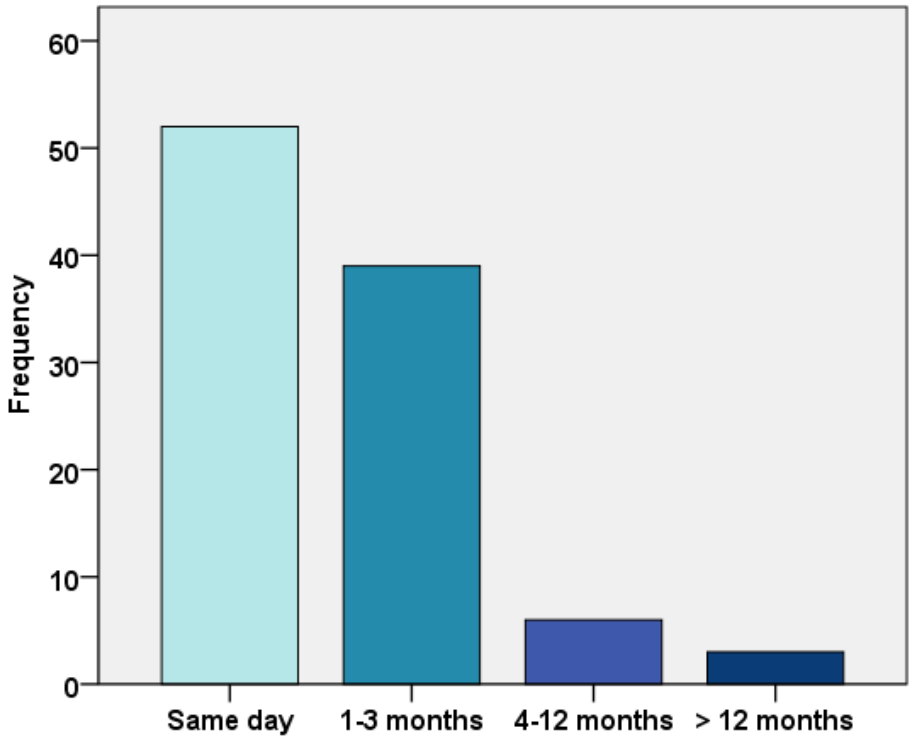

Figure 5.5: The provisional diagnostic time (PDT) for 100 PMD patients.

As can be seen in Table 5.1, cases experiencing a delay in incisional biopsy included one mild, one moderate and one severe dysplasia, however all of them were DF at the most recent patients follow-up.

Using Kruskal-Wallis testing, no significant differences were found in the mean provisional diagnosis time (first presentation to incisional biopsy) amongst different grades of dysplasia (p=0.758) and clinical outcomes (p=0.177).

Also, no correlation was found between the provisional diagnostic time and the degree of dysplasia for both WHO and binary grading systems (r=-0.019, n=100, p=0.855), (r=-0.009, n=100, p=0.926), respectively.

Table 5.1: Degree of dysplasia in relation to provisional diagnostic time.

Degree of dysplasia	Time between first presentation and incisional biopsy (months)				Total
	Same day biopsy	1-3	4-12	> 12	
Mild	**20** *48%*	**20** *48%*	**1** *2%*	**1** *2%*	**42** *100%*
Moderate	**14** *58%*	**6** *25%*	**3** *13%*	**1** *4%*	**24** *100%*
Severe-CIS	**18** *53%*	**13** *38%*	**2** *6%*	**1** *3%*	**34** *100%*
Total	**52**	**39**	**6**	**3**	**100**

There was no significant association between the clinical appearance of PMDs and the time between first presentation and undertaking incisional biopsy (p=0.062; Chi-Square test), although 63% of erythroplakias and 50% of speckled non-homogenous leukoplakias underwent same day incisional biopsies; Table 5.2.

Table 5.2: Clinical appearance of PMDs in relation to provisional diagnostic time.

Clinical type of PMDs		Time between first presentation and incisional biopsy (months)				Total
		Same day biopsy	1-3	4-12	> 12	
Homogenous leukoplakia		**34** *51%*	**28** *42%*	**4** *6%*	**1** *1%*	**67** *100%*
Non-homogenous leukoplakia	Speckled	**8** *50%*	**5** *31%*	**1** *6%*	**2** *13%*	**16** *100%*
	Nodular	-	**1** *50%*	**1** *50%*	-	**2** *100%*
	Exophytic	**4** *80%*	**1** *20%*	-	-	**5** *100%*
	Ulcerated	**1** *50%*	**1** *50%*	-	-	**2** *100%*
Erythroplakia		**5** *63%*	**3** *38%*	-	-	**8** *100%*
Total		**52**	**39**	**6**	**3**	**100**

The time between the first presentation of the patient and laser treatment or the *diagnosis and management time* was similarly investigated and classified into 3 groups: 1-3 months, 4-12 and > 12 months. In this study, this time ranged from 1-163 months with a mean of 16.96 months (SD: 26.262). Thirty-three percent of patients waited 4-12 months for the laser intervention after initial presentation, with a similar percentage of patients experienced the shortest time for laser to take place 1-3 months, followed by > 12 months waiting time (34%); Figure 5.6.

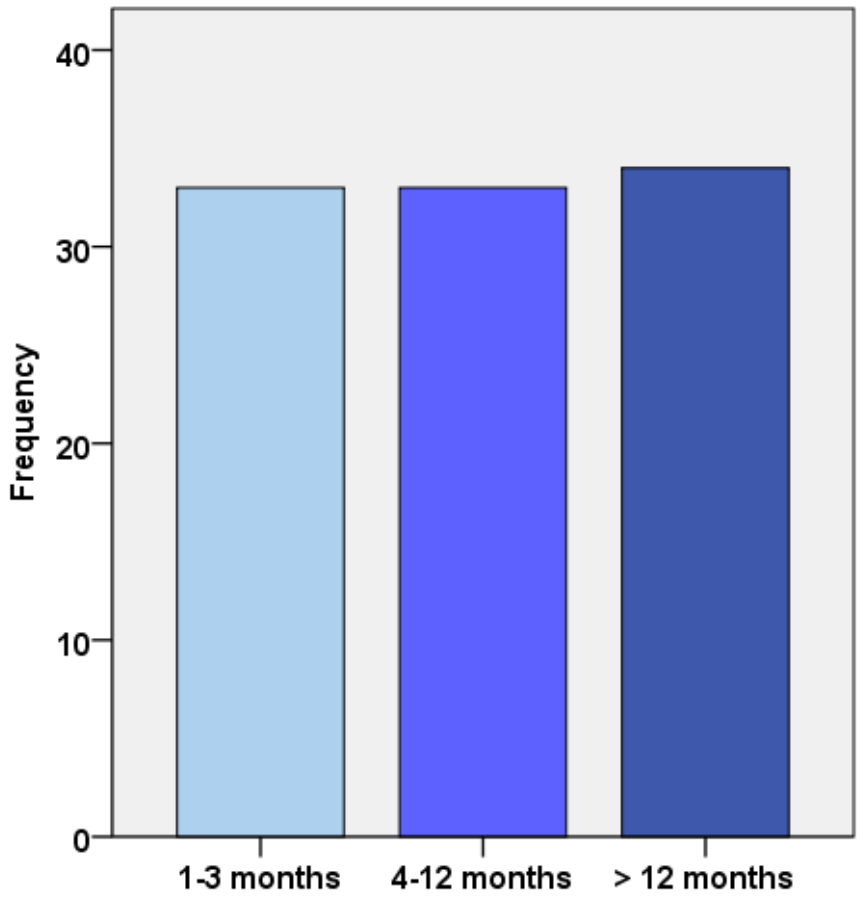

Figure 5.6: First presentation to laser surgery time among 100 PMD patients.

A significant relation was found between first presentation to laser time and the degree of dysplasia (p=0.0001; Chi-Square test), with the longest wait for laser intervention (> 12 months) seen in mild dysplasia cases 57% (24/42), followed by 4-12 months wait for 38% (9/24) of moderate dysplasia cases, and the shortest waiting time (1-3 months) in 59% (20/34) of patients with severe dysplasia-CIS; Table 5.3.

Table 5.3: Degree of dysplasia in relation to first presentation to laser time.

Degree of dysplasia	Time between first presentation and laser intervention (PDT) (months)			Total
	1-3	4-12	> 12	
Mild	5 *12%*	13 *31%*	24 *57%*	42 *100%*
Moderate	8 *33%*	9 *38%*	7 *29%*	24 *100%*
Severe-CIS	20 *59%*	11 *32%*	3 *9%*	34 *100%*
Total	33	33	34	100

Table 5.4 and Table 5.5 show the mean total clinical management time intervals (mean time of the provisional diagnostic time, first presentation to laser surgery, definitive diagnosis and follow-up time) in relation to the degree of dysplasia and the clinical outcome, respectively.

In general, Kruskal-Wallis testing showed no significant differences in clinical outcome with varying time from first presentation to laser intervention (p=0.175). However, pair-wise comparison using Mann-Whitney U test showed a significant differences in time to laser from the first presentation between DF and DA patients (p=0.034), with DA cases experienced a shorter mean time to laser surgery than DF patients. Similarly, patients with further disease showed significantly a shorter mean time for the laser to take place after first presentation than further disease-free patients (p=0.030); Table 5.5.

Table 5.4: Mean total clinical management time intervals in relation to the grades of dysplasia.

Degree of dysplasia	Provisional diagnostic	First presentation to laser	Definitive diagnostic	Follow-up	Total
Mild	1.57	25.55	23.69	50.50	42
Moderate	2.29	18.21	10.96	46.42	24
Severe	1.57	6.65	4.78	66.91	23
CIS	1.55	3	1.18	66.36	11
Total mean	1.74	16.96	13.81	55.04	100

Table 5.5: Time to treatment consideration in relation to clinical outcome with *p*-value of Mann-Whitney U test.

Treatment outcome	N	Provisional diagnostic mean time	*p*-value	First presentation to laser mean time	*p*-value	Definitive diagnostic mean time	*p*-value	Follow-up mean time	*p*-value
Disease-active	38	1.11	0.479	14.26	0.034	10	0.004	81.42	0.0001
Disease-free	62	2.13		18.61		16.15		38.87	
Recurrent disease	17	0.88	0.076	22.41	0.740	15	0.428	86.94	0.002
Recurrence-free	83	1.92		15.84		13.7		48.51	
Further disease	14	1.36	0.143	6.43	0.030	4.57	0.004	87.21	0.001
Further disease-free	86	1.80		18.67		15.31		49.80	
OSCC (new site)	2	3	0.630	6.50	1.000	4	0.663	79.50	0.328
OSCC-free	98	1.71		17.17		14.01		54.54	
MT (same site)	5	0.40	0.230	11.60	0.480	10.60	0.848	47.20	0.937
MT-free	95	1.81		17.24		13.98		55.45	

The time between incisional biopsy and laser excision was defined as the *definitive diagnosis time (DDT)*, and ranged from 3 weeks-163 months with a mean of 13.81 months (SD: 25.81).

The majority of patients in this study (44%) waited between 1-3 months for laser interventions, 26% waited 4-12 months and a further 25% waited for more than 12 months; 5% of patients actually waited less than one month to undergo laser surgery.
Thirty-three percent and 45% of mild dysplasia cases waited for 4-12 and more than 12 months, respectively. In contrast, 68% of severe dysplasia-CIS and 50% of moderate dysplasia only waited between 1-3 months for laser excision; Table 5.6.

A highly significant difference in the mean time for definitive diagnosis (incisional to excisional time) between different grades of dysplasia was found (p=0.001; Kruskal-Wallis test). Subsequently, a Mann-Whitney U test was performed which showed a highly significant difference between mild and CIS (p=0.0001), mild and severe dysplasia (p=0.0001), mild and moderate (p=0.002) and moderate-CIS (p=0.042). The milder the dysplasia, the longer the waiting time for laser treatment. Mild dysplasia exhibited the longest mean time between incisional biopsy and excisional laser treatment compared to moderate, severe dysplasia and CIS.

Further statistical analysis showed a negative significant correlation between the time of definitive diagnosis and degree of dysplasia (r=-0.554, n=100, p=0.0001). The higher the degree of dysplasia, the shorter the waiting time for laser treatment after incisional biopsy was reported; Figure 5.7. Similarly, for the binary grading system (r=-0.466, n=100, p=0.0001), low grade dysplasia was associated with a longer waiting time from incisional biopsy to laser treatment.

Table 5.6: Degree of dysplasia in relation to definitive diagnostic time (DDT).

Degree of dysplasia	Time between incisional biopsy and laser intervention (months)				Total
	< 1	1-3	4-12	> 12	
Mild	-	9 *21%*	14 *33%*	19 *45%*	42 *100%*
Moderate	1 *4%*	12 *50%*	7 *29%*	4 *17%*	24 *100%*
Severe-CIS	4 *12%*	23 *68%*	5 *15%*	2 *6%*	34 *100%*
Total	5	44	26	25	100

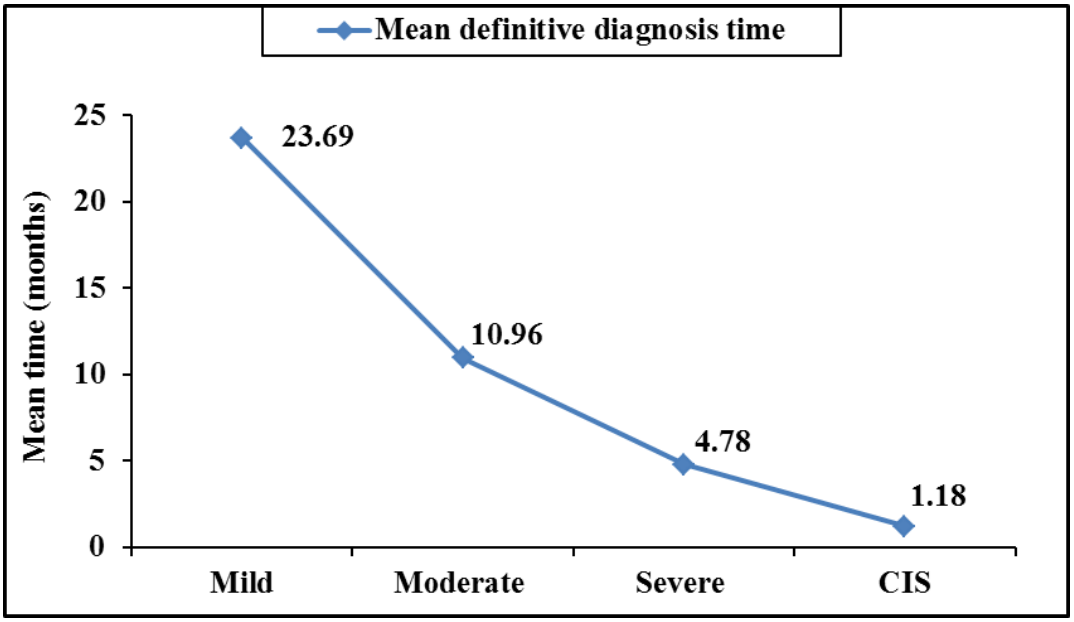

Figure 5.7: Mean time between incisional biopsy and laser intervention (DDT) and degree of dysplasia.

Kruskal-Wallis testing showed a significant difference in the DDT (incisional to excisional time) among clinical outcome groups (p=0.026). Subsequently, a Mann-Whitney U test showed a highly significant difference in the mean time to laser intervention after incisional biopsy (DDT) between DF patients and DA patients (p=0.004) who had a shorter mean time to laser intervention, compared to DF patients (10 vs. 16.15 months).

Patients developing further disease showed a significantly shorter mean time to laser treatment following incisional biopsy (DDT) compared to those who did not (4.57 vs. 15.31 months, p=0.004). However, no significant differences were seen in the mean time to laser intervention after incisional biopsy between patients who developed recurrent disease or those who did not, patients who developed OSCC (new-site) or did not, patients undergoing MT or who did not, and between patients who had recurrence and those who developed further (new-site) dysplasia (p=0.173).

With respect to sex, male patients experienced a shorter mean time to laser intervention after incisional biopsy (DDT) compared to female patients (12.48 vs. 16.38 months), however the differences did not reach significance (p=0.107; Mann-Whitney U test).

The time between laser intervention and the most recent follow-up was defined as the *follow-up time*, and was measured from the date of definitive diagnosis to the most recent clinical contact with the patient.
In this study, patients were followed-up after laser treatment from between 6-183 months, with a mean period of 55.49 months (SD: 41.667).
Using a Kruskal-Wallis test, no significant differences in the mean follow-up time (laser to last visit) was found among different grades of dysplasia (p=0.438), which was similar for high and low grade dysplasia grading (p=0.868; Mann-Whitney U test). However, a highly significant difference in the mean follow-up time was found for treatment outcomes (p=0.0001). DF patients showed the shortest mean follow-up time, followed by MT (same site), OSCC (new-site), recurrent dysplasia (same site) and further dysplasia formation (new-site) (38.87, 47.20, 79.5, 86.94 and 87.21 months); Table 5.5.

Subsequently, Mann-Whitney U testing showed a significant difference in mean follow-up times between patients who developed recurrence (same site) and those who were recurrence-

free (p=0.002), and between patients developing further dysplasia and those who did not (p=0.001).

Patients with recurrence were followed-up for a longer time than recurrence-free patients (86.94 vs. 48.51 month) and this was similar for patients with further disease compared to further disease-free patients (87.21 vs. 49.80 months). However, no significant differences were seen between patients who developed OSCC (new-site) or those who underwent MT (same site) and those who were OSCC-free or MT-free.

Considering the clinical appearance of oral dysplastic PMDs, no significant difference was seen in the mean follow-up time between homogenous and non-homogenous leukoplakia (p=0.0648; Mann-Whitney U test), although non-homogenous type did exhibit longer follow-up time compared with homogenous leukoplakia (62.32 vs. 54.06 months).

Table 5.7 shows the mean follow-up time for homogenous and non-homogenous leukoplakia subtypes. Although a Kruskal-Wallis test was not significant (p=0.395), the longest mean follow-up time was reported for exophytic subtypes (90.40 months; range 46-133), followed by speckled (57.19 months; range 4-183), ulcerated (53.00 months; range 24-82) and nodular PMDs (42.50 months; range 16-69).

Table 5.7: Mean follow-up time in relation to clinical appearance of leukoplakia.

Leukoplakia clinical appearance	Mean Follow-up time (month)	N	Range	SD
Homogenous	54.06	67	3-168	39.432
Non-homogenous	62.32	25	4-183	48.487
Total mean	56.30	92	3-183	41.969
Non-homogenous subtypes				
Speckled	57.19	16	4-183	53.755
Nodular	42.50	2	16-69	37.477
Exophytic	90.40	5	46-133	34.166
Ulcerated	53.00	2	24-82	41.012
Total mean	62.32	25	4-183	48.487

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With respect to *total clinical management time*, which was calculated from the first presentation to the most recent follow-up time, a significant difference in clinical outcome was found (p=0.0001; Kruskal-Wallis test). Subsequently, using a Mann-Whitney U test, a significant difference in the mean total clinical management time was found between DF and recurrent disease groups (p=0.0001), with a significantly shorter total clinical management time for DF patients compared to those undergoing recurrence (57.95 vs. 108.25 months).
Similarly, a significant shorter mean management time for DF patients was observed compared to patients developing further disease (57.95 vs. 94.21 months, p=0.002).
No significant differences in total management time were seen, however between the remaining clinical outcome groups.
Table 5.8 presents the mean total clinical management time for all five clinical outcomes; DF showed the shortest mean time compared to the others.

Table 5.8: Mean total clinical management time in relation to clinical outcomes.

Total clinical management time (First presentation to the most recent follow-up) (months)				
Clinical outcome	**Mean**	**N**	**Range**	**SD**
DF	**57.95**	**62**	**5-189**	**42.045**
Recurrent disease	**108.25**	**17**	**17-184**	**47.460**
Further disease	**94.21**	**14**	**31-135**	**31.232**
MT (same site)	**59.20**	**5**	**26-103**	**29.064**
OSCC (new site)	**87.00**	**2**	**84-90**	**4.243**
Total mean	**71.86**	**100**	**5-189**	**45.077**

Disease-Free (Clinical resolution)

Disease-free (DF) cases were defined as such by clinical examinations and/or pathological evidence, with the time between definitive diagnosis and the confirmation of any disease active state defined as disease-free time.

After laser treatment and at the most recent follow-up, 62/100 patients were DF (39 males and 23 females); these had been followed-up from 4 to 122 months (mean of 38.87 months), with 5-42 follow-up appointments.
The total clinical management time of patients in this group was between 5-189 months, with a mean of 57.95 months (SD: 42.045).

All patients in this group underwent one laser intervention and between 0 to 4 observational biopsies before laser surgery and between 0 to 2 follow-up biopsies after laser intervention.

Fifty-two percent (32/62) of DF patients were middle age (41-62 years), followed by 34% (21/62) old age (63-84 years), 12% (8/62) young age (≤ 40) with only one patient older than 85 years; Figure 5.2. No significant association was found between clinical outcome and age group (p=0.316; Chi-Square test).

Disease-free survival was the time between the diagnosis and confirmation of the DF event. Kaplan-Meier survival analysis method showed that overall disease-free survival rates for the total PMDs cohort at 1-year, 2, 3, 5 and 10-years were 88%, 75%, 68%, 47% and 42% postoperatively; Figure 5.8.

Table 5.9 summarises the clinicopathological characteristics of the 62 DF patients.

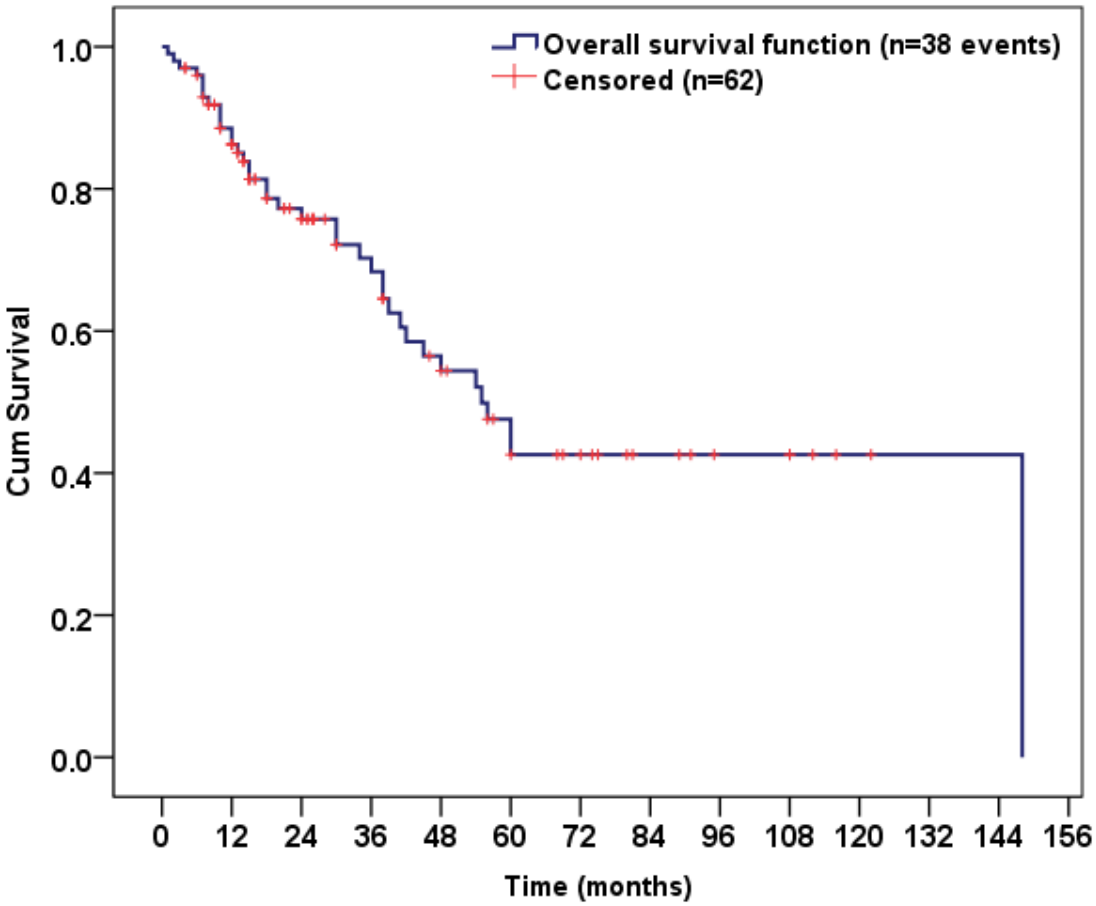

Figure 5.8: Overall disease-free survival curve by Kaplan-Meier analysis.

Table 5.9: Clinicopathological features of 62 disease-free patients.

Case	Age	Sex	Site	Clinical types	Hpath WHO	Hpath Binary	Size (mm^2)	Resection Margins	Smoking (cig./day)	Drinking (u/w)	Follow-up (months)	Medical conditions
1	36	F	FOM	Non-homogenous speckled	Md	LG	<200	Normal	Ex-smoker	1-14	15	Hypertension, DM II
2	62	M	FOM	Homogenous leukoplakia	Modd	HG	200-600	Modd	>20	>28	74	Hypertension, DM
3	33	F	FOM	Homogenous leukoplakia	Md	LG	<200	Md	10-20	1-14	4	
4	66	F	FOM	Homogenous leukoplakia	Modd	HG	<200	Normal	>20	1-14	21	Hypertension
5	52	M	FOM	Homogenous leukoplakia	CIS	HG	>600	Normal	>20	>28	48	Hypertension, DM
6	59	F	FOM	Homogenous leukoplakia	Md	LG	<200	Normal	10-20	1-14	16	
7	67	M	FOM	Non-homogenous speckled	Modd	HG	200-600	Normal	Ex-smoker	1-14	6	
8	35	M	FOM	Homogenous leukoplakia	Md	LG	200-600	Md	10-20	>28	81	
9	42	M	FOM	Homogenous leukoplakia	Modd	HG	200-600	Md	>20	>28	116	Hypertension
10	59	M	FOM	Homogenous leukoplakia	CIS	HG	200-600	Md	10-20	>28	12	Hypertension
11	64	F	FOM	Homogenous leukoplakia	Md	LG	200-600	Md	10-20	1-14	14	Hypertension, DM II
12	48	M	FOM	Homogenous leukoplakia	Md	LG	<200		10-20	1-14	122	
13	54	F	FOM	Homogenous leukoplakia	Md	LG	<200	Md	>20	Non-drinker	15	Hypertension, DM II
14	48	F	FOM	Homogenous leukoplakia	Md	LG	200-600		10-20	1-14	68	
15	45	F	FOM	Homogenous leukoplakia	Md	LG	200-600	Md	10-20	1-14	30	Hypertension, DM
16	56	F	FOM	Homogenous leukoplakia	Md	LG	<200	Normal	Ex-smoker	Non-drinker	89	
17	64	M	FOM	Homogenous leukoplakia	Md	LG	<200	Normal	10-20	1-14	26	Hypertension
18	71	F	FOM	Homogenous leukoplakia	Md	LG	200-600		Ex-smoker	Non-drinker	56	
19	55	F	FOM	Homogenous leukoplakia	Md	LG	<200	Normal	10-20	Non-drinker	18	Hypertension
20	49	M	FOM	Homogenous leukoplakia	Md	LG	<200	Normal	>20	Ex-drinker	8	Hypertension

F=female; M=males; FOM=Floor of the mouth; Hpath=histopathology; Md=mild dysplasia; Modd=moderate dysplasia; Sd=severe dysplasia; CIS=carcinoma *in situ*; LG=low grade dysphasia; HG=high grade dysphasia; DM II=diabetes mellitus type 2, DM=diabetes mellitus.

Continued

Case	Age	Sex	Site	Clinical types	Hpath WHO	Hpath Binary	Size (mm^2)	Margin	Smoking (Cig./day)	Drinking (U/W)	Follow-up (months)	Medical conditions
21	40	F	FOM	Homogenous leukoplakia	Md	LG	<200	Md	10-20	1-14	112	
22	71	M	FOM	Erythroplakia	CIS	HG	<200	CIS	Ex-smoker	>28	13	Hypertension
23	37	M	FOM	Homogenous leukoplakia	Md	LG	<200	Normal	>20	>28	12	
24	45	M	FOM	Homogenous leukoplakia	Md	LG	<200	Md	10-20	15-28	108	
25	30	M	FOM	Homogenous leukoplakia	Md	LG	<200	Normal	10-20	>28	95	
26	58	M	FOM	Non-homogenous speckled	Sd	HG	200-600		10-20	>28	38	
27	65	M	FOM	Homogenous leukoplakia	Modd	LG	200-600	Md	10-20	15-28	60	Anaemia
28	58	F	FOM	Homogenous leukoplakia	Modd	HG	<200	Md	>20	1-14	72	Hypertension
29	71	M	FOM	Homogenous leukoplakia	Mdd	LG	<200	Modd	>20	1-14	80	Hypertension, anaemia
30	55	F	FOM	Homogenous leukoplakia	Sd	HG	<200	Md	10-20	1-14	108	Hypertension, DM II, anaemia
31	61	M	FOM	Homogenous leukoplakia	Md	LG	<200	Normal	Ex-smoker	>28	14	Hypertension
32	58	F	FOM	Homogenous leukoplakia	Modd	HG	<200	Md	10-20	Non-drinker	24	Hypertension
33	65	M	FOM	Homogenous leukoplakia	Md	LG	<200	Normal	10-20	>28	28	Hypertension
34	57	M	FOM	Homogenous leukoplakia	Md	LG	<200	Mild	Ex-smoker	Ex-drinker	26	Hypertension
35	42	M	FOM	Homogenous leukoplakia	Md	LG	200-600	Normal	>20	1-14	26	
36	42	F	Lateral tongue	Homogenous leukoplakia	Md	LG	<200	Normal	Non-smoker	1-14	15	
37	75	M	Lateral tongue	Homogenous leukoplakia	Md	LG	<200	Md	Ex-smoker	1-14	25	Hypertension
38	69	M	Lateral tongue	Erythroplakia	Modd	HG	200-600	Modd	Ex-smoker	1-14	21	Hypertension
39	94	F	Lateral tongue	Non-homogenous speckled	Md	LG	200-600	Normal	Non-smoker	Non-drinker	30	Hypertension
40	50	M	Lateral tongue	Homogenous leukoplakia	Modd	HG	200-600	Normal	10-20	1-14	10	
41	69	M	Lateral tongue	Homogenous leukoplakia	Sd	HG	>600	Normal	Non-smoker	1-14	8	Hypertension, DM

Continued

Case	Age	Sex	Site	Clinical types	Hpath WHO	Hpath Binary	Size (mm^2)	Margin	Smoking (Cig./day)	Drinking (U/W)	Follow-up (months)	Medical conditions
42	54	F	Lateral tongue	Homogenous leukoplakia	Md	LG	200-600	Sd	Non-smoker	1-14	49	
43	69	F	Lateral tongue	Homogenous leukoplakia	Modd	HG	>600	Modd	Non-smoker	1-14	75	Hypertension
44	51	F	Lateral tongue	Non-homogenous speckled	Modd	HG	200-600	Normal	>20	15-28	4	Hypertension
45	47	M	Lateral tongue	Homogenous leukoplakia	Md	LG	200-600	Normal	Non-smoker	Non-drinker	16	
46	68	F	Lateral tongue	Erythroplakia	Sd	HG	200-600	Normal	Ex-smoker	1-14	25	
47	79	F	Ventral tongue	Homogenous leukoplakia	Modd	HG	<200	Normal	Ex-smoker	1-14	26	DM II
48	76	M	Ventral tongue	Non-homogenous/nodular	Sd	HG	>600	Normal	Non-smoker	1-14	12	
49	51	M	Ventral tongue	Homogenous leukoplakia	Md	LG	200-600	Normal	10-20	1-14	24	Hypertension
50	60	M	Ventral tongue	Homogenous leukoplakia	Sd	HG	<200	Normal	>20	15-28	38	Hypertension, Oral Candida
51	77	M	Ventral tongue	Homogenous leukoplakia	Modd	HG	<200	Normal	10-20	1-14	9	
52	47	M	Soft palate	Erythroplakia	Modd	HG	<200	Sd	>20	>28	9	
53	39	M	Soft palate	Homogenous leukoplakia	Modd	HG	<200	Modd	10-20	>28	46	
54	70	M	Soft palate	Homogenous leukoplakia	Modd	LG	<200	Normal	Ex-smoker	Non-drinker	18	Hypertension
55	47	M	Soft palate	Homogenous leukoplakia	Md	LG	<200	Normal	10-20	>28	57	Hypertension, DM
56	46	M	Soft palate	Non-homogenous speckled	Sd	HG	<200	Sd	10-20	Ex-drinker	10	Hypertension
57	56	M	Fauces	Non-homogenous speckled	Modd	HG	<200	Modd	10-20	>28	13	
58	58	M	Fauces	Homogenous leukoplakia	CIS	HG	<200	Sd	10-20	>28	7	Hypertension
59	81	M	Alveolar mucosa	Non-homogenous exophytic	Modd	LG	<200	Normal	Ex-smoker	1-14	91	DM II
60	79	M	Alveolar mucosa	Homogenous leukoplakia	Md	LG	Vaporization	Normal	Ex-smoker	15-28	22	
61	81	M	Buccal mucosa	Non-homogenous speckled	Sd	HG	200-600	Modd	Non-smoker	1-14	9	Hypertension
62	37	M	Buccal mucosa	Non-homogenous nodular	Md	LG	200-600	Md	10-20	15-28	69	Oral Candida

Clinical Appearance

Clinically, 93% (58/62) of PMDs seen in DF patients were leukoplakias, whilst the remaining 7% (4/62) were erythroplakias. Of the leukoplakia cases, 70% (47/67) seen in DF patients were homogenous leukoplakia compared to only 44% (11/25) of non-homogenous leukoplakias; Table 5.10.

Considering non-homogenous categories, speckled was the main reported subtype in the DF group 73% (8/11), followed by nodular 18% (2/11) and exophytic 9% (1/11); Table 5.11 and Figure 5.9.

The relation between the clinical appearance of PMDs and treatment outcomes was found to be significant (p=0.042; Chi-Square test).

In this study, homogenous leukoplakia was predominant in all clinical outcome groups. While 50% (4/8) of erythroplakias, 50% (8/16) of speckled leukoplakias, and all nodular subtypes were reported as DF at the most recent patient follow-up, the majority of exophytic non-homogenous leukoplakias exhibited recurrent disease 60% (3/5).

Table 5.10: Type of leukoplakia in relation to clinical outcomes.

Leukoplakia type	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
Homogenous	**47** *70%*	**10** *15%*	**7** *10%*	**2** *3%*	**1** *1%*	**67** *100%*
Non-homogenous	**11** *44%*	**6** *24%*	**6** *24%*	**2** *8%*	**-**	**25** *100%*
Total	**58** *63%*	**16** *17%*	**13** *14%*	**4** *4%*	**1** *1%*	**92** *100%*

Table 5.11: Clinical outcome in relation to clinical appearance of PMDs.

Clinical type of PMDs		Clinical outcome					Total
		DF	Recurrent disease	Further disease	MT	OSCC	
Homogenous leukoplakia		**47** *70%*	**10** *15%*	**7** *10%*	**2** *3%*	**1** *1%*	**67** *100%*
Non-homogenous leukoplakia	Speckled	**8** *50%*	**2** *13%*	**4** *25%*	**2** *13%*	**-**	**16** *100%*
	Nodular	**2** *100%*	**-**	**-**	**-**	**-**	**2** *100%*
	Exophytic	**1** *20%*	**3** *60%*	**1** *20%*	**-**	**-**	**5** *100%*
	Ulcerated	**-**	**1** *50%*	**1** *50%*	**-**	**-**	**2** *100%*
Erythroplakia		**4** *50%*	**1** *13%*	**1** *13%*	**1** *13%*	**1** *13%*	**8** *100%*
Total		**62**	**17**	**14**	**5**	**2**	**100**

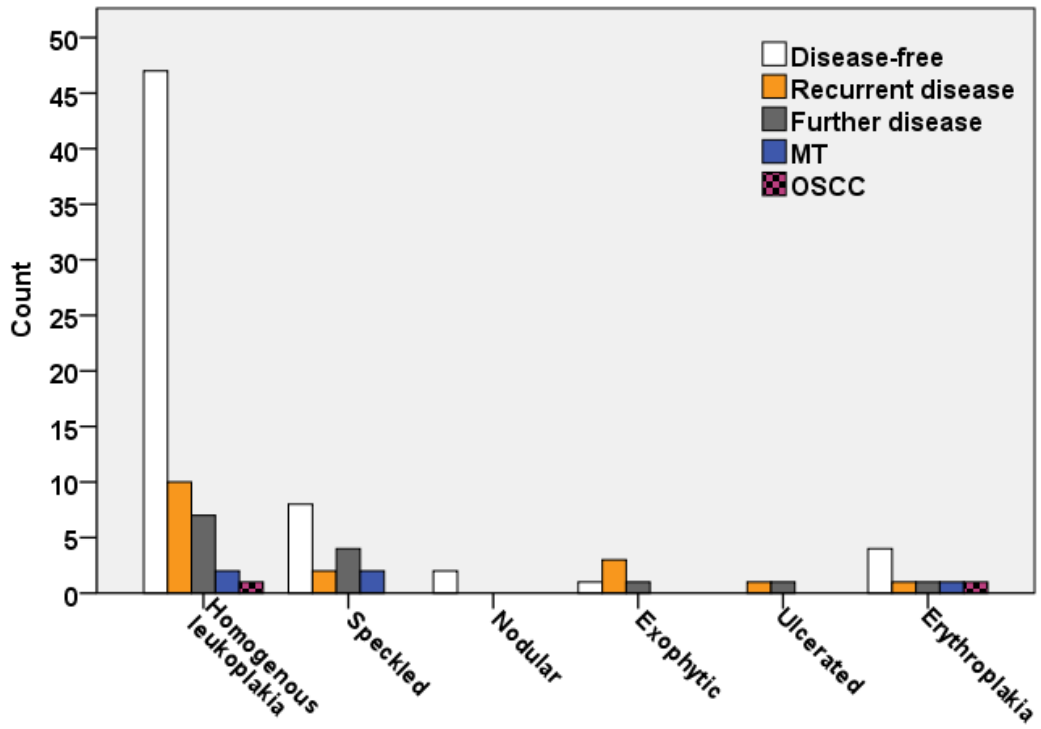

Figure 5.9: Clinical outcome in relation to clinical appearance of PMDs.

Anatomical Site

Seventy-six percent (35/46) of patients with FOM dysplasia were reported as DF, followed by 58% (11/19) with lateral tongue, 56% (5/9) soft palate, 50% (2/4) faucial of pillars, 40% (2/5) buccal mucosa and 36% (5/14) with ventral tongue.
All cases of alveolar mucosa 100% (2/2) were DF at the most recent follow-up of patients; Table 5.12 and Figure 5.10.

The relation between clinical outcome and anatomical site of dysplastic PMDs was significant (p=0.020; Chi-Square test).
The majority of both DF (57%; 35/62) and recurrent cases (29%; 5/17) were observed in the FOM. Both FOM and ventral tongue were equally affected by further disease formation (29%; 4/14). In this study, the case of retromolar area was affected with MT and OSCC developments distant from the primary dysplasia (2/2) were only seen in the tongue.

Size of Dysplasia

The size of dysplasia in DF patients ranged from 36 to 1,040 mm^2 with a mean size of 251.22 mm^2. 74% (31/42) of minor sized cases were DF, followed by 56% (25/45) intermediate and 40% (4/10) major size.

Table 5.13 and Figure 5.11 summarise the clinical outcome in relation to PMD size categories.

A significant relation was found between clinical outcome and size (p=0.010; Chi-Square test).
DF (52%; 31/60) and recurrent cases (44%; 7/16) were mainly minor sized dysplasia, whilst both patients with MT (80%; 4/5) and those developing new site dysplasias (64%; 9/14) were mainly intermediate size. OSCC development distant from the primary dysplasia was only seen in intermediate or major sized dysplasias (100%; 2/2).

Table 5.12: Clinical outcome in relation to PMD anatomical site.

Anatomical site	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
FOM	35 *76%*	5 *11%*	4 *9%*	2 *4%*	-	46 *100%*
Lateral tongue	11 *58%*	4 *21%*	1 *5%*	2 *11%*	1 *5%*	19 *100%*
Ventral tongue	5 *36%*	4 *29%*	4 *29%*	-	1 *7%*	14 *100%*
Buccal mucosa	2 *40%*	1 *20%*	2 *40%*	-	-	5 *100%*
Soft palate	5 *56%*	3 *33%*	1 *11%*	-	-	9 *100%*
Fauces	2 *50%*	-	2 *50%*	-	-	4 *100%*
Retromolar area	-	-	-	1	-	1 *100%*
Alveolar mucosa	2	-	-	-	-	2 *100%*
Total	62	17	14	5	2	100

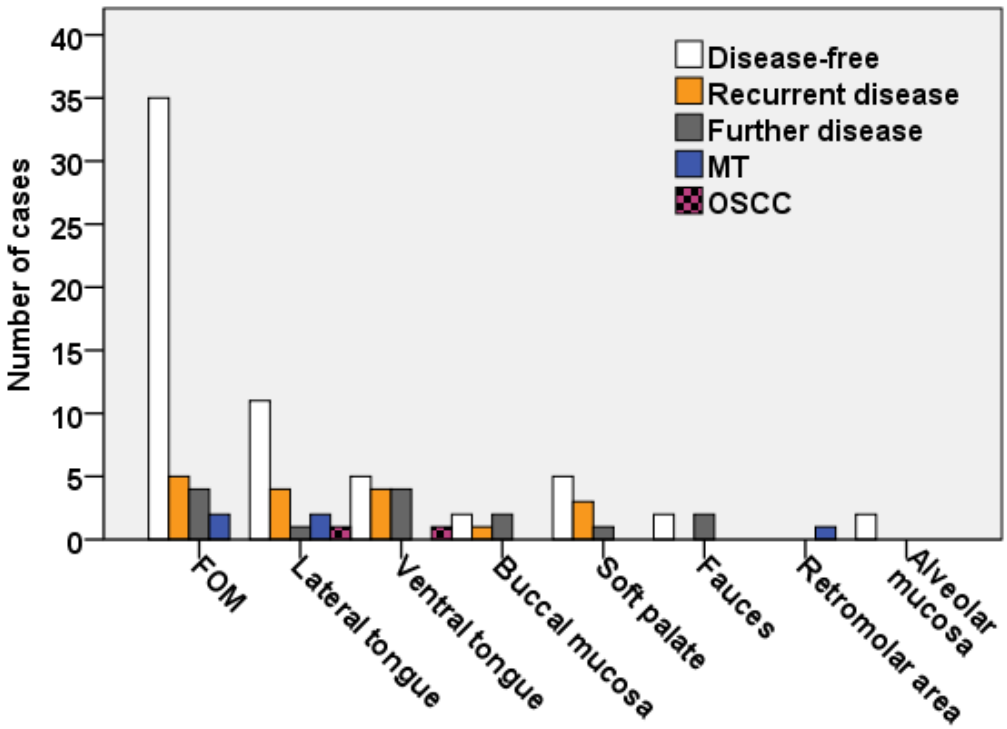

Figure 5.10: Clinical outcome in relation to PMD anatomical site.

Table 5.13: Clinical outcome in relation to PMD size (mm^2).

PMD size category (mm^2)	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
Minor < 200	**31** *74%*	**7** *17%*	**3** *7%*	**1** *2%*	**-**	**42** *100%*
Intermediate (200-600)	**25** *56%*	**6** *13%*	**9** *20%*	**4** *9%*	**1** *2%*	**45** *100%*
Major > 600	**4** *40%*	**3** *30%*	**2** *20%*	**-**	**1** *10%*	**10** *100%*
Total	**60** *62%*	**16** *16%*	**14** *14%*	**5** *5%*	**2** *2%*	**97** *100%*

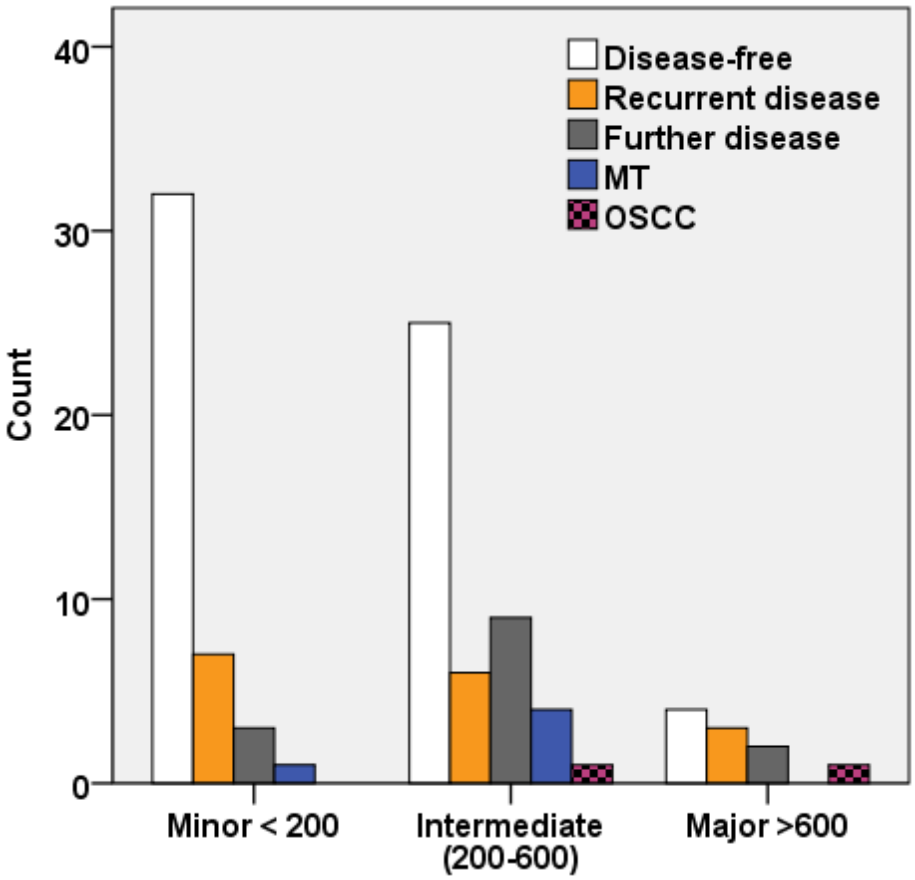

Figure 5.11: Clinical outcome in relation to PMD size category (mm^2).

Dysplasia Grading

Considering the histopathological diagnosis of laser excised tissue, the majority of mild dysplasia 76% (32/42) was seen in DF patients, followed by 71% (17/24) of moderate, 39% (9/23) of severe dysplasia and 36% (4/11) of CIS; Table 5.14 and Figure 5.12.

A significant relation was found between the degree of dysplasia and clinical outcome (p=0.003; Chi-Square test). DF (32/62) and further disease (6/14) were mainly reported in patients affected with mild dysplasia. Recurrent cases were predominantly and equally observed in moderate-severe dysplasia (6/17, for each). MT was equally seen in mild and severe dysplasia cases (2/4, for each), with one case of CIS, whilst developing OSCC (new site) was only reported in severe dysplasia-CIS (2/2).

Similarly, a significant relation was found between clinical outcome and high/low grade dysplasia (p=0.004; Chi-Square test). The majority of low grade dysplasias were seen in DF patients, compared to the high grade dysplasias (74%; 35/47 vs. 50%; 27/53), respectively. In contrast, high grade dysplasias were predominantly observed in cases which underwent recurrences, further dysplasia and MT, with OSCCs (new-site) were only reported in patients affected with high grade dysplasia; Table 5.15 and Figure 5.13.

Table 5.14: Clinical outcome in relation to degree of dysplasia.

Dysplasia WHO system	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
Mild	32 *76%*	2 *5%*	6 *14%*	2 *5%*	-	42 *100%*
Moderate	17 *71%*	6 *25%*	1 *4%*	-	-	24 *100%*
Severe	9 *39%*	6 *26%*	5 *22%*	2 *9%*	1 *4%*	23 *100%*
CIS	4 *36%*	3 *27%*	2 *18%*	1 *9%*	1 *9%*	11 *100%*
Total	62	17	14	5	2	100 *100%*

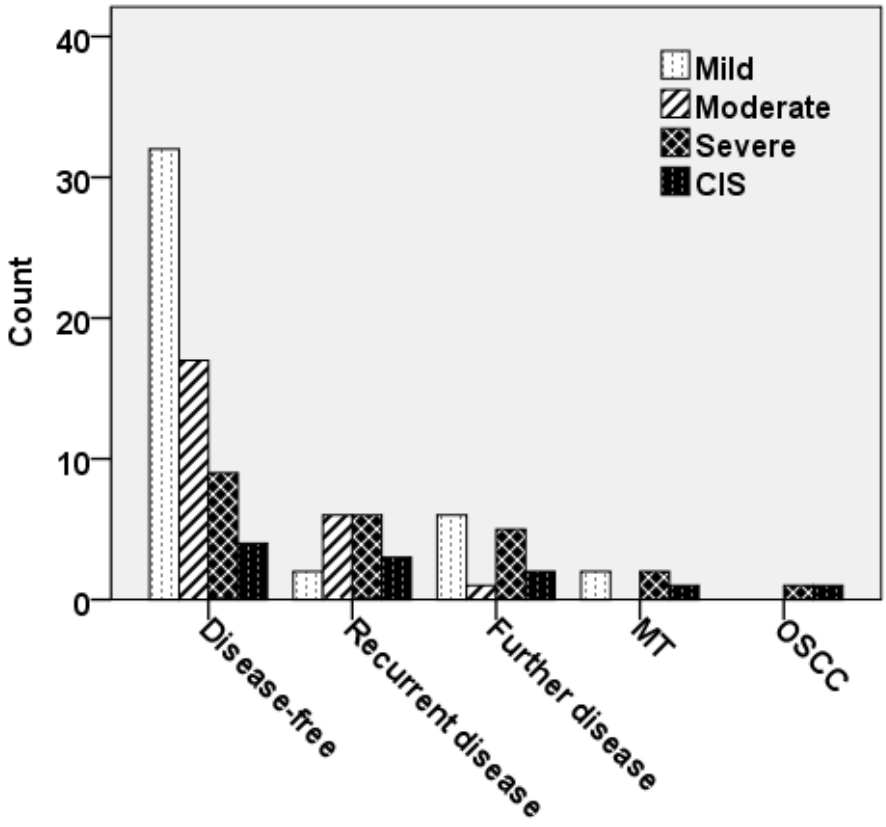

Figure 5.12: Grade of epithelial dysplasia in relation to clinical outcome.

Table 5.15: Clinical outcome in relation to binary grading system.

Dysplasia Binary grading	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
High grade	**27** *50%*	**13** *25%*	**8** *15%*	**3** *6%*	**2** *4%*	**53** *100%*
Low grade	**35** *74%*	**4** *9%*	**6** *13%*	**2** *4%*	**-**	**47** *100%*
Total	**62** *63%*	**17** *16%*	**14** *14%*	**5** *5%*	**2** *2%*	**100** *100%*

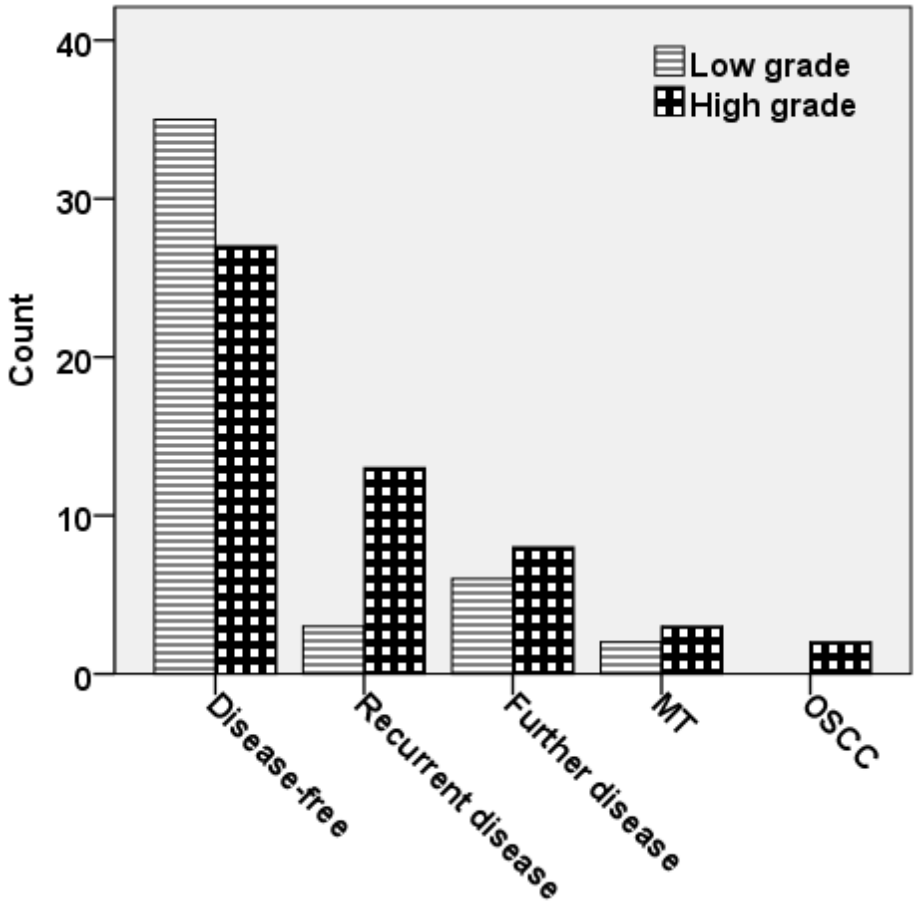

Figure 5.13: High/low grade dysplasia in relation to clinical outcome.

Smoking Behaviour

With respect to smoking behaviour, the majority of DF patients were current smokers 64% (40/62), followed by ex-smokers 21% (13/62) and non-smokers 15% (9/62).
DF current tobacco smokers were mainly intermediate smokers who smoked between 10-20 cigarettes/day (43%; 27/62), followed by heavy smokers who smoked more than 20 cigarettes/day (21%; 13/62), however no light smokers were seen in this group; Table 5.16 and Figure 5.14.

A significant relation was found between smoking status and clinical outcome (p=0.014; Chi-Square test). All OSCC patients 100% (2/2) were non-smokers. Seventy-one percent of patients who developed further disease (10/14), 59% (10/17) of patients with recurrence and 56% (40/62) of DF patients were reported as current tobacco smokers.

With respect to the extent of smoking, Chi-Square test showed no significant relation between clinical outcome and the number of cigarettes smoked per day (p=0.139).

Regarding smoking history, no significant relation was found between patients' smoking history in term of years as a smoker and clinical outcome (p=0.565; Chi-Square test).
Fifty-nine percent of DF patients had a smoking history of 31-50 years, followed by 36% who smoked for 10-30 years, with only one case in our patient series who smoked for more than 50 years and this patient was DF at the most recent follow-up; Table 5.17 and Figure 5.15.

The current smoking behaviour of patients who were DF showed a significant change at the most recent follow-up (p=0.0001; Sign test). Ex-smokers increased from 13 (21%) to 32 (52%), current smokers decreased from 40 (65%) to 21 (34%), light smokers increased from 0 to 12 cases, intermediate smokers decreased from 27 to 8 cases and heavy smokers decreased dramatically from 13 cases to only one case at the most recent follow-up time; Figure 5.16.

Table 5.16: Clinical outcome in relation to smoking behaviour.

Smoking behaviour		Clinical outcome					Total
		DF	Recurrent disease	Further disease	MT	OSCC	
Non-smoker		9 *15%*	2 *12%*	1 *7%*	1 *20%*	2 *100%*	15
Current smoker (cigarettes/day)	< 10	-	1 *6%*	1 *7%*	-	-	2
	10-20	27 *43%*	4 *24%*	5 *36%*	2 *40%*	-	38
	> 20	13 *21%*	5 *29%*	4 *29%*	1 *20%*	-	23
Ex-smoker		13 *21%*	5 *29%*	3 *21%*	1 *20%*	-	22
Total		62 *100%*	17 *100%*	14 *100%*	5 *100%*	2 *100%*	100

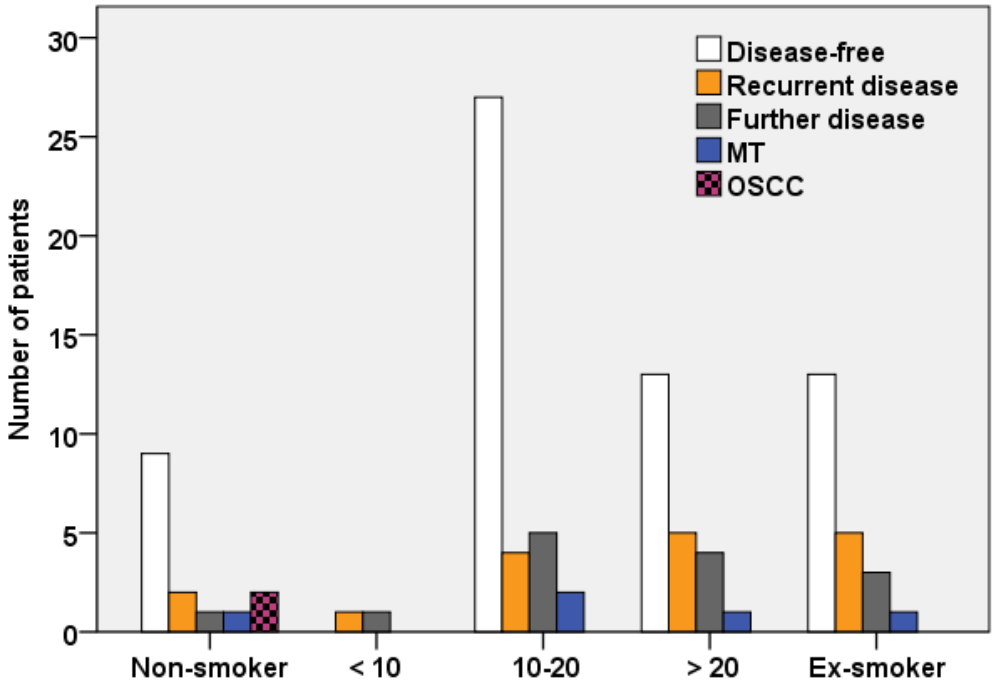

Figure 5.14: Clinical outcome in relation to smoking behaviour.

Table 5.17: Clinical outcome in relation to smoking history.

Smoking history (years)	Clinical outcome				Total
	DF	Recurrent disease	Further disease	MT	
10-30	**8** *36%*	**3** *50%*	**2** *33%*	**1** *50%*	**14** *39%*
31-50	**13** *59%*	**3** *50%*	**4** *67%*	**1** *50%*	**21** *58%*
> 50	**1** *5%*	-	-	-	**1** *3%*
Total	**22** *100%*	**6** *100%*	**6** *100%*	**2** *100%*	**36** *100%*

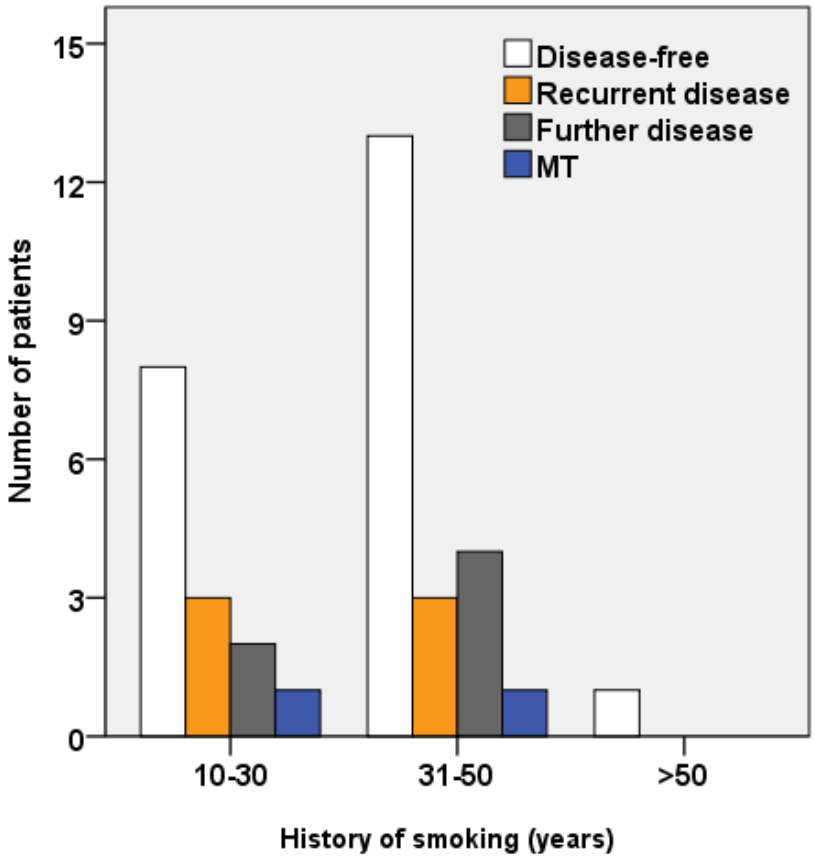

Figure 5.15: Clinical outcome in relation to smoking history.

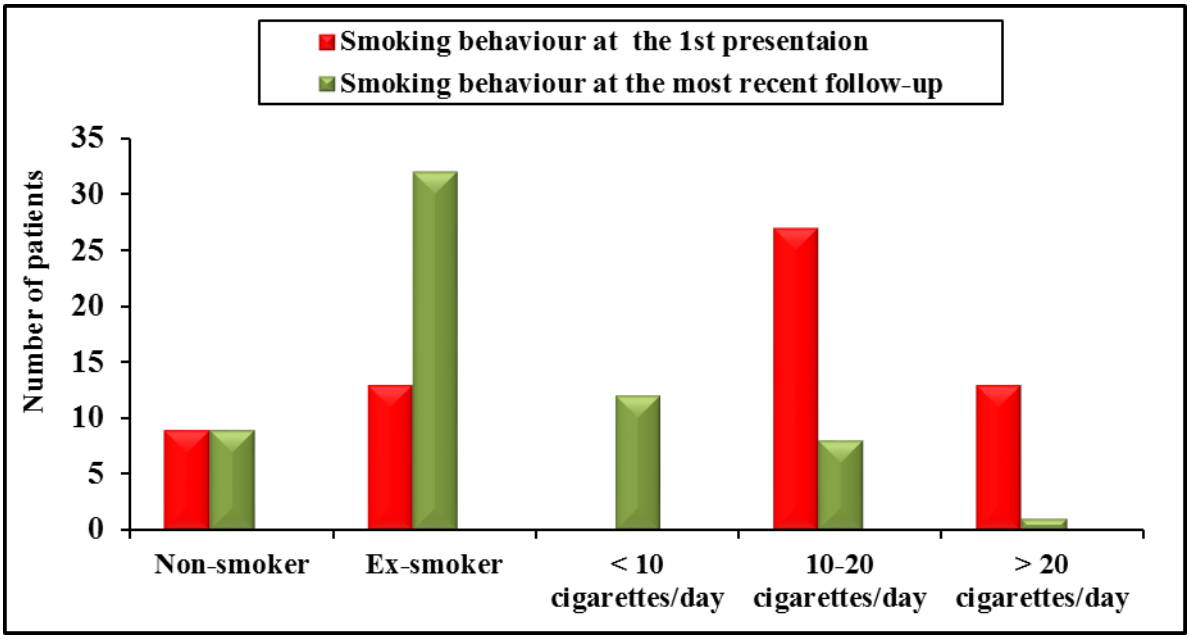

Figure 5.16: Comparison between smoking status at initial presentation and at most recent follow-up for 62 DF patients (*p*=0.0001; Sign test).

Alcohol Use

Table 5.18 and Figure 5.17 show the association between alcohol drinking and clinical outcome. DF cases were predominant in all groups of drinking behaviour.
The majority of DF patients were current alcohol drinkers 83% (51/62), followed by non-drinkers 13% (8/62) and ex-drinkers 4% (3/62).
Of the current drinkers, the highest rate of DF was reported in the light drinkers 44% (27/62), followed by heavy 26% (16/62) and intermediate drinkers 13% (8/62), whilst all ex-drinkers 100% (3/3) were DF at the most recent follow-up.

Using Chi-Square test, no significant relation was found between clinical outcomes and drinking status (p=0.080); however, a significant association was found with the amount of alcohol consumed in term of units per week (p=0.013). The majority of DF patients (44%; 27/62) and 100% (2/2) of patients who developed OSSC at distant site were light drinkers. The majority of patients who underwent recurrences (53%; 9/17), further disease (50%; 7/14) and those who underwent MT (40%; 2/5) were all reported as heavy drinkers.

At the most recent follow-up appointment, alcohol drinking behaviour showed no significant change (p=0.161; Sign test). Whilst non-drinkers and ex-drinkers remained the same, heavy drinkers decreased from 16 to 11 cases, intermediate drinkers increased from 6 to 10 and light drinkers increased from 29 to 30 cases; Figure 5.18.

Dental Prosthesis Wear

Regarding the relationship between clinical outcome and the presence of a dental prosthesis or not, the majority of non-wearers 70% (33/47) were DF compared to 55% (29/53) of wearers who were DF; Table 5.19 and Figure 5.19.

Using Chi-Square test, a significant relation was found between the presence of a dental prosthesis and clinical outcome (p=0.004). 100% (5/5) of patients underwent MT and 71% (12/17) of those who had recurrent dysplasia were dental prosthesis wearers. In contrast, 57% (8/14) of patients who developed further dysplasia and 53% (33/62) of DF patients did not wear dental prostheses.

Table 5.18: Clinical outcome in relation to alcohol drinking behaviour.

Drinking behaviour		Clinical outcome					Total
		DF	Recurrent disease	Further disease	MT	OSCC	
Non-drinker		**8** *13%*	**3** *18%*	**1** *7%*	**2** *40%*	-	**14**
Current drinker (units/week)	**1-14**	**27** *44%*	**3** *18%*	**4** *29%*	**1** *20%*	**2**	**37**
	15-28	**8** *13%*	**2** *11%*	**2** *14%*	-	-	**12**
	> 28	16 *26%*	9 *53%*	7 *50%*	2 *40%*	-	34
Ex-drinker		**3** *4%*	-	-	-	-	**3**
Total		**62** *100%*	**17** *100%*	**14** *100%*	**5** *100%*	**2** *100%*	**100**

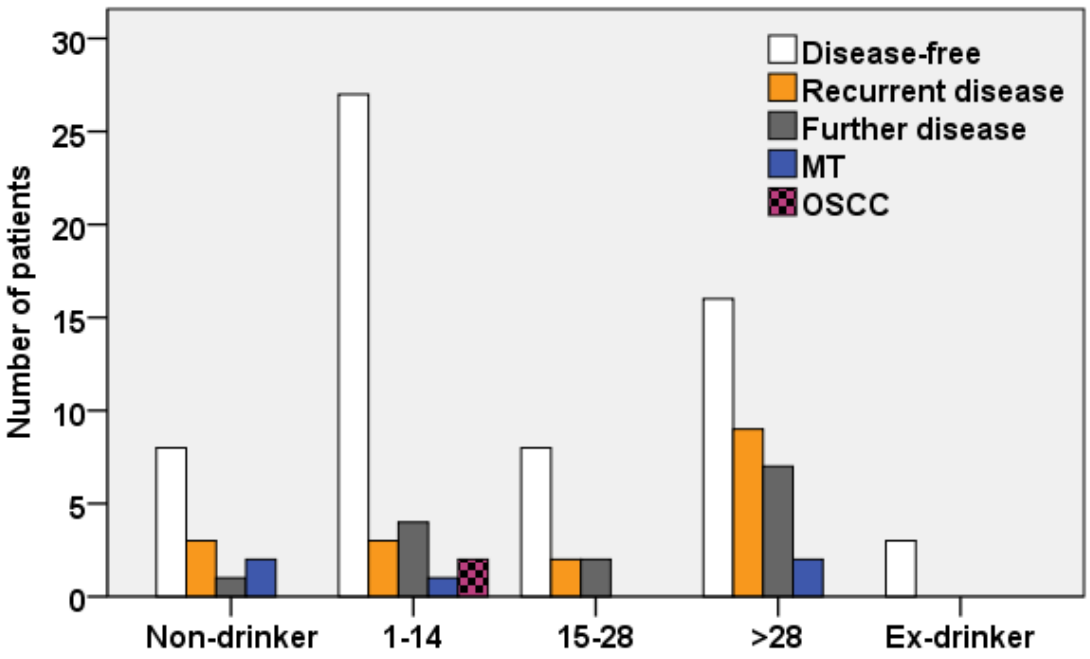

Figure 5.17: Clinical outcome in relation to alcohol drinking behaviour.

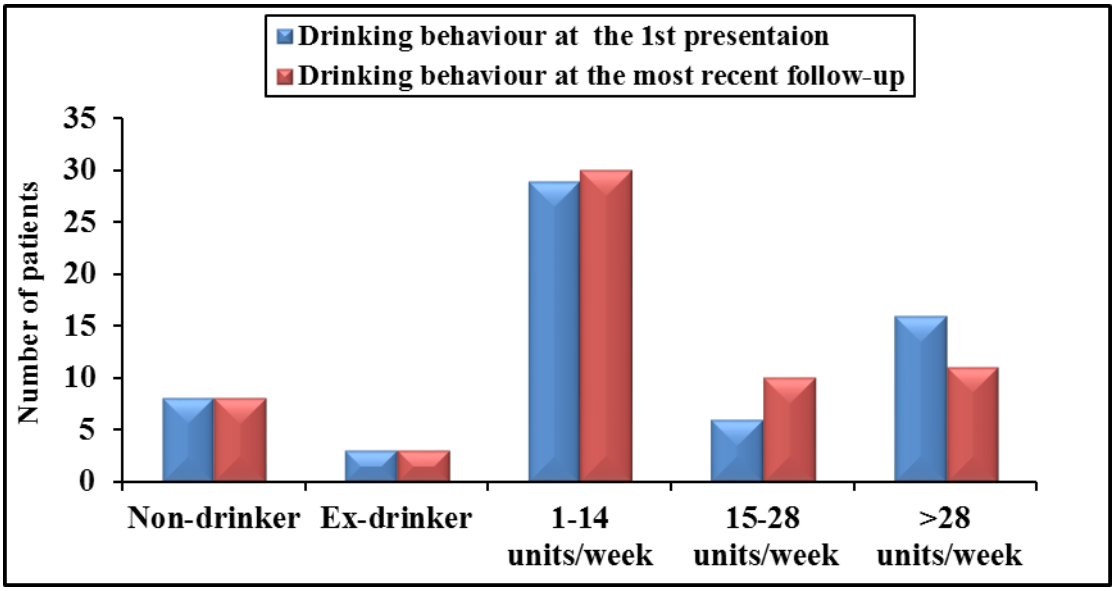

Figure 5.18: Comparison between alcohol drinking status at initial presentation and at most recent follow-up for 62 DF patients (p=0.161; Sign test).

Table 5.19: Clinical outcome in relation to dental prostheses wear.

Dental prosthesis	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
Wearer	**29** *55%*	**12** *23%*	**6** *11%*	**5** *9%*	**1** *2%*	**53** *100%*
Non-wearer	**33** *70%*	**5** *11%*	**8** *17%*	**-**	**1** *2%*	**47** *100%*
Total	**62**	**17**	**14**	**5**	**2**	**100**

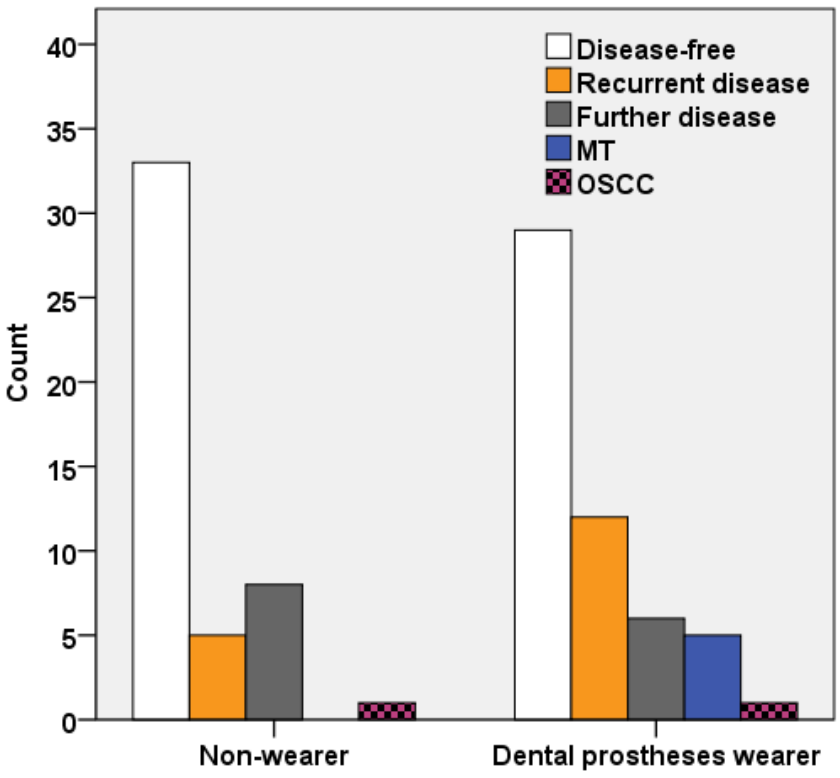

Figure 5.19: Clinical outcome in relation to dental prostheses wear.

Disease Active (DA)

This group involves all patients with unfavourable events following laser treatment including recurrence, new site dysplasia, MT and OSCC development at sites distant from primary dysplasia.

Dysplasia Grading

The degree of dysplasia had a significant effect on patients developing an unfavourable outcome (DA). Severe dysplasia-CIS showed a significantly shorter mean time to develop active disease state compared with patients with moderate or mild dysplasia respectively (40 vs. 78.8, 87.83 months). Also, lower 2- and 5-year disease-free survival rates were seen for severe dysplasia-CIS than for moderate and mild dysplasia (63% vs. 76%, 85%) and (14% vs. 59%, 62%), respectively (p=0.006; Log-Rank test); Figure 5.20.

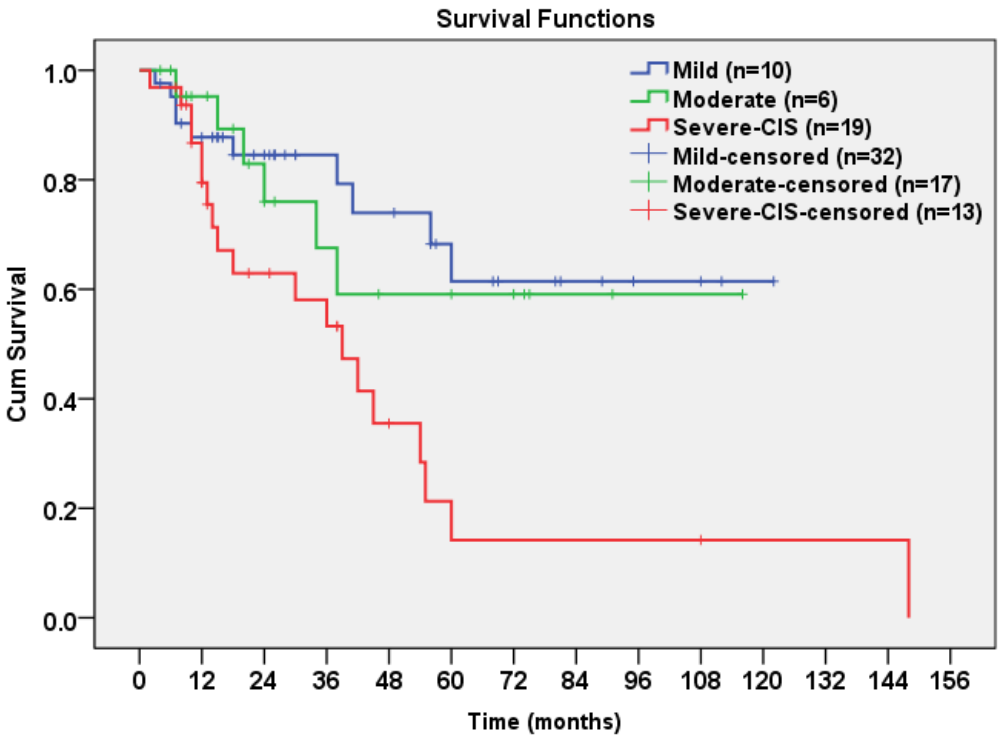

Figure 5.20: Disease-free survival according to the WHO grading of dysplasia (p=0.006; Log-Rank test).

With respect to binary grading of histopathological diagnosis, patients with high grade dysplasia had significantly a shorter mean time to develop an unfavourable outcome (DA), compared to patients with low grade dysplasia (64 vs. 88.7 months). Also, lower 2- and 5-year disease-free survival rates for high grade than low grade dysplasia was found (68% vs. 83%) and (29% vs. 63%), respectively (p=0.013; Log-Rank test); Figure 5.21.

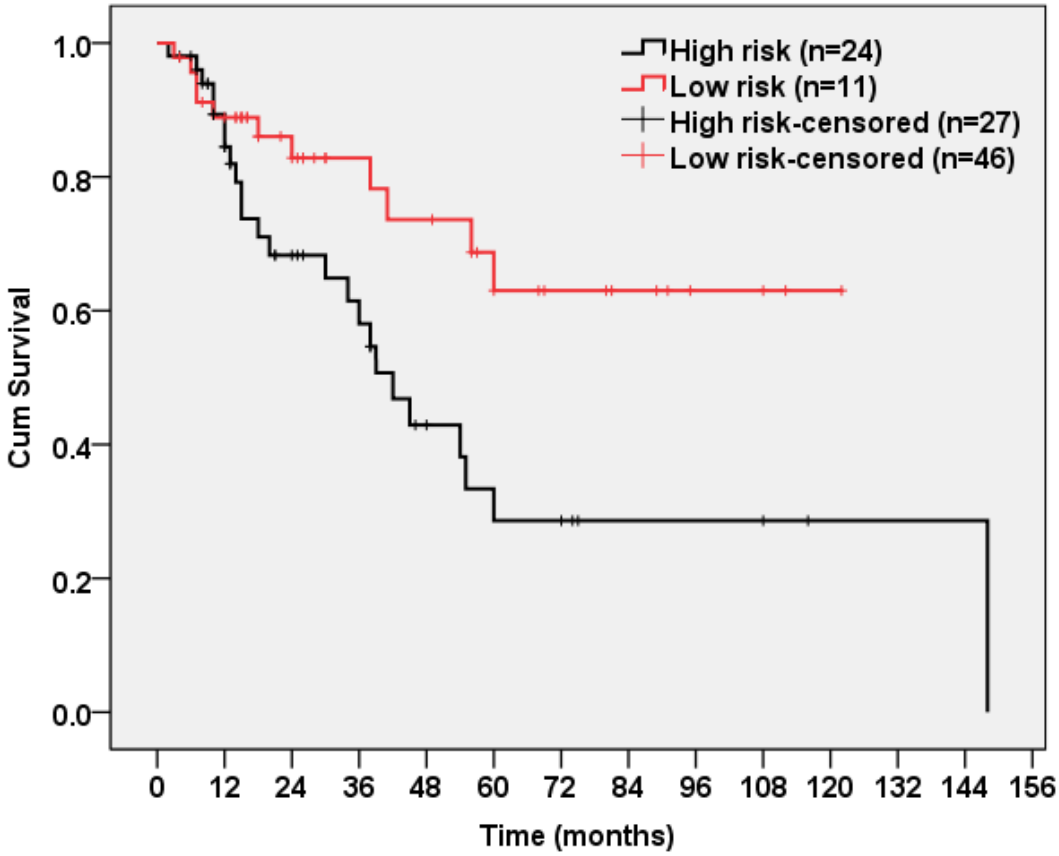

Figure 5.21: Disease-free survival according to high and low grade dysplasia (p=0.013; Log-Rank test).

Smoking Behaviour

Figure 5.22 shows the cumulative survival analysis of smoking status subgroups. Disease-free survival rate in relation to smoking status 1-year post laser surgery was 71% for ex-smokers, 86% for non-smokers and 91% for current smokers. The percentage dropped to 36% for ex-smokers at 4 years post laser treatment and to 49% for current and 18% for non-smokers at 5 years post laser treatment.

Survival analysis showed that ex-smokers presented the shortest estimated mean time to develop unfavourable outcome (DA) (49.4 months), followed by non-smokers (50.7 months), whilst current smokers exhibited the longest estimated mean time for DA state to develop (87.7 months), however, Log-Rank testing was not significant (p=0.511).

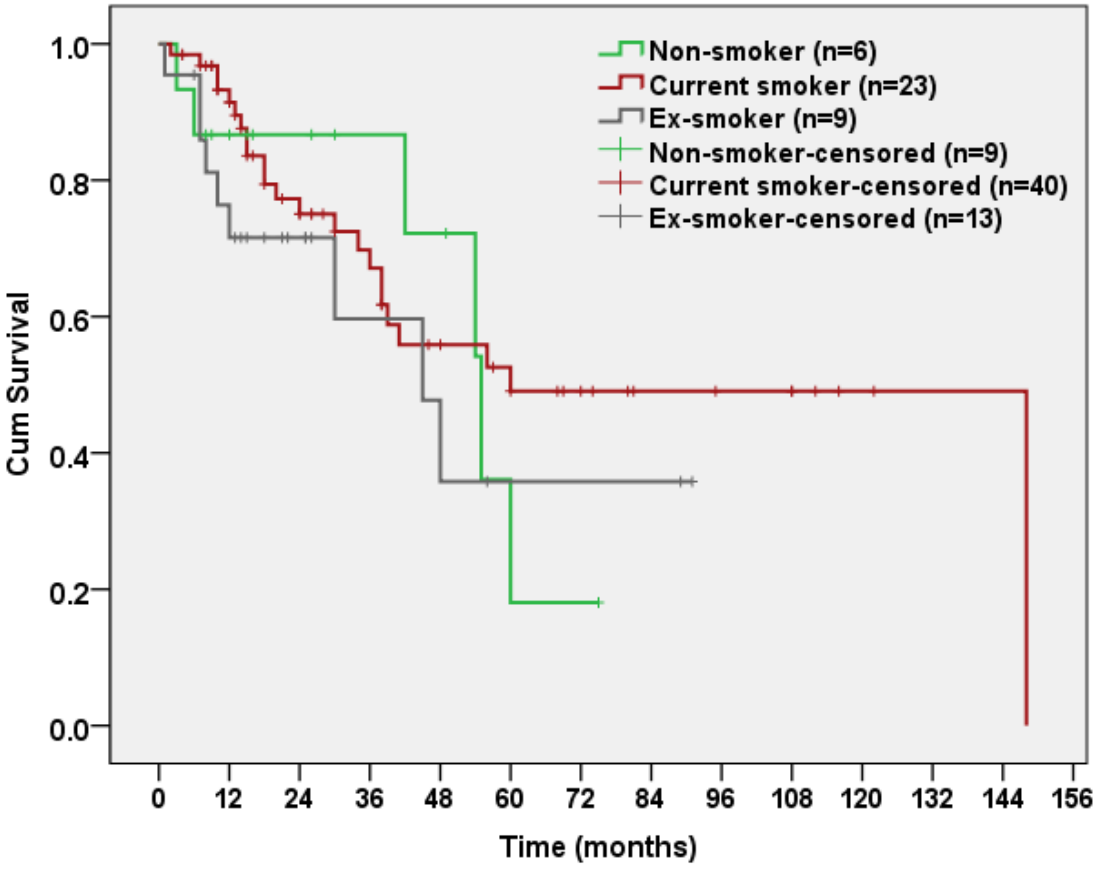

Figure 5.22: Disease-free survival in relation to smoking status.

Alcohol Use

Considering drinking behaviour, Figure 5.23 shows the Kaplan-Meier analysis for alcohol drinkers and non-drinkers.

Disease-free survival rate 1-year post laser surgery was 78% for non-drinkers and 89% for drinkers. The rate dropped to 49% and 55% for non-drinkers and drinkers at 4 years post laser treatment, and to 33% for non-drinker and 44% for drinkers at 5 years post laser surgery.

Non-drinkers of alcohol showed a shorter estimated mean time to develop unfavourable outcome (DA) (50.62 months) compared with alcohol drinkers (82.2 months), however, Log-Rank testing was not significant (p=0.552).

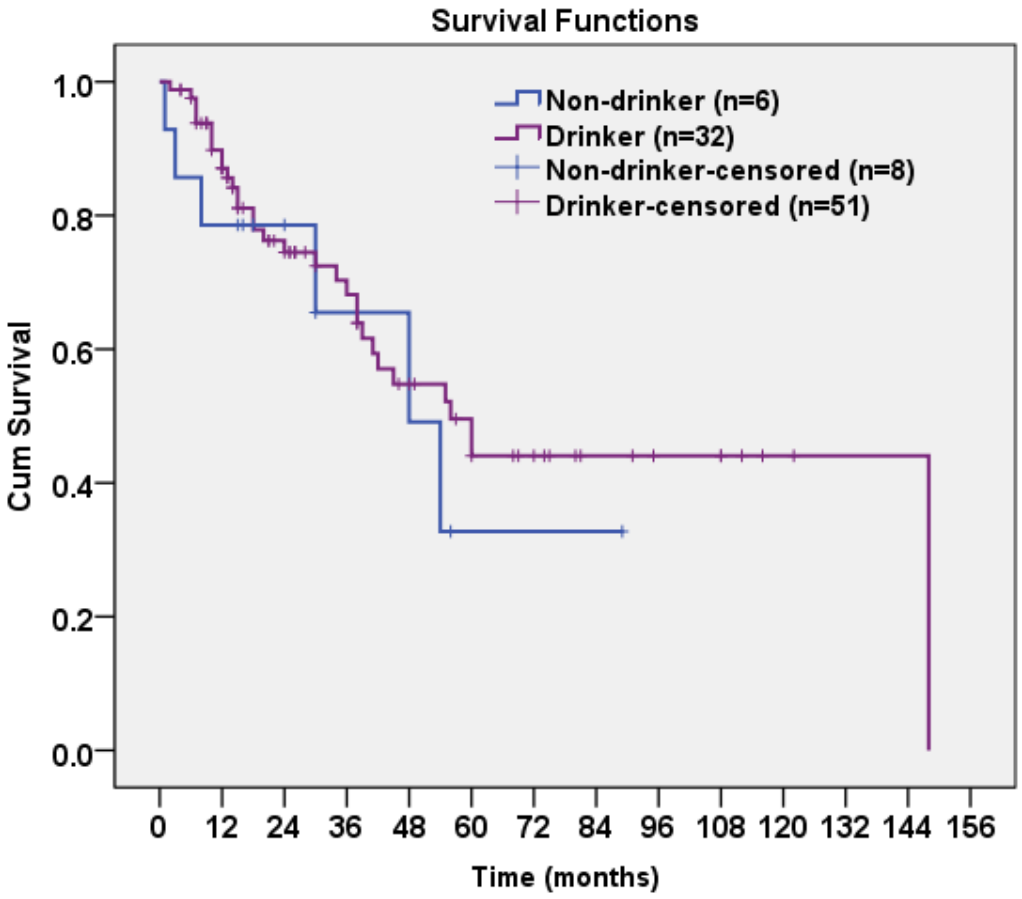

Figure 5.23: Disease-free survival in relation to alcohol drinking status.

Logistic Regression Analysis

Further statistical analysis was performed using logistic regression analysis to try to predict significant factors determining clinical outcome.

Age of patients, size of dysplasia, number of cigarettes/day, length of smoking history, pack score, tobacco grams/week and number of alcohol units/week were used as covariates. Sex, clinical appearance, anatomical site of PMD, wearing of dental prostheses, smoking status, drinking status and histopathological diagnosis (WHO and binary grading system) were entered as independent variables to try to predict the most important factor (s) determining treatment outcome.

For single factor analysis, age was entered as a continuous variable to perform a trend test.

Considering the anatomical site of dysplasia, oral subsites were stratified into 3 subgroups to facilitate the regression analysis: FOM, tongue and "other remaining sites".

The role of several independent factors for unfavourable outcome (DA) including recurrence, new site dysplasia, MT and OSCC development were estimated by means of univariate and multivariate logistic regression analysis.

Table 5.20 shows OR and 95% CI, with significant levels for single variables and multivariable final model.

Factors included in the univariate regression analysis were: age, sex, PMD clinical types, anatomical site of disease, histopathological diagnosis (WHO and binary grading system), resection margins status (dysplastic/clear), size of dysplasia and medical history (having systemic disease or not).

The analysis showed that patients' age showed no effect on disease active status (OR=1.007, 95% CI, 0.976-1.040) (p=0.646).

Males were at increased risk for disease active status being 1.448 times more likely to occur, compared with females (95% CI, 0.806-3.455), but this did not reach significance (p=0.405).

Non-homogenous leukoplakia was identified as a significant predictor for disease active status (p=0.023); this increased the risk by 2.991 times, compared with homogenous leukoplakia (95% CI, 1.160-7.713).

Logistic regression analysis showed that tongue, as an anatomical site of origin, was a significant predictor for disease active status to develop (p=0.013), increasing risk by 3.381-time compared to the FOM as a reference category (95% CI, 1.292-8.845).

The analysis showed that severe dysplasia was a highly significant predictor for DA status to develop (p=0.007); severe dysplasia showing 4.622 times the risk of mild dysplasia (95% CI, 1.527-13.990). CIS exhibited 4.8 times the risk of mild dysplasia for disease active (95% CI, 1.123-20.479) (p=0.034), however moderate dysplasia was a non-significant predictor for DA status to develop (OR=1.129) (95% CI, 0.350-3.641) (p=0.839).

Also, high grade dysplasia was found to be a significant predictor for the DA state (p=0.020), increasing the risk by 2.828 times compared with low grade dysplasia (95% CI, 1.182-6.768).

Regarding the status of surgical resection margins, dysplasia present in margins was identified as a significant prognostic factor for the disease active state (p=0.035), dysplastic margins increasing risk by 2.812 times compared with clear-margins (95% CI, 1.073-7.371). The major sized dysplasia (> 600 mm^2) appeared as a significant predictor (p=0.045) for disease active status; major size increasing risk by 4.464 times compared to minor sizes (< 200 mm^2) (95% CI, 1.035-18.394). Whilst intermediate size (200-600 mm^2) was 2.327 times the risk of minor size for disease active events to occur (95% CI, 0.944-5.740), but did not reach significance (p=0.067).

A 7.980 times increased risk for a disease active state to develop in patients with systemic disease (positive medical history) was found compared to patients with no medical problems (95% CI, 0.987-64.547), but this had border line significance (p=0.051).

The final model to try to predict disease active events included margin status, clinical type of leukoplakia, degree and anatomical site of dysplasia, although only dysplastic margins (p=0.011), severe dysplasia (p=0.023) and CIS (p=0.004) were identified as significant predictors. Dysplastic margins showed 6.562 times the risk for clear margins (95% CI, 1.545-27.878); severe dysplasia 5.994 times the risk of mild dysplasia (95% CI, 1.282-128.018), whilst CIS exhibited 17.104 times increased risk compared to mild dysplasia (95% CI, 2.427-120.561).

Table 5.20: Logistic regression models for disease active status.

Outcome	Risk Factors	Uni-variable analysis		Multi-variable analysis	
		Odds (95% CI)	**p-value**	**Odds (95% CI)**	**p-value**
Disease active:	Age	1.007 (0.976-1.040)	0.646		
Recurrence, New-site dysplasia, MT and OSCC	Sex				
	Females	Reference category			
	Males	1.448 (0.806-3.455)	0.405		
	Leukoplakia types				
	Homogenous	Reference category			
	Non-homogenous	**2.991 (1.160-7.713)**	**0.023**	3.319 (0.799-13.779)	0.099
	PMDs site				
	FOM	Reference category			
	Tongue	**3.381 (1.292-8.845)**	**0.013**	3.323 (0.775-14.241)	0.106
	Other remaining site	**2.893 (0.971-8.620)**	**0.057**	0.944 (0.171-5.218)	0.947
	Histopathology (WHO grading)				
	Mid dysphasia	Reference category			
	Moderate	1.129 (0.350- 3.641)	0.839	1.960 (0.419- 9.167)	0.393
	Severe	**4.622 (1.527- 13.990)**	**0.007**	**5.994 (1.282- 28.018)**	**0.023**
	CIS	**4.800 (1.123-20.479)**	**0.034**	**17.104 (2.427-120.561)**	**0.004**
	Binary grading				
	Low grade	Reference category			
	High grade	**2.828 (1.182-6.678)**	**0.020**		
	Resection margin				
	Free-margins	Reference category			
	Dysplastic-margins	**2.812 (1.073-7.371)**	**0.035**	**6.562 (1.545- 27.878)**	**0.011**
	PMDs size (mm^2)				
	Minor < 200	Reference category			
	Intermediate 200-600	2.327 (0.944- 5.740)	0.067		
	Major > 600	**4.464 (1.035- 18.394)**	**0.045**		
	Medical history				
	Negative history	Reference category			
	Positive history	**7.980 (0.987-64.547)**	**0.051**		

Recurrent (same site) Disease

The overall incidence of same site recurrent PMD disease in this study was 17/100; 13 males and 4 females. Patients with recurrence were followed- up from 14-183 months (mean 83.75 months).
The mean age of patients who underwent recurrence was 58.06 years (SD: 12.044) but ranged from 40-77 years. As can be seen in Figure 5.2, 59% (10/17) of patients who developed recurrent PMD were middle age, followed by 35% (6/17) old age and only one patient 6% was younger than 40 years. In this group of patients, 77% (13/17) were married and 47% (8/17) were retired.

The mean time between primary treatment and first recurrence was 28.47 months, ranging from 6-148 months (SD: 33.88), whilst a second recurrence occurred at a mean time of 40.875 months ranged from 18-96 months (SD: 27.80).
In this study, out of the 17 patients with recurrent dysplasia, 8 had 2 or more recurrences.

Recurrence of dysplasia after laser treatment had a male predilection, 76% (13/17) of males underwent recurrences compared to 24% (4/17) of females; Figure 5.3. However, the association between having a recurrence or not and sex was not significant (p=0.406; Fisher's Exact test). Females showed a shorter mean time to recurrence than males (26.25 vs. 29.15 months), but statistically the difference was not significant (p=0.88; Independent t-test). Males who experienced recurrences were younger than females (56.08 vs. 64.50 years), but the difference did not reach significance (p=0.233; Independent t-test).

The total clinical management time for patients developing recurrent disease was between 17-184 months (SD: 45.931) with a mean of 110.47 months.
A significantly longer mean management time of patient with recurrence compared to that of DF patients was found (110.47 vs. 60.63 months) (p=0.0001; Mann-Whitney U test).

During their clinical management time, patients in this group experienced 0 to 7 observational biopsies, underwent 1 to 4 laser interventions, between 1 to 7 recurrences and 0 to 10 follow-up biopsies after laser surgery.

Sixty-five percent (11/17) of patients in this group underwent between 2 to 4 further laser interventions, whilst the remaining 35% (6/17) underwent only one further laser surgery. Patients developing recurrent disease experienced a higher number of follow-up visits compared with other outcome groups, between 9 to 78 appointments.

Kaplan-Meier survival analysis showed that, 1-year, 2, 3 and 5-years recurrence-free survival rates were 92%, 86%, 80% and 73%, respectively; Figure 5.24.
Recurrence and recurrence-free cumulative survival function were compared in Figure 5.25; the majority of recurrences 65% (11/17) were more likely to take place in the first 2 years following laser treatment, whilst the remaining 35% (6/17) occurred after 2-years.
A significantly lower disease-free survival rate of patients underwent recurrences 1-year, 2-, and 4-years after laser treatment compared to the recurrence-free patients (71% vs. 93%), (35% vs. 86%) and (12% vs. 68%), respectively (p=0.0001; Log-Rank test).

The clinicopathological features of recurrent cases are summarized in Table 5.21.

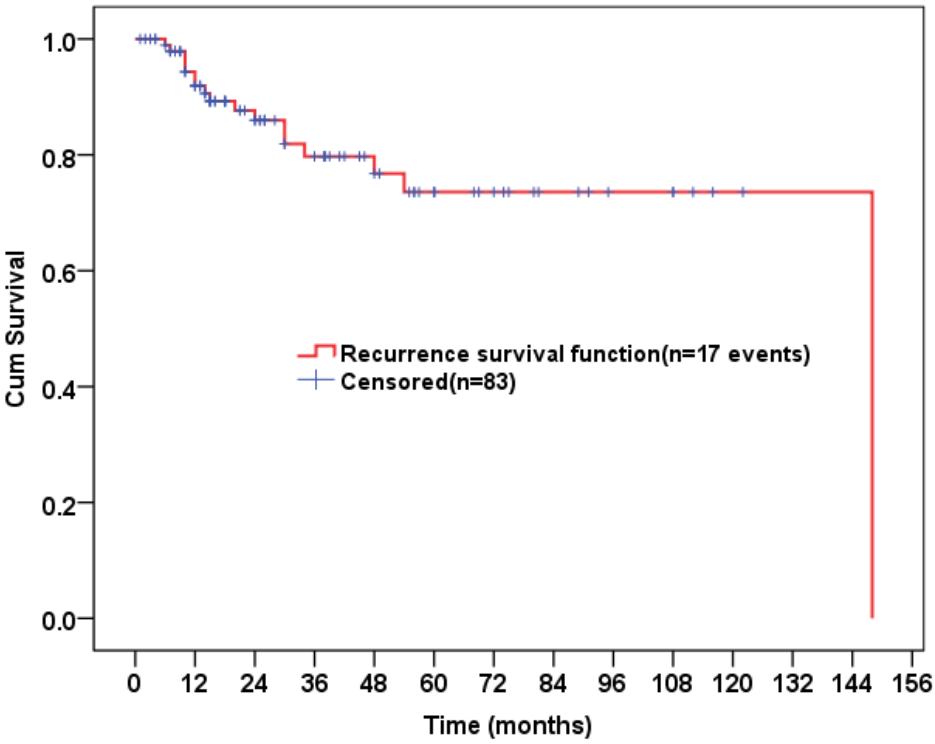

Figure 5.24: Recurrence–free survival functions by Kaplan–Meier analysis.

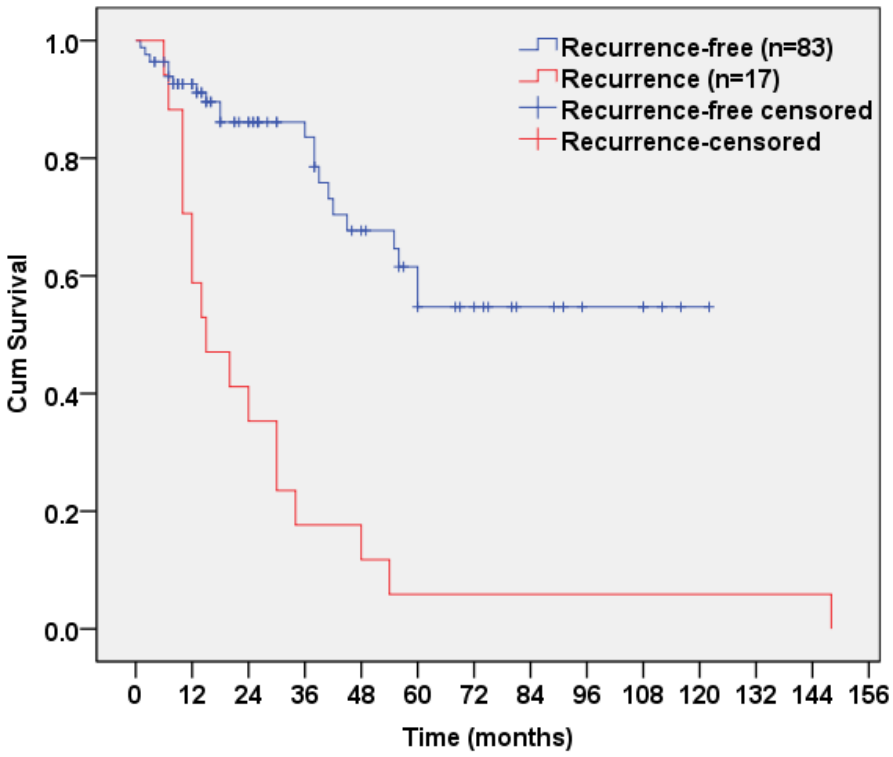

Figure 5.25: Kaplan-Meier survival functions of recurrence and recurrence-free patients (*p*=0.0001; Log-Rank test).

Table 5.21: Clinicopathological features of patients developing recurrent disease.

Case	Age (year)	Sex	Site	Clinical types	Hpath WHO	Hpath Binary	Size (mm^2)	Margin status	1st RT	2nd RT	Smoking	Drinking	Follow-up (months)	Medical conditions
1	40	F	FOM	Homogenous leukoplakia	Modd	HG	<200	Normal	15	20	10-20 (cig./day)	1-14 (u/w)	35	Hypertension
2	48	M	FOM	Homogenous leukoplakia	Sd	HG	200-600	Sd	148	-	>20 (cig./day)	>28 (u/w)	168	Hypertension
3	53	M	FOM	Homogenous leukoplakia	CIS	HG	200-600	Sd	10	-	10-20 (cig./day)	>28(u/w)	109	Hypertension
4	47	M	FOM	Erythroplakia	Sd	HG	<200	Laser damaged	30	96	>20 (cig./day)	>28(u/w)	110	Hypertension
5	63	M	FOM	Non-homogenous exophytic	CIS	HG	200-600	Normal	14	-	<10 (cig./day)	>28 (u/w)	70	Hypertension
6	68	F	Lateral tongue	Non-homogenous exophytic	Md	LG	<200	Md	6	18	Non-smoker	1-14(u/w)	46	Hypertension
7	56	M	Lateral tongue	Non-homogenous ulcerated	Modd	HG	>600	Modd	7	22	Ex-smoker	15-28(u/w)	24	Digestive system
8	62	M	Lateral tongue	Homogenous leukoplakia	Md	LG	200-600	Normal	10	39	Ex-smoker	>28(u/w)	81	Hypertension
9	74	M	Lateral tongue	Homogenous leukoplakia	CIS	HG	>600	CIS	12	-	Ex-smoker	1-14(u/w)	112	Hypertension-DM
10	77	F	Ventral tongue	Homogenous leukoplakia	Sd	HG	<200	Modd	54	-	Non-smoker	Non-drinker	124	Hypertension
11	48	M	Ventral tongue	Homogenous leukoplakia	Sd	HG	>600	Sd	10	-	10-20 (cig./day)	15-28(u/w)	13	Hypertension
12	73	F	Ventral tongue	Non-homogenous speckled	Sd	HG	<200	Sd	30	68	Ex-smoker	Non-drinker	183	Hypertension, DM oral candida
13	60	M	Ventral tongue	Homogenous leukoplakia	Sd	HG	200-600	Modd	12	23	>20 (cig./day)	>28 (u/w)	88	CVS, liver problem
14	56	M	Soft palate	Non-homogenous speckled	Modd	HG	200-600	Modd	34	-	10-20 (cig./day)	>28(u/w)	73	Hypertension
15	46	M	Soft palate	Homogenous leukoplakia	Modd	LG	<200	Normal	24	-	>20 (cig./day)	>28 (u/w)	26	-
16	74	M	Soft palate	Homogenous leukoplakia	Vaporization			Normal	48	41	Ex-smoker	Non-drinker	104	Hypertension
17	48	M	Buccal mucosa	Non-homogenous exophytic	Modd	HG	<200	Md	20	-	>20 (cig./day)	>28(u/w)	112	Oral candida, anaemia

F=female; M=males; FOM=Floor of the mouth; Hpath=histopathology; Md=mild dysplasia; Modd=moderate dysplasia; Sd=severe dysplasia; CIS=carcinoma *in situ*; LG=low grade; HG=high grade; DM=diabetes mellitus; 1st RT=1st recurrence time; 2nd RT=2nd recurrence time.

Clinical Appearance

Table 5.10 and Table 5.11, together with Figure 5.9 summarise the clinical outcome in relation to the clinical appearance of PMDs. Ninety-four percent (16/17) of recurrent cases were leukoplakias (10 homogenous and 6 non-homogenous), with only 6% (1/17) erythroplakia. Half of the cases of non-homogenous leukoplakia (3/6) were exophytic subtypes.

A significant relation was found between recurrence status and PMDs clinical appearance (p=0.028; Chi-Square test). Non-homogenous leukoplakia showed a higher rate of recurrence than homogenous leukoplakia 24% (6/25) vs. 15% (10/67), with the risk estimate showed that non-homogenous leukoplakia was 1.6 times higher risk to develop recurrence after laser surgery than homogenous leukoplakia (95% CI, 0.653-3.963).

Also, Kaplan-Meier survival analysis showed that non-homogenous leukoplakia cases exhibited a shorter estimated mean time to develop recurrences compared with homogenous leukoplakia (63.643 vs. 123.008 months), but this was non-significant (p=0.094; Log-Rank test); Figure 5.26.

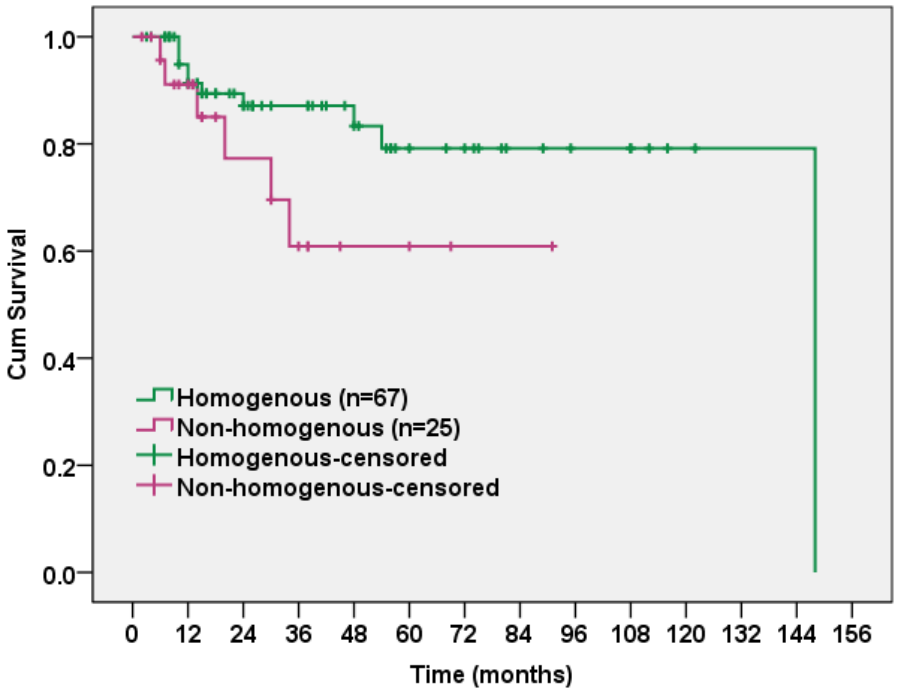

Figure 5.26: Kaplan-Meier analysis of recurrence in relation to homogenous and non-homogenous leukoplakia.

Anatomical Site

The FOM showed the highest recurrence rate 29% (5/17), followed equally by lateral and ventral surfaces of the tongue 24% (4/17), soft palate 17% (3/17) and buccal mucosa which showed the least recurrence rate 6% (1/17). However, no recurrences were seen in the pillar of fauces, retromolar area and alveolar mucosa; Table 5.12 and Figure 5.10. The relation between recurrence status (yes, no) and the primary affected anatomical site approached significance (p=0.051; Chi-Square test).

Size of Dysplasia

Recurrent disease developed in cases with a mean size of 393.63 mm^2 and a range from 32-1,800 mm^2. Forty-four percent (7/16) of recurrent cases were seen in sites primarily affected by minor sized PMDs, followed by those affected with intermediate 37% (6/16) and major size 19% (3/16); Table 5.13 and Figure 5.11.

A comparison between recurrence and recurrence-free cases showed a higher recurrence rate in patients affected with major size dysplasias compared to recurrence-free patients 19% (3/16) vs. 9% (7/82). However, the association between recurrence status and the size of dysplastic PMDs was not significant (p=0.241; Chi-Square test); Figure 5.27.
A higher mean size of dysplasia in patients with recurrence (393.63 mm^2) compared to those who were recurrence-free (281.70 mm^2) was seen, but also the difference did not reach statistical significant (p=0.356; Independent t-test).

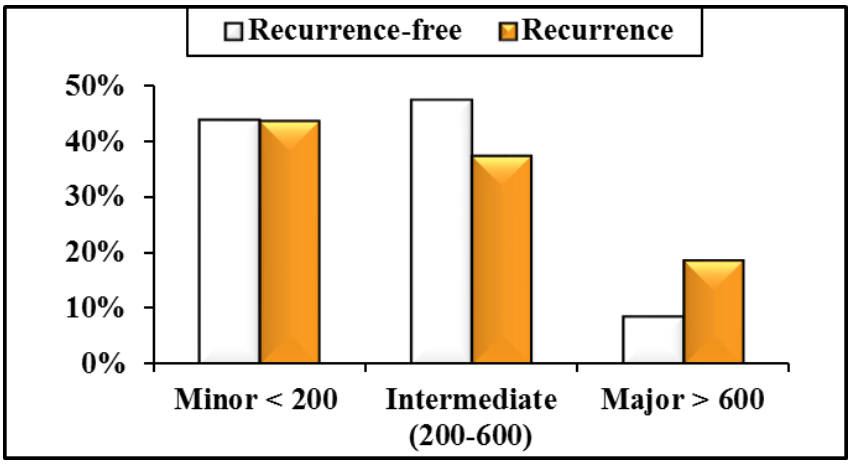

Figure 5.27: Recurrence and recurrence-free in relation to PMD size (mm^2).

Dysplasia Grading

A significant association was found between recurrence status (yes, no) and the grade of dysplasia (p=0.008; Chi-Square test), with CIS showing the highest recurrence rates 27% (3/11), followed by severe 26% (6/23) and moderate dysplasia 25% (6/24), with mild dysplasia exhibiting the least recurrence rates 5% (2/42).

Recurrence-free patients exhibited primarily mild dysplasia 48% (40/83), followed by moderate 22% (18/83), severe 21% (17/83) and CIS 10% (8/83); Figure 5.28.

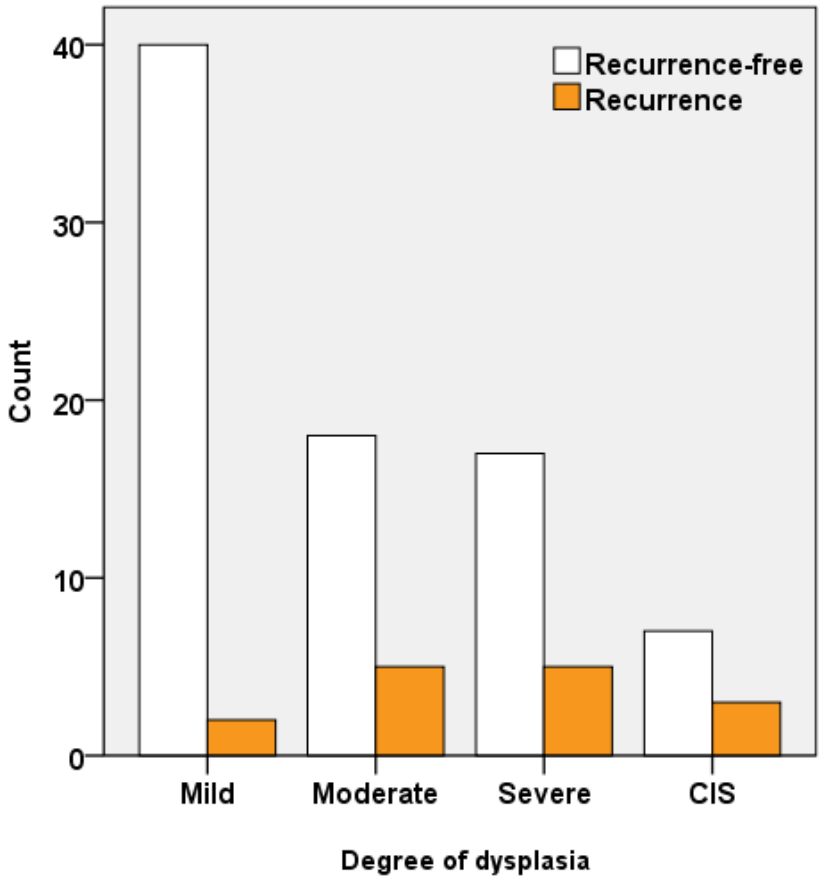

Figure 5.28: Degree of dysplasia in relation to recurrence status.

Similarly, the highest recurrence rate 77% (13/17) was seen in cases with high grade dysplasias compared with low grade dysplasias 23% (4/17). Patients with high grade dysplasia had a significantly higher recurrence incidence than low grade dysplasia, reflected by the significant relation found between high/low grade dysplasia and recurrence status (p=0.025; Fisher's Exact test).

Recurrence-free cases were slightly more commonly seen in patients with low grade dysplasia 52% (43/83) compared to high grade dysplasia 48% (40/83); Figure 5.29.

Of those patients who developed recurrence, 3 (18%) developed recurrent PMD with an increased grade of dysplasia, 8 (47%) developed recurrences with less severe dysplasia, whilst 6 cases (35%) recurred with the same degree of dysplasia as the initially presenting disease.

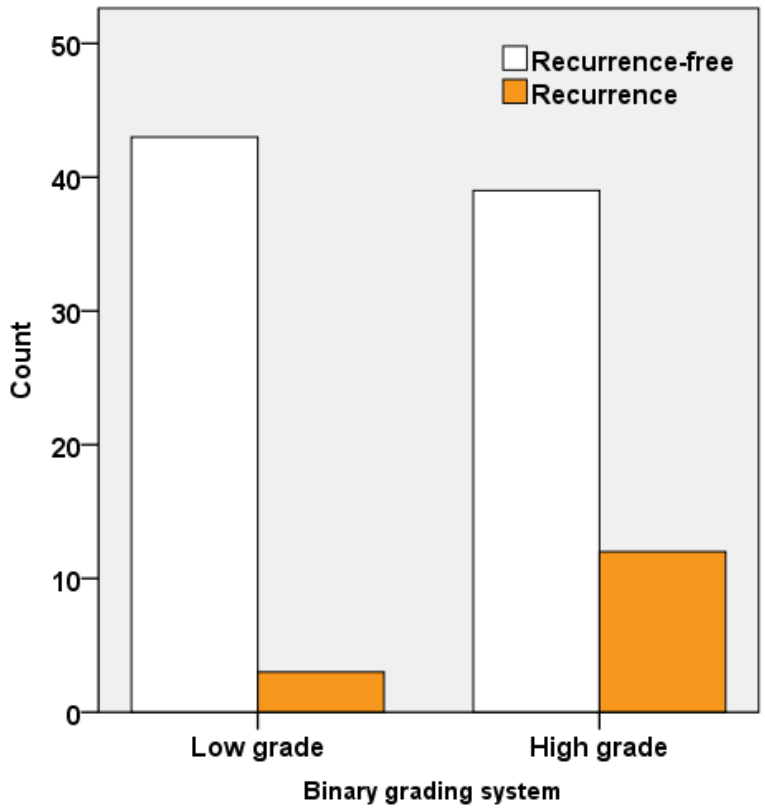

Figure 5.29: High/low grade dysplasia in relation to recurrence status.

Comparing the recurrence-free survival rates of patients with mild, moderate, severe dysplasia, and CIS, Kaplan Meier survival analysis showed that patients with CIS showed the shortest estimated mean time for recurrence to develop, followed by moderate, severe and mild dysplasia (38.84, 85.39, 102.20 and 116.06 months), respectively; however the differences did not reach significance (p=0.085; Log-Rank test); Figure 5.30. Patients with severe dysplasia showed lower recurrence-free survival rates at 1, 3 and 5-year postoperatively (88%, 78% and 59%). Similarly, for patients with moderate dysplasia, a lower recurrence–free survival rate 2-and 5-year post-laser surgery was seen (76% and 68%). Also, moderate dysplasia cases showed a higher recurrence-free survival rate at 1-year compared to patients with both severe dysplasia and CIS (95% vs. 88%, 77%).

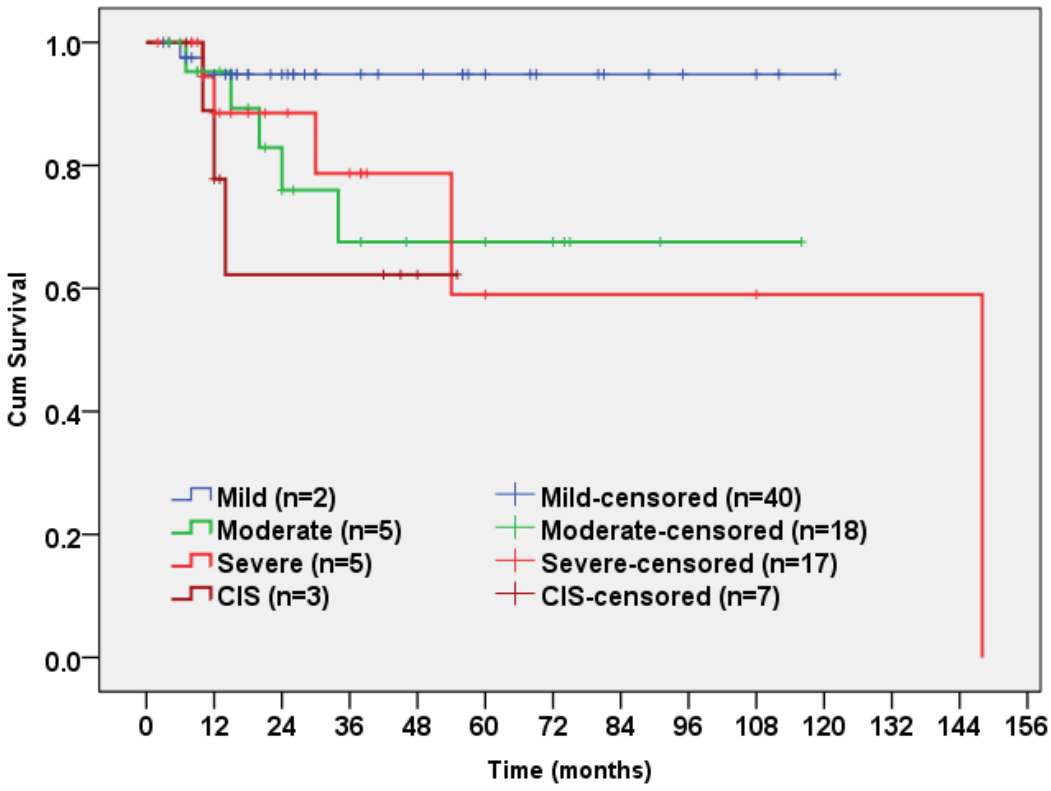

Figure 5.30: Kaplan-Meier analysis of patients with recurrence in relation to grade of dysplasia.

Considering the binary grading system, Figure 5.31 compares recurrence-free survival for high and low grade dysplasia. Patients who underwent recurrences primarily affected by high grade dysplasia had significantly lower 2-and 5–year recurrence-free survival rates than those with low grade dysplasia (79% vs. 91%), (63% vs. 91%), respectively.

Also, the estimated mean time for recurrence to occur in high grade dysplasia was significantly shorter than those with low grade dysplasia (103.13 vs. 113.16 months) (p=0.023; Log-Rank test).

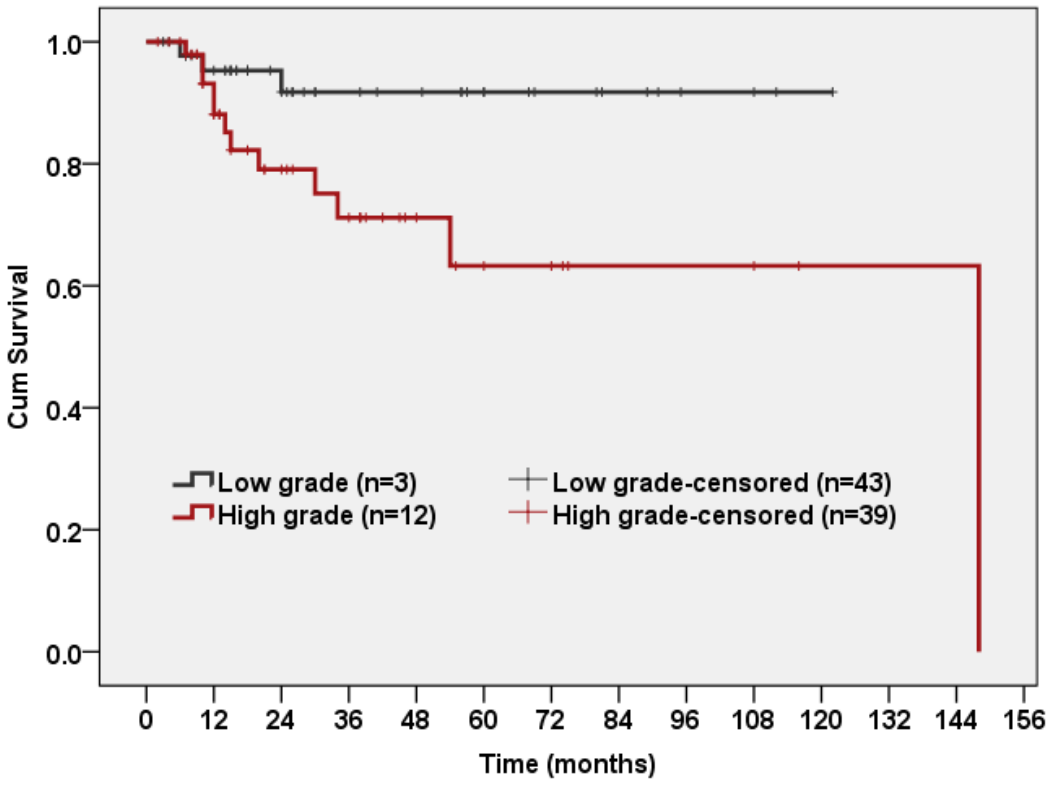

Figure 5.31: Kaplan-Meier analysis of patients with recurrence in relation to high/low grade dysplasia (p=0.023; Log-Rank test).

Recurrence and Resection Margin Status

Table 5.22 and Table 5.23 show the recurrence status in relation to the surgical margins status and margins histopathology, respectively.

Histopathological reports showed that 44% (38/87) of cases showed clear resection margins, whilst 56% (49/87) displayed residual dysplasia. Out of the cases with clear-margins, 11% (4/38) developed recurrences, but this increased to 20% (10/49) in cases with dysplastic margins. However, there was no significant relation between recurrence status (recurrence/recurrence free) and resection margins status (clear/dysplastic) (p=0.252; Fisher's Exact test).

There was a significant relation between recurrence status and the histopathology diagnosis of residual dysplasia in the resection margins (p=0.004; Chi-Square test). Four (29%) of recurrent cases showed moderate residual dysplasia in the margins with a similar number of severe residual dysplasia, and only one case with CIS and one case with mild residual dysplasia. Whilst the majority of patients who were recurrence-free (89%; 34/38) showed clear resection margins, compared to only 11% (4/38) of those who underwent recurrence but with clear margins.

Table 5.22: Recurrence status in relation to surgical margin status.

Resection margin status	Recurrence status		Total
	Recurrence-free	Recurrence	
Clear-margin	**34** *89%*	**4** *11%*	**38** *100%*
Dysplastic margins	**39** *80%*	**10** *20%*	**49** *100%*
Total	**73** *84%*	**14** *16%*	**87** *100%*

Table 5.23: Recurrence status in relation to surgical margin histopathology.

Histopathology of surgical margin	Recurrence status		Total
	Recurrence-free	Recurrence	
Clear-margin	**34** *47%*	**4** *29%*	**38** *44%*
Mild	**19** *26%*	**1** *7%*	**20** *23%*
Moderate	**9** *12%*	**4** *29%*	**13** *15%*
Severe	**9** *12%*	**4** *29%*	**13** *15%*
CIS	**2** *3%*	**1** *7%*	**3** *3%*
Total	**73** *100%*	**14** *100%*	**87** *100%*

With respect to the anatomical sites of the recurrent cases in relation to resection margins, Table 5.24, out of the 4 cases with clear resection margins, 2 were in the FOM and 2 in the soft palate. Seven cases with residual dysplasias in the margins were observed in the tongue (3 in the lateral and 4 in the ventral tongue), followed by 2 in the FOM and one in the soft palate.

Table 5.24: Recurrent cases in relation to anatomical site.

Histopathology of surgical margin	Anatomical site of the recurrent cases				Total
	FOM	Lateral tongue	Ventral tongue	Soft palate	
Clear-margin	2	-	-	2	4
Mild	-	1	-	-	1
Moderate	-	1	2	1	4
Severe	2	-	2	-	4
CIS	-	1	-	-	1
Total	4	3	4	3	14

Smoking Behaviour

Regarding smoking behaviour and recurrence of dysplasia, 88% (15/17) of patients who underwent recurrence were tobacco users, whilst the remaining 12% (2/17) were non-smokers. Of the 15 tobacco users, 67% (10) were current smokers and 33% (5) were ex-smokers. Current smokers with recurrences smoked between 7- 40 cigarettes/day (mean of 23.60). Of the current tobacco smokers, 50% (5/10) were heavy smokers, 40% (4/10) intermediate and only one patient was a light smoker 10% (1/10); Table 5.16 and Figure 5.14.

Using Chi-Square test, no significant association was found between recurrent status and either smoking status (p=0.509) or number of cigarettes smoked per day (p=0.062). However, heavy smokers showed a higher recurrence rate 50% (5/10), whilst intermediate smokers represented the majority of the recurrence-free group; Figure 5.32.

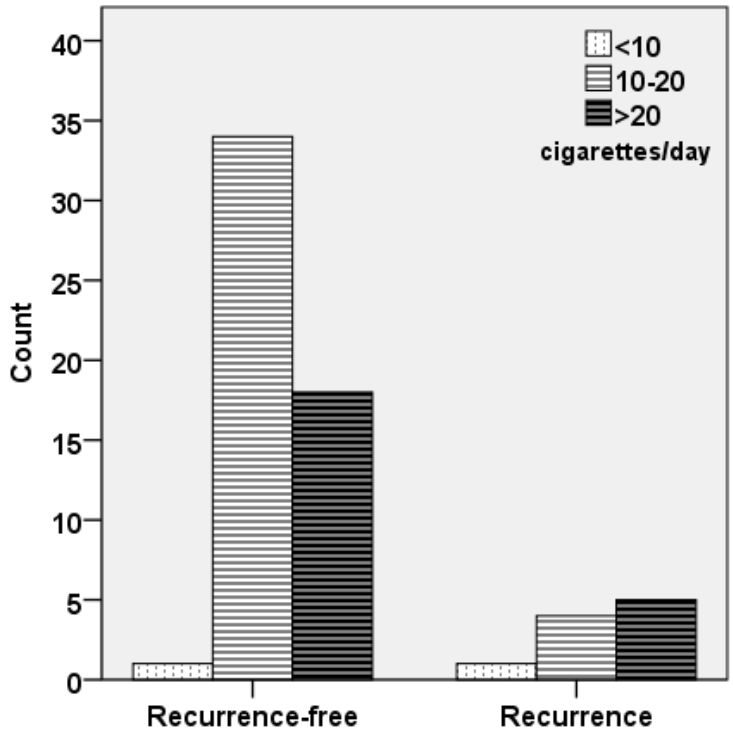

Figure 5.32: Recurrence status in relation to the number of cigarettes smoked per day.

Patients with recurrence had a smoking history between 10- 48 years with a mean of 32.50 years. Recurrence rate was the same for both groups of patients who smoked for either a long duration of time (31-50 years) or a relatively long duration (10-30 years). However, 60% (18/30) of recurrence-free cases were associated with a long smoking history (31-50) compared with those who smoked for 10-30 years, but the relation between smoking history and recurrence status was not significant (p=0.415; Chi-Square test); Figure 5.33.

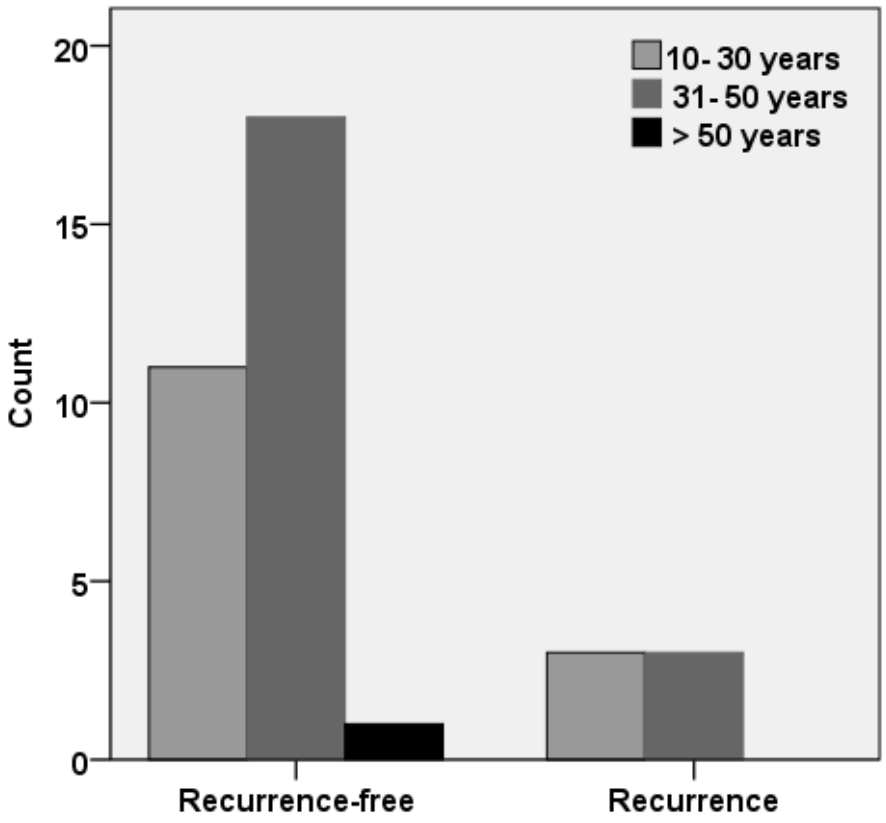

Figure 5.33: Recurrence status in relation to smoking history.

Taking into consideration the smoking status in patients who underwent recurrences, Kaplan-Meier analysis showed that recurrence-free survival rates of patients with recurrences 1-year postoperatively was 94%, 93% 84% for current smokers, non-smokers and ex-smokers, respectively. Two-years after treatment, the percentage for current smokers decreased to 85% but remained the same for both non-smokers and ex-smokers. The 3-year recurrence-free survival rate of current smokers dropped to 80%, whilst the 5-year rate was the same, but dropped to 70% and 52% for non-smokers and ex-smokers, respectively; Figure 5.34.

Also, ex-smokers experienced the shortest estimated mean time for recurrence to occur (61.82 months) compared to both non-smokers (65.19 months) and current smokers (121.67 months), however, a Log-Rank test was not significant (p=0.396).

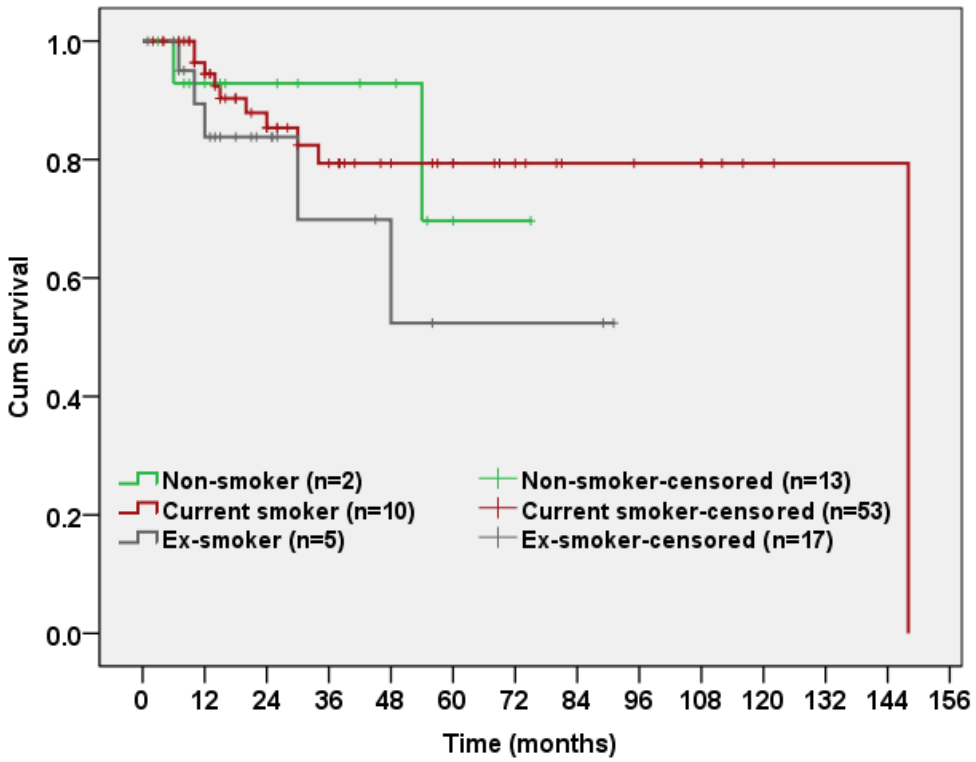

Figure 5.34: Kaplan-Meier analysis of patients with recurrence in relation to smoking status.

In general, Chi-Square testing showed no significant relation between clinical outcome and smoking status, both at the first presentation and the most recent follow-up (p=0.509, p=0.516, respectively). Whilst non-smokers remained the same, current smokers who underwent recurrence decreased from 10 to 7 cases, ex-smokers increased from 5 to 8 cases; Figure 5.35 and Figure 5.36.

Similarly, no significant association was found between clinical outcome and smoking intensity (cigarettes/day) either at the first or most recent follow-up (p=0.06, p=0.252, respectively). However, light smokers with recurrences increased from one patient at initial presentation to 5 at the most recent follow-up, whilst intermediate smokers decreased from 4 to 2 patients and no heavy smokers were seen at the most recent follow-up from the 5 cases at first presentation.

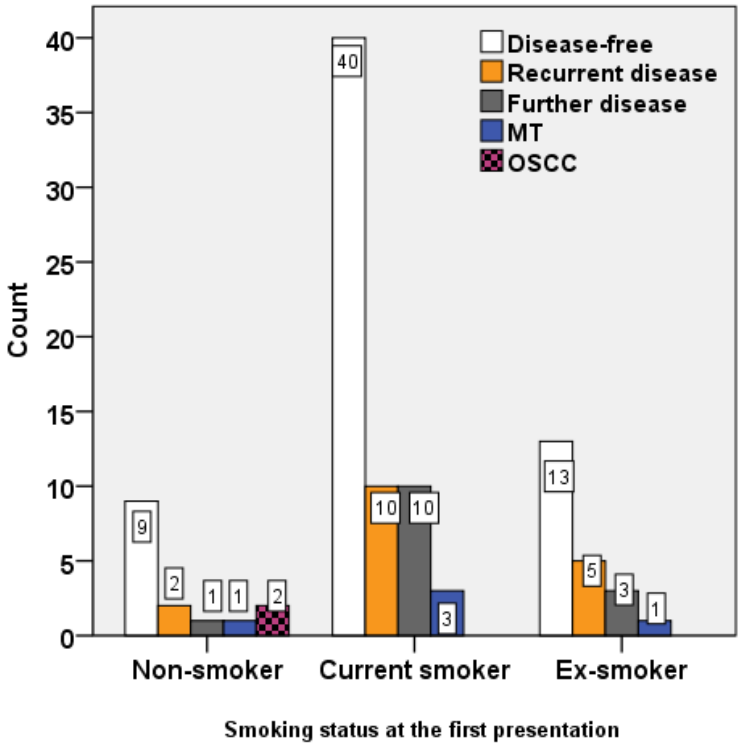

Figure 5.35: Smoking status at first presentation in relation to clinical outcome.

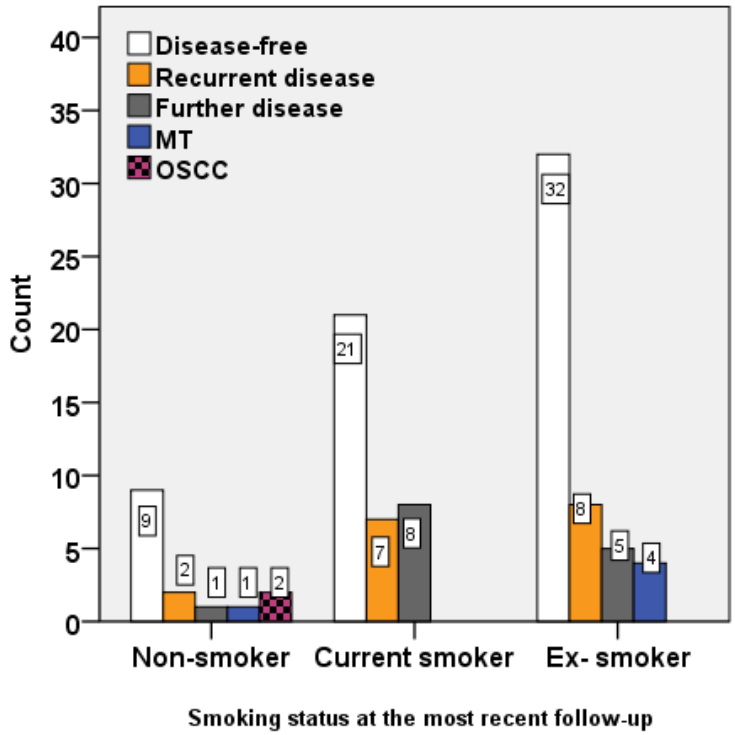

Figure 5.36: Smoking status at most recent follow-up in relation to clinical outcome.

Alcohol Use

Out of the 17 patients with recurrence, 82% (14/17) were current drinkers and the remaining 18% (3/17) were non-drinkers, though no ex-drinkers were seen in this group; Table 5.25. Patients who developed recurrent disease drank between 2 and 80 units/week with a mean of 36.2 (units/week).

Table 5.25: Recurrence status in relation to drinking status.

Recurrence status	Drinking status at initial presentation			Total
	Non-drinker	Current drinker	Ex-drinker	
Recurrence-free	**11** *13%*	**69** *83%*	**3** *4%*	**83** *100%*
Recurrence	**3** *18%*	**14** *82%*	**-**	**17** *100%*
Total	**14**	**83**	**3**	**100**

A significant relation was found between recurrence status and the amount of alcohol consumed in terms of units per week (light, moderate, heavy drinkers) (p=0.019; Chi-Square test). Heavy drinkers exhibited the highest recurrences rates 64% (9/14), whilst recurrence-free patients were light drinkers 52% (36/69); Figure 5.37.

Patients consuming > 28 units/week experienced higher recurrence rates 64% (9/14) after laser treatment compared to those who consumed ≤ 28 units/week 36% (5/14). However, no significant relation was found between the amount of alcohol consumed in terms of units/week and recurrence (p=0.074; Fisher's Exact test). However, risk estimate showed that consumption of more than 28 units/week was a 2.594 times higher risk for recurrence to develop post-laser intervention than in those consuming ≤ 28 units/week (95% CI, 0.953-0.7065).

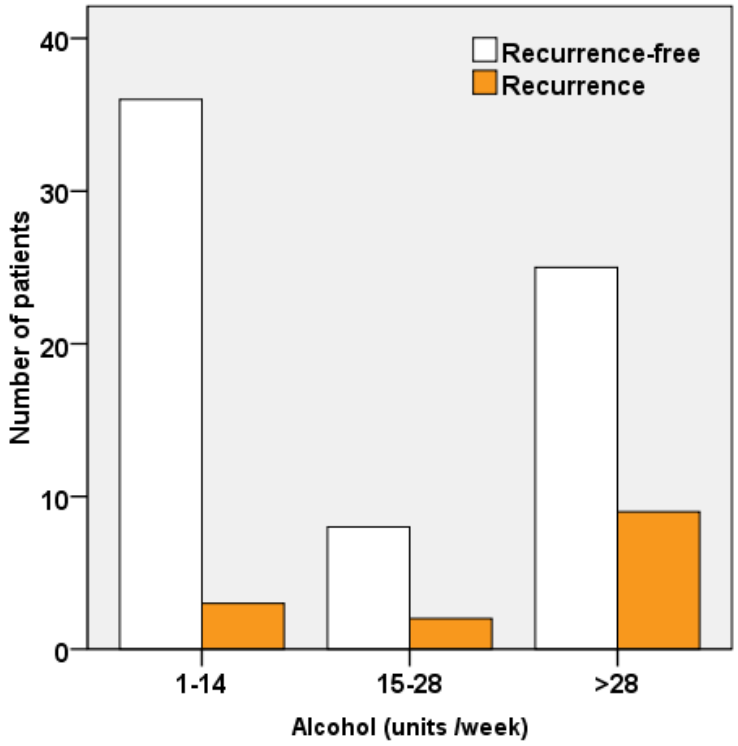

Figure 5.37: Recurrence and recurrence-free in relation to alcohol intake.

Although the majority of patients who underwent recurrence were drinkers, a higher recurrence rate was seen among non-drinkers 21% (3/14) compared with 17% (14/83) in drinkers; the relation, however, was not significant (p=0.707; Fisher's Exact test).
Risk estimate showed that non-drinkers had an increased risk of 1.27 times of developing recurrence compared to current drinkers (95% CI, 0.418-3.859).

Furthermore, Kaplan-Meier analysis showed that non-drinkers had a shorter estimated mean time to develop recurrence compared to drinkers (63.33 vs. 120.39 months), but Log-Rank testing was not significant (p=0.566). Recurrence-free survival rate of non-drinkers who underwent recurrence was 80% at 30 months (2.5 years) after laser surgery, which dropped to 53% at 54 months (4.5 years); Figure 5.38.

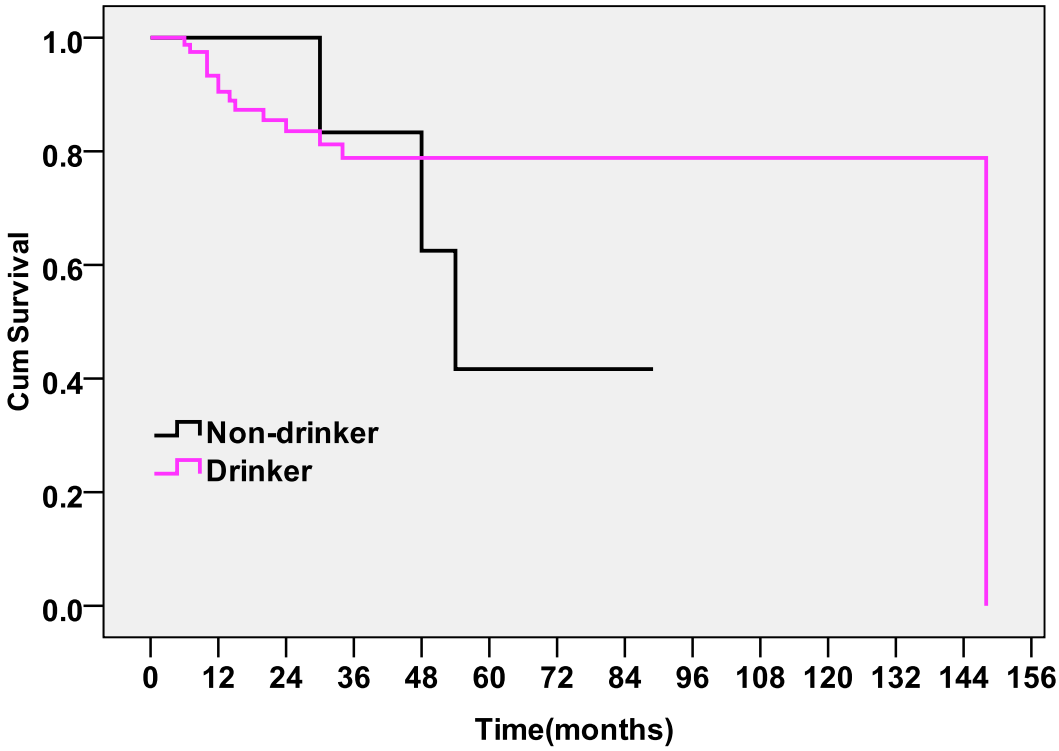

Figure 5.38: Kaplan-Meier analysis of patients with recurrence in relation to alcohol drinker and non-drinker.

Patients consuming > 28 and ≤ 28 units/week had the same recurrence-free survival rates 1-year after laser treatment (90%), but at 2-years the rate dropped to 78% for patients consuming more than 28 units/week compared to those consuming ≤ 28 (88%).

At 5-years post-laser intervention the survival rate was the same for patients consuming ≤ 28 units/week, but decreased to 69% for those who drank more than 28 units/week; however the Log-Rank test was not significant (p=0.206); Figure 5.39.

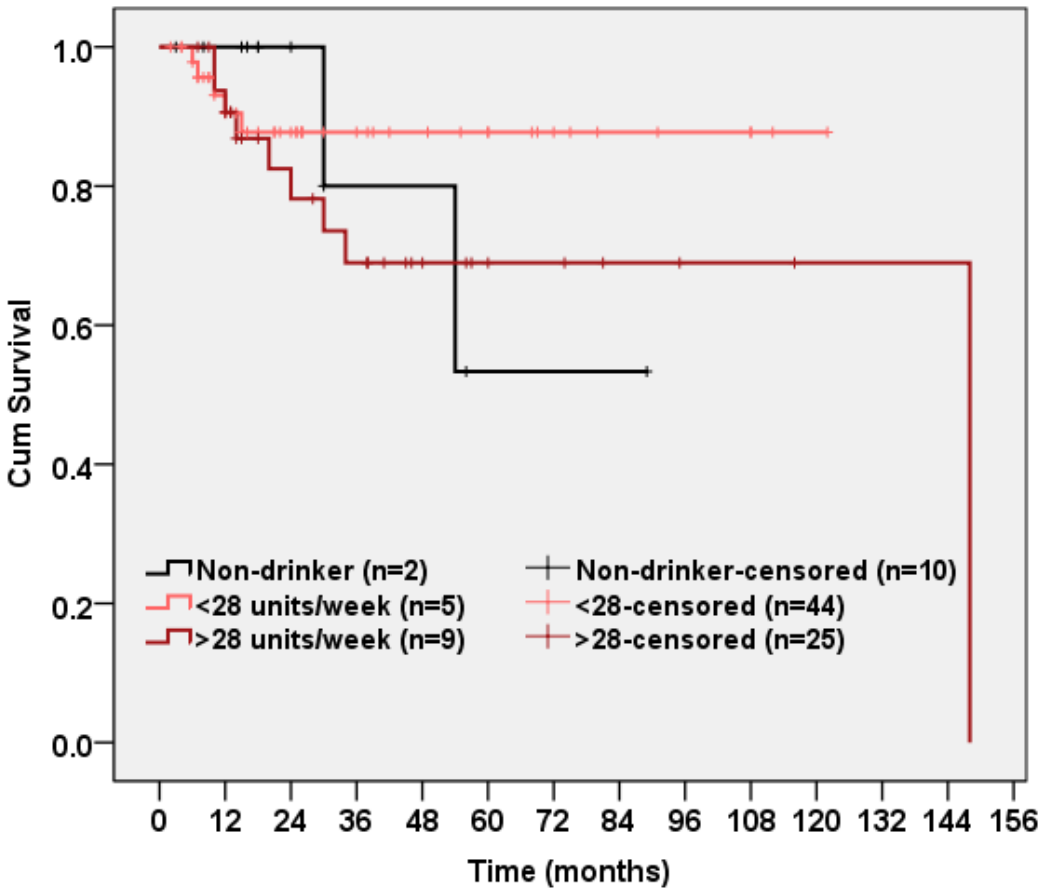

Figure 5.39: Kaplan-Meier analysis of patients with recurrence in relation to drinking behaviour.

Dental Prosthesis Wear

Overall, the majority of patients who underwent recurrence (71%; 12/17) were dental prostheses wearers, whilst the majority of recurrence-free patients were non-wearers (51%; 42/83). Out of the 17 recurrent cases, 12 were dental prosthesis wearers (6 wore full dentures, 4 with crowns & bridges and 2 with upper or lower set of denture) and 5 were non-wearers; Figure 5.40 and Table 5.26. Fisher's Exact test showed no significant association between recurrence status and the wearing of a dental prosthesis (p=0.181).

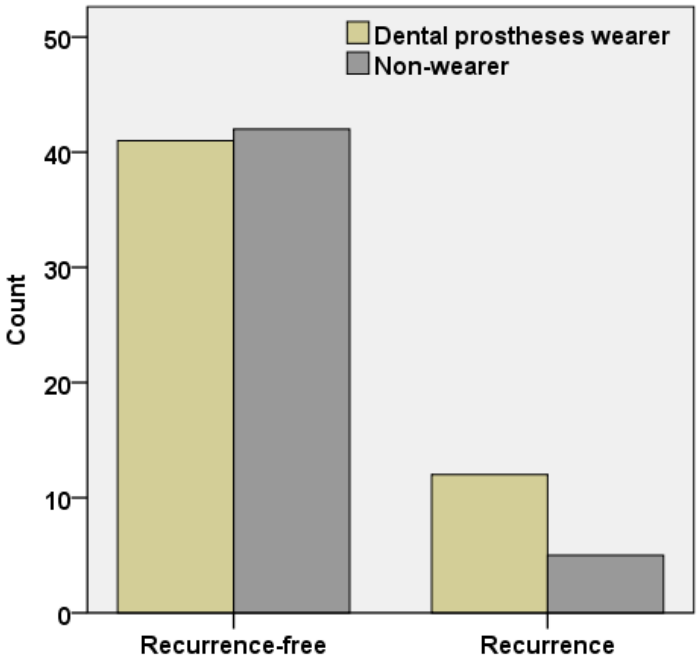

Figure 5.40: Recurrence status in relation to dental prostheses wear.

Table 5.26: Recurrence status in relation to dental prostheses wear.

Recurrence status	Dental prosthesis		Total
	Wearer	Non-wearer	
Recurrence-free	**41** *49%*	**42** *51%*	**83** *100%*
Recurrence	**12** *71%*	**5** *29%*	**17** *100%*
Total	**53** *53%*	**47** *47%*	**100** *100%*

Logistic Regression Analysis

To explore the most important significant predictors (independent risk factors) associated with same site recurrence of dysplasia after laser surgery, age, sex, clinical types of leukoplakia, anatomical site, size, degree of epithelial dysplasia, resection margin histopathology, smoking and drinking status, dental prosthesis wearer and a history of systemic disease were incorporated into the regression analysis separately. The -2 log likelihood ratio test statistic was used to reach the best fit for the final model.

Table 5.27 shows the final logistic regression models for recurrence of dysplasia after laser treatment of dysplastic PMDs.

The age of the patient had no effect on recurrence of dysplasia (OR=1.002, 95% CI, 0.963-1.044) (p=0.905). Males exhibited 1.840 times the risk of females for recurrence (95% CI, 0.550-6.149), but this was not significant (p=0.322).

The clinical type of leukoplakia seems to influence recurrence, so that non-homogenous leukoplakia was 1.8 times more likely to recur than homogenous leukoplakia (95% CI, 0.577-5.614), but did not reach significance (p=0.311).

Major size PMDs were 2.204 times more likely to recur than minor size PMDs (95% CI, 0.456-10.661), but this was non-significant (p=0.326).

Using mild dysplasia as a reference category, moderate dysplasia increased the risk of recurrence by 5.556 times (95% CI, 0.983-31.387) but with a border line significant (p=0.052). Severe dysplasia increased the risk of recurrence 5.882 times (95% CI, 1.037-33.354); and was a significant predictor for recurrence to develop (p=0.045). Similarly, CIS increased the risk for recurrence to occur by 8.571 times compared to mild dysplasia (95% CI, 1.206-60.920); and was as a significant predictor of recurrence (p=0.032).

With respect to histopathology of resection margins, those displaying severe dysplasia-CIS increased the risk of recurrence by 3.864 times compared to the risk for clear-margins (95% CI, 0.879-16.975) (p=0.073). Margins with moderate dysplasia were 3.778 times more likely

to develop recurrence than clear-margins (95% CI, 0.787-18.133), but was non-significant (p=0.097).

Using non-smoker and non-drinker as reference categories, smoking ≤ 20 cigarettes/day produced 1.114 times the risk of non-smokers for recurrence (95% CI, 0.199-6.237) (p=0.902), whilst smoking > 20 cigarettes/day caused 1.444 times the risk of non-smokers (95% CI, 0.229-9.106) (p=0.695). For ex-smokers, the risk was 1.912 times higher of recurrence compared with non-smokers (95% CI, 0.319-11.471) (p=0.478).
Patients who drank > 28 units/week were 1.320 times more likely to develop recurrence than non-drinkers (95% CI, 0.298-5.838) (p=0.714).

The lateral and ventral surfaces of the tongue have an increased risk of recurrence by 2.624 times compared with the recurrence risk for the FOM (95% CI, 0.772-8.915), although this was non-significant (p=0.122).
Wearing a dental prosthesis showed an increased risk by 2.459 times for recurrence compared with non-wearers (95% CI, 0.795-7.600), but this was not significant (p=0.118).

Chronic medical problems increased the risk for recurrence by 2.444 times compared with patients who had no systemic disease (95% CI, 0.294-20.316), but this was non-significant (p=0.408).

After the model-fitting technique, final model selection utilized the goodness of fit statistic -2 log likelihood ratio and revealed that the histopathology of the margins, degree of epithelial dysplasia, clinical type of leukoplakia and site of dysplasia were the most important predictors of recurrent disease, although no one was a significant predictor.
CIS showed an 11.902 times increased risk for recurrence compared to mild dysplasia. Dysplastic margins with severe dysplasia-CIS exhibited a 4.312 times risk of recurrence.
The tongue showed a 6.4 times increased risk compared to the FOM and drinking an excess of 28 units per week increased the risk of recurrence 2.327 times compared to non-drinkers.

Table 5.27: Logistic regression models of local recurrence of dysplasia.

Clinical outcome	Risk Factors	Uni-variable analysis		Multi-variable analysis	
		Odds (95% CI)	p-value	Odds (95% CI)	p-value
Recurrence (same site)	Age	1.002 (0.963- 1.044)	0.905		
	Sex				
	Females	Reference category			
	Males	1.840 (0.550-6.149)	0.322		
	leukoplakia types				
	Homogenous	Reference category			
	Non-homogenous	1.8 (0.577-5.614)	0.311	1.627 (0.315- 8.407)	0.561
	PMDs site				
	FOM	Reference category			
	Tongue	2.624 (0.772-8.915)	0.122	6.400 (0.434- 94.305)	0.176
	Other remaining sites	1.929 (0.461-8.072)	0.368	0.543 (0.056-5.237)	0.598
	Histopathology (WHO grading)				
	Mid dysphasia	Reference category			
	Moderate	5.556 (0.983- 31.387)	0.052	6.726 (0.538-84.109)	0.139
	Severe	5.882 (1.037-33.354)	0.045	3.892 (0.302-50.217)	0.298
	CIS	8.571 (1.206- 60.920)	0.032	11.902 (0.817- 173.431)	0.070
	Resection margins histopathology				
	Free-margins	Reference category			
	Mild dysplasia	0.45 (0.047- 4.296)	0.486	1.056 (0.074-15.023)	0.968
	Moderate	3.778 (0.787- 18.133)	0.097	3.954 (0.421-37.146)	0.229
	Severe-CI	3.864 (0.879- 16.975)	0.073	4.312 (0.518- 35.911)	0.177
	PMDs size (mm^2)				
	Minor < 200	Reference category			
	Intermediate 200-600	0.791 (0.242-2.577)	0.697		
	Major > 600	2.204 (0.456-10.661)	0.326		

Continued

Clinical outcome	Risk Factors	Uni-variable analysis		Multi-variable analysis	
		Odds (95% CI)	p-value	Odds (95% CI)	p-value
Recurrence (same site)	Smoking status				
	Non-smokers	Reference category			
	Smoking ≤ 20 cigarettes/day	1.114 (0.199- 6.237)	0.902		
	Smoking > 20 cigarettes/day	1.444 (0.229- 9.106)	0.695		
	Ex-smokers	1.912 (0.319-11.471)	0.478		
	Drinking status				
	Non- drinker	Reference category			
	≤ 28 units/week	0.417 (0.86-2.016)	0.276	0.459 (0.024-8.757)	0.605
	> 28 unite/week	1.320 (0.298-5.838)	0.714	2.327 (0.112-48.475)	0.586
	Medical history				
	Negative	Reference category			
	positive	2.444 (0.294-20.316)	0.408		
	Oral dental prosthesis				
	Non-wearer	Reference category			
	Wearer	2.459 (0.795-7.600)	0.118		

Further Disease (new-site PMDs)

Further disease was defined as new dysplastic lesions developing at oral sites, distant from the primary dysplasia.
At the most recent follow-up of patients, 14/100 of the study cohort patients had undergone new-site dysplasia formation.

The total clinical management time of patients in this group was between 33-139 months, with 15-68 follow-up appointments. The mean follow-up time was 93 months, with a range from 25-133 months. Patients in this group developed between 1 to 3 new dysplasias during their clinical course and experienced between 0 to 3 observational biopsies.
Overall, patients in this group underwent between 1 to 6 further lasers interventions, with the majority of them 64% (9/14) undergoing 2 to 6 laser surgeries; the remaining 36% (5/14) underwent only one additional laser surgery. Patients in this group experienced between 0 to 13 follow-up biopsies post-laser surgery.

After laser treatment, the first new-site dysplasia appeared at an average time of 30.35 months with a range of 7-60 months, whilst the second new dysplasia occurred after an average time of 48.25 months and a range of 41-63 month.

The mean age of patients with further disease was 58.93 years (SD: 11.579), with a range from 39-76 years. Patients were predominantly in their middle age 64% (9/14), followed by old age 29% (4/14), whilst patients ≤ 40 years showed the least tendency for new-site disease formation 7% (1/14); Figure 5.2. However, no significant relation was found between developing new-site dysplasia and patient age groups (p=0.210; Chi-Square test).

Development of new-site dysplasia had a male predilection: 71% (10/14) compared to 29% (4/14) in females; as shown in Figure 5.3. However, a Chi-Square test showed no significant relation between sex and developing new-site dysplasia (p=0.767). Females showed a shorter mean time to develop new disease compared with males (25.50 vs. 32.30 months), but this difference was not significant (p=0.549; Independent t- test).

Also, no significant difference was found in the mean age between males and females who underwent new-site dysplasia (p=0.399; Independent t-test), although females presented at a higher mean age than males (63.25 vs. 57.20 years).

Kaplan-Meier survival analysis showed that new-site dysplasia-free survival rates of patients 1-, 2-, 3 and 5-years after laser treatment were 97%, 92%, 89% and 69%, respectively.
Patients were more likely to develop new-site dysplasia 3-years after laser treatment (8/14), compared with first and second post-operative years; Figure 5.41.

Figure 5.42 compares the disease-free survival rates of new-site dysplasia and new-site dysplasia-free group of patients, significantly a lower disease-free survival rates of patients experienced new-site dysplasia formation compared to those who were new-site dysplasia-free at 1-year and~3 years (38 months) post-laser surgery (50% vs. 94%), (36% vs. 72%), respectively (p=0.0001; Log-Rank test).

The clinicopathological features of this group of patients are summarized in Table 5.28.

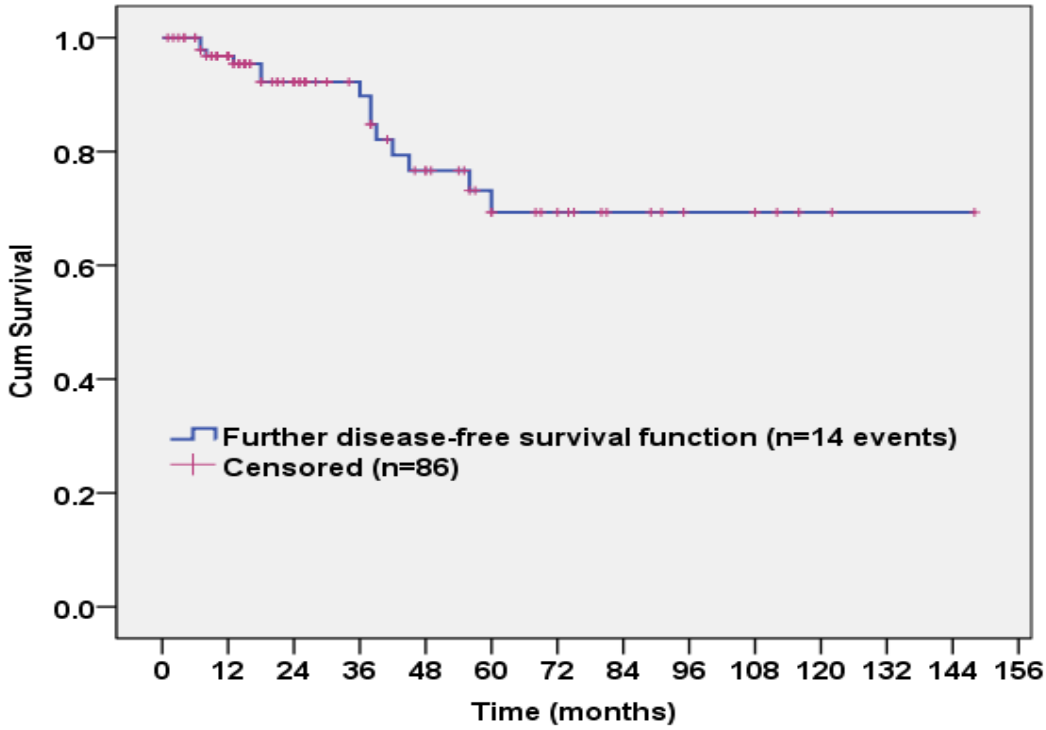

Figure 5.41: Overall new site dysplasia-free survival functions by Kaplan–Meier analysis.

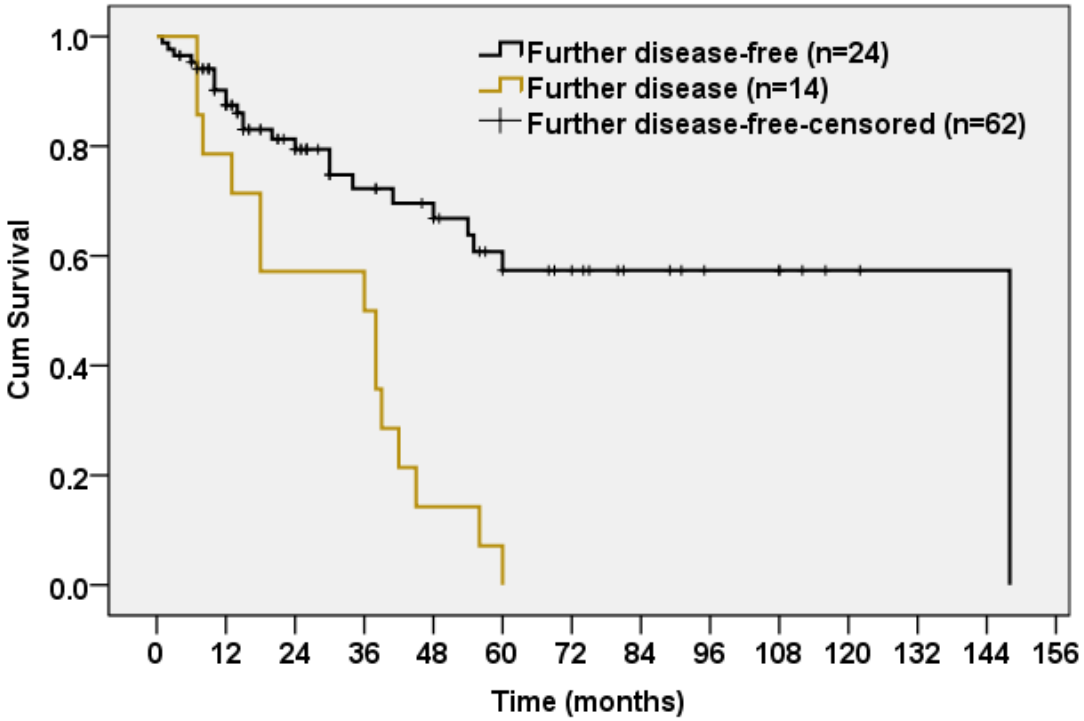

Figure 5.42: Kaplan-Meier analysis of patients with further disease and further disease-free (*p*=0.0001; Log-Rank test).

Table 5.28: Clinicopathological features of patients developing further (new site) PMDs.

Case	Age	Sex	Primary site	Clinical types	1st new dysplasia	1st NT months	Hpath WHO	Hpath Binary	Margin	Smoking	Drinking	Follow-up months	Medical conditions
1	53	M	R.FOM	Homogenous leukoplakia	L.FOM	38	Modd	HG	Md	10-20 cig./day	>28 (u/w)	82	Hypertension
2	39	M	L.FOM	Non-homogenous/speckled	L.Ventral tongue	18	Sd	HG	CIS	>20 cig./day	>28 (u/w)	119	Hypertension
3	76	M	FOM	Homogenous leukoplakia	L.Buccal mucosa	8	Sd	HG	Sd	Ex-smoker	Non-drinker	27	Hypertension
4	61	M	FOM	Homogenous leukoplakia	R.Lateral tongue	56	Md	LG	Modd	10-20 cig./day	>28 (u/w)	77	Hypertension
5	59	M	Ventral tongue	Homogenous leukoplakia	L.Ventral tongue	7	Md	LG	Md	10-20 cig./day	15-28 (u/w)	107	Cervical myelopathy Bladder cancer
6	74	M	Ventral tongue	Homogenous leukoplakia	L.Lateral tongue	7	Md	LG	Laser damaged	Ex-smoker	1-14 (u/w)	69	Oral Candida
7	76	F	Ventral tongue	Homogenous leukoplakia	L.Lateral tongue	42	CIS	HG	Normal	Non-smoker	1-14 (u/w)	124	Hypertension
8	68	M	Ventral tongue	Non-homogenous/speckled	L.Lateral tongue	45	CIS	HG	Sd	Ex-smoker	>28 (u/w)	114	Hypertension
9	52	M	Lateral tongue	Non-homogenous/ulcerated	L.FOM	36	Sd	HG	Sd	>20 cig./day	1-14 (u/w)	24	Hypertension
10	60	M	R. Buccal mucosa	Non-homogenous/exophytic	L.Buccal mucosa	60	Md	LG	Md	<10 cig./day	>28 (u/w)	133	Oral Candida
11	59	F	L. Buccal mucosa	Homogenous leukoplakia	R.Buccal mucosa	39	Sd	HG	Sd	10-20 cig./day	1-14 (u/w)	77	Skeletomuscular Rheumatoid arthritis
12	55	M	Fauces	Non-homogenous/speckled	Anterior FOM	38	Md	LG	Modd	10-20 cig./day	>28 (u/w)	104	Hypertension
13	42	F	Fauces	non-homogenous/speckled	L.FOM	13	Sd	HG	Md	>20 cig./day	>28 (u/w)	106	CVS, Respiratory
14	68	M	Soft palate	Erythroplakia	L.Labial mucosa	18	Md	LG	Normal	>20 cig./day	15-28 (u/w)	25	Hypertension Oral candida

R=right side, L=left side; FOM=floor of mouth; 1st NT=1st new dysplasia time; Hpath=histopathology; cig =cigarettes; u/w=units of alcohol per week
Modd=moderate dysplasia; Sd=severe dysplasia; Md=mild dysplasia; CIS=carcinoma *in situ*; HG=high grade dysplasia; LG=low grade dysplasia.

Clinical Appearance

Out of the 14 patients who developed new-site dysplasia, 13 were leukoplakias with 1 erythroplakia. Of the 13 leukoplakias, 7 were homogenous and 6 non-homogenous, in which the majority were speckled (4/6), one exophytic and one ulcerated; Table 5.11 and Figure 5.9. No significant relation was found between new-site dysplasia formation and leukoplakia type either homogenous or non-homogenous (p=0.174; Fisher's Exact test), although a significant relation was found overall between clinical appearance of PMDs and the development of new dysplasia (p=0.021; Chi-Square test).

Ninety-percent (60/67) of patients with homogenous leukoplakia were free from new dysplasia compared to 10% (7/67) that developed new-site dysplasia. New-site dysplasia formation rate of homogenous leukoplakia was 54% (7/13) compared to 46% (6/13) of non-homogenous type. Among other non-homogenous leukoplakia, patients with the speckled subtype showed the highest rate of new dysplasia formation 31% (4/13); Table 5.29.
Risk estimate showed that patients with non-homogenous leukoplakia had 2.297 times the risk to develop new-site dysplasia compared to those with homogenous leukoplakia (95% CI, 0.854-6.176).

Table 5.29: New site dysplasia status in relation to clinical appearance of PMDs.

PMDs appearance		New site dysplasia-free	New site dysplasia	Total
Homogenous leukoplakia		**60** *90%*	**7** *10%*	**67** *100%*
Non-homogenous leukoplakia	**Speckled**	**12** *75%*	**4** *25%*	**16** *100%*
	Nodular	**2** *100%*	**-** **-**	**2** *100%*
	Exophytic	**4** *80%*	**1** *20%*	**5** *100%*
	Ulcerated	**1** *50%*	**1** *50%*	**2** *100%*
Erythroplakia		**7** *88%*	**1** *12%*	**8** *100%*
Total		**86**	**14**	**100**

Anatomical Site

A significant relationship was found between developing new-site dysplasia and the primary affected oral subsites (p=0.002; Chi-Square test). New-site dysplasia formation was mainly seen in patients with FOM and ventral tongue lesions equally at 29% (4/14), followed by buccal mucosa and fauces 14% (2/14), lateral tongue and soft palate in equal distribution (1/14), whilst no new dysplasia was seen in patients initially affected at retromolar area or alveolar mucosa sites; Table 5.30.

Table 5.30: New site dysplasia status in relation to PMD anatomical site.

PMDs anatomical site	New site dysplasia-free	New site dysplasia	Total
FOM	**42** *91%*	**4** *9%*	**46** *100%*
Lateral tongue	**18** *95%*	**1** *5%*	**19** *100%*
Ventral tongue	**10** *71%*	**4** *29%*	**14** *100%*
Buccal mucosa	**3** *60%*	**2** *40%*	**5** *100%*
Soft palate	**8** *89%*	**1** *11%*	**9** *100%*
Fauces	**2** *50%*	**2** *50%*	**4** *100%*
Retromolar area	**1** *100%*	**-**	**1** *100%*
Alveolar mucosa	**2** *100%*	**-**	**2** *100%*
Total	**86** *86%*	**14** *14%*	**100** *100%*

Size of Dysplasia

Patients developing new-site dysplasia primarily presented with lesions of a mean size of 342.79 mm^2 (SD: 217.493) with a range of 21-704 mm^2.

A significant relation was found between developing new-site dysplasia and the size of the primary dysplastic PMDs affecting the patients (p=0.049; Chi-Square test); patients with dysplasia sized between 200-600 mm^2 showing the highest rate of new dysplasia formation 64% (9/14); Table 5.31.

Table 5.31: New site dysplasia status in relation to the primary PMD size category.

PMD size category (mm^2)	New site dysplasia-free	New site dysplasia	Total
Minor < 200	**40** *48%*	**3** *21%*	**43** *44%*
Intermediate (200-600)	**36** *43%*	**9** *64%*	**45** *46%*
Major > 600	**8** *10%*	**2** *14%*	**10** *10%*
Total	**84** *100%*	**14** *100%*	**98** *100%*

Dysplasia Grading

Regarding histopathology diagnosis, the relation between the degree of epithelial dysplasia and developing new-site dysplasia was significant (p=0.040; Chi-Square test).

A higher rate of new dysplasia formation was observed in patients exhibiting mild dysplasia 43% (6/14), followed by severe 36% (5/14) and CIS 14% (2/14), with patients with moderate dysplasia showing the least rate of new-site dysplasia formation 7% (1/14); Table 5.32.

Considering the binary grading system, high grade dysplasia showed the highest rate of new-site dysplasia formation compared with low grade dysplasia (57%; 8/14 vs. 43%; 6/14), however, Fisher's Exact test was not significant (p=0.778); Table 5.33.

For patients who developed new-site dysplasia, 57% (8/14) developed new-site disease with the same degree of initial dysplasia, 36% (5/14) showed a decreased degree of dysplasia and only one case (7%) of new-site dysplasia showed an increased grade of dysplasia.

Table 5.32: New site dysplasia status in relation to degree of primary dysplasia.

Degree of dysplasia (WHO grading)	New site dysplasia-free	New site dysplasia	Total
Mild	**36** *43%*	**6** *43%*	**42** *43%*
Moderate	**22** *27%*	**1** *7%*	**23** *24%*
Severe	**17** *20%*	**5** *36%*	**22** *23%*
CIS	**8** *10%*	**2** *14%*	**10** *10%*
Total	**83** *100%*	**14** *100%*	**97** *100%*

Table 5.33: New site dysplasia status in relation to high/low grade dysplasia.

Binary grading dysplasia	New site dysplasia-free	New site dysplasia	Total
Low grade	**40** *48%*	**6** *43%*	**46** *47%*
High grade	**43** *52%*	**8** *57%*	**51** *53%*
Total	**83** *100%*	**14** *100%*	**97** *100%*

Smoking Behaviour

Patients showing new-site dysplasia smoked between 8-50 cigarettes/day with a mean of 26.80. A higher rate of new dysplasia formation was seen in tobacco users; 71% (10/14) in current smokers and 21% (3/14) in ex-smokers, whilst only one case (7%) of a non-smoker developing new-site dysplasia was seen; Table 5.34.

No significant association was found between the development of new-site dysplasia and smoking status (p=0.284; Chi-Square test).

Table 5.34: New site dysplasia status in relation to smoking status.

Smoking status	New site dysplasia-free	New site dysplasia	Total
Non-smoker	**14** *16%*	**1** *7%*	**15** *15%*
Current smoker	**53** *62%*	**10** *71%*	**63** *63%*
Ex-smoker	**19** *22%*	**3** *21%*	**22** *22%*
Total	**86** *100%*	**14** *100%*	**100** *100%*

No significant relation was found between the development of new-site dysplasia and smoking intensity in term of cigarettes per day (p=0.093; Chi-Square test). However, the highest rates of new dysplasia formation were seen in patients who smoked intermediate and heavy amounts of tobacco 50% (5/10) and 40% (4/10), respectively, followed by those who consumed small numbers of cigarettes per day 10% (1/10) who showed the lowest rate of new-site dysplasia; Table 5.35.

A higher rate of new dysplastic PMD presentation was seen amongst patients with a long smoking history (31-50) years (67%; 4/6) compared to those who had smoked for a relatively shorter duration of time (10-30) years (33%; 2/6), however this relation was not significant (p=0.446; Chi-Square test); Table 5.36.

Table 5.35: New site dysplasia status in relation to number of cigarettes per day.

Smoking intensity (cigarettes/day)	New site dysplasia-free	New site dysplasia	Total
< 10	**1** *2%*	**1** *10%*	**2** *3%*
10-20	**33** *62%*	**5** *50%*	**38** *60%*
> 20	**19** *36%*	**4** *40%*	**23** *37%*
Total	**53** *100%*	**10** *100%*	**63** *100%*

Table 5.36: New site dysplasia status in relation to smoking history.

Smoking history (years)	New site dysplasia-free	New site dysplasia	Total
10-30	**12** *40%*	**2** *33%*	**14** *39%*
31-50	**17** *57%*	**4** *67%*	**21** *58%*
> 50	**1** *3%*	**-**	**1** *3%*
Total	**30** *100%*	**6** *100%*	**36** *100%*

Kaplan-Meier survival function regarding smoking status in relation to new-site dysplasia formation showed that ex-smokers demonstrated a shorter estimated mean time to develop new-site dysplasia compared with current smokers (74.17 vs. 112.3 months). Lower new-site dysplasia-free survival rates were seen amongst ex-smokers 1-and 4-year post-laser intervention compared to current smokers (89% vs. 98%) and (71% vs. 77%), respectively, although Log-Rank testing was non-significant (p=0.687); Figure 5.43.

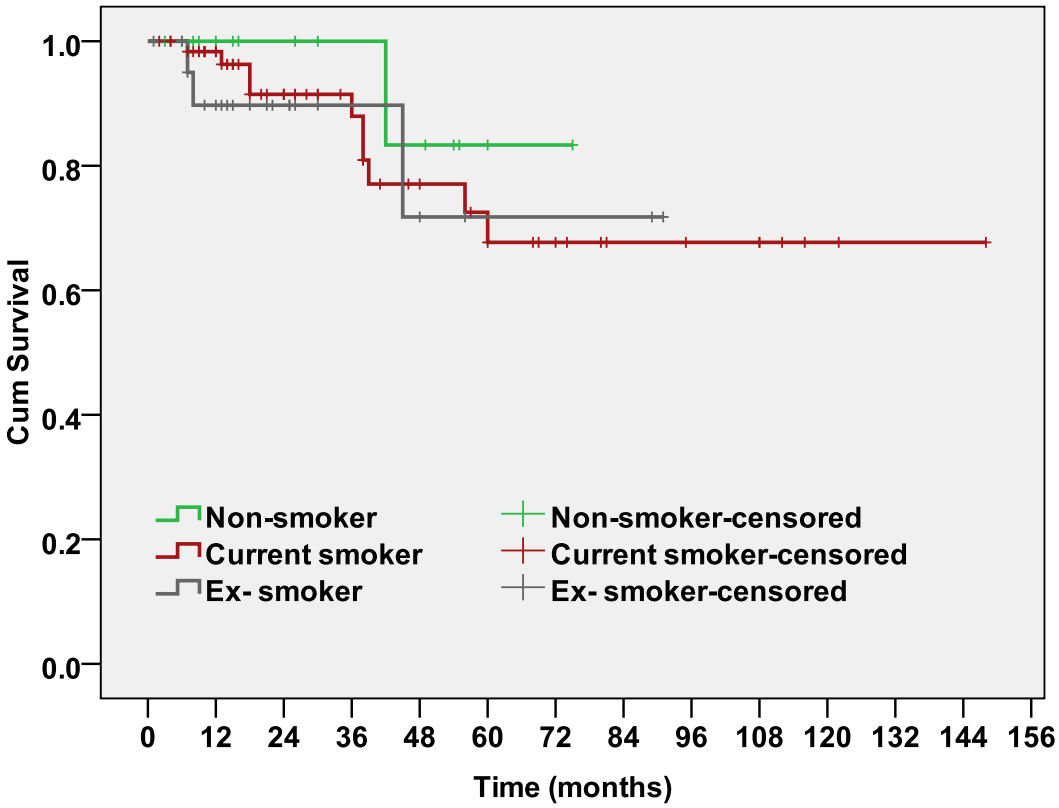

Figure 5.43: Kaplan-Meier analysis of patients with new site dysplasia according to smoking status.

Alcohol Use

The majority of patients who developed new-site dysplasia were current drinkers 93% (13/14), with only one non-drinker (7%) reported in this group; Table 5.37. No significant relation was found, however, between developing new dysplasia and drinking status (p=0.218; Chi-Square test).

Also, no significant association was found between new dysplasia formation and the number of alcohol units consumed per week (p=0.387; Chi-Square test). However, heavy drinkers who consumed > 28 units/week showed higher rates of new dysplasia formation (54%; 7/13), followed by light drinkers (31%; 4/13) and intermediate drinkers (15%; 2/14).
Patients who were new-site dysplasia-free were mainly light drinkers who consumed between 1-14 units/week (50%; 35/70); Table 5.38.

Table 5.37: New site dysplasia status in relation to drinking status.

Drinking status	New site dysplasia-free	New site dysplasia	Total
Non-drinker	**13** *15%*	**1** *7%*	**14** *14%*
Current drinker	**70** *81%*	**13** *93%*	**83** *83%*
Ex-drinker	**3** *3%*	**-**	**3** *3%*
Total	**86** *100%*	**14** *100%*	**100** *100%*

Table 5.38: New site dysplasia status in relation to alcohol intake.

Alcohol (units/week)	New site dysplasia-free	New site dysplasia	Total
1-14	**35** *50%*	**4** *31%*	**39** *47%*
15-28	**8** *11%*	**2** *15%*	**10** *12%*
> 28	**27** *39%*	**7** *54%*	**34** *41%*
Total	**70** *100%*	**13** *100%*	**83** *100%*

With respect to drinking status and new-site dysplasia formation, Kaplan-Meier survival analysis showed that current drinkers showed a clear fall in disease-free survival rates 1-, 2-, 3-, 4- and 5-years after laser treatment (97%, 92%, 89%, 74% and 67%, respectively), compared to non-drinkers. A fall in disease-free survival rate of current drinkers compared to non-drinkers 4 years and 5 years post-laser surgery was found (74% vs.92%), (67% vs. 92%) respectively, although Log-Rank testing was not significant (p=0.773); Figure 5.44.

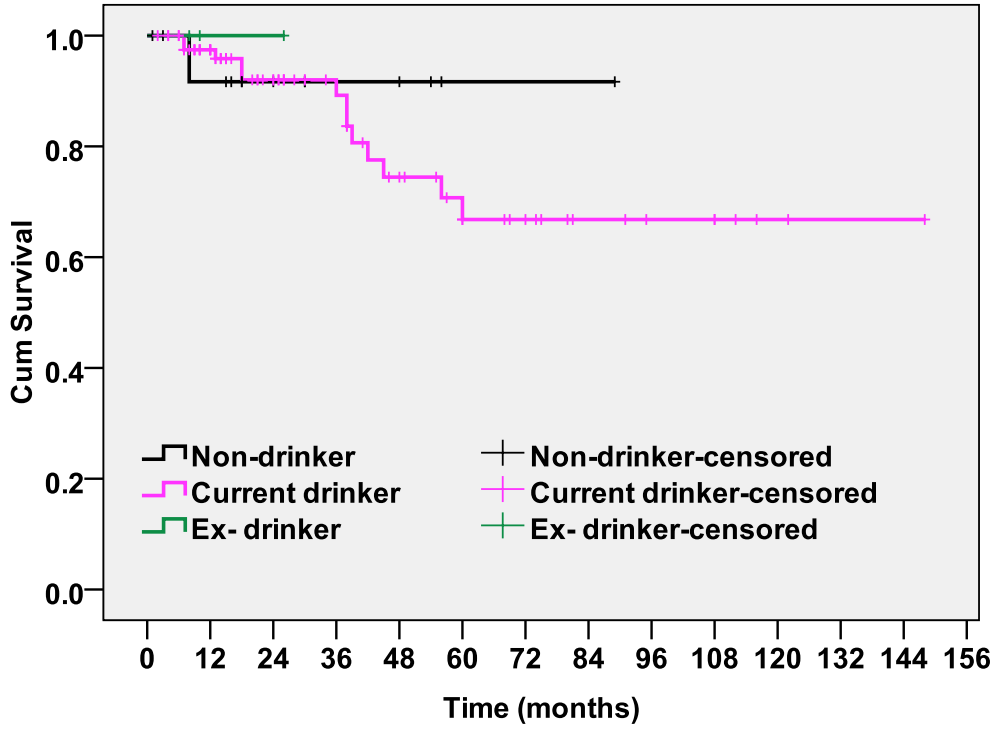

Figure 5.44: Kaplan-Meier analysis of patients with new site dysplasia in relation to drinking status.

Dental Prosthesis Wear

A higher proportion of new-site dysplasia formation was seen amongst patients not wearing dental prostheses (57%; 8/14) compared to wearers (43%; 6/14); however, Fisher's Exact test showed no significant association between wearing a dental prosthesis and new-site dysplasia formation (p=0.565); Table 5.19 and Figure 5.19.

Logistic Regression Analysis

To explore the most important predictors for new-site dysplasia formation, a logistic regression analysis was performed. Age, sex, binary histopathological diagnosis, clinical type of leukoplakia, smoking and drinking behaviour, anatomical site of dysplasia and history of medical problems were included in the analysis separately, then the goodness of fit statistic -2 log likelihood ratio was utilized to select the final regression model.
Table 5.39 shows the logistic regression models of new-site dysplasia formation.

Whilst no effect of patients' age on new-site dysplasia formation was found (OR=1.009, 95% CI, 0.965-1.054) (p=0.704), males displayed 1.339 times the risk of females for new-site dysplasia development (95% CI, 0.387-4.635), but this was non-significant (p=0.645).

Non-homogenous leukoplakia showed a 2.707 times increased risk for new dysplasia to occur compared with homogenous leukoplakia (95% CI, 0.810-9.044); statistically, this was non-significant (p=0.106).

High grade dysplasia was at 1.240 times the risk of low grade dysplasia for new-site PMDs to develop (95% CI, 0.396- 3.889), but again this was not significant (p=0.712).

Smoking ≤ 20 cigarettes/day increased the risk for new-site dysplasia to occur 2.4 times more compared with the risk of non-smokers (95% CI, 0.264-21.787), whereas smoking > 20 cigarettes/day showed a 3.111 times increased risk compared with non-smokers (95% CI, 0.312-31.028); both were non-significant (p=0.333, p=0.437, respectively). Ex-smokers increased the risk for new-site dysplasia to occur by 2.211 compared with non-smokers (95% CI, 0.207-23.555), but again this was not significant (p=0.511).

Drinking alcohol ≤ 28 units/week posed a 1.814 times higher risk for new-site dysplasia to occur compared with non-drinkers (95% CI, 0.200-16.470), but this was not significant (p=0.597), whilst drinking > 28 units/week increased the risk for new-site dysplasia to develop by 3.370 compared with non-drinkers (95% CI, 0.374-30.335), but again non-significant (p=0.278).

The best fit for the final model for multivariable regression analysis that utilized the goodness of fit statistic -2 log likelihood ratio was for leukoplakia type, anatomical site, binary histopathological diagnosis, smoking and drinking, all of which may be considered as important predictors, although none reach statistical significance.

Table 5.39: Logistic regression models of new site dysplasia formation.

Outcome	Risk factors	Uni-variable analysis		Multi-variable analysis	
		Odds (95% CI)	p-value	Odds(95% CI)	p-value
Further Disease New site PMD	Age	1.009 (0.965-1.054)	0.704		
	Sex Females Males	 Reference category 1.339 (0.387- 4.635)	 0.645		
	Leukoplakia type Homogenous Non-homogenous	 Reference category 2.707(0.810- 9.044)	 0.106	 2.212 (0.538-9.098)	 0.271
	Histopathology -binary system Low grade High grade	 Reference category 1.240 (0.396- 3.889)	 0.712	 1.281 (0.317-5.180)	 0.728
	PMD site FOM Tongue Remaining sites	 Reference category 1.875 (0.463-7.596) 3.281 (0.781-13.785)	 0.379 0.105	 3.564 (0.652-19.483) 2.181 (0.398- 11.934)	 0.143 0.369
	Smoking status Non-smokers Smoking ≤ 20 cig/day Smoking > 20 cig/day Ex-smokers	 Reference category 2.400 (0.264- 21.787) 3.111 (0.312- 31.028) 2.211 (0.207- 23.555)	 0.437 0.333 0.511	 3.638 (0.240-55.203) 3.530 (0.209- 59.512) 4.589 (0.349-60.355)	 0.240 0.209 0.349
	Drinking status Non-drinker ≤ 28 u/w > 28 u/w	 Reference category 1.814 (0.200- 16.470) 3.370 (0.374- 30.335)	 0.597 0.278	 0.658 (0.054- 8.052) 1.506(0.110-20.656)	 0.743 0.759

Comparison between the Recurrent Disease and the Development of Further Dysplasia

Male patients developed more recurrent disease 57% (13/23) than further disease at sites distant from their primary dysplasia 43% (10/23), whereas females developed recurrences and new-site dysplasia equally; the relation was not significant, however (p=1.000; Fisher's Exact test); Figure 5.45.

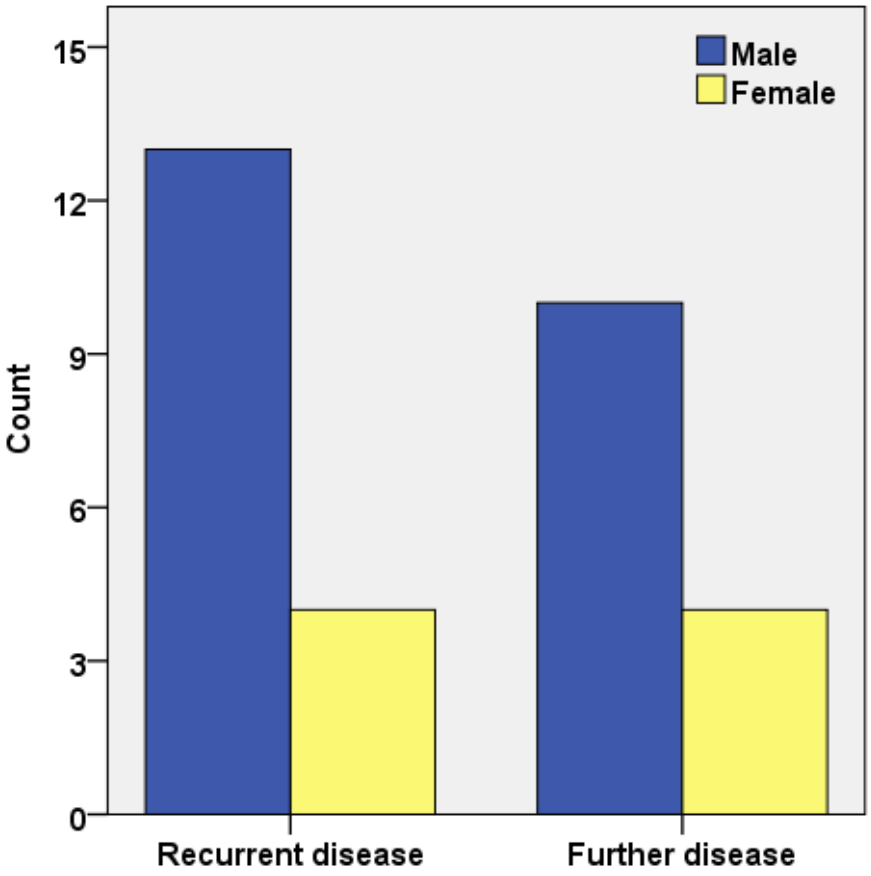

Figure 5.45: Sex in relation to recurrent (same site) disease and new site dysplasia formation.

In middle age patients, a higher recurrence rate compared to new-site dysplasia formation was seen (10/17 vs. 9/14). Similarly, in old age a higher rate of recurrence (6/17) was seen compared to new dysplasia formation (4/14). Patients ≤ 40 years showed an equal rate of both recurrence and new-site dysplasia formation, although the relation was not significant (p=0.634; Chi-Square test); Figure 5.46.

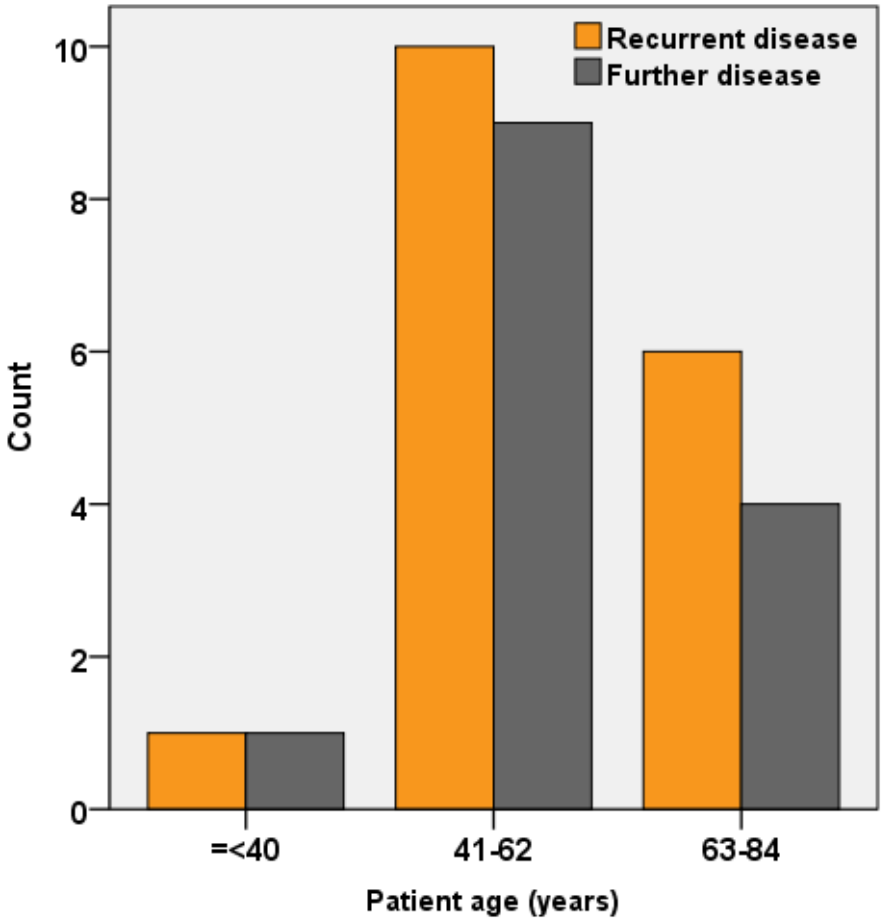

Figure 5.46: Age in relation to recurrent (same site) disease and new site dysplasia formation.

A significant relationship was found between the degree of dysplasia and whether same site or new-site dysplasia occurred (p=0.048; Chi-Square test).

Cases that were diagnosed initially as mild dysplasia, showed a higher rate of new-site dysplasia compared to recurrence (75%; 6/8 vs. 25%; 2/8), whereas moderate dysplasia showed a higher recurrence rate compared to new-site dysplasia (86%; 6/7 vs. 14%; 1/7). Both CIS and severe dysplasia showed higher rates of recurrence compared with new-site dysplasia (60%; 3/5 vs. 40%; 2/5), (55%; 6/11 vs. 45%; 5/11), respectively; Table 5.40.

Table 5.40: Recurrent and new site disease formation in relation to degree of dysplasia.

Degree of dysplasia	Recurrent disease	Further disease	Total
Mild	**2** *25%*	**6** *75%*	**8** *100%*
Moderate	**6** *86%*	**1** *14%*	**7** *100%*
Severe	**6** *55%*	**5** *45%*	**11** *100%*
CIS	**3** *60%*	**2** *40%*	**5** *100%*
Total	**17** *55%*	**14** *45%*	**31** *100%*

Current smokers showed equal rates for both recurrence and new-site dysplasia formation (10/20). Recurrence rates were higher than new-site disease in both non-smokers (2/3 vs.1/3) and ex-smokers (5/8 vs. 3/8), but Chi-Square testing was not significant (p=0.693); Figure 5.47 and Table 5.41.

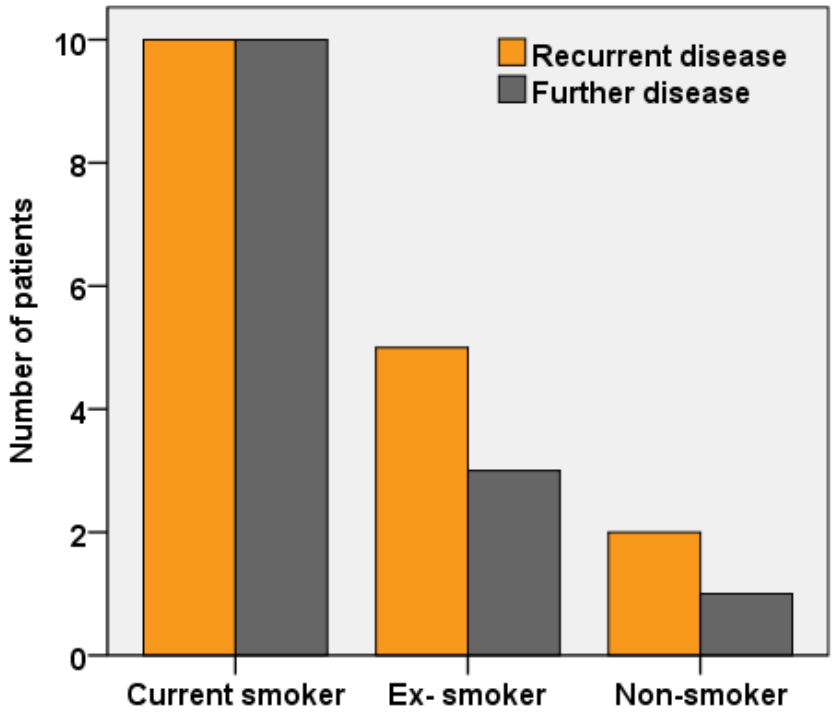

Figure 5.47: Recurrent and new dysplasia in relation to smoking status.

Table 5.41: Recurrent and new dysplasia in relation to smoking status.

Smoking status	Recurrent disease	Further disease	Total
Non-smoker	**2** *67%*	**1** *33%*	**3** *100%*
Current smoker	**10** *50%*	**10** *50%*	**20** *100%*
Ex-smoker	**5** *63%*	**3** *38%*	**8** *100%*
Total	**17** *55%*	**14** *45%*	**31** *100%*

A higher mean tobacco consumption in terms of both cigarettes smoked per day (26.80 vs. 23.60) and grams of tobacco per week in patients developing new-site dysplasia compared to recurrent disease cases was seen (189.09 vs. 165.20), however the differences did not reach significance (p=0.614, p=0.561; Mann-Whitney U test).

Patients who smoked for a long duration of time (31-50 years) showed a higher rate of new-site dysplasia formation compared to recurrence (4/7 vs. 3/7), whilst patients with a shorter smoking history (10-30 years) showed higher recurrence rates (3/5) than new-site disease (2/5); Fisher's Exact test was not significant (p=1.000); Table 5.42.

Considering the intensity and duration of smoking, pack-years score was higher in patients developing new-site dysplasia than those with recurrent disease (44.17 vs. 36.7), but the differences did not reach significance (p=0.532; Independent t-test).

Table 5.42: Recurrent and new dysplasia in relation to smoking history.

Smoking history (years)	Recurrent disease	Further disease	Total
10-30	**3** *60%*	**2** *40%*	**5** *100%*
31-50	**3** *43%*	**4** *57%*	**7** *100%*
Total	**6** *50%*	**6** *50%*	**12** *100%*

The majority of patients who underwent both recurrences (14/17) and new-site dysplasia formation (13/14) were current drinkers, but the relation was not significant (p=0.607; Fisher's Exact test); Table 5.43.

Also, patients with new-site dysplasia showed a higher mean of alcohol consumed in terms of units per week (38.92) compared to patients who suffered recurrent disease (36.21); however these differences did not reach significance (p=1.000; Mann-Whitney U test).

Table 5.43: Recurrent and new dysplasia in relation to drinking status.

Drinking status	Recurrent disease	Further disease	Total
Non-drinker	**3** *18%*	**1** *7%*	**4**
Current drinker	**14** *82%*	**13** *93%*	**27**
Total	**17** *100%*	**14** *100%*	**31** *100%*

Seventy-one percent of patients (12/17) who developed recurrence wore dental prostheses, whilst the majority of patients with new-site dysplasia were non-dental prosthesis wearers (57%; 8/14); Table 5.44. However, no significant relation was found between the presence of a dental prosthesis and the occurrence of active disease; (p=0.157 Fisher's Exact test).

Table 5.44: Recurrent and new dysplasia in relation to dental prosthesis wear.

Dental prosthesis	Recurrent disease	Further disease	Total
Wearer	**12** *71%*	**6** *43%*	**18** *58%*
Non-wearer	**5** 29%	**8** 57%	**13** 42%
Total	**17** *100%*	**14** *100%*	**31** *100%*

Malignant Transformation (same site)

MT was defined as OSCC arising at a site of pre-existing epithelial dysplasia and which was proven histologically. In our hospital-based population, MT was only seen in 5/100 patients (3 males and 2 females).

The total clinical management time for this group of patients was between 35 and 115 months with a mean follow-up time of 47.2 months (range, 24-65 months), and between 10-35 follow-up appointments.

After laser intervention, MT occurred between 1 to 41 months post-surgery (mean, 12.4 months). Patients in this group experienced between 0 to 3 observational biopsies before laser surgery and between 1 to 3 laser interventions, with 1 to 7 follow-up biopsies after laser treatment.

The mean age of patients in this group was 63 years (range, 58-76 years). Out of the 5 transformed cases, 3 (60%) were middle aged (41-62 years) and 2 (40%) old age (63-84 years), but no MT was identified in patients ≤ 40 years of old; Figure 5.2. All males were middle aged (3/3), whilst all females were old age (2/2). Males undergoing MT (mean age 58 years) were younger than females (mean age 70.50 years), with Independent t-test approaching a significant difference (p=0.055).

Females showed a higher percentage of MT (5.9%; 2/34) compared to males (4.5; 3/66), with a risk estimate that showed females were 1.294 times more likely to develop MT compared with males (95% CI, 0.227-7.378); Table 5.45.

Table 5.45: Sex and malignant transformation (MT).

Sex	MT-free	MT	Total
Male	**63** *95.5%*	**3** *4.5%*	**66** *100%*
Female	**32** *94.1%*	**2** *5.9%*	**34** *100%*
Total	**95** *95%*	**5** *5%*	**100**

Kaplan-Meier survival analysis showed that the MT-free survival rates, 1-, 2 and 5-years following laser intervention were 97%, 96% and 93%, respectively; Figure 5.48.

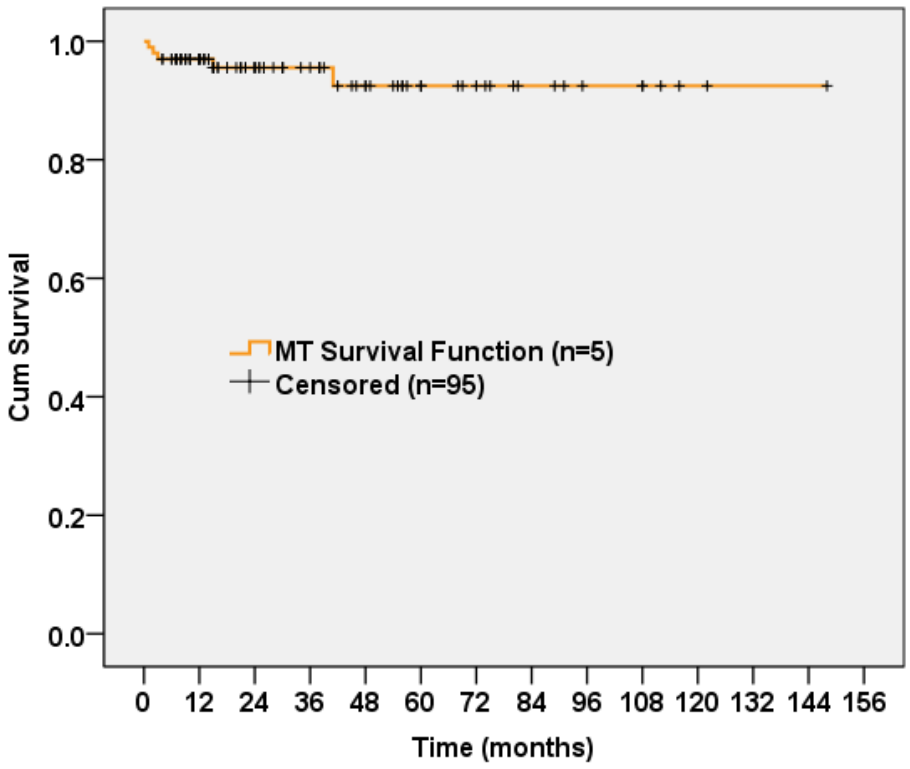

Figure 5.48: Overall disease-free survival functions of patients with MT by Kaplan–Meier analysis.

Figure 5.49 compares the survival function of patients underwent MT and those who were MT-free. A significantly lower disease-free survival rate of patients underwent MT after laser treatment than patients free from MT. At 15-months post-laser surgery, the disease-free survival rate was 20% for patient underwent MT compared to 85% for patients free from MT (p=0.0001; Log-Rank test).

Clinicopathological characteristics of this group of patients are summarized in Table 5.46.

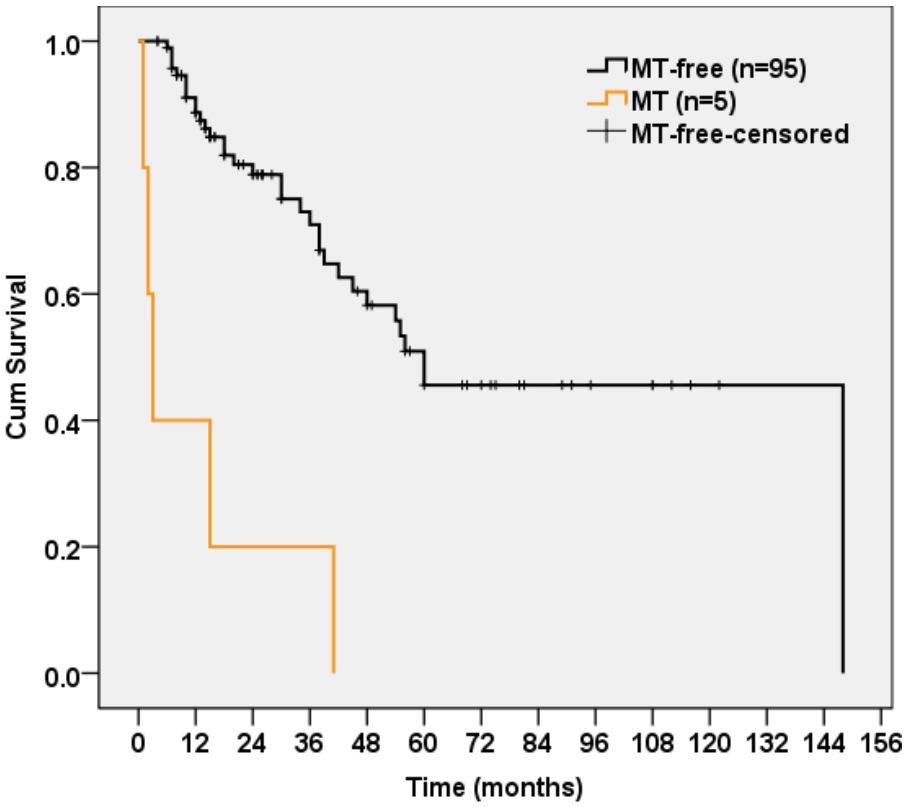

Figure 5.49: Kaplan-Meier analysis of patients with MT and MT-free (*p*=0.0001; Log-Rank test).

Table 5.46: Clinicopathological features of patients developing OSCC at same site (MT) and new distant site.

Case	Age	Sex	Primary site	Cancer outcome	Clinical types	Size (mm^2)	Time (months)	Hpath incision	Hpath excision	Margin status	Smoking (cig./day)	Drinking (u/w)	Follow-up months	Medical conditions
1	58	M	FOM	MT	Non-homogenous speckled	200-600	15	Sd	Sd	Sd	> 20	> 28	24	Hypertension
2	58	M	Lateral tongue	MT	Homogenous leukoplakia	200-600	41	Md	SCC	-	10-20	> 28	62	Depression
3	76	F	Lateral tongue	MT	Homogenous leukoplakia	200-600	3	Md	SCC	-	Non-smoker	Non-drinker	44	Hypertension
4	65	F	FOM	MT	Non-homogenous speckled	200-600	2	Sd	SCC	-	10-20	1-14	65	Hypertension DM
5	58	M	Retromolar area	MT	Erythroplakia	< 200	1	CIS	CIS	Normal	Ex-smoker	Non-drinker	41	Hypertension
6	47	F	Ventral tongue	OSCC (new site)	Homogenous leukoplakia	200-600	55	CIS	CIS	Normal	Non-smoker	1-14	87	DM
7	48	M	Lateral tongue	OSCC (new site)	Erythroplakia	> 600	60	Modd	Sd	-	Non-smoker	1-15	82	Immunodeficiency Hypertension

F=female; M=male; FOM=Floor of the mouth; MT=malignant transformation; H path=histopathology; Md=mild dysplasia; Modd=moderate dysplasia Sd=severe dysplasia; CIS=carcinoma *in situ*; cig=cigarettes; u/w=units per week; DM=diabetes mellitus.

Clinical Appearance

The clinical type of PMDs associated with MT was 80% (4/5) leukoplakia (2 homogenous, 2 non-homogenous speckled) and 20% (1/5) erythroplakia; Table 5.11 and Figure 5.9.
A higher proportion of non-homogenous leukoplakia 8% (2/25) was seen in transformed cases compared to homogenous ones 3% (2/67); Table 5.47. Risk estimate showed that non-homogenous leukoplakia had 2.680 times the risk of homogenous leukoplakia for MT to occur (95% CI, 0.399-18.015).

Table 5.47: Malignant transformation (MT) in relation to leukoplakia type.

Leukoplakia type	MT-free	MT	Total
Non-homogenous	**23** *92%*	**2** *8%*	**25** *100%*
Homogenous	**65** *97%*	**2** *3%*	**67** *100%*
Total	**88** *96%*	**4** *4%*	**92** *100%*

Anatomical Site

MT occurred in 40% (2/5) of FOM cases, 40% (2/5) of lateral tongue and 20% (1/5) of the retromolar area, but no transformed cases were seen in the remaining oral subsites.
The lateral border of the tongue showed a higher rate of MT compared to the FOM (11%; 2/19 vs. 4%; 2/46); Table 5.12 and Figure 5.10.

Although MT was mainly seen in high risk oral sites (FOM and tongue) (80%; 4/5), the high risk sites exhibited no increased risk for MT compared to low risk sites (OR=1.063; 95% CI, 0.125-9.018).

Size of Dysplasia

Eighty-percent (4/5) of the transformed dysplasias were intermediate in size (200-600 mm^2) and one was minor, with a range from 60-570 mm^2 (mean size of 316 mm^2); Table 5.13 and Figure 5.11.

Although no significant association was found between MT and the size of PMD (p=0.593; Chi-Square test), risk estimate showed that if the size of dysplasia exceeded or equalled to 425 mm^2 (equivalent to the third quartile), the OR for MT was 2.056 times higher than for smaller sizes (95% CI, 0.365-11.582).

Dysplasia Grading

Out of the 5 cases that underwent MT, 2 (40%) were severe dysplasia, 2 (40%) mild dysplasia and 1 (20%) CIS.

Severe dysplasia 9% (2/23) and CIS 9% (1/11) were the histological features associated with the highest percentages of MT, whilst mild dysplasia accounted for 5% (2/42) of MT cases; Table 5.48. This reflects a tendency for more severe dysplastic features to develop malignancies, compared to mildly dysplastic ones, however, the relation between MT and degree of dysplasia was not significant (p=0.124; Chi-Square test).

Table 5.48: Malignant transformation (MT) in relation to degree of dysplasia.

Degree of dysplasia	MT-free	MT	Total
Mild	**40** *95%*	**2** *5%*	**42** *100%*
Moderate	**24** *100%*	**-**	**24** *100%*
Severe	**21** *91%*	**2** *9%*	**23** *100%*
CIS	**10** *91%*	**1** *9%*	**11** *100%*
Total	**95**	**5**	**100**

MT was higher in high grade dysplasia compared to low grade lesions (60%; 3/5 vs. 40% 2/5). High grade dysplasia accounted for 6% (3/53) of transformed cases, which was higher than low grade transformed cases 4% (2/47), but the relation between binary grading and MT was not significant (p=1.000; Fisher's Exact test); Table 5.15 and Figure 5.13.

Smoking Behaviour

Sixty-percent (3/5) of transformed cases were current smokers consuming an intermediate amount of tobacco (15-28 cigarettes/day), followed by ex-smokers and non-smokers equally, one case for each (20%); Table 5.16 and Figure 5.14.

Non-smokers showed a higher percentage of MT 7% (1/15) compared to both current smokers 5% (3/63) and ex-smokers 5% (1/22). However, the relation between smoking status and MT was not significant (p=0.771; Chi-Square test); Table 5.49.

The rate of MT in females was seen equally in both current and non-smokers, whilst in males, MT was higher in current smokers, but MT was not seen in one male non-smoker.

Table 5.49: Malignant transformation (MT) in relation to smoking status.

MT status	Smoking status			Total
	Non-smoker	Current smoker	Ex-smoker	
MT-free	**14** *93%*	**60** *95%*	**21** *95%*	**95** *95%*
MT	**1** *7%*	**3** *5%*	**1** *5%*	**5** *5%*
Total	**15** *100%*	**63** *100%*	**22** *100%*	**100**

Alcohol Use

Patients undergoing MT were mainly current drinkers 60% (2 heavy drinkers and 1 light drinker), with the remaining 2 cases (40%) non-drinkers; Table 5.18 and Figure 5.17.

Non-drinkers demonstrated a higher rate of MT 14% (2/14) compared to current drinkers 4% (3/83), but the relation between drinking status and MT was not significant (p=0.255; Chi-Square test); Table 5.50.

Table 5.50: Malignant transformation (MT) in relation to drinking status.

MT status	Drinking status			Total
	Non-drinker	Current drinker	Ex-drinker	
MT-free	**12** *86%*	**80** *96%*	**3** *100%*	**95** *95%*
MT	**2** *14%*	**3** *4%*	**-**	**5** *5%*
Total	**14** *100%*	**83** *100%*	**3** *100%*	**100**

OSCC Development (new site from primary dysplasia)

Development of OSCC at new sites distant from the primary dysplasia was reported in only 2/100 of our study population (47 years female and 48 years male) at 55 months and 60 months following laser intervention.
Table 5.46 summarises the clinicopathological characteristics of the two patients in this group.

The total clinical management time was 84 and 90 months, whilst follow-up after laser intervention was 82 and 87 months. Patients in this group underwent only one observational biopsy, but between 5 to 13 follow-up biopsies after laser surgery which followed 2 to 6 laser interventions, with 23-39 follow-up appointments.

Both cases of OSCC occurred in the tongue, the ventral and lateral surfaces. Development of OSCC at sites distant from the primary dysplasia was only seen in patients affected with high grade dysplasia. One was seen in a patient who presented with a severe major sized dysplasia (945 mm^2), whilst the other one was an intermediate sized CIS (450 mm^2).

In addition to developing OSCC, these patients also suffered from recurrence and new-site dysplasia. One patient displayed 1 recurrence and 3 new-site dysplasias whilst the other patient developed 5 recurrences and 1 new dysplasia formation. Both patients were non-smokers and light drinkers (social drinkers) who consumed 4 units per week. The female patient was diabetic and the male was hypertensive with an immunodeficiency syndrome.

For statistical purposes and because of the small number of samples, cases with MT (n=5) and those who developed OSCC at distant sites from the primary dysplasia (n=2), were combined into one group for analysis.

Clinical Appearance

Risk estimate showed that non-homogenous leukoplakia increased the risk of developing oral cancer by 1.787 times compared with that for homogenous leukoplakia (95% CI, 0.317-10.07).

Smoking Behaviour

Patients who developed oral cancer smoked small amounts of tobacco compared to OSCC-free patients, in terms of both cigarettes per day (21.67 vs. 23.32) and also grams of tobacco per week (151.67 vs. 165.37), however the differences were not significant (p=0.881, p=0.987; Mann-Whitney U test, respectively).

Also, patients who developed oral cancer had a shorter smoking history compared with OSCC-free patients (27.50 vs. 34.04 months), with a lower pack-year score (27.50 vs. 41.56), but differences did not reach significance for either history of smoking (p=0.399) or pack-year score (p=0.356; Independent t-test).

Kaplan-Meier survival analysis showed that oral cancer-free survival rate 1-, 2 and 5-year after laser treatment were 97%, 95% and 83%, respectively; Figure 5.50.

Non-smokers experienced the shortest mean time to develop oral cancer (59.31 months) compared to both ex-smokers (86.90 months) and current smokers (137.56 months). Non-smokers who developed oral cancer showed a lower oral cancer-free survival rate 5-year post-laser surgery compared to the first-year (31% vs. 93%). Also, non-smokers with oral cancer showed a lower 5-years survival rates compared with current smokers (31% vs. 91%), however Log-Rank test was not significant (p=0.108); Figure 5.51.

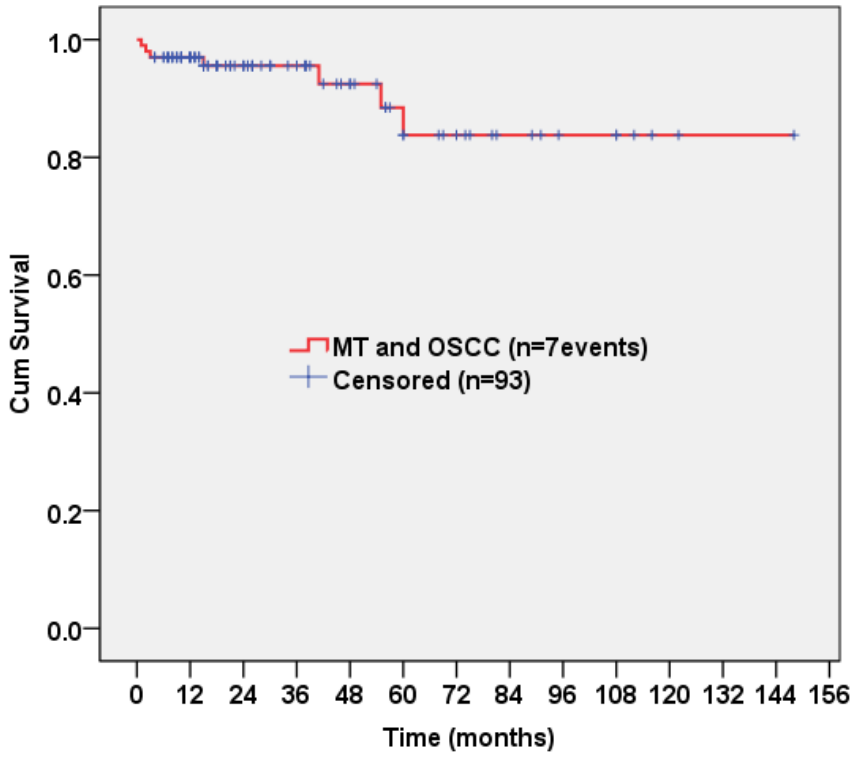

Figure 5.50: Oral cancer-free survival functions for patients by Kaplan–Meier analysis.

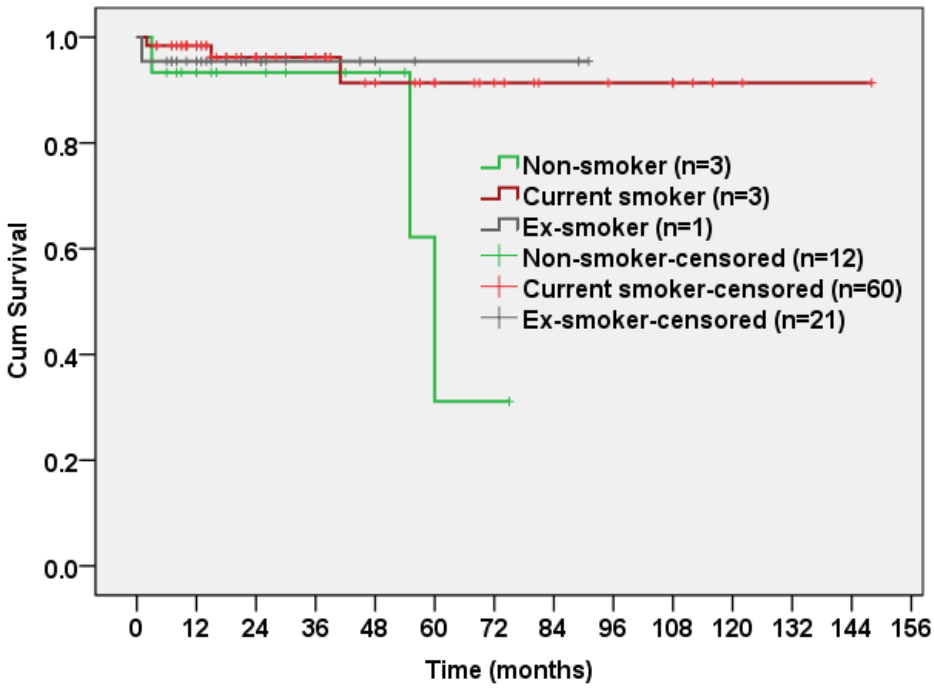

Figure 5.51: Kaplan-Meier analysis of patients with oral cancer in relation to smoking status.

Alcohol Use

Patients developing oral cancer consumed a higher amount of alcohol in terms of units per week compared with OSCC-free patients (38 vs. 29.54), although Mann-Whitney U testing was not significant (p=0.715).

Kaplan-Meier survival analysis showed that non-alcohol drinkers demonstrated a shorter mean time to develop oral cancer, compared with current drinkers but Log-Rank test was not significant (p=0.382); Figure 5.52.

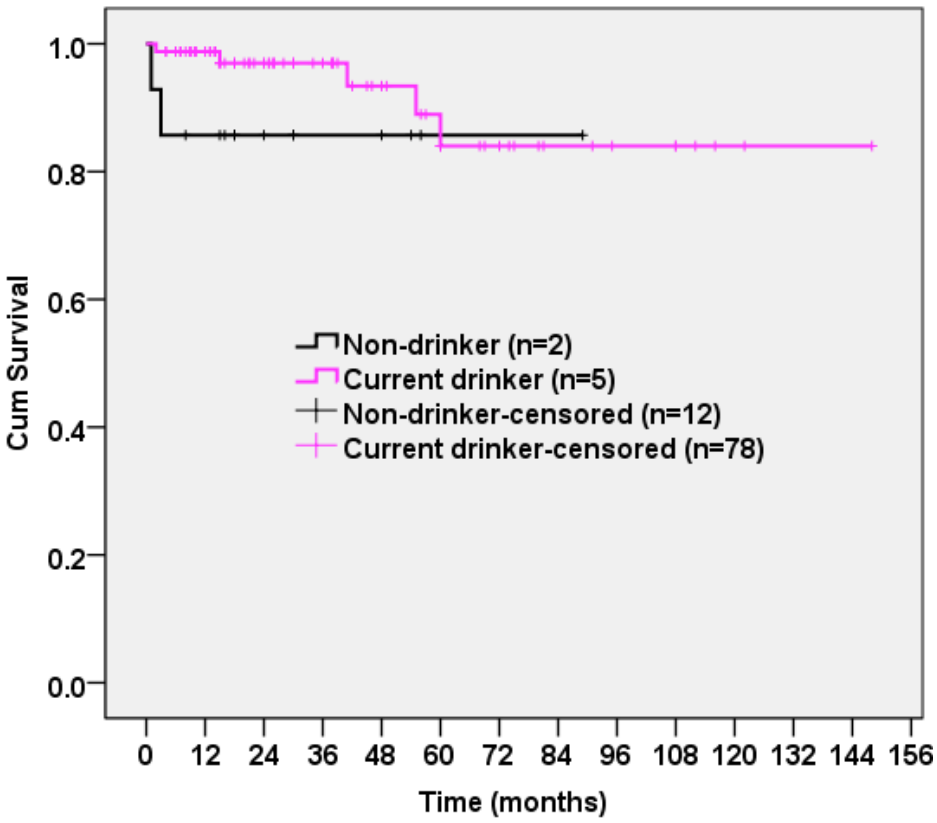

Figure 5.52: Kaplan Meier analysis of patients with oral cancer in relation to drinking status.

Logistic Regression Analysis

Logistic regression analysis was performed to explore important and significant predictors for oral cancer development. Because of the small number of samples and to facilitate regression analysis the size of dysplasia was entered as < 425 mm^2 and ≥ 425 mm^2 (equivalent to the third quartile), smoking habit was entered as either smokers (past and current tobacco users) or non-smokers, drinking was entered as drinkers (past and current alcohol drinkers) and non-drinkers, sites was entered as high risk (FOM and tongue) or low risk (the remaining oral sites).

Table 5.51 presents the included independent variables for oral cancer development in the logistic regression models.

Considering univariate statistical analysis, tobacco smoking was found to be the most significant prognostic factor for oral cancer to develop (p=0.049), whilst clinical type (erythroplakia) and size of PMDs (425 mm^2) were identified as marginally significant predictors (p=0.053). No other significant predictors were identified.

The best fit for the final model for multivariable regression depending on the -2 log likelihood ratio included: clinical type of PMD, resection margin status and smoking status. Although no significant predictors were identified from the multivariable models, non-homogenous leukoplakia was 3.147 times more likely than homogenous leukoplakia to develop oral cancer (95% CI, 0.174-56.659). Erythroplakia increased the risk for oral cancer to develop by 18.625 times compared with homogenous leukoplakia (95% CI, 0.733-473.301), although both were non-significant (p=0.438, p=0.076, respectively).

Table 5.51: Logistic regression models of oral cancer development.

Outcome	Risk factors	Uni-variable analysis		Multi-variable analysis	
		Odds (95% CI)	**p-value**	**Odds (95% CI)**	**p-value**
Cancer development (MT and OSCC)	Age	1.006 (0.947-1.067)	0.856		
	Sex				
	Females	Reference category			
	Males	0.667 (0.140-3.166)	0.610		
	Clinical types				
	Homogenous	Reference category			
	Non-homogenous	1.855 (0.291-11.816)	0.513	3.147 (0.174-56.959)	0.438
	Erythroplakia	**7.111 (0.987-51.258)**	**0.053**	18.625 (0.733-473.301)	0.076
	PMD site				
	Low risk site	Reference category			
	High risk site	1.644 (0.187-14.455)	0.654		
	Size of PMDs (mm^2)				
	< 425	Reference category			
	≥ 425	**4.733 (0.978-22.912)**	**0.053**		
	Histopathology				
	Low grade	Reference category			
	High grade	1.872 (0.327-10.737)	0.482		
	Margin status				
	Free-margin	Reference category			
	Dysplastic	0.375 (0.033-4.298)	0.431	0.414 (0.032-5.348)	0.499
	Smoking status				
	Non-smokers	Reference category			
	Smoker (past & present)	**0.198 (0.039-0.993)**	**0.049**	0.225 (0.012-4.100)	0.314
	Drinking status				
	Non-drinker	Reference category			
	Drinkers	0.385 (0.067-2.211)	0.284		

5.3.2. Analysis by Clinicopathological Profiling

Age and Sex

No significant association was found between clinical outcome and patient age (p=0.316; Chi-Square test), although middle age patients were predominant in all outcome groups and no OSCC development at distant sites was seen in patients younger than 40 years. Similarly, no significant association was found between the clinical outcome and sex (p=0.118; Chi-Square test), although males were most common in DF, recurrence, new-site dysplasia and MT categories.

Clinical Appearance

A significant relation was found between the clinical type of leukoplakia (homogenous and non-homogenous) and clinical outcome (p=0.016; Chi-Square test); non-homogenous leukoplakia showed a higher rate of recurrence 24% (6/25) compared to homogenous leukoplakia 15% (10/67), which was similar for new-site dysplasia formation 24%; 6/25 vs. 10%; 7/67 and MT 8%; 2/25 vs. 3%; 2/67; Table 5.10.

Anatomical Site

Table 5.12 and Figure 5.10 summarise the relation between anatomical site and clinical outcome, which was significant (p=0.020; Chi-Square test). At the most recent clinical follow-up, the majority of FOM cases 76% (35/46) were DF. However, FOM also showed an equal rate for recurrent disease of 29% (5/17) and new-site disease formation 29% (4/14).

Whilst no OSCC developed at distant sites in FOM cases, an equal MT rate was seen in the FOM and lateral tongue surface (2/5), followed by the retromolar region (1/5).

The ventral and lateral surfaces of the tongue were the only sites for the development of OSCC at distant site from the primary dysplasia. The lateral surface of the tongue showed a

MT rate of 11% (2/19), which also showed a 21% (4/19) recurrent disease and 5% (1/19) new-site dysplasia formation.

Half of the cases in the pillars of fauces were DF (2/4) with the other half developing new-site dysplasia formation (2/4); however, this site showed no recurrent disease or oral cancer development.

The soft palate showed the highest rate of DF cases 56% (5/9), with 33% (3/9) recurrence rate followed by new-site dysplasia formation 11% (1/9), but no cancer development was observed at this site. Buccal mucosa showed equal rates of DF and new-site dysplasia formation 40% (2/5), with a recurrence rate of only 20% (1/5).

In this study, the only reported case of retromolar area underwent MT, with the two cases of the alveolar mucosa were reported as a DF (2/2). No cancer development was seen in the buccal or alveolar mucosa.

Size of Dysplasia

A significant relation was found between clinical outcome and the size of presenting dysplastic PMD (p=0.009; Chi-Square test). The majority of DF cases were reported as minor size 52% (32/61). Also, minor size dysplasias showed the highest recurrence rate 44% (7/16). Dysplasia with intermediate size exhibited the highest rate in both MT 80% (4/5) and new-site dysplasia formation 64% (9/14).

The two dysplasia cases that developed OSCC at sites distant from primary dysplasia were major and intermediate in size; Table 5.13 and Figure 5.11.

Dysplasia Grading

A significant association was found between clinical outcome and the degree of dysplasia (p=0.004; Chi-Square test); Table 5.14 and Figure 5.12. Whilst mild and moderate dysplasia showed no development of OSCC at distant sites, severe dysplasia and CIS both showed

OSCC development with a higher percentage in CIS 9% (1/11), compared to 4% (1/23) in severe dysplasia. Mild dysplastic PMDs showed a higher rate of new-site dysplasia formation 14% (6/42) compared with the development of recurrent disease and MT rates, which were both seen in only 5% of cases (2/42). Moderate dysplasia showed a higher recurrence rate 25% (6/24) compared with new-site dysplasia formation 4% (1/24). Similarly, severe dysplasia showed a higher rate of recurrence 26% (6/23), followed by new-site dysplasia 22% (5/23), MT 9% (2/23) and OSCC development 4% (1/23). CIS cases showed a higher rate of recurrence 27% (3/11), followed by new-site dysplasia 18% (2/11), with MT and OSCC both occurring in 9% of cases (1/11).

Considering the binary grading system, a similar, significant relationship was found between clinical outcome and high/low grade dysplasia (p=0.004; Chi-Square test).
A higher rate of DF status was seen in patients with low grade dysplastic PMD compared to those with high grade dysplasia, 75% (35/47) vs. 51% (27/53).
High grade dysplasia showed a higher rate of recurrence 25% (13/53) compared with low grade dysplasia 9% (4/47). Similarly, new-site dysplasia was more common in high grade dysplasia 15% (8/53) compared with low grade dysplasia 13% (6/47).
Also, a higher rate of MT was seen in high grade dysplasia 6% (3/53) compared to low grade dysplasia 4% (2/47). Whilst no OSCC development at distant sites was seen in low grade dysplasia cases, the two OSCCs in this study arose in patients previously showing high grade dysplasia.

Smoking Behaviour

Table 5.16 and Figure 5.14 show the relation between smoking behaviour and clinical outcome. In this study, the two patients who developed OSCC were both non-smokers, but ex-smokers showed a higher rate of recurrence 23% (5/22) compared to new-site dysplasia formation 14% (3/22).

The two light smokers in this study exhibited recurrence and new-site dysplasia. Heavy smokers showed a lower rate of DF status compared with intermediate smokers, 57% (13/23)

vs. 71% (27/38). Heavy smokers also showed a higher recurrence rate (22%; 5/23) compared with intermediate smokers (11%; 4/38).
The majority of MT 40% (2/5) and new-site dysplasia formation 36% (5/14) were seen in intermediate smokers. A Chi-Square test showed no significant relation between clinical outcome and number of cigarettes smoked per day, however (p=0.139).

Regarding the length of smoking history, an equal distribution of both recurrence and MT was seen in patients with a long smoking history (31-50 years) and a relatively shorter smoking history (10-30 years). Increased smoking history was associated with a higher rate of new-site dysplasia compared to those with shorter smoking histories (67%; 4/6 vs. 33%; 2/6); Table 5.17. No significant relation was found, however, between smoking history and clinical outcome (p=0.565; Chi-Square test).

Alcohol Use

Table 5.18 and Figure 5.17 summarise the relation between alcohol drinking and clinical outcome. Ex-drinkers were all DF, but whilst non-drinkers showed no OSCC, a higher rate of recurrence 22% (3/14) compared to new-site dysplasia formation was seen 7% (1/14).

DF status was predominant in all groups of drinking, with the highest rate seen in light drinkers 44% (27/62), who also showed a higher rate of new-site dysplasia compared with recurrence (11%; 4/37 vs. 8%; 3/37). Intermediate drinkers also showed no OSCC development, although both recurrent and new-site dysplasias were observed equally in this group (17%; 2/12).

In contrast, a higher recurrence rate compared to new-site disease was observed in heavy drinkers (26%; 9/34 vs. 21%; 7/34). Also, heavy drinkers showed a much higher recurrence rate 26% (9/34) compared with light drinkers 8% (3/37). However, no significant association was found between clinical outcome and the amount of alcohol consumed, both at first presentation and at most recent clinical follow-up (p=0.267, p=0.277; Chi-Square test, respectively).

Dental Prosthesis Wear

A significant relation was found between wearing dental prosthesis and clinical outcome (p=0.004; Chi-Square test). The majority of non-dental prosthesis wearers 70% (33/47) were DF. Although OSCC was equally seen in both wearers and non-wearers (1/2), MT was only reported in dental prostheses wearers (5/5).

Whilst the recurrence rate was much higher in dental prostheses wearers 71% (12/17) compared to non-wearers 29% (5/17), the rate of new-site dysplasia formation was higher in non-wearers 57% (8/14) compared with wearers 43% (6/14); Table 5.19 and Figure 5.19.

Resection Margin Status and Clinical Outcome

Table 5.52 and Figure 5.53 display the resection margin status in relation to treatment outcome which was found to be statistically significant (p=0.001; Chi-Square test). DF was mainly seen in patients with clear, non-dysplastic margins 52% (30/58), whilst new-site dysplasia and recurrences were predominantly seen in cases with dysplasia present in excision margins (83%; 10/12) and (71%; 10/14), respectively.

MT was seen equally in both dysplasia-free and positive margins (1 for each), whereas OSCC development at distant sites was only seen in patients with clear margins.

Using Fisher's Exact test, no significant relation between margin status and recurrence status was seen (p=0.252), although the majority of recurrent cases (71%; 10/14) were seen in patients with dysplastic margins compared to 29% (4/14) in patients with clear resection margins. Similarly, 83% (10/12) of patients who developed new-site dysplasia showed dysplasia in resection margins, compared to 17% (2/12) with non-dysplastic margins, but this was not significant (p=0.060).

With respect to sex and resection margin status, males showed a higher rate of dysplastic margins compared to females (65%; 32/49 vs. 35%; 17/49), however the association was not significant (p=1.000; Fisher's Exact test).

Table 5.52: Resection margin status in relation to clinical outcomes.

Clinical Outcome	Resection margin status		Total
	Free-margin	Dysplastic	
DF	**30** *52%*	**28** *48%*	**58** *100%*
Recurrent disease	**4** *29%*	**10** *71%*	**14** *100%*
Further disease	**2** *17%*	**10** *83%*	**12** *100%*
MT	**1** *50%*	**1** *50%*	**2** *100%*
OSCC	**1** *100%*	**-**	**1** *100%*
Total	**38** *44%*	**49** *56%*	**87** *100%*

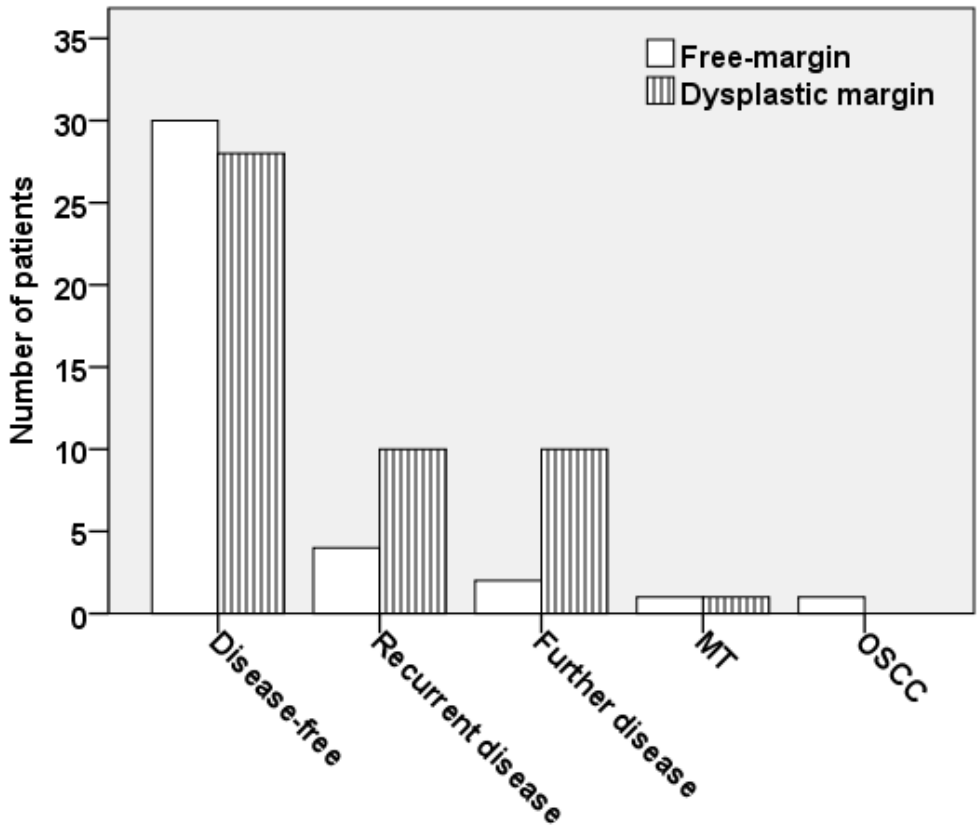

Figure 5.53: Resection margin status in relation to clinical outcomes.

Although dysplastic margins were equally reported for recurrent and new-site disease (10/20 for each), patients with dysplastic margins showed a higher proportion of new-site dysplasia formation compared with those exhibiting recurrence (83%; 10/12 vs. 71%; 10/14); but Chi-Square test was not significant, however (p=0.652); Table 5.53.

A significant association was found between the histopathological diagnosis of resection margin status and recurrence (p=0.004; Chi-Square test). Patients with recurrent disease were equally diagnosed with clear margins, or margins with moderate and severe dysplasia (29%; 4/14). Recurrence was equally seen in margins with mild dysplasia and CIS (7%; 1/14).
The majority of recurrence-free patients were diagnosed with clear margins, followed by margins with mild, moderate-severe dysplasia and CIS, respectively; Table 5.54.

Table 5.53: Recurrent and new site dysplasia in relation to margin status.

Margin status	Recurrent disease	Further disease	Total
Clear-margin	**4** *29%*	**2** *17%*	**6** *23%*
Dysplastic margin	**10** *71%*	**10** *83%*	**20** *77%*
Total	**14** *100%*	**12** *100%*	**26** *100%*

Table 5.54: Histopathology of surgical margin in relation to recurrence status.

Resection margin histopathological diagnosis	Recurrence status		Total
	Recurrence-free	Recurrence	
Clear-margin	**34** *47%*	**4** *29%*	**38** *44%*
Mild	**19** *26%*	**1** *7%*	**20** *23%*
Moderate	**9** *12%*	**4** *29%*	**13** *15%*
Severe	**9** *12%*	**4** *29%*	**13** *15%*
CIS	**2** *3%*	**1** *7%*	**3** *3%*
Total	**73** *100%*	**14** *100%*	**87** *100%*

A significant relation was found between new-site dysplasia and the histopathology of resection margins (p=0.050; Chi-Square test). A higher rate of new-site dysplasia were reported in cases which showed severe dysplasia in excision margins (33%; 4/12), whilst patient free from new-site dysplasia had predominantly clear-surgical margins (48%; 36/75); Table 5.55.

Table 5.55: Histopathology of surgical margin in relation to the new site dysplasia status.

Resection margin histopathological diagnosis	New site dysplasia status		Total
	New dysplasia-free	New dysplasia	
Clear-margin	**36** *48%*	**2** *17%*	**38** *44%*
Mild	**17** *23%*	**3** *25%*	**20** *23%*
Moderate	**11** *15%*	**2** *17%*	**13** *15%*
Severe	**9** *12%*	**4** *33%*	**13** *15%*
CIS	**2** *3%*	**1** *8%*	**3** *3%*
Total	**75** *100%*	**12** *100%*	**87** *100%*

Table 5.56 compares recurrent (same site) disease and further (new-site) dysplasia formation in relation to histopathology of resection margins; a higher rate of new-site dysplasia formation was reported in patients with severe dysplasia in the margins 33% (4/12), whilst a higher recurrence rate was seen equally frequently in moderate and severely dysplastic margins 29% (4/14).

Lesions with surgical margins showing mild dysplasia were more at risk of developing new-site dysplasia formation than having recurrences (25%; 3/4 vs. 7%; 1/4), whereas clear margins showed a higher recurrence rate than new dysplasia formation (29%; 4/6 vs. 17%; 2/6); but Chi-Square test was not significant, however (p=0.216).

Table 5.56: Histopathology of surgical margin in relation to recurrent and new site dysplasia formation.

Resection margin histopathological diagnosis	Recurrent disease	Further disease	Total
Clear-margin	**4** *29%*	**2** *17%*	**6** *23%*
Mild	**1** *7%*	**3** *25%*	**4** *15%*
Moderate	**4** *29%*	**2** *17%*	**6** *23%*
Severe	**4** *29%*	**4** *33%*	**8** *31%*
CIS	**1** *7%*	**1** *8%*	**2** *8%*
Total	**14** *100%*	**12** *100%*	**26** *100%*

Further statistical analysis was performed considering the association between clinical outcome and the extent of residual dysplasia in resection margins. One margin involvement was the most common (30/49), followed by four margins (8/49); an equal presentation of one margin involvement was seen in cases with DF and DA status; Table 5.57. One margin involvement was equally seen in patients with recurrent and new-site dysplasia formation; Table 5.58.

No significant association was found between extent of dysplasia in the margins and the clinical outcome (DF/DA) (p=0.163) and as DF, recurrent disease, further disease and MT (p=0.388; Chi-Square test). This suggests that the extent of resection margin involvement has no influence on the clinical outcome of patients.

Table 5.57: Disease-free and disease active in relation to the extent of dysplasia in margins.

Resection margins involvement status	Clinical outcome		Total
	DF	DA	
One/margin	**15** *50%*	**15** *50%*	**30** *100%*
Two/margins	**5** *83%*	**1** *17%*	**6** *100%*
Three/margins	**3** *60%*	**2** *40%*	**5** *100%*
Four/margins	**5** *63%*	**3** *38%*	**8** *100%*
Total	**28** *57%*	**21** *43%*	**49** *100%*

Table 5.58: Clinical outcomes in relation to the extent of dysplasia in margins.

Resection margins involvement status	Clinical outcome				Total
	DF	Recurrent disease	Further disease	MT	
One/margin	**15** *50%*	**7** *23%*	**7** *23%*	**1** *3%*	**30** *100%*
Two/margins	**5** *83%*	**1** *17%*	**-**	**-**	**6** *100%*
Three/margins	**3** *60%*	**-**	**2** *40%*	**-**	**5** *100%*
Four/margins	**5** *63%*	**2** *25%*	**1** *13%*	**-**	**8** *100%*
Total	**28** *57%*	**10** *20%*	**10** *20%*	**1** *2%*	**49** *100%*

Logistic Regression Analysis

In order to investigate important predictors of positive dysplastic resection margins, logistic regression analysis was performed; Table 5.59. Univariate analysis showed that age (OR=0.987, 95% CI, 0.955-1.020) (p=0.429) and sex had no influence on dysplastic margins to occur (OR=1.022, 95%, CI 0.401-2.388) (p=0.962).

The appearance of non-homogenous leukoplakia increased the risk for dysplastic margins to occur by 1.750 times compared with homogenous PMDs (95% CI, 0.619-4.948), but this was not significant (p=0.291).

A reduced risk of approximately 38% was observed in tongue PMDs compared to the FOM (OR=0.619, 95% CI, 0.231-1.658), but this was not significant (p=0.340).
Size of dysplastic PMDs, between 200 to 600 mm^2, was a significant predictor for dysplastic margins to occur compared with smaller sizes (< 200 mm^2) (p=0.010), with an increased risk of dysplastic margin 3.518 times higher than smaller sizes (95% CI, 1.345-9.203). Dysplasia > 600 mm^2 had a 2.706 times higher risk than minor sized PMDs (95% CI, 0.591-12.385), but this was not significant (p=0.200).

Regarding degree of dysplasia, severe dysplasia-CIS showed a 2.118 times increased risk for dysplastic margins occurrence, compared with mild dysplasia (95% CI, 0.749-5.986), but this was not significant (p=0.157). Moderate dysplasia exhibited a 1.529 times higher risk for dysplastic margins to occur compared with mild dysplasia (95% CI, 0.521-4.494), but this was also not significant (p=0.440).

The multivariate logistic regression final model selection included the clinical type of leukoplakia, anatomical site, size, degree of dysplasia and sex. These factors were considered the most important to be associated with the presence of residual dysplasia in surgical margins. However, size was the only significant predictor identified in both univariable and multivariable regression analyses. Size of dysplasia exceeding 600 mm^2 increased the risk for dysplastic margins to occur by 12.247 times, compared with minor sizes (< 200 mm^2) (p=0.026). Also, sizes between 200 to 600 mm^2 exhibited an increased risk for dysplastic margins to occur by 5.875 times compared with minor sizes (p=0.006).

Table 5.59: Logistic regression models for resection margin with dysplasia.

Outcome	Risk Factors	Uni-variable analysis		Multi-variable analysis	
		Odds (95% CI)	p-value	Odds(95% CI)	p-value
Resection margin status Dysplastic margin	Age	0.987 (0.955-1.020)	0.429		
	Sex				
	Females	Reference category			
	Males	0.979 (0.401-2.383)	0.962	0.362 (0.102-1.281)	0.115
	Leukoplakia types				
	Homogenous	Reference category			
	Non-homogenous	1.750 (0.619-4.948)	0.291	0.649 (0.176-2.400)	0.517
	PMDs site				
	FOM	Reference category			
	Tongue	0.619 (0.231-1.658)	0.340	0.302 (0.080-1.138)	0.077
	Remaining sites	1.000 (0.334-2.991)	1.000	2.813 (0.541-14.618)	0.219
	Size of PMDs (mm^2)				
	Minor < 200	Reference category			
	Intermediate (200-600)	**3.518 (1.345- 9.203)**	**0.010**	**5.875 (1.668-20.701)**	**0.006**
	Major > 600	2.706 (0.591- 12.385)	0.200	**12.247 (1.350-111.090)**	**0.026**
	WHO histopathological diagnosis				
	Mid	Reference category			
	Moderate	1.529 (0.521-4.494)	0.440	1.246 (0.348- 4.458)	0.735
	Severe-CIS	2.118 (0.749- 5.986)	0.157	1.408 (0.388- 5.106)	0.603

Medical History and Clinical Outcome

A significant relation was found between systemic health status (having a systemic disease or not) and clinical outcome (p=0.022; Chi-Square test). At the most recent follow-up, the majority of patients with a clear medical history (92%; 11/12) were reported as DF, compared to 58% (51/88) of patients with a positive medical history.

All patients who developed oral cancer (7/7), new-site dysplasia (14/14) and 94% (16/17) of recurrent cases and 82% (51/62) of DF patients, were seen to have a positive medical history, having one or more chronic, systemic diseases; Table 5.60.

Table 5.60: Clinical outcome in relation to medical history.

Medical history status	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
Negative	**11** *92%*	**1** *8%*	-	-	-	**12** *100%*
Positive	**51** *58%*	**16** *18%*	**14** *16%*	**5** *6%*	**2** *2%*	**88** *100%*
Total	**62**	**17**	**14**	**5**	**2**	**100**

A much higher recurrence rate was observed in patients with a positive medical history (94%; 16/17), compared to systemic disease-free patients (6%; 1/17). However, no significant association was found between the presence of a systemic disease and recurrence status (p=0.472; Fisher's Exact test); Table 5.61.

Table 5.61: Medical history in relation to recurrence status.

Medical history status	Recurrence status		Total
	Recurrence-free	Recurrence	
Negative	**11** *13%*	**1** *6%*	**12** *12%*
Positive	**72** *87%*	**16** *94%*	**88** *88%*
Total	**83** *100%*	**17** *100%*	**100**

Table 5.62 shows the distribution of the systemic diseases according to clinical outcome. Out of the 60 hypertensive patients, 33 (55%) were DF, 13 (21%) underwent recurrence, 9 (15%) showed new-site dysplasia, 4 (7%) underwent MT and only one case (2%) developed OSCC at a site distant from the primary dysplasia.

Table 5.62: Systemic disease in relation to the clinical outcome.

Medical disease	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
Immunodeficiency	**-**	**-**	**-**	**-**	**1**	**1**
Anaemia	**3**	**1**	**-**	**-**	**-**	**4**
Diabetes mellitus	**11**	**2**	**-**	**1**	**1**	**15**
Candida infection	**3**	**2**	**3**	**-**	**-**	**8**
Hypertension	**33** *55%*	**13** *21%*	**9** *15%*	**4** *7%*	**1** *2%*	**60** *100%*

Comparing *hypertensive* with normotensive patients, a higher rate of MT, recurrence and new-site dysplasia formation were all found in hypertensive patients, whilst OSCC development was seen equally in both hypertensive and normotensive patients; Table 5.63. However, no significant association was found between clinical outcome and hypertension status (presence or absence of hypertension) (p=0.060; Chi-Square test).

Table 5.63: Clinical outcome in relation to hypertension.

Hypertension status	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
Normotensive	**29** *73%*	**4** *10%*	**5** *13%*	**1** *3%*	**1** *3%*	**40** *100%*
Hypertensive	**33** *55%*	**13** *22%*	**9** *15%*	**4** *7%*	**1** *2%*	**60** *100%*
Total	**62**	**17**	**14**	**5**	**2**	**100**

Considering *diabetes mellitus* (DM), a significant relation was found between having DM or not and clinical outcome (p=0.024; Chi-Square test).
A higher rate of DF was seen in diabetics; out of 15 diabetics, 73% (11) were reported as DF. Recurrence was seen in 13% (2/15) of diabetics at 12 and 30 months following laser treatment, whilst MT and OSCC development were seen equally (7%; 1/15) at 2 months and 55 months after laser treatment, respectively. Diabetics showed no new-site dysplasia formation.

Comparing diabetic and diabetic-free patients, higher proportions of MT and OSCC development were observed in diabetics compared to non-diabetics (7% vs. 5%), (7% vs. 1%) respectively, whereas a lower recurrence rate was seen in diabetics compared to non-diabetic patients (13% vs. 18%); Table 5.64.

No further statistical analysis was performed because of the small numbers of cases in the related subgroups.

Table 5.64: Clinical outcome in relation to diabetes mellitus.

Diabetes mellitus status	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
Diabetes-free	**51** *60%*	**15** *18%*	**14** *16%*	**4** *5%*	**1** *1%*	**85** *100%*
Diabetic	**11** *73%*	**2** *13%*	**-**	**1** *7%*	**1** *7%*	**15** *100%*
Total	**62**	**17**	**14**	**5**	**2**	**100**

With respect to *candida infection*, in this study, amongst 8 patients with positive candida infection, no MT or OSCC development was seen.

At the most recent follow-up, 3 out of the 8 patients were DF, whilst 3 developed new-site dysplasia at 7.45, 60 and 18 months post-laser intervention, and the remaining 2 exhibited recurrent disease at 20 and 30 months postoperatively.

Patients with candida infection exhibited a higher rate of new-site dysplasia (3/8) compared to recurrence (2/8). However, no significant relationship was found between candida infection and clinical outcome (p=0.164; Chi-Square test); Table 5.65.

Table 5.65: Clinical outcome in relation to candida infection.

Candida infection status	Clinical outcome					Total
	DF	Recurrent disease	Further disease	MT	OSCC	
Candida infection-free	**59** *95%*	**15** *88%*	**11** *79%*	**5**	**2**	**92** *92%*
Candida infection	**3** *5%*	**2** *12%*	**3** *21%*	**-**	**-**	**8** *8%*
Total	**62** *100%*	**17** *100%*	**14** *100%*	**5** *100%*	**2** *100%*	**100**

In this study, *anaemia* was seen in 4 cases, 3 of them were DF at the most recent follow-up and 1 (a 48 year old male) underwent recurrence at 20 months after laser surgery. This patient was diagnosed with minor size (150 mm^2) moderate dysplasia which presented as an exophytic leukoplakia in the buccal mucosa. The patient was a heavy smoker (40 cigarettes/day), and a heavy drinker (70 units/week) and was also affected by candida infection.
No significant relation was found between the presence of anaemia and clinical outcome (p=0.243; Chi-Square test).

Regarding *family cancer history*, out of 10 patients who reported a negative familial history, seven were DF, 2 had recurrence and 1 developed new-site dysplasia, whilst no cancer development was seen.

On the other hand, out of 5 patients with a positive cancer history, 2 were DF, 2 developed recurrence and 1 exhibited new-site dysplasia formation. However, no significant relation between familial cancer history status (negative or positive) and clinical outcome was found (p=0.668, Chi-Square test).

No further statistical analysis was conducted because of the small number in these related subgroups.

In the current study there was only one patient who suffered from *immunodeficiency* who developed OSCC at a site distant from the primary dysplasia, at 60 months following laser intervention. This patient presented with a 945 mm^2 sized erythroplakia, diagnosed initially with moderate dysplasia in the incisional biopsy and severe dysplasia in the excisional biopsy. Although suffering from hypertension, this patient was a non-smoker and a light drinker (4 units/week). In addition, this patient underwent 5 recurrences and one new-site dysplasia formation.

5.4. Discussion

Investigating treatment outcomes requires standardisation of the length of follow-up time and a defined study population (van der Waal and Axell, 2002; Holmstrup et al., 2006).

In this study, our hospital-based 100 patients were diagnosed with single oral epithelial dysplastic lesions, underwent laser intervention, and were followed-up by the same team in Newcastle Oncology and Dysplasia Clinics.

5.4.1. Analysis by Clinical Outcome

Time Considerations

The overall clinical management time in this study was 189 months (15.75 years) with an average of 71.86 months (5.98 years).

The average time between the initial presentation and incisional biopsy was 1.74 months with a maximum time of 24 months (2 years). In this study, 52% of patient undergoing same day incisional biopsies as first presentation and 39% within the first 3 months of presentation, however a delay of more than one year was reported in 3 cases, which was mainly due to patients' delays.

This may suggest a greater degree of suspicious appearance of the majority of cases attended the Oncology/Dysplasia Clinics at their first presentation and/or the efficient management protocol used in this clinic for patients with PMDs.

The time from first presentation to incisional biopsy showed no significant differences among different grades of dysplasia. Of the 3 cases who underwent initial diagnostic delay, one showed mild dysplasia, one moderate and one severe dysplasia.

The time from the first presentation to incisional biopsy showed no influence on treatment outcomes (no significant differences between the different treatment outcomes) which was supported by the fact that a DF state was seen in all cases that had experienced delay in their incisional biopsies.

The time between the initial diagnostic biopsy and laser excision (definitive diagnosis) was significantly associated and correlated with the degree of dysplasia; the higher the severity of dysplasia confirmed histopathologically the shorter the waiting time for laser intervention. The majority of moderate, severe dysplasia and CIS patients had a short waiting time compared with the majority of mild dysplasia cases which had a delay of more than one year to have their laser interventions. Despite the attempts to modify risk factors behaviour in these apparently low risk cases, continued to tobacco and alcohol consumption and the resultant persistent of oral lesions ultimately led to formal laser excision. During these periods of observation it was also observed that a much higher number of observational biopsies, which found to have a negative significant correlation with degree of dysplasia; with the majority of observational biopsies were reported in cases of mild dysplasia (73%), compared to higher grades dysplasias (27%).

Since epithelial dysplasia is considered one of the most important predictors of MT (Reibel, 2003), and the association between MT and the presence of dysplasia is significant (Schaaij-Visser et al., 2010), early removal of dysplasia is the fundamental aim to prevent MT. It is increasingly accepted that the higher the severity of epithelia dysplasia, the more tendency for MT and this explains the short waiting time to laser surgery after initial diagnosis for cases with high grade dysplasias.

In general, the study showed that patients with disease active state showed a significantly shorter mean time to have laser surgery after incisional biopsy as compared to disease-free patients. In particular, new-site dysplasia formation experienced a significantly shorter mean time to laser intervention compared to patients free from new-site dysplasia. This may be due to the fact that 50% of patients with new dysplasia formation were diagnosed primarily with high grade dysplasia: severe dysplasia 36% (5/14) and CIS 14% (2/14) which obviously require early laser intervention than lower grade dysplasia depending on the general accepted rule of more dysplasia severity more MT rate. Also, 64% of these cases were relatively large sized disorders (200-600 mm^2) which require early treatment.

With respect to sex, males experienced a shorter mean time to have their laser surgery compared to females, but this was not significant. This may be due to the fact that 55% of males were diagnosed with high grade dysplasia compared to females, and thus suggesting

higher risk of disease active state compared to females (OR=1.45). Males were also more likely to develop both recurrences and new-site dysplasia (76% and 71%), respectively. In light of these findings male patients may need to have their laser surgery arranged in a shorter time period compared to females.

Follow-up was measured from the time of definitive diagnosis to the most recent clinic follow-up of patients (Jaber, 2010). Although no universally accepted guidelines are available with respect to duration and period of intervals of follow-up appointments, lifelong follow-up is generally advised for PMD patients both for surgically treated and non-treated cases (supervised) (van der Waal and Axell, 2002; Weijers et al., 2008; Jaber, 2010).

In the current study all patients were reviewed on a regular basis post-laser intervention, at varying intervals of 2, 3, 4, 6 or 12 months based upon both clinical and pathological features, to monitor the clinical course of disease and patients' outcome.
At each follow-up appointment, updating of the medical history of patients and review of risk factors, disease status determined clinically and via follow-up biopsy if appropriate was confirmed.

Patients in this study were followed-up for up to 183 months (15.25 years) after laser intervention with a mean of 55.49 months (4.62 years). Patients who developed recurrences demonstrated a significantly longer average follow-up time compared to those who were recurrence-free which was similar for patients suffering from new-site dysplasia compared to those who were new-site dysplasia-free. The extended surveillance duration for DA patients (recurrence and new-site dysplasia) suggests a more complicated clinical course in these patients.

Although development of OSCC at sites distant from the primary dysplasia experienced a longer follow-up time compared to OSCC-free patients (79.50 vs. 54.54 months), the differences did not reach statistical significance, probably because of the very small number of patient in this group (2/100).

MT cases showed a shorter mean follow-up time as opposed to MT-free patients, however this was non-significant. There is no clear reason why the transformed cases demonstrated a

shorter follow-up time compared to patients free from MT, unless this observation highlight inherent instability of the oral mucosa in patients at high risk of neoplastic transformation.

Clinical Outcome

Considering treatment outcomes, time intervals were calculated from the time of laser intervention to the subsequent event of interest, such as recurrent (same site) disease, further (new-site) dysplasia, MT or development of OSCC at a site distant from the primary dysplasia.

After laser intervention and at the most recent follow-up appointment there were 62 disease-free patients with complete clinical resolution, with a mean follow-up time of 38.87 months (3.24 years) (range, 4-122 months) (0.4-10.16 years). This finding is quite similar to a previous study performed by Thomson *et al.* (2008) on 40 patients with single dysplastic PMDs treated by laser excision, in which 24 patients (60%) were disease-free after laser intervention.

A large retrospective study used the CO_2 laser to treat 282 leukoplakias in 200 patients and achieved a cure rate of 89% during a mean follow-up period of 52 months (4.3 years, range 1-219 months). This high "cure" rate, however, was related to the fact that approximately 64% of cases in this study were actually non-dysplastic with only 36% showing dysplasia (van der Hem et al., 2005).

In the present study, 75% of patients were disease-free 2-years after laser treatment which dropped to 63% and 47%, at 3-years and 5-years postoperatively. In an Australian study, with a mean follow-up time of 47 months (3.91 years), disease-free survival 3-year postoperatively was 55.4% which dropped to 33.9%, 5 years after treatment (Chandu and Smith, 2005).
The marked fall in disease-free survival after 5-years in this study was associated with long-term recurrence, the longest of which occurred at 183 months (15.25 years, with a range of 13-183 months). This emphasises the need for long-term follow-up of patients and this has been noted in several previous studies (Chu et al., 1988; Roodenburg et al., 1991; Schoelch et al., 1999; Thomson and Wylie, 2002; Chandu and Smith, 2005).

In a systematic review with meta-analysis of treatment and follow-up of oral dysplasia, Mehanna *et al*. (2009) suggested that after diagnosis, patients should be kept under surveillance for up to 20 years. It may be feasible to tailor the duration and frequency of this surveillance, depending on clinicopathological factors including the grade of dysplasia and the type of management.

In this study, significantly lower disease-free survival status was seen in high grade dysplasia compared to low grade dysplasia at 2 years (68% vs. 83%) and 5 years (29% vs. 63%) postoperatively. This was supported by a significantly shorter mean time for high grade dysplasia to develop further disease compared to low grade dysplasia (5.33 vs. 7.39 years).
This clearly reflects an association between dysplasia severity and the time to develop disease active state, with a much shorter the time for disease active state development with more severely dysplastic tissue.

Logistic regression analysis was employed to predict the significant factors for disease active status. Univariate analysis revealed that high grade dysplasia, localization in the tongue, the clinical appearance of non-homogenous leukoplakia, dysplasia present in resection margins, size of dysplasia exceeding 600 mm^2 and patients with a positive history of systemic disease were strongly associated with active disease. Subsequently, the final model of multivariate regression analysis identified CIS-severe dysplasia and dysplastic margins as the most significant predictors of further disease.
This may reflect the importance of these parameters in the prediction of unsuccessful treatment outcome and emphasises urgent intervention and close follow-up of patients with high grade dysplasia and dysplasia in resection margins.

Recurrent (same site) Disease

One of this study's aims was to investigate the recurrence prevalence and to identify significant clinicopathological predictors associated with an increased risk for local failure or recurrence in a cohort of patients with PMDs.

Local recurrence was defined as reappearance of dysplasia in the same site of the primary dysplasia which has been previously successfully treated by laser and whose recurrence was then confirmed by a follow-up biopsy.

Follow-up data were obtained, including pathologically proven recurrence and time to first recurrence, which was defined as the time period between the date of laser surgery and the date of recurrence. After surgical excision, recurrences may occur at the same site of the primary dysplasia, regardless of the duration of time between laser excision and the recurrence (van der Waal and Axell, 2002). The overall recurrence rate in this study was 17%, 14-183 months (1.16-15.25 years) after laser intervention, with a mean follow-up of 83.75 months (~7 years). This is similar to a study conducted by Yang *et al.* (2011) to evaluate factors associated with recurrence in patients who received laser surgery for dysplastic leukoplakia who found a recurrence rate of 17.5%, 1.75-9.1 years (21-109.2 months) after treatment with a mean follow-up time of 3.4 years (40.8 months).
Also, this finding is similar to another recent study conducted by Jerjes *et al*. (2012) on the CO_2 laser of oral dysplasia with a recurrence rate of 19.5% after laser surgery and with patients followed-up for a mean of 6.4 years (76.8 months). This emphasises the long-term nature of recurrences in oral PMDs and necessitates close and long term follow-up to manage early recurrent disease.

The rate of recurrence is different between different studies. These variations may be attributed to different surgical techniques, such as the precise modality of laser beams, different follow-up times, patient factors (Frame, 1985; Ishii et al., 2004), differences in reporting recurrence per patient or per lesion, and different definitions of recurrent disease. Thus, comparing the recurrence rates between different studies is problematic, difficult and sometimes unfeasible (Yang et al., 2011).

Reporting recurrence per patient may result in higher rates of recurrences than might be expected as one patient may be affected by multiple PMDs, only one of which may actually undergo recurrence (Chandu and Smith, 2005). However, this study investigation was presented to single PMD disease so that recurrence was reported per patient not per lesion, to avoid any controversy regarding patient/lesion numbers and recurrence rates.

A comparable study was conducted by Thomson *et al.* (2008) in 40 patients with single dysplastic PMDs treated by laser excision. Outcome was classified as no recurrence or further disease which included recurrences at the same site or new disease at distinct intraoral sites. 24 patients had no recurrence after laser intervention, while 16 developed further disease, 7 (18%) at new intra oral sites and 9 with same site recurrence (23%). The most episodes of further disease occurred during the first 18 months following treatment.

To assess the clinical usefulness of CO_2 laser surgery for OL, Ishii *et al.* (2004), stated that the rate of recurrence in laser surgery was 29.3% over a mean time period of 3.4 years (0.6-11.3 years). The higher recurrence rate compared to the present study may be due to the use of laser vaporization technique for the majority of cases (98/154) restricting laser excision to only 14 cases. The author concluded that vaporization technique is very suitable for non-dysplastic homogenous leukoplakia on the gingiva which is anatomically coved by 1-1.5 mm thick gingiva with a low risk of MT, whilst laser excision was indicated for non-keratinized sites.

In this study, with the exception of three cases, all dysplastic PMDs were excised using CO_2 laser with additional 5 mm margins outside the obvious clinical edge of each lesion. By defocusing the laser beam, laser vaporization can be used to treat some dysplasia which cannot be excised or accessed easily, such as those in the gingiva or hard palate (Thomson and Wylie, 2002). However, the risk of laser vaporization is that pathological tissue may not be completely removed (Pinheiro and Frame, 1996) and also it prevents acquisition of a postoperative specimen for histopathological assessment leaving the decision about complete resection and more definitive diagnosis uncertain.

A large retrospective study conducted by van der Hem *et al.* (2005) who studied a group of 200 patients with 282 OLs reported a 10% local recurrence rate in a mean period of 87 months (5-168 months) post-laser treatment which were confirmed by biopsy, but 63% of specimens were originally non-dysplastic. This finding is quite similar to previous studies, such as a study conducted by Chu *et al.* (1988) with an initial recurrence rate of 10.8% (4/37) of leukoplakias varied from hyperkeratosis to CIS in an average time of 5 years and a follow-up ranged from 3 to 10 years. Roodenburg *et al.'s* (1991) study with a recurrence rate of 10%

in 70 patients with 103 OLs followed for an average of 3.9 years after operation but again 68% of treated cases were non-dysplastic.

However, a recent study conducted by Lim *et al.* (2010) showed a higher crude recurrence rate of 39.5% in an average time of 26.16 months (2-80 months) in CO_2 laser treated patients with different dysplastic grades, lichenoid changes and hyperplasia and actinic changes. The higher recurrence rate may possibly due to the wide range of different oral disorders that were included.

Different explanations for local recurrences have been suggested: recurrence may occur, according to Lim *et al.* (2010) if deep nests are left in place after any surgical treatment, so it is important to remove the full thickness of epithelium during laser surgery (Cantarelli Morosolli et al., 2006).

Frame *et al.* (1985) hypothesized that recurrence of leukoplakia following excision might be due to migration of new epithelium from the periphery of the wound originating from surrounding, potentially unstable oral mucosa explaining the recurrence of dysplastic leukoplakia via the concept of field change cancerization (Lim et al., 2010).

Clinically, oral epithelium within the area of field change might appear morphologically normal and in such patients, recurrence may be inevitable, regardless of treatment methods, because the surgical margins might be located within a wider area of field change (Thomson and Wylie, 2002; Chandu and Smith, 2005).

Genetically altered epithelial cells uncovered by routine histopathological examination and even in areas with normal histology (Tabor et al., 2003) might explain why recurrence is almost unavoidable (Yang et al., 2011).

Some investigators have advocated the use of vital staining, such as toluidine blue (Martin et al., 1998; Onofre et al., 2001) or iodine (Nakanishi et al., 1997) before laser intervention to ensure clear resection margins and to reduce local recurrences and MT, but little benefit of these vital staining techniques has been reported (Kerawala et al., 2000) as well as alterations in the amount of the energy absorbed from the laser in dyed tissue which may reduce its effectiveness (Ishii et al., 2003).

This study showed that the mean time to the first recurrence was 28.47 months (2.37 years), whilst the mean time to the second recurrence was 40.875 months (3.4 years). Sixty-five percent (11/17) of recurrent cases developed within the first 2 years (24 months) following

laser interventions, with the remaining 35% (6/17) arising after 2 years. These findings are consistent with Thomson *et al.*'s (2008) study which found that most episodes of further disease occurred during the first 18 months following laser treatment.
These observations are also quite similar to the findings of a study conducted by Chandu and Smith (2005) who reported a mean time to first recurrence/new lesion of 19.1 months (~1.6 year) and the mean time for a second recurrence of 22.5 months (~1.88 year).

The average total clinical management time (1st presentation to the most recent follow-up) of patients with recurrence was 108.25 months (9 years), which was significantly longer than that in the DF group (57.95 months), with more frequent follow-up appointments (9-78) vs. (5-42).
Compared to DF patients who underwent only one laser surgery and one-fifth of the follow-up biopsies, only 35% of patients in this group underwent one laser surgery, whilst the remaining 65% experienced up to 4 laser interventions and up to10 follow-up biopsies.
This may suggest the problematical clinical course of this group of patients and the need of longer and close follow-up protocol. This is supported by what has been previously reported by Chiesa *et al.* (1990) that recurrence rate of leukoplakia increased as time passed with higher risk of malignant potential.

In this study, males suffered from a higher rate of recurrent disease (76%; 13/17) compared with females. This finding was further supported by univariate regression analysis, which demonstrated an increased the risk of 1.8 times in males compared to females, although this was not statistically significant. Similarly, in a study conducted by Jerjes *et al*. (2012) out of 15 patients who underwent recurrence, 13 (86%) were males with only 2 females.
There is no obvious reason why males have a higher rate of recurrence compared to females, but it may be influenced by the fact that the 65% of male cases exhibited dysplasia in resection margins. Although recurrence of oral epithelial dysplasia had a male predominance, females did exhibit a shorter (albeit non-significant) mean time to develop recurrence.

Overall, a significant relation was found between the site of origin of dysplasia and clinical outcome classified as DF, recurrence, new-site dysplasia, MT and OSCC, although only borderline significance was found when considering recurrent disease and anatomical site.

The FOM and tongue showed the higher rate of recurrences, whilst buccal mucosa showed the least number of recurrences.

These findings are consistent with a study conducted by Thomson *et al.* (2008) who found no significant association between clinical outcome, and site of presenting lesion. Also, Jerjes *et al*. (2012) in a prospective study of 123 oral dysplasias from 77 consecutive patients treated with CO_2 laser resection and/or ablation found no association between the rate of recurrence and the location of dysplasia affecting ventral tongue, buccal mucosa or retromolar sites. The difference in oral sites affected by recurrence of dysplasia may be related to different study populations and varying risk factor and lifestyle habits.

Non-homogenous leukoplakia showed a higher recurrence rate compared to homogenous (24%; 6/25 vs. 15%; 10/67). This was supported by a higher risk of recurrence (OR=1.8 times) in non-homogenous compared to homogenous leukoplakia, which was further supported by the results of a Kaplan-Meier survival analysis that showed a shorter mean time to recurrence of non-homogenous leukoplakia cases compared to homogenous ones (63.6 vs. 123 months), although this did not reach significance. The higher recurrence rate in non-homogenous leukoplakia is in agreement with other recent studies. Yang *et al.* (2011) investigated treatment outcomes for dysplastic OL and found that non-homogenous leukoplakia was a significant risk factor for recurrence. Jerjes *et al*. (2012) also reported that recurrence of oral dysplasia was mainly seen in non-homogenous leukoplakias. The reason behind the higher tendency for non-homogenous leukoplakia to recur is not clear, but is probably due to the fact that this type of leukoplakia is mainly associated with higher grades of dysplasia and larger sized PMDs.

The high recurrence rate seen in patients with major sized dysplastic PMDs compared to recurrence-free patients (19% vs. 9%) was also supported by the finding of a larger mean size of dysplasia affecting patients with recurrence compared to those free from recurrence (393.6 vs. 281.7 mm^2). Also, univariate statistical analysis showed that dysplasia of a major size (> 600 mm^2) increased the risk for recurrence 2.2 times more than minor size (< 200 mm^2), although this did not reach significance. This may underline the link between the clinical extent of PMD and the development of recurrence. A larger sized dysplasia may be subjected to the risk of incomplete removal allowing the residual diseased tissue to grow from the border of the wound, perhaps accounting for treatment failure (Napier et al., 2003).

Recognising the extent of mucosal involvement by PMDs might help in identifying patients at high risk of unsuccessful treatment, such as developing recurrent disease and who thus require close clinical follow-up in conjunction with the assessment of other important clinicopathological parameters such as site, clinical appearance, extent of dysplasia and lifestyle risk factors.

In the present study, a significant association was found between recurrence status and the degree of dysplasia. Interestingly, CIS showed the highest recurrence rate, followed by severe and moderate dysplasia, with mild dysplasia showing the least recurrence rate (30%, 23%, 22% and 5%, respectively) compared to recurrence-free patients who showed a higher proportion of mild, followed by moderate, severe dysplasia and CIS (49%, 22%, 21% and 9%, respectively).

Kaplan-Meier survival analysis showed a clear fall in disease-free survival rate in patients with recurrence associated with an increased dysplasia severity: 1-year survival rate after initial intervention for patients with CIS was 77%, compared to 88%, 95% and 94% of severe, moderate and mild dysplasia, respectively. Further, CIS was found to have the shortest mean time to develop recurrence compared with other grades of dysplasia, albeit non-significant.

Univariate statistical analysis showed that CIS, followed by severe dysplasia, were identified as significant predictors for recurrence to develop (p=0.032) and (p=0.045), respectively, and increased the risk of recurrence 8.6 and 5.88 times compared to mild dysplasia. Moderate dysplasia was 5.56 times more likely to develop recurrence compared to mild dysplasia, but with only borderline significance (p=0.052).
These findings were further confirmed by multivariate regression analysis which showed that CIS had a 12 times higher risk for recurrence to develop compared with mild dysplasia.

With respect to binary grading, a similar significant association was found between recurrence status and high/low grade dysplasia (p=0.025); 80% of recurrence cases were diagnosed initially as high grade dysplasia.

Survival analysis showed that patients with high grade dysplasia experienced a significantly lower disease-free survival rate at 2 and 5-years post-operatively (79% and 63%, respectively) compared with low grade dysplasia and also experienced a shorter mean time to develop recurrence than low grade dysplasia (p=0.023). Similar findings were noted in other studies: Yang *et al.* (2011) reported that OL showing high grade dysplasia was significantly associated with increased post-operative recurrence compared with intermediate and low grade dysplasia. Chandu and Smith (2005) found that recurrent disease occurred with all grades of dysplasia but particularly so when severe dysplasia and CIS were involved, although this was not significant and they explained this in light of an inadequate number of cases to analyse. Hamadah and Thomson's (2009) study showed similar findings, notably that recurrence was seen in all dysplasia categories but was especially frequent in severe dysplasia, albeit statistically non-significant.

A recent prospective study carried out by Jerjes *et al*. (2012), analysing 123 oral dysplasias treated with CO_2 laser, showed a significant association between the severity of dysplasia and recurrence after treatment; recurrence was doubled when severely dysplastic PMDs were compared with moderate dysplasia. The same group also observed that complete response to treatment was significantly lower in severe dysplasia compared to mild or moderate dysplasia cohorts. These results compare favourably with our findings of lower DF rates found in CIS, severe and moderate dysplasia compared to mild dysplasia. These findings reflect the association between development of recurrence and the severity of presenting dysplasia and clearly confirm the importance of epithelial dysplasia, in particular high grade dysplasia, in the prediction of postoperative recurrence. Thus, patients with high grade dysplasia should be considered as high risk patients requiring careful intervention with close follow-up and life-long surveillance.

In this study, one of our aims was to investigate the importance of laser resection margin status on both local recurrence and overall disease-free survival for PMD patients.
Complete removal of all dysplastic tissue is of fundamental importance in any planed surgical intervention for PMD treatment. The histopathology of resection margins was found to be significantly associated with recurrence status. Of the 14 recurrent cases, 4 were reported with clear resection margins (2 in the FOM and 2 in the soft palate), whereas the remaining 10 cases showed foci of dysplasia within excision margins. The majority were in the tongue

(7/14), 2 in the FOM and one in the soft palate (1 mild, 4 moderate, 4 severe dysplasias and 1 CIS). We found that the degree of residual dysplasia seen in the resection margins was significantly associated with definitive histopathological diagnosis. Mild dysplasia cases thus showed mainly clear margins whilst more severe dysplasia was more likely to exhibit dysplasia in resection margins which was usually the same grade as the excised dysplasia. This almost certainly reflects incomplete removal of the presenting dysplasia in these cases.

Furthermore, in this study, a significant association was found between the clinical extent of the excised dysplastic lesion and resection margin status and grades of residual dysplasia. Minor sized lesions usually had clear margins, whilst intermediate and major sizes often showed dysplasia in the margins. Also, minor size lesions with dysplastic margins were predominantly seen in mild dysplasias, whereas severe dysplasia-CIS and moderate dysplasia in margins were more frequently seen in major and intermediate sized PMDs. This clearly underlines the association between the clinical extent of dysplasia and the margins status; higher dysplastic features in excision margins are associated with larger sized dysplasia.

The extent of dysplasia in the resection margins and the clinical outcome was also investigated; whilst one margin involvement was most common in DF patients, it was equally seen in patients who developed recurrent and new-site dysplasia. 24% of cases with four margin involvement developed recurrent disease, although the relation was not significant. This suggests that the number of margins involved by residual dysplasia does not significantly influence patients' clinical outcome. However, further study with a larger sample size is required.

Histopathological data confirmed that 44% of cases had dysplasia-free-margins. Subsequently, 11% developed same site recurrences, but this increased to 25% when excision margins exhibited residual dysplasia, although the difference did not approach significance. These findings were confirmed by both univariate and multivariate analysis which emphasised that resection margins with severe-CIS residual dysplasia increased the risk for recurrence 3.9 times compared with margins with mild dysplasia; this remained statistically non-significant, however.

These data appear to reflect incomplete removal of the presenting focal dysplasia in 10/14 cases as compared to entirely removed dysplasia from the remaining 4 cases. Thus, same site recurrence in this study may be explained in two ways: firstly, field change cancerization in cases with clear resection margins with migration of new epithelium from surrounding unstable mucosa (Lim et al., 2010), in which case margin status means clearly very little; this is supported by mounting evidence that suggests that complete resection of dysplasia may have little impact on recurrences or cancer development (Lippman and Hong, 2001). Secondly, recurrence may be due to incomplete removal of focal dysplasia with the possibility of residual dysplasia extending from the border of the wound to develop a recurrence (Frame et al., 1984).

Recurrence in the presence of clear excision margins may thus be inevitable regardless of the effectiveness of the treatment modality if resection margins are in an area of field change (Thomson and Wylie, 2002; Chandu and Smith, 2005). Margins with residual dysplasia, however, may reflect both inadequate surgical excision and difficulties in removing the entire focal dysplasia which may be influenced by biological or anatomical factors in the affected region. There are also no accepted guidelines for the amount of normal tissue to be removed to achieve clear excision margins which may have a substantive influence on both disease-free and patient survival.

Single variable statistical analysis in the present study showed that the recurrence rate following laser surgery was higher in tobacco users; 67% of patients undergoing recurrences were current smokers and 33% were ex-smokers, whilst only 12% were non-smokers.
Heavy smokers were the most common subgroup amongst current smokers to exhibit recurrent disease. Using survival analysis, a decrease in disease-free survival rate of current smokers from 94% to 80%, 1-year and 3 years post-treatment was seen.

Interestingly, ex-smokers who experienced same site recurrence showed a clear fall in disease-free survival 5-years post-laser treatment compared to 1-year (84% vs. 52%); this was also supported by the shortest mean time to develop recurrence compared to other smoking subgroups, albeit non-significant. Also, univariate regression analysis showed that ex-smokers and heavy smokers showed a higher risk to develop recurrence, 1.9 and 1.4 times

higher, respectively compared with the risk for non-smokers, although this was not statistically significant.

These data confirm a strong relation between smoking behaviour and recurrence of dysplasia after treatment. This may be explained by chronic mucosal irritation by tobacco agents that may affect the entire oral mucosa well before the clinical appearance of any disease (Tepperman and Fitzpatrick, 1981). This concept was investigated by van Oijen *et al*. (1998) who showed that cell proliferation indices of oral epithelium from histopatholgically normal mucosa taken from smokers and non-smokers, were significantly higher in smokers than non-smokers.

In the current study, the higher rate and risk of recurrence in ex-smokers is not surprising since the accumulation of irreversible biological damage may be seen in oral mucosa even after long-term quitting (van Oijen et al., 1998). Thus tobacco smoking behaviour both past and current was found to be closely associated with recurrence of dysplasia after laser treatment. It has been previously estimated that quitting of tobacco smoking may take 10–15 years before risks lessen significantly (Jaber et al., 1999). This is also in line with a recent study conducted by Warnakulasuriya *et al.* (2010) who showed that the risk of oral cancer in former smokers almost matched that of never smokers but only after 10 years cessation.

In the current study, the confirmed higher potential for current smokers to develop recurrence is in agreement with the findings of several other studies. For example, Jerjes *et al*. (2012) found that heavy long-life smoking (> 20 cigarettes/day) was significantly likely to lead to recurrence of dysplasia following laser surgery. Yang *et al.* (2011) emphasised that recurrence is of higher clinical importance than simple treatment failure. Although it may reflect inadequate surgical excision, it may also indicate continuous exposure to risk factors after treatment and an enhanced malignant potential. They also found that recurrence rates after laser surgery were significantly higher in patients who continued to smoke or chew betel quid and continuation of smoking was identified in multivariate logistic regression as an independent prognostic factor for disease recurrence.

These observations were supported by Chandu and Smith's (2005) study in which a significant effect of continued smoking on recurrence was found, treatment failure probably because of oral mucosa field changes due to oral habits. It was concluded by the same group

that during overall treatment of oral PMDs, removal of risk factors such as smoking and alcohol must be given a strong emphasis as well as surgical removal of individual lesions. According to Arduino *et al.* (2009), patients who have continued exposure to risk factors after treatment are more likely to have recurrence during the follow-up period.

Smoking cessation is associated with a decrease in tobacco-related leukoplakia (Jaber et al., 1999; Roosaar et al., 2007). However, no controlled studies are available to assess the impact of cessation of risk habits on the recurrence of dysplasia or induced malignant transformation (Lodi and Porter, 2008). A number of studies have investigated the effectiveness of tobacco cessation in patients with PMDs: Roed-Petersen (1982) reported a resolution of OL following smoking cessation, whilst another study conducted in a dysplasia clinic in London provided a brief cessation advice and achieved a 30% cessation rate, with ultimately three patients out of 180 managed cases developed invasive carcinoma in a mean follow up time of 4.2 years (Poate and Warnakulasuriya, 2006). Thus, recommendations to reduce alcohol intake and smoking cessation may have the potential to reduce the incidence of recurrence. Encouraging tobacco cessation is an effective modality in achieving better outcomes and this requires informational exchange between specialist smoking cessation services and dental/oral clinicians which will be of high importance during patient follow-up (Warnakulasuriya, 2011). However, whether recurrence is related to continued exposure to risk factors or due to an underlying disease predisposition that initiated the original presentation remains unclear. Patients, however, should be closely monitored for recurrent disease regardless (Jaber, 2010).

A growing body of evidence has thus shown that disease-free survival rate, risk of recurrence, susceptibility to new-site disease and overall treatment outcomes are affected by persistent smoking. However, a considerable number of patients continue to smoke both during and after interventional treatment.

Overall, 85% of our study population were tobacco users (63% current and 22% ex-smokers) and only 15% were non-smokers. Out of the 63% current smokers, 36% were still tobacco smokers at their most recent follow-up. It is difficult to know what else can be done to encourage smoking cessation, but it does seem to be the case that ready access to smoking cessation services helps improve quit rates (Hamadah et al., 2007).

Considering alcohol drinking, 82% of patients who developed dysplasia recurrence were regular drinkers. A significant relation was found between drinking intensity in term of units of alcohol per week and recurrent status; heavy drinkers (> 28 units/week) showed the highest rate of recurrence (64%) and increased their risk of recurrence 2.6 times compared to those who consumed ≤ 28 units/week.

Further, Kaplan-Meier survival analysis showed that current drinkers who consumed > 28 units/week displayed a fall in their recurrent-free survival rate from 90% to 69%, 1-and 5 years post-laser treatment. A similar trend for a fall in recurrence-free survival in non-drinkers was also observed, 2.5 and 4.5 years post-treatment, at 80% and 53%, respectively. Univariate analysis showed that heavy drinkers increased their risk of recurrence by 1.3 times the risk of non-drinkers, whilst in multivariate analysis, where multiple risk factors were entered in the model, heavy drinkers showed an even stronger risk of 2.3 times for recurrence, but this was not significant.

The increased risk of heavy drinkers to develop recurrence was confirmed in multivariate analysis, probably associated with the well-known synergistic and multiplicative effects of combining both tobacco smoking and alcohol drinking (Salaspuro and Salaspuro, 2004; Salaspuro, 2007). These findings are consistent with other studies, for example Chandu and Smith (2005), who investigated factors affecting recurrence and found that alcohol consumption was associated with significant adverse effects on disease-free survival post-laser treatment. Similarly, Goodson *et al.* (2010) observed that a heavy alcohol intake of more than 28 units/week was significantly associated with an increased risk of further dysplastic disease particularly recurrence at the same site of primary disease after treatment; continued exposure to alcohol may thus have encouraged further dysplastic change in susceptible healing oral mucosa. A recent study carried out by Jerjes *et al*. (2012) found that heavy long-life drinking (> 21 units/week) was significant and likely to lead to recurrence of dysplasia.

An eighty-three percent of our study cohort was active drinkers, 14 were non-drinkers and 3% were ex-drinkers. At the most recent contact with patients, no significant changes in drinking behaviour were noticed. Although heavy drinkers had decreased from 34 to 23%,

intermediate drinkers actually increased from 12 to 21% whilst ex-drinkers increased from 3 to 5%.

Unfortunately, the population of North-East England has one of the worst health deprivation indicators in the UK, with 71% of men and 56% of women (aged 16 and over) reported as drinking an alcoholic drink on at least one day in the week, with 21% of men and 20% of women also reporting cigarette smoking (The NHS Information Centre, 2010).

With respect to oral health, a significant association was found between clinical outcome and the wearing of a dental prosthesis or not. Seventy-percent of DF patients did not wear dental prostheses. More than fifty-percent of recurrence-free patients did not wear dental prostheses and a higher rate of recurrent disease was seen in patients who wore dental prostheses compared to non-wearers (71% vs. 29%), but this was non-significant. Further, univariate analysis confirmed that wearing a dental prosthesis increased the risk of recurrence 2.5 times compared with non-wearers, but again this was non-significant.

To the best of our knowledge, this is the first study to investigate the relation between dental prosthesis wear and recurrence of dysplasia after laser treatment.
There is no direct explanation of the higher risk of recurrence of dysplasia in patients wearing dental prosthesis, including complete/partial denture or crowns and bridges, and further detailed investigations are required to confirm this finding. It may be the presence of prosthetic dental appliance reflects a poor, pre-existing oral health status with loss of the natural dentition or perhaps cause local mucosal trauma irritation and/ or infection rendering the oral mucosa vulnerable to further dysplastic change.

Considering systemic health, univariate statistical analysis showed that patients with systemic disease had an increased risk of recurrence 2.4 times higher than patients without systemic diseases, albeit non-significant. Ninety-four percent of patients who developed recurrences exhibited systemic disease, although the relation between having systemic disease and recurrent disease remained not significant.

To the best of our knowledge this is the first study that has investigated the relationship between recurrence of dysplasia after laser treatment and systemic disease status.

The higher risk for recurrence in patients with systemic disease may be explained in two local ways: firstly, patients with positive systemic diseases were initially affected with high grade dysplasia compared to patients without systemic diseases, which may make recurrence more likely, and secondly, the higher risk of recurrence may be due to the fact that more patients in this group had dysplastic margins (88%; 43/49) compared to only 12% (6/49) of patients without systemic diseases. However, the relevance of general patients' fitness, well-being and possible immunosuppression consequent upon long-term chronic illness may also be an important systemic mechanism rendering patients with systemic disease susceptible to unforeseen clinical outcome and progression of dysplasia.

Further (new site) Disease

Further disease was defined as the development of new dysplastic changes at sites distant from the primary dysplasia.

The overall rate of new-site dysplasia formation in this study was 14/100, 25-133 months (2-11 years) after laser intervention, with a mean follow-up of 93 months (7.75 years).

The mean time for the first new-site dysplasia was 30.4 months (2.5 years), with the second new dysplasia 48.3 months (4 years) after laser. During their clinical course, patients in this group developed from 1 to 3 new-site dysplasias, 64% underwent 2-6 laser surgeries, with up to 13 follow-up biopsies. These data reflect the complicated clinical course experienced by this group of patients, emphasising an inherent instability in their oral mucosa.

Survival analysis showed a clear fall in disease-free survival rate of patients who underwent new-site dysplasia compared to patients free from further disease, 1 year and ~3 years post-laser intervention (50% vs. 94%), (36% vs. 72%), respectively. Most episodes of new-site dysplasia formation were seen at 3-years post-laser treatment (57%; 8/14) compared to first (3/14) and second years (3/14) after laser.

Patients younger than 40 years showed the least tendency to develop new-site dysplasia, but the relation between age groups and further disease formation was not significant, and this was confirmed by univariate analysis.

This study showed that development of new-site dysplasia had a male predilection compared to females (71% vs. 29%), which was confirmed by a higher risk estimate of 1.3 times for new-site dysplasia in males compared with females.
This higher tendency for further disease in males may be explained by the association that higher grade dysplasia occurs 1.13 times more commonly in males and that new-site dysplasia was seen mainly in patients with high grade dysplasia. Females, however, exhibited a shorter mean time for new-site dysplasia to develop compared with their male counterparts (25.5 vs. 32.3 months), but this was non-significant. It may be that disease progression in susceptible females is much quicker, although there are no clear reasons to explain this.

Significantly, FOM and ventral tongue showed the highest rates of new-site dysplasia compared to other oral subsites. This may be due to the fact that high grade dysplasias were mainly seen in those high-risk sites (FOM and tongue); severe dysplasia and CIS were thus seen more frequently in the FOM (41%, 50%), followed by the ventral tongue (23%, 30%).
This is supported by Waldron and Shafer's (1975) study who found a higher prevalence of severe dysplasia or CIS in the FOM and tongue reflecting the higher risk of these regions.

A significant association was found in this study between the development of new-site dysplasia and high grade lesions with new-site dysplasia more frequently affecting patients with high grade dysplasia. It has been shown that dysplasia arising in high-risk sites (FOM, ventro-lateral tongue and the soft palate complex) contain a significantly higher frequency of loss of heterozygosity at chromosomes 3 p, 9 p, and 17 p, compared with low-risk sites (Zhang et al., 2001b).

Widespread field cancerization in the oral cavity was originally proposed by Slaughter in 1953 to explain the occurrence of multiple dysplastic lesions both synchronously and metachronously. Field carcinogenesis suggests that dysplastic change may occur in any area of mucous membrane exposed to a carcinogen, so patients with dysplasia are at risk of developing multiple primary lesions within the upper aerodigestive tract due to the accumulation of genetic alterations of oncogenes and tumour suppressor genes (Choi and Myers, 2008). Thus, development of new disease at new-sites remains a problem resulting in both complex and difficult clinical management situations (Thomson, 2002; Thomas et al., 2003; Thomson and Hamadah, 2007).

Overall, 93% (13/14) of PMDs that developed further (new-site) dysphasia were leukoplakias (7 homogenous and 6 non-homogenous) with only one erythroplakia.

Although numerically there were more cases of homogenous leukoplakia that subsequently developed new-site lesions, non-homogenous leukoplakia was statistically more significant exhibiting 2.3 times increasing risk of further disease development compared to homogenous ones. A higher risk for non-homogenous leukoplakia to develop new-site dysplasia was supported by univariate regression analysis, which demonstrated that non-homogenous leukoplakia had a 2.7 times increased risk for further disease development compared to homogenous leukoplakia, albeit non-significant.

The higher risk for new-site dysplasia formation in non-homogenous leukoplakia in general and in the speckled subtype in particular observed in this study is supported by the findings of other studies. Lee *et al.* (2006) showed that non-homogenous leukoplakia increased the risk for dysplasia 5.69 times compared with homogenous leukoplakia. Arduino *et al.* (2009) reported that the speckled subtype was a significant predictor for high grade dysplasia to occur. More recently, Vazquez-Alvarez *et al.* (2010) conducted a study to establish correlation between clinical and pathological diagnosis for OL and found that non-homogenous leukoplakia was significantly associated with an increased degree of dysplasia.
The relation between dysplastic non-homogenous leukoplakia and new-site dysplasia formation is clearly important and of clinical value and may be related to field change concept discussed previously. Since non-homogenous leukoplakias were frequently associated with more extensive dysplastic changes which may subsequently subject patients to the field cancerization from unstable oral mucosa and ultimately developing of a new-site disease.

Patients initially affected with dysplastic PMDs sized 200-600 mm^2 showed a significantly higher rate of new-site dysplasia formation. Out of the 14 cases affected with further disease, 9 cases were (64%) intermediate in size.
To the best of our knowledge, this is the first study to investigate the relation between the size of dysplastic PMDs and new-site dysplasia formation and it seems clear that PMD size is one of the important clinical parameters that helps identify high risk patients who require close follow-up.

The current study showed a higher rate of new-site dysplasia formation in patients who were initially diagnosed with high grade dysplasia compared to low grade dysplasia (57% vs. 43%). This was further confirmed by univariate analysis which showed that high grade dysplasia increased the risk for new-site dysplasia formation by 1.2 times compared with low grade dysplasia, this was non-significant, however. This higher tendency for new-site dysplasia to occur in high grade dysplasia cases is, however in agreement with the concept of field change carcinogenesis.

Considering subgroup analysis, mild dysplasia showed a significantly higher rate for new-site dysplasia formation. This unpredictable association, however, may simply reflect the higher frequency of mild dysplasia in our study population (43%).

Overall, tobacco users were more likely to develop new-site dysplasia, with the majority of cases being current smokers (71%). Intermediate and heavy smokers showed the highest rates of new-site dysplasia especially in patients who had smoked for a long duration of time, although this was non-significant. These data were further supported by univariate regression analysis which showed that patients who smoked more than 20 cigarettes/day increased their risk for new-site dysplasia by 3 times, followed by an increased risk for those who smoked less than 20 cigarettes/day (2.4 times) and then ex-smokers (2.2 times) compared with non-smokers, although this was non-significant.

It has been suggested that second primary malignancies in tobacco users may reflect the chronic mucosal irritation by carcinogens which subjects the entire oral epithelial tissue to the risk of neoplastic change even before the morphological appearance of disease (Tepperman and Fitzpatrick, 1981). This may explain the higher risk of tobacco users to develop new-site dysplasia compared to non-users observed in the current study.

Interestingly, Jaber (2010) found that the rates of development of second dysplastic lesions was more common in non-smokers compared to smokers (13.5% vs.7.7%), although this was not significant. Perhaps the non-smokers in Jaber's study possessed other aetiological factors responsible for the higher rate of new-site dysplasia seen in this group compared with tobacco users. Alternatively, patients who develop oral dysplasia in the absence of identifiable risk

factors may exhibit substantial pre-existing molecular and genetic abnormalities resulting in field cancerization.

A clear fall in the disease-free survival rate of current drinkers compared to non-drinkers was observed, but this was non-significant. Similar to smoking behaviour, current drinkers showed the highest rate of new-site dysplasia formation, with patients who drank > 28 units/week exhibiting the highest rate of new-site dysplasia compared to light and intermediate drinkers. These findings were further supported by univariate analysis: high alcohol intake increased the risk for new-site dysplasia formation by 3.4 times compared to non-drinkers whilst, patients who drank < 28 units/week increased their risk for new-site dysplasia by 1.8 times, but the difference was non-significant.

These data are in agreement with other studies, for example Maserejian *et al.* (2006a) who found that an alcohol intake of more than 4 units per day was associated with a 2.5 times increased risk of developing PMDs. Goodson *et al.* (2010) investigated the role of alcohol in oral precancer in a North-East England population and found that an alcohol consumption of more than 28 units/week was significantly associated with an increased risk of further disease. This may be explained by the chronic irritation of carcinogens subjecting the oral mucosa to the risk of new-site dysplasia by the effect of field change phenomenon.

Comparing recurrent (same site) and further (new-site) dysplasia, showed that males were more likely to develop recurrences, whilst females were equally affected by recurrence and new-site dysplasia, although this was not statistically significant. This may be explained in light of this study findings, that males more commonly showed dysplasia in the margins than females (32/49 vs. 17/49) and they also exhibited higher grades of dysplasia in excision margins with more margin involvement. These observations may explain why males had more recurrences than females.

Patients developing new-site dysplasia showed a higher mean number of cigarettes smoked per day, a longer history of smoking and a higher pack-year score with a higher amount of alcohol consumed in terms of units/week compared to patients who underwent same-site recurrence. Whilst recurrent disease was more commonly seen in non-smokers and ex-smokers compared to new-site dysplasia, this was non-significant.

Dental prostheses wearers were most often affected by recurrent dysplasia, whereby new-site dysplasia formation was mainly seen in non-prosthesis wearers.
Interestingly, and at a statistically significant level, patients who developed new-site dysplasia were mainly those showing mild dysplasia on initial presentation, whilst patients with severe dysplasia were affected equally by recurrence or new-site dysplasia.
These observations are difficult to explain in the current study analysis and probably further investigation is required utilizing molecular investigations to obtain precise genetic profiling for different grades of dysplasia in relation to treatment outcome.

Malignant Transformation (same site) and OSCC Development (new site)

The main clinical significance of PMDs is their ability to undergo MT which was defined in this study as the appearance of OSCC at the same site as pre-existing PMD.
The time to MT was defined as the time between definitive diagnosis and progression to OSCC, confirmed histologically.
MT rates of PMDs vary from population to population and from study to study probably due to both individual disease and population differences. It is likely that MT rates of hospital-based study are consistently higher than those of community-based ones because of sampling bias (Napier and Speight, 2008). However, identified risk factors remain useful in patient assessment, whatever the patient base, to help alert clinicians to patients at increased risk.

In the present study the overall MT rate was 5/100, approximately 12.4 months (~1 year) (1-41 months) after laser intervention, with a mean follow-up of 47.2 months (3.93 years) (24-65 months) (2-5.41 years). This is similar to a retrospective laboratory-based study conducted by Cowan *et al.* (2001) who investigated the malignant potential of oral mucosal disorders in Northern Ireland and found an overall MT of 3% which although lower than in our study probably reflects the wide range of both dysplastic and non-dysplastic oral disorders included. However, if dysplasia only patients were considered, MT occurred in 6% of cases at an average interval of 48 months following diagnosis, which is quite similar to our study findings.

In a study from Taiwan conducted by Hsue *et al.* (2007) a 3% MT rate was reported at an average interval of 42.64 months post-diagnosis. However, the same group suggested that at least 6 months latency time should be given for transformation to exclude the possibility of a concomitant presentation of oral cancer. However, 6 months for MT may be a long time for consideration as rapid progression of some dysplastic cases may take place.

It was reported by Cowan *et al.* (2001) that in a number of cases a dysplastic lesions was associated with an adjacent invasive tumour, there was a short clinical history of less than 12 months (often only 1 or 2 months before transformation). Interestingly, this is supported by earlier studies (Pindborg et al., 1968; Silverman et al., 1984; Lind, 1987; Schepman et al., 1998) who found that a significant number of leukoplakias progress to oral cancer during the first year of follow-up of early cancer diagnosis following laser excision of precancerous lesions.

In their study, Goodson and Thomson (2011) reported that the longest time interval between incisional biopsy and laser excision was 6 weeks, which suggested that foci of oral SCC seen in 9% of cases were present but missed at incisional biopsy, probably because of sampling errors during initial diagnostic biopsies. It has been reported previously that 3% of leukoplakias might already be carcinomas at the time of biopsy (Waldron and Shafer, 1975). These cases may represent either unrepresentative tissue sampling during biopsy or perhaps long-standing disorders in the last stages of MT (Cowan et al., 2001).

It is possible that areas of dysplasia or early carcinoma are missed at initial biopsy due to the inhomogeneity of dysplastic lesions or that some biopsies are not representative of the worst areas of the lesion especially when small specimens are taken (Cowan et al., 2001). Biopsy samples should be representative, of course, and it has been recommended that biopsies should be taken from the most suspicious area, such as erosions or verrucus areas, with larger lesions requiring more than one biopsy (van der Hem et al., 2005; Sloan, 2011). Moreover, assessment of the histopathological features of dysplasia can be relatively subjective and may not always be reliable (Pindborg et al., 1985; Abbey et al., 1995).

In the current study, there were 3 cases in which oral cancer became evident on surgical laser excision of the dysplastic lesions, respectively 1, 2 and 3 months after incisional biopsies

showing mild, severe and mild dysplasia. Two of these cases underwent same day incisional biopsy of clinically suspicious lesions with the remaining biopsy 2 months after first presentation.

The time between incisional and excisional biopsy was 3 weeks, 2 months and 11 months, respectively. The time between 1st presentations to laser was 1, 3 and 13 months, respectively. These observations suggested the presence of foci of oral cancer in the first 2 cases which were missed at incisional biopsies (non-representative sample), whilst long waiting time for laser surgery from the time of initial diagnostic biopsy in the third case explained the progression to oral cancer.

Patients exhibiting MT experienced 1-3 laser interventions and 1-7 follow-up biopsies, reflecting the difficult clinical course followed by those patients. Also, this group of patients showed a significantly lower disease-free survival rate compared to MT-free patients, 15 months (1.25 years) after laser treatment the survival rate dropped to 20% in patients with MT compared to 85% in patients free from MT.

Interestingly, MT was not seen in patients younger than 40 years, but males who suffered MT were younger than females (58 vs. 70.5 years; p=0.055).
This is supported by other studies: a higher MT rate in patients aged over 50 years was reported by Amagasa *et al.* (2006), Banoczy (1977) and Chiesa *et al.* (1993) also reported a higher risk of MT among 60-70 years old patients.

Our study showed that females were at an increased risk of 1.3 times for MT compared to males. This is similar to a study conducted by Lind (1987) who reported that females were more prone to MT than males. Amagasa *et al.* (2006) also reported a higher MT rate in females than males (11.2% vs. 6.2%), but the difference was not significant. It remains unclear why the risk for MT is higher in females compared to males. Female patients in spite of less exposure to the risk of tobacco and alcohol may have an increased susceptibility to carcinogenic effects with different genetic and hormonal factors; further studies in molecular biology and genetic susceptibility are required.

Our results are in contrast to other studies which found a male predilection for MT in India and Taiwan (Silverman et al., 1976; Gupta et al., 1980; Hsue et al., 2007). This may be explained in the light of populational, environmental, disease and oral habits differences. Males in these studies may be more frequently subjected to the risk from smokeless tobacco, betel-quid, areca nut, in addition to cigarette smoking and alcohol drinking, compared to females.

Schepman *et al.* (1999) suggested that almost 50% of oral cancers were associated with or preceded by OL. Similarly, Bouquot *et al.* (1988) in a retrospective laboratory-based study of oral and oropharyngeal cancers in the USA reported that leukoplakia and erythroplakia were associated with 41% of intra-oral carcinomas.

In this study, leukoplakia was the most common clinical type accounting for MT; 80% compared with 20% for erythroplakia. Although MT was equally seen in homogenous and non-homogenous leukoplakia, a higher percentage of non-homogenous leukoplakias underwent MT compared to the homogenous type (8%; 2/25 vs. 3%; 2/67) was seen. A higher risk of MT for non-homogenous leukoplakia by 2.7 times was seen compared with homogenous leukoplakia.

The higher risk associated with non-homogenous leukoplakia is consistent with other previous studies (Pindborg et al., 1968; Banoczy, 1977; Silverman et al., 1984; Lind, 1987; Gupta et al., 1989; Schepman et al., 1998). Also, our findings agree with a more recent study conducted by Holmstrup *et al.* (2006) who found that the clinical type of leukoplakia was a significant predictor for MT; non-homogenous leukoplakia increased the risk for oral cancer 7 times compared to homogenous leukoplakia. They showed that the clinical type accounting for most MT was non-homogenous leukoplakia (20%) compared to homogenous leukoplakia (3%).

The current study also showed that MT was more predominant in high risk sites (FOM and tongue) compared to low risk ones (80%; 4/5 vs. 20%; 1/5). The lateral border of the tongue (11%; 2/19) showed a higher rate of MT compared to the FOM (4%; 2/46). This is supported by a previous study conducted by Flynn *et al.* (1988) who reported that the lateral tongue surface is characterised by a high risk of MT.

It has been reported previously that the anatomical site of origin of PMDs has great influence on MT, with the chance of carcinoma 2.72 times higher in tongue and FOM leukoplakias compared to the buccal mucosa (Lee et al., 2006). This is supported by other studies which showed that these locations were liable to exhibit severe dysplasia (Jaber et al., 1999) and that PMDs at these locations have a higher potential for MT (Mashberg and Meyers, 1976; Silverman et al., 1984; Gupta et al., 1989).

Also, in a cross-sectional study of dysplasia and OSCC in the USA, the highest prevalence of severe dysplasia or CIS was seen in the FOM (13.5%) and tongue (5%) emphasising the high risk nature of these locations (Waldron and Shafer, 1975).

The higher tendency of tongue and FOM to undergo MT may be related to the thinner, non-keratinized oral mucosa covering these sites which offers less protection and higher permeability (Mashberg and Meyers, 1976; Lesch et al., 1989) enhancing the effects of local carcinogens pooling in saliva (Lederman, 1964). As adjacent epithelia in early cancer or OL cases may alter to promote cell migration and molecular alteration of basal cells, it has been suggested that tongue leukoplakia should be completely excised with confirmation of histopathology diagnosis and subsequent close and long-term follow-up (Ishii et al., 2004).
Further, Zhang *et al.* (2001a) performed a genetic study and analysed 127 oral dysplasias for loss of heterozygosity on 3 chromosome arms. They found that loss of heterozygosity frequencies were elevated at high risk sites among both sexes and amongst both smokers and non-smokers and they concluded that the anatomic location of mild and moderate oral dysplasias in Western populations may be an important diagnostic indicator because lesions at high risk sites have a greater tendency to include genetic alterations associated with an elevated risk of progression.

In this study, the lack of a higher risk for MT at high risk sites is consistent with the findings of Holmstrup *et al.* (2006) who reported that sites considered to be at risk such as the lateral and ventral tongue and the FOM exhibited no increased risk of MT compared with other oral sites. This was supported by Schepman *et al.* (1998) who investigated MT of OL in a hospital-based population from Netherlands and found that no oral subsites were associated with an increased risk of MT and that malignant potential was independent of dysplasia site.

However, in this study the high risk sites seems to carry a higher tendency for malignant progression as cancer development was mainly seen in these anatomical sites (FOM and tongue), thus a careful thorough and regular examination of high risk site is mandatory.

The size of the majority of cases that underwent MT were between 200-600 mm^2 with a mean of 380 mm^2; dysplasia size greater than or equal to the third quartile (425 mm^2) increased the risk for MT two times compared to smaller sizes. This is in agreement with Holmstrup *et al.* (2006), who found that if lesion size exceeded 200 mm^2, the risk of having oral cancer was 5.4 times the risk of smaller lesions. In another study following 331 PMDs for more than 1 year, Roed-Petersen (1971) reported that 8 out of 9 leukoplakias that developed cancer were greater than a mean size of 5.5 cm^2.

It is generally accepted that dysplastic disorders carry a greater risk of MT than non-dysplastic ones, and the more dysplasia the greater the risk (Banoczy and Csiba, 1976). Nevertheless, it should be recognized that not all dysplasias will eventually transform to cancer and some may even spontaneously regress particularly after discontinuation of smoking and drinking and changes in life style habits (Axell et al., 1996).
MT may also arise in non-dysplastic lesions (Pindborg et al., 1977; Gupta et al., 1980; Silverman et al., 1984; Schepman et al., 1998) and OSCC can occur without preceding dysplasia (Schoelch et al., 1999).

The present study demonstrated that patients diagnosed with high grade dysplasia tended to have greater transformation rates than those with low grade dysplasia: 6% (3/51) of high grade dysplasia underwent MT compared to 4% (2/46) of low grade dysplasia, although this was non-significant. This is in agreement with the findings of several other studies, which showed that high grade dysplasia carried a greater risk of progression to carcinoma (Schepman et al., 1998; Neville and Day, 2002; Kujan et al., 2006; Rosin et al., 2007).

In this study, MT occurred often in severe dysplasia and CIS (9%) compared to mild dysplasia (5%); but no cancer development was seen in moderate dysplasia. It has been reported that severe epithelial dysplasia can show a wide range of MT, from 7%–50% (Silverman et al., 1984; Lumerman et al., 1995; Schepman et al., 1998; Jaber et al., 2003);

our findings are comparable to this, with severe dysplasia cases exhibiting a 9% transformation rate.

Similar to our findings, Chandu and Smith (2005) found that the majority of MT cases occurred in severe dysplasia-CIS and Mehanna *et al*. (2009) in a meta-analysis reported a MT rate for mild-moderate lesions of 10.3% compared to 24.1% for severe dysplasia and CIS.
A hospital-based study conducted by Holmstrup *et al.* (2006) showed that the histological features most associated with the highest percentage of MT following surgical treatment was CIS 33%, compared to 9% moderate/severe or 11% mild dysplasia. However, the degree of dysplasia did not correlate precisely with MT and it was suggested that the lack of correlation may be explained by biopsy techniques being unrepresentative of the affected area. They reported that mild dysplasia exhibited a MT rate similar to severe dysplasia, which challenges previous assumptions that mild dysplasia can be considered harmless. This is in agreement with our findings that 2 out of 5 cases undergoing MT were mild dysplastic. Thus, mild dysplasia should be considered important and should be managed similarly to other grades of dysplasia.

At the present time, it is generally accepted that the more severe the dysplasia, the higher the tendency is for malignant change; however, MT rates based upon grade of dysplasia are difficult to establish (Brennan et al., 2007) because of the inherent subjectivity of the histopathological grading system and inter-observer disagreement. The latter is one of the main restrictions in using existing histological criteria to predict transformation of dysplasia (de Vet et al., 1995). Nevertheless, histopathological dysplasia grading remains a fundamental guide to relative risk of malignant change in individual lesions (Lumerman et al., 1995; Cowan et al., 2001; Reibel, 2003). The presence of significantly dysplastic areas in oral epithelium is believed to be associated with progression to cancer (van der Waal et al., 1997; Arduino et al., 2009; Schaaij-Visser et al., 2010), possibly because of accumulation of chromosomal, genomic and molecular alterations (Warnakulasuriya et al., 2008; Garnis et al., 2009).

In the current study, patients undergoing MT were mainly tobacco users (3 current smokers and 1 ex-smoker) with only 1 non-smoker. However, non-smokers showed a higher rate of MT (7%; 1/15) compared to both current smokers (5%; 3/63) and ex-smokers (5%; 1/22).

While smoking tobacco is associated with an increased risk of OL (Hashibe et al., 2000a; Banoczy et al., 2001; Lee et al., 2003; Dietrich et al., 2004), follow-up studies have reported that the risk of MT of oral PMDs is higher in non-smokers compared to smokers. It is, however, often difficult to obtain reliable information about the exact quantity of tobacco intake, the type of tobacco product used, and the number of pack years for individual patients (van der Waal and Axell, 2002).

Lower rates of MT in smokers compared with non-smokers is supported by previous studies (Gupta et al., 1980; Silverman et al., 1984; Lind, 1987; Schepman et al., 1998; Reibel, 2003). In a study of 257 patients with OL, 12% of 183 smokers developed carcinoma, whereas 32% of 74 non-smokers developed carcinoma (Silverman et al., 1984). In another study of 166 patients with OL, non-smoking women had a significantly higher risk of MT than female smokers (Schepman et al., 1998). In a study performed by Jaber (2010), 4% of tobacco users and 10.8% of non-users transformed to malignancy after a mean follow-up of 4 years.
The reasons of why non-smokers at increased risk of MT remain unclear and further studies are required to investigate why oral mucosa remains prone to further disease and oral cancer development in the absence of known carcinogens.

The present study showed that out of 5 patients undergoing MT, 3 were current drinkers (2 heavy, 1 light) and 2 were non-drinkers. Non-drinkers exhibited a higher rate of MT (14%; 2/14) than current drinkers (4%; 3/83), but not significantly.

All alcohol use was assessed by self-reporting, however, and this may not always be reliable. Controversy about the role of alcohol alone in PMD transformation exists (Andre et al., 1995; La Vecchia et al., 1997). The non-significant relation between MT and drinking status agrees with a study conducted by Schepman *et al.* (1998) who found no association between alcohol consumption and MT.

It has been reported previously that in heavy drinkers (over 30 gram ethanol/day), alcohol increases the relative risk for MT of oral PMDs after controlling for tobacco smoking by 2.5 times compared with non-drinkers and is independent of the drink or drinking pattern (La Vecchia et al., 1997). In our study we found a higher rate of MT in non-drinkers compared to alcohol drinkers.

Further studies are thus required with larger sample sizes to investigate this to provide more accurate alcohol measurements other than self-reporting.

In the current study, in 2 patients, cancer developed at distant sites to the primary dysplasia, at 4.58 and 5 years after laser intervention with means of 6.83 years and 7.75 years, respectively. The 2 OSCC cases were seen in patients affected with ventral tongue severely dysplastic erythroplakia (945 mm^2) and lateral tongue homogenous leukoplakia with CIS (450 mm^2).

These patients underwent 2 to 6 laser interventions, 5 to 13 follow-up biopsies and 23-39 follow-up visits. They also exhibited multiple recurrences and new-site dysplasias both synchronously and metachronously, suggesting a complicated clinical course and highly unstable oral mucosa. Both of these patients were non-smokers and social drinkers consuming only 1-4 units/week; one showed clear excision margins and the other only mild dysplasia in the margin.

Developing OSCC at sites distant from the primary dysplasia supports the concept of field change cancerization which is important in oral carcinogenesis (Chiesa et al., 1990; Thomson and Wylie, 2002) and reflects an inherently unstable nature of these patients' oral mucosa and emphasises the need for close and long term clinical follow-up.

For statistical purposes, cancer development at both same site (5) and new sites (2) were combined into one group and compared to cancer-free patients.

Patients developing oral cancer had lower tobacco intake, shorter smoking histories and lower pack-year scores compared to oral cancer-free patients and non-smokers exhibited the shortest time to develop oral cancer compared to both ex-smokers and current smokers.

Although patients developing oral cancer consumed a higher amount of alcohol in terms of units per week compared to cancer-free patients, this did not reach the significance and non-drinkers who developed cancer exhibited a shorter time to cancer presentation with a lower 2-year disease-free survival rate compared to current drinkers (85% vs. 97%); but this did not reach significance.

Single variable statistics showed that non-homogenous leukoplakia had a 1.8 times increased risk for oral cancer compared to homogenous leukoplakia.

To predict the most significant factor(s) for oral cancer development, univariate regression analysis was performed and showed that tobacco smoking was a significant predictor, with size and clinical type of PMDs only marginally significant. A multivariate model showed no significant predictors, although non-homogenous leukoplakia increased the risk for oral cancer by 3 times and erythroplakia a stronger risk of 18.6 times. It is possible that erythroplakia rather than leukoplakia is a truly pre-invasive disorder, and this is supported by an earlier study conducted by Mashberg *et al.* (1973) who found 96% of asymptomatic OSCCs were erythroplastic in appearance. This is supported by a study conducted by Shafer and Waldron (1975) who found a higher rate of dysplasia in erythroplakia: 91% of 65 erythroplakic biopsies showed invasive carcinoma, CIS or severe dysplasia.

The multi-step process of oral cancer development is well developed, with progression from hyperplasia, through increasing grades of dysplasia to CIS and ultimately invasive OSCC attributed to a series of genetic abnormalities. The main difficulty in early diagnosis of high risk tissue is our limited ability to differentiate lesions at risk of progressing into cancer from those at low risk (Guillaud et al., 2008).

In the light of the present study findings, there is a need for improved management of dysplastic PMDs to prevent MT and recurrence after treatment. This may be through identification of significant predictors of local failure (recurrence), new-site dysplasia, MT and oral cancer formation, and implementation of these in the assessment of patients at high risk. Further, there is a real need to develop understanding of these mechanisms at a bimolecular level.

5.4.2. Analysis by Clinicopathological Profiling

The relation between clinical outcome and location of dysplasia was significant. Whilst most FOM lesions were DF following laser, the FOM also showed the highest proportion of recurrent, new-site and MT cases. Although FOM lesions were affected by MT, no distant site OSCC development as a result of field cancerization was seen. The number of cases was

small, but this is an important clinical observation suggesting the FOM is subjected to direct effects of local carcinogens from tobacco smoking and alcohol drinking at the site where the greatest dose is supplied pooled in saliva.

The ventral and lateral surfaces of the tongue were the only sites to develop OSCC, whilst buccal mucosa showed a high rate of new-site dysplasia formation. The faucial of pillars showed no recurrence or cancer development whilst the soft palate showed a higher rate of recurrence compared to new-site dysplasia formation with no cancer development. The 2 cases arising on alveolar mucosa were DF following treatment.

The tongue was the only site affected by OSCC development as a distant site phenomenon presumably due to field change. There is no reason why the tongue influenced by the field cancerization phenomenon rather than MT, however, this clearly demonstrated and in a very small number of cases that the tongue is a high risk site for cancer development.

The relation between the size of dysplasia and the treatment outcome was significant; the majority of DF patients originally had minor sized PMDs. Unexpectedly, minor sized dysplasias underwent the highest rate of MT among all size groups.
Interestingly, out of the 7 minor size dysplasias that underwent recurrence 4 had dysplastic margins which suggest that biological activity rather than size and zone is important in local control of disease.

Non-homogenous leukoplakias showed significantly higher recurrence, new-site and MT rates which may be because most non-homogenous lesions showed severe dysplasia and were larger in size. Larger dysplasias may be at risk of incomplete removal allowing residual disease to remain active, undergo clonal expansion and replace adjacent morphologically normal oral epithelium (Napier et al., 2003).

A significant relationship was found between clinical outcome and epithelial dysplasia. Higher rates of DF status were seen in low grade dysplasias, whilst much higher recurrence and new-site dysplasia rates were observed in high grade dysplasias. A higher MT rate was seen in high than low grade dysplasia, whilst 2 cases of OSCC development were only seen in high grade dysplasia.

MT, however, was seen in low grade dysplasia and this emphasises that removal of mildly dysplastic tissue together with close and long follow-up is also relevant in planning treatment protocols.

Ex-smokers exhibited a higher incidence of recurrent disease compared to new-site dysplasia, so that past users of tobacco may also suffer from treatment failure. Although no controlled studies are available to assess the impact of cessation of tobacco on recurrence of dysplasia or MT (Lodi and Porter, 2008), recommendation to stop smoking does have potential to reduce the incidence of recurrence and may decrease the risk of new PMD formation (Poate and Warnakulasuriya, 2006). However, it has been estimated that a significant decrease in risk after smoking cessation may take 10-15 years (Jaber et al., 1999).

Light smokers may also be affected by recurrence and new-site dysplasia and this is in agreement with a study performed by Castellsague *et al.* (2004) who reported an increased risk for oral carcinogenesis amongst both light smokers (1-10 cigarettes/day) and short-term smokers (1-10 years). So, all smokers and recent ex-smokers require close and careful follow-up.

All MT and the majority of recurrent disease were seen in dental prostheses wearers, whilst new-site dysplasia and OSCC were more commonly seen in non-wearers. This may be due to the fact that the mean size of dysplasia in patients with dental prosthesis was larger than in patients without prosthesis (328 vs. 269.5 mm^2), subjecting patients with prosthesis to the risk of incomplete lesions removal and subsequent recurrence of dysplasia and/or MT.

Resection Margin Status and Clinical Outcome

The study showed a significant relation between surgical margins status and treatment outcome. The majority of DF patients exhibited clear surgical margins, whilst the majority of patients who remained DA showed dysplastic margins.

The relation between histopathological diagnosis of resection margins and recurrence status was significant. Patients who were recurrence-free were more frequently reported to have

clear surgical margins, followed by those with mild dysplasia in the margins, and then moderate, severe and CIS, respectively with cases of moderate-severe residual dysplasia in the margins showing a higher recurrence rate (29%). Of interest in this study is the observation of a significant relation between residual dysplasia in excision margins and definitive histopathological diagnosis of dysplasia. Clear margins were mainly seen in cases of mild dysplasia, whilst higher grades of dysplasia were more likely to exhibit dysplastic margins.

Higher recurrence rates in cases with clear surgical margins compared to new-site dysplasia formation was an interesting finding which may suggest that in spite of apparent complete removal of dysplasia, biomolecularly altered fields may remain nearby to the original presenting lesions (Choi and Myers, 2008).

The most significant predictor(s) for dysplastic margins was investigated using regression analysis: the presence of non-homogenous leukoplakia was found to increase the risk for dysplastic margins by 1.8 times compared with that of homogenous leukoplakia. It is unclear why non-homogenous leukoplakia was associated with a higher risk of recurrence; however, this may be because non-homogenous leukoplakias are mainly high grade dysplasias and major size, both of which are associated with high rates of recurrent disease.

A 38% reduction in risk for dysplastic margins to occur was seen in the tongue compared to the FOM. There is no clear reason why the positive margins were lower in the tongue compared to the FOM.

Severe dysplasia-CIS increased the risk of dysplastic margin to occur 2 times more than mild dysplasia, whilst moderate dysplasia increased the risk 1.5 times. Severe dysplasias were mainly associated with major sized PMDs which may increase the risk of incomplete removal with a subsequent higher risk of recurrence.

The only significant predictor for the presence of dysplasia in excision margins in both univariate and multivariate analysis was the size of the treated dysplastic lesion.

Univariate analysis showed that if the size of the dysplasia was 200-600 mm^2 the risk for dysplastic margins to occur was 3.5 times compared to minor sizes, and when the size exceeded 600 mm^2 the risk was 2.7 times.

The final multivariate model showed that size exceeding 600 mm^2 strongly increased the risk for dysplastic margins by 12 times compared to the risk for minor sized dysplasia (p=0.026), and those between 200-600 mm^2 increased the risk 5.8 times compared to minor sizes (p=0.006). This clearly emphasises the close relationship between the extent of focal dysplasia and the risk of dysplastic margins. This suggests either incomplete removal of dysplastic tissue or that large sized dysplasias are biologically different from smaller lesions due to clonal expansion and replacement of adjacent' normal' oral epithelium (Napier et al., 2003).

Regression analysis revealed that the clinical type of leukoplakia, the site of oral lesion and the degree of dysplasia may be important factors in determining the likelihood of dysplastic margins, but was not statistically significant.

Removal of local dysplasia with clear surgical margins greatly benefits disease-free patient survival. However, ensuring clear surgical margins depends on innate biological features such as the clinical type of leukoplakia, the degree of dysplasia and the anatomical site affected. Although there is no accepted, standard excision margin for oral PMDs, 3-5 mm is most widely used by surgeons.

Systemic Health and Clinical Out come

The systemic health of patients was significantly associated with treatment outcome. DF was mainly seen in patients without systemic disease, whilst all cancer development and new-site dysplasia and the majority of recurrent PMD cases were seen in patients with a history of one or more systemic diseases.
This may reflect an association between oral and systemic health and emphasises that patients with a history of systemic disease should be considered potentially high risk requiring a careful management protocol with close and life-long follow-up.

Hypertension and diabetes mellitus were the most common systemic diseases in our series. The higher frequency of patients with hypertension and diabetic mellitus might simply be coincidental due to the relatively advanced ages of the patients in this study as many of these disorders tend to occur in the later decades of life.

A higher rate of MT, recurrences and new-site dysplasia formation were reported in hypertensive patients compared to the normotensive, but this was non-significant.
There is no clear reason why hypertensive patients underwent both MT and further disease development more than the normotensive patients, although our study showed that hypertensive patients were significantly associated with more dysplastic lesions (moderate, severe dysplasia and CIS) compared to normotensive individuals which may explain the higher rates of MT and further disease formation.

A significant relation was found between diabetes mellitus and clinical outcome, with the majority of diabetic patients reported as DF (73%; 11/15) with only 2 recurrent cases and 2 cancer development (MT and OSCC at new-site).

A lower recurrence and a higher cancer development rate were thus observed in diabetic patients compared with non-diabetics, but for no obvious reason. However, the 2 diabetic patients who suffered from recurrent disease exhibited residual dysplasia in resection margins which may explain the higher recurrence rate. Further investigations are required, however, with larger number of patients to establish any detailed inter-relationships.

5.5. Conclusions

5.5.1. Time Considerations

1. More than half of patients had their incisional biopsies on the same day as first presentation.
2. Clinical appearance of PMDs did not influence the waiting time to have the incisional biopsy.
3. Degree of dysplasia did not influence the time between the 1st presentation and incisional biopsy.
4. Time between the 1st presentation and incisional biopsy did not influence the treatment outcome.
5. Degree of dysplasia significantly influenced the time between incisional biopsy and laser excision, the higher the severity the shorter the waiting time for laser surgery.
6. Patients with DA demonstrated a significantly shorter time to have laser surgery after incisional biopsy compared to DF patients.
7. Males experienced a shorter waiting time to undergo laser surgery than females.
8. An extended follow-up time was seen for patients who suffered from recurrences, further disease and oral cancer compared to disease-free patients.
9. PMD patients should be kept under long-term surveillance taking into consideration clinicopathological features and the method of treatment.
10. High grade dysplasia was significantly associated with a shorter time to develop DA state.

5.5.2. Disease-Free (clinical resolution)

1. Seventy-percent of patients with homogenous leukoplakias were DF compared to 44% of non-homogenous leukoplakias.
2. The majority of DF cases were with FOM PMDs.
3. More than 50% of DF cases were minor sized dysplasias.
4. Seventy-six percent of patients with mild dysplasias were reported as DF at the most recent patients follow-up.
5. High grade dysplasias were associated with lower rate of DF.
6. The majority of DF patients were intermediate smokers and light drinkers.
7. DF patients changed their smoking behaviour significantly at the most recent clinical contact, with current smokers decreased from 40 to 21 patients and ex-smokers increased from 13 to 32 patients.
8. Seventy-percent of non–dental prosthesis wearers were DF patients.

5.5.3. Disease Active

1. Logistic regression analysis revealed that high grade dysplasia (severe dysplasia-CIS), lateral-ventral aspect of the tongue, non-homogenous leukoplakia, dysplasia in resection margins, size exceeding 600 mm^2 and a positive medical history were all significant predictors for DA states to develop. The final model of multivariate analysis confirmed that CIS, severe dysplasia and dysplasia in the margins were still significant predictors for DA to develop after laser surgery. Severe dysplasia was 6 times the risk of mild dysplasia; dysplasia at the resection margins was approximately 7-time the risk of clear margins, with CIS showing 17-time increased risk for DA to occur compared to the risk of mild dysplasia.

Recurrent (same site) Disease

1. Residual dysplasias in resection margin and field change phenomenon may account for recurrence of dysplasia after removal.
2. Approximately two-thirds of recurrences developed within the first two years after laser treatment, with the remaining one-third developing after that time.
3. Approximately two-thirds of recurrent cases underwent multiple laser interventions and multiple follow-up biopsies.
4. Male sex increases the risk of recurrences after laser treatment compared to females.
5. Females experienced a shorter time to recurrence after laser treatment than males.
6. The FOM and tongue were significantly more common oral sites to be affected by dysplasia recurrence after removal.
7. Non-homogenous leukoplakias showed a shorter time and the greatest risk for recurrence (1.8 times) compared to homogenous leukoplakias.
8. Size of dysplasia exceeding 600 mm^2 increased the risk for recurrence by more than 2 fold.
9. Significantly, CIS presented the shortest time and the highest recurrence rate, followed by severe, moderate and mild dysplasia, respectively. CIS increased the risk for recurrence by more than 12-fold, severe dysplasia increase the risk by approximately 6-fold and moderate dysplasia increased the risk by more than 5-fold.
10. Severe dysplasia or CIS at the resection margins increased the risk for recurrence by approximately 4-fold compared to clear margins.
11. Current tobacco smokers, in particular heavy smokers, showed a higher rate of recurrence with increased risk (1.4 fold) compared to non-smokers.
12. Ex-smokers exhibited the shortest time to undergo recurrences with a 1.9 fold increased risk of recurrence compared to non-smokers.
13. More than half of current smokers continued to smoke after laser surgery.
14. Ninety-eight percent of current drinkers continued to drink after laser surgery.
15. Heavy drinking significantly showed the highest recurrence rate with a 2.6 times increased risk.
16. The joint effect of tobacco and alcohol presented a strong intensifier risk factor for recurrence.

17. A higher rate of recurrence was seen in patients wearing dental prostheses; 2.5 times higher risk compared to non-wearers.
18. Ninety-four percent of patients who suffered from recurrence also had systemic disease which increased the risk of recurrence by 2.4 times compared to patients without systemic disease.

Further (new site) Disease

1. Patients who suffered from new-site dysplasia formation experienced a more complicated clinical course compared to those with recurrences, reflected by more laser interventions and more follow-up biopsies.
2. Most episodes of new-site dysplasia formation were reported 3-years post-laser intervention.
3. Males showed a higher tendency for new-site dysplasia formation than females.
4. Females developed new-site dysplasia in a shorter time than males.
5. The FOM and ventral tongue significantly showed the highest rate of new-site dysplasia formation.
6. Non-homogenous leukoplakias increase the risk of new-site dysplasia formation by more than 2.7 fold compared to homogenous leukoplakias.
7. Size of dysplasia was an important prognostic parameter, significantly associated with high rates of new-site dysplasia formation.
8. High grade dysplasias carry both higher rates and higher risk for new-site dysplasia to develop compared to low grade dysplasias.
9. Current tobacco smokers with long smoking history, in particular heavy smokers (> 20 cigarettes/day) were associated with a higher rate of new-site dysplasia formation and an increased risk of 3 fold compared to non-smokers.
10. Current drinkers and in particular heavy drinkers have the highest rate of new-site dysplasia with an increased risk of more than 3-fold compared to non-drinkers.
11. Males were more likely to have recurrences, whilst females were affected equally by recurrence and new-site dysplasia formation.
12. Wearing dental prostheses was associated with recurrent disease, but not with new-site dysplasia formation.

13. Patients affected with mild dysplasia were significantly associated with a higher rate of new-site dysplasia compared to recurrences.
14. Patients affected with severe dysplasia exhibited equal rates of recurrences and new-site dysplasia formation.

Malignant Transformation

1. Patients with MT showed significantly a lower disease-free survival rate compared to patients free from MT.
2. No MT in patients younger than 40 years.
3. Males with MT were younger than females (58 vs. 70.5 years).
4. Female sex increased the risk for MT by 1.3-fold compared to males.
5. Non-homogenous leukoplakias increased the risk for MT by 2.7-fold compared with homogenous leukoplakias.
6. The FOM and lateral tongue exhibited a higher tendency for MT, with the lateral aspect of the tongue demonstrating the highest rate.
7. Size of dysplasia $\geq$ 425 mm^2 increased the risk for MT by 2-fold compared to smaller sizes.
8. Mild and severe dysplasias exhibited equal rates of MT.
9. High grade dysplasias (severe-CIS) have a greater tendency for MT than low grade dysplasias (mild dysplasia).
10. No MT reported in moderate dysplasia.
11. Non-smokers have a higher rate of MT compared to current and ex-smokers.
12. Non-drinkers have a higher rate of MT compared to current drinkers.

OSCC (new site) Development

1. Patients developing oral cancers at distant sites were non-smokers and social drinkers.
2. Non-homogenous leukoplakia increased the risk of oral cancer by 3-fold compared to homogenous leukoplakia.
3. Erythroplakias increased risk of oral cancer to develop by 19-times.
4. Size of dysplasia was an important predictor for oral cancer development.
5. The FOM is only affected with MT, but not oral cancer at new-site.
6. Cancer development in the lateral and ventral tongue surfaces were not from MT of dysplasia, but resulted from field change.
7. Non-smokers have the shortest time to develop oral cancer compared to current and ex-smokers.
8. Smokers who developed oral cancer were those reporting low tobacco intake, shorter smoking histories and lower pack-year scores.
9. Tobacco smoking is a significant predictor for oral cancer development.
10. Drinkers who develop oral cancer were associated with a higher intake of alcohol.
11. Non-drinkers have the shortest time to develop oral cancer.

5.5.4. Resection Margin

1. Margin status and outcome were significantly related; with the majority of DF cases were associated with clear margins, whilst the majority of DA states were associated with dysplastic margins.
2. Recurrence-free patients showed significant relationship with clear resection margins
3. A higher rate of recurrence was associated with moderate-severe dysplasia in the margins.
4. Patients with clear resection margins showed a higher rate of recurrence compared to new-site dysplasia formation.
5. Severe-CIS and moderately dysplastic PMDs were 2 and 1.5 times, respectively, more likely to exhibit dysplasia in their resection margins compared to mildly dysplastic ones.

6. Size of dysplasia was the only significant predictor for dysplastic margins to occur, with size exceeding 600 mm^2 increasing the risk of dysplastic margins by 12 times and those 200-600 mm^2 increasing the risk by approximately 6-times.
7. Degree of dysplasia, sites and clinical type of leukoplakia were all important in predicting dysplasia in resection margins.

5.5.5. Medical History

1. Hypertension and diabetes mellitus were the most common systemic disease in PMD patients.
2. Hypertensive patients were affected with higher rates of recurrences and MT compared to normotensive patients.
3. Diabetic patients were predominantly DF.

5.5.6. Dental Prosthesis

1. All patients with MT and the majority of patients who underwent recurrence were dental prostheses wearers.
2. Developing further dysplasia and oral cancer were not statistically associated with wearing dental prostheses.

Chapter Six: Raman Spectroscopy

Study 4

6.1. Introduction

The major aim in cancer research is the early detection and management of oral cancer and precancer to improve quality of life, and to increase the survival rate of patients. There is real clinical need for a diagnostic technique that can detect early changes, and to fully understand the biological process associated with carcinogenesis.

The conventional method for tissue diagnosis is through histopathological evaluation of morphological abnormalities based on hematoxylin and eosin (H&E) staining after tissue fixation and sectioning. Histopathology is somewhat subjective, and associated with inter-observer variations. It is both costly and unable to provide biochemical information on cellular tissue structure, in addition to its lack of immediate results (Manoharan et al., 1996).

Tissue undergoing transformation towards dysplasia and subsequently toward malignancies undergoes structural and metabolic changes which change their light interaction properties (Gobinet et al., 2007). Substantial changes in cellular biochemistry in all stages of tissue cancer is often driven by underlying genetic changes, these changes may either initiate disease or occur as the result of the disease process (Swain et al., 2008). Qualitative and quantitative analysis of these changes may provide important information about specific diagnosis and/or the grade of disease process (Manoharan et al., 1996).

Raman spectroscopy (RS) is a potentially powerful diagnostic technique known for its high sensitivity to subtle molecular and biochemical changes associated with the process of carcinogenesis (Shafer-Peltier et al., 2002b; Lieber and Mahadevan-Jansen, 2003; Swain et al., 2008). It has the ability to detect early biochemical changes associated with carcinogenesis before the morphological clinical manifestation (Papamarkakis et al., 2010) and this may greatly improve early diagnosis of cancers (Harris et al. 2010).

RS is a laser based system applied to a wide range of biological applications (Lambert et al. 2006). This system is able to provide chemical characterisation of tissue molecules under examination, providing detailed information about tissue constituents including proteins, nucleic acids and lipids (Chan et al., 2006). Biological molecules, in general, exhibit their own characteristic spectral features (Bakker Schut et al., 2000; Huang et al., 2003b;

Koljenovic et al., 2004; Stone et al., 2004). The Raman spectrum represents molecular information about all cellular components, including DNA, RNA, proteins, lipids, and carbohydrates (Swain et al., 2008). In this spectrum of bands, each Raman band is characteristic of specific molecular motion within an investigated area of the sample (Krafft, 2004; Yu et al., 2006). RS is a useful tool to analyse the molecular composition and the structure of a sample on the basis of unique Raman signatures specific to each molecular constituent. Therefore, the positions, relative intensities and shapes of the bands in a Raman spectrum carry detailed information about the molecular composition of the sample (Oliveira et al., 2006; Gobinet et al., 2007; Gobinet et al., 2009).

Analysing the differences between the acquired Raman spectra from morphologically normal and pathological tissues may be used for real-time clinical diagnosis (Gniadecka et al., 1997; Bakker Schut et al., 2000; Stone et al., 2000; Venkatakrishna et al., 2001; Stone et al., 2002b; Krishna et al., 2004; Romeo et al., 2006). This allows the differentiation of cancerous from normal tissue and may allow the detection of precancerous changes at early stages. RS has been successfully used for distinguishing tissue pathologies in various tissues including cervical (Mahadevan-Jansen et al., 1998), oesophageal (Stone et al., 2002a) and oral tissue (Venkatakrishna et al., 2001; Malini et al., 2006).

Recent years have seen a great interest in the use of RS for clinical applications. Additionally, it has been shown to be a promising method for the classification of tissue samples based on spectral biochemical information, rather than on the distribution of stains and cellular or nuclear morphology for diagnosis. The spectroscopic measurements identify the changes in spectral patterns of the tissue, and are analysed using multivariate statistical data analysis methods (Kendall et al., 2011). Unlike conventional histopathology, this does not necessarily require fixation, labelling and staining of tissue. It can provide real-time biochemical tissue analysis, automated information, an objective, reproducible diagnosis and is independent of inter-observer variability and morphological changes (Viehoever et al., 2003; Swain and Stevens, 2007; Papamarkakis et al., 2010).

In this study, RS has been assessed as an objective potential diagnostic system due to its ability to detect subtle biochemical tissue changes at a cellular level objectively and reproducibly (Baena and Lendl, 2004).

This system may improve the already existing gold standard of histopathological analysis being utilised alternatively or conjointly with conventional histopathology.

6.2. Current Diagnostic Method/Limitations

Presently, the current diagnosis of cancer is through histopathological assessment which includes removal of a tissue biopsy from a suspicious area, subjecting it to many steps of tissue processing (fixation, sectioning and staining) followed by pathologist examination. This is largely based on the measurement of structural and morphological changes to detect cancer cells which are mainly due to biochemical changes within the diseased tissue (Stone et al., 2004). Currently, histopathology is often subjective, suffering from both inter- and intra-observer variability (Kujan et al., 2007). Further, it requires sample preparation, associated with cost, and delay of both diagnosis and treatment accordingly (de Veld et al., 2005a; Upile et al., 2007; Kendall et al., 2009). Finally, sampling errors which cause unnecessary repetition of biopsies (Kendall et al., 2003; Muller et al., 2003) are often associated with patient morbidity and discomfort (Lau et al., 2003).

Successful treatment increases the survival rate and improves the life quality of patients. This can be obtained by early detection of the PMDs and early diagnosis of malignant lesions which are often diagnosed at advanced stages (de Veld et al., 2005a; Ahmed et al., 2009). In fact, there is real need for an objective, real-time, accurate, reliable diagnostic method without prior tissue processing that it is either complementary or alternative to the conventional histopathology. Further, it should efficiently and rapidly diagnose malignant and PMDs even before their clinico-morphological detection.

6.3. New Diagnostic Techniques: Optical Spectroscopy

Recently, optical spectroscopy techniques have been used to enable the identification of early neoplastic changes by probing the tissue biochemically, providing detailed information about biochemical tissue components. Optical diagnostic techniques have shown a considerable promise as complementary and possible alternatives to the current diagnostic technique (Hutchings, 2009). These techniques could potentially complement the conventional histopathology by guiding the biopsy and assessing the resection margins during surgery. Alternatively, they may be used as a non-invasive, real time *in situ* tool for tissue diagnosis through a fibre optic device.

Essentially, all spectroscopy techniques have the same mode of action, based on the interaction of light with matter and dependent on the fact that the optical spectrum displays biochemical constitutes of tissue under examination by measuring the signals of fluorescence, absorption, and scattering (Bigio and Bown, 2004; Schwarz et al., 2008). Qualitative and quantitative analysis of the biochemical changes can be performed by studying spectral features and measuring their intensities over the spectral range providing important information about disease diagnosis and disease stages (Manoharan et al., 1996; Mourant et al., 2005).

The advantages of optical spectroscopy as an intended objective diagnostic technique have been demonstrated by many authors (Ingrams et al., 1997; Bigio and Bown, 2004; Stone et al., 2004; Upile et al., 2007; Bagan and Scully, 2008). In summary, they potentially could:

- Reduce pathological observers' disagreement by providing reliable biochemical tissue measurements used as standard for diagnosis of dysplasia and cancer stages.
- Reduce missed lesions (sampling errors) by guiding biopsy location.
- Reduce the number of multiple biopsies required for patient follow-up which will probably reduce both costs and patient anxiety.
- Provide a simple portable tool with a reduced need for skilled interpretation.
- Increase the ability to assess resection margins during surgical operation.
- Provide diagnosis for lesions in higher risk areas such as the central nervous system and vascular system, in which surgical biopsy is dangerous.

In addition to diagnosis, optical spectroscopy could be useful in disease management through adaptation of chemodynamic and photodynamic therapy dosage (Upile et al., 2007) as well as monitoring the response of the patient to treatment (dosage customisation) (Bigio and Bown,

2004). There are many types of optical spectroscopy systems which have been used in cancer research, such as fluorescent spectroscopy, infrared spectroscopy, elastic scattering spectroscopy and Raman inelastic scattering spectroscopy (Muller et al., 2003; Swinson et al., 2006; Harris et al., 2010).

6.3.1. Fluorescence Spectroscopy (FS)

Fluorescence happens by excitation of a biological molecule (fluorophores) by a light at a wavelength that is located within the absorption spectrum of that molecule causing molecular excitation from the ground to an excited state, and returning back to the first position with an associated emitted fluorescence (Ramanujam, 2000). Basically, the fluorescent features of a tissue may occur either naturally by light stimulation, or by topical or systemic application of exogenous photosensitiser such as 5-aminolevulinic acids (ALA) (Upile et al., 2007). Since different tissues have different biochemical components and different naturally occurring endogenous fluorophores, FS has the ability to detect these fluorophores by providing distinctive spectra, which represent biochemical changes characteristic of dysplastic or cancerous conditions (Swinson et al., 2006; Upile et al., 2007). The endogenous fluorophores which are expected to change in the malignant transformation include: amino acids (tryptophan, tyrosine) structural proteins, collagens, elastin, porphyrins and co-enzymes, such as nicotinamide adenine dinucleotide hydrate and flavins (Ramanujam, 2000; Bigio and Bown, 2004). This concept was initially used for detection of cancer by Alfano and co-workers (1984) using normal and cancerous animal tissues. The spectral profiles of normal and cancerous tissue showed substantial differences related to flavins and porphyrins fluorphorse. Following, several researchers have studied the use of FS using various experimental techniques in differentiating normal from malignant and pre-malignant tissues of different human organs. Ingrams *et al.* (1997) studied the fluorescence characteristics features of twelve histologically normal and ten dysplastic and malignant oral mucosa samples and found that using a 410 nm excitation wavelength and spectral range of 250–500 nm was most significant. They correctly discriminated 20 of 22 samples and indicated that the emissions in this region may be generally attributed to porphyrins and particularly to protoporphyrin IX.

Although FS seems to be accurate for differentiating healthy from pathological oral mucosa, this method is less specific when discriminating between different types of pathologies and is limited by false positive results (Pfefer et al., 2003; De Veld et al., 2005c).

6.3.2. Elastic Scattering Spectroscopy (ESS)

This technique is based on backscattered photons which have the same wavelength as the incident photons. When the scattering light has the same frequency as the incident light, it is called elastic or Rayleigh scattering (Sharwani et al., 2006; Swinson et al., 2006; Upile et al., 2007). According to Swinson *et al.* (2006), nucleus, chromatin concentration and subcellular organelles are the main scattering centres in the tissue. Additional scattering centres include structural proteins, lipids and erythrocytes (Sharwani et al., 2006). ESS provides a wavelength dependant spectrum which reflects morphological and structural alterations in the examined tissue (Sharwani et al., 2006; Swinson et al., 2006; Upile et al., 2007). It is sensitive to cellular crowding, nuclear cytoplasmic ratio, nuclear size and the chromatin content of the nucleus (Upile et al., 2007) which in fact is similar to criteria used by pathologists in the diagnosis of malignant cells (Mourant et al., 1998; Mourant et al., 2000; Lovat et al., 2006; Swinson et al., 2006; Upile et al., 2007). This technique is limited by both sampling volume and morphologically probing tissue. Specifically, it can examine only 1 mm^3 of tissue volume (Lovat et al., 2006) and probe only the morphology of the tissue not their biochemical changes (Hutchings, 2009).

6.3.3. Infrared Spectroscopy

Infrared spectroscopy (IRS) is an absorption analytical tool for detecting biomedical constituents such as nucleic acids, proteins, lipids and carbohydrates in cells, tissues, and biological fluids in diseases or disease progression states (Wang and Mizaikoff, 2008). IRS uses a polychromatic source of radiation (Hutchings, 2009). The frequency of the IRS absorption provides information relating to structure/conformation and intermolecular interaction (Krishnakumar et al., 2008).

This technique has been applied by a variety of research groups and in different fields such as cancer detection. However, it is hindered by water absorption which disturbs the resulting spectra and prevents *in vivo* application for clinical implementation (Hutchings, 2009).

IRS has been utilized to study a number of normal and pathological tissues. For example, detection of epithelial dysplasia and neoplasia in cervical tissue (Wood et al., 2004), distinction and grading of malignant brain tumours tissues (Krafft et al., 2004) and analysis of normal and malignant oral epithelial tissue (Romeo et al., 2006; Krishnakumar et al., 2008). With multivariate statistical analysis, IRS has the ability to provide information on individual cell type, level of maturity and the state of disease which can be exploited for diagnostic purposes (Romeo et al., 2006).

Studies on oral epithelial tissue have shown that peaks associated with lipids dominate normal epithelial tissue spectra (Krishnakumar et al., 2008), while proteins and nucleic acids dominate the malignant tissue spectra, confirming the increase in protein content in the cancerous tissues, compared to the normal tissues (Krishnakumar et al., 2008). This was proposed to be due to the production of large amounts of surface proteins, like receptor proteins, enzymes, antigens and antibodies (Malini et al., 2006). Also, the increased cell proliferation and decreased apoptotic activity of malignant cells may lead to an increase in number and size of the differentiated oral malignant cells, due to enlargement of the cellular nuclei and increased nuclear-cytoplasm ratio. These changes result in higher concentrations of proteins in the cells and increased thickness of the epithelium. This is reflected by an intense absorption of the nucleic acid bands at 1240, 1338 and 1715 cm^{-1} (Krishnakumar et al., 2008).

In a recent study, a spectral cytopathological approach has been applied using IRS as a novel approach for diagnosis of disease in individual exfoliated cells by collecting spectral biochemical information, followed by multivariate data analysis. In this approach, cellular deviations from the natural composition produces specific spectral features of a disease state that are reproducible and can be used to identify cells compromised at the molecular level by dysplasia, neoplasia, drug metabolites or viral infection (Papamarkakis et al., 2010).

6.3.4. Raman Spectroscopy

Among other optical diagnostic techniques, Raman spectroscopy (RS) in particular is well suited to probe the cellular biochemical changes when comparing between numerous healthy and diseased samples (Lambert et al., 2006). It has the ability to explore many specific chemical bonds present within tissue, providing detailed biochemical information with molecular specificity, hence providing a highly specific spectral fingerprint (Viehoever et al., 2003). Although RS has weak signals, with recent technological advances this problem may no longer be considered as a limitation for its use (Hutchings, 2009).

RS is a laser-based system using a monochromatic source of excitation and relying on molecular vibration frequencies (Hutchings, 2009). Raman system can provide detailed qualitative and quantitative information about a sample being studied (Shafer-Peltier et al., 2002a). Basically, different tissues have individual molecular structures and accordingly they have specific Raman spectra, pathologic conditions associated with chemical and structural tissue changes will also have particular vibrational spectra (Koljenovic et al., 2005; Krafft et al., 2009). RS is an objective diagnostic system providing accurate and very detailed biochemical analysis of the target materials. Additionally, it requires very little sample preparation (Harris et al., 2010).

A Brief History of Raman Spectroscopy

The Raman effect was first observed experimentally in 1928 by Sir Chandrasekhara Venkata Raman and is due to the interaction between light and matter (Smith and Dent, 2005). Raman, along with K.S. Krishnan found that when a beam of light crosses a transparent chemical compound, a small fraction of that light is scattered at right angles and different wavelengths from the original beam (Raman and Krishnan, 1928-in Smith and Dent, 2005). In the original experiment, sunlight was focused by telescope onto a sample which was either a purified liquid or a dust-free vapour, with a second lens placed to collect the scattered radiation (Smith and Dent, 2005). However, the modern era of RS began in 1960s along with the development of commercial lasers and detectors (Manoharan et al., 1996).

Biological applications of RS started with first report of proteins and amino acids performed by Tobin (1968), while the first cellular spectra were reported in early 1980s to study antibiotic interactions with nucleic acid (Manfait et al., 1982). In the early 1990s several research groups used RS to study normal and malignant tissues. Subsequently, with the development of instrumentation and multivariate spectral data analysis techniques, RS has been increasingly used to investigate biological systems including cells (Shafer-Peltier et al., 2002a; Krishna et al., 2005b; Taleb et al., 2006; Harris et al., 2009b), complex tissues (Stone et al., 2000; Venkatakrishna et al., 2001; Krishna et al., 2004; Stone et al., 2004) and body fluids (Harris et al., 2009a; Sikirzhytski et al., 2010).

There are four types of Raman system, each detailed in the following sections:

Surfaces Enhance Raman Spectroscopy (SERS)

In spite of the effective diagnostic ability of RS to provides quantitative and qualitative biochemical tissue information, it is considered as a low efficacy system with a limited ability to detect very low molecular concentrations (Petry et al., 2003). Therefore, using special resonators, the magnitude of the Raman cross-section can be significantly increased to about 15 times that of the primary RS, thus enabling measurement of very low concentrations, even a single molecular spectrum (Kudelski, 2008). In this system, a molecule is placed on resonators of rough surfaces of gold, silver or copper and the enhancement is either performed by formation of electromagnetic field at the metal surface or by charge transfer between the metal and the adsorbed molecules (Petry et al., 2003; Kudelski, 2008).

The potential applications of SERS such as the recognition of bacteria, bacteria spores, viruses and fungi (Kudelski, 2008) might provide an accurate and rapid identification of the biological agents in cases of disease diagnosis. In addition, SERS is able to offer a highly sensitive and rich information for trace analysis (Petry et al., 2003). The heavy metal surface used in this system may lead to damage of sensitive biological specimens; however, this can be managed by using a metal-coated glass fibre tip which also has the benefit of accurately positioning the device (Kiefer et al., 2003). Recently, this system has been developed and modified to a small probe-head attached to an optical fibre connected to a portable Raman

device (tip-enhanced RS) (Kudelski, 2008). This can be implanted as sensor inside a human and animal body such as a glucose sensor to analyse glucose level in the body directly by measuring the spectra of the compound under investigation (Lyandres et al., 2005).

Resonance Raman Spectroscopy (RRS)

This system uses the benefit of emission from the excited molecules at a wavelength near their transition positions and is occasionally hampered by fluorescence interference (Baena and Lendl, 2004). This technique is widely used in the analysis of a variety of chromophoric biological samples such as enzymes. It is mostly due to increased electronic excitation of the vibrational mode in the area surrounding the chromophores leading to signal enhancement (Kudelski, 2008). RRS has been used to determine the haemoglobin oxygen saturation in living tissue by measuring the ratio of intensities due to oxygenated and de-oxygenated haemoglobin (Ward et al., 2007). Also, a combination of both SERS and RRS can reduce fluorescence contamination and provide significantly sharp spectrographs which are highly useful for detection of DNA (Faulds et al., 2005).

Coherent Anti-Stokes Raman Scattering System (CASRS)

This system has widely used the non-linear Raman technique in which the incident radiation has either one monochromatic wave of frequencies (the scattered radiation may involve other frequencies) or two or more coherent monochromatic beams. It is rarely used because of the complexity of instrumentation (Kudelski, 2008). The incident radiation of a typical coherent anti-Stokes Raman scattering consist of two overlapping coherent monochromatic beams of frequencies, F1, F2 when F1 is greater than F2 and F1 minus F2 is equal to the frequency of the molecule. According to Kudelski (2008), this system has two important benefits: strong signal level and significant spatial resolution, in which laser light is focused into a sample and the signal is produced in a small excitation volume less than 1 μm^3 (Baena and Lendl, 2004). The advantages of this technique over the conventional Raman are well documented in its important recent application in cell analysis such as embryonic stem cells. For example, Konorov *et al.* (2007) reported that coherent anti-Stokes Raman scattering microscopy is a

sensitive and non-destructive method that can provide spectroscopic markers to discriminate between differentiated and undifferentiated cells. Further, CASRS reduces the fluorescence interference by creating a highly strong single laser beam obtained by a combination of beams from two laser sources, so that Raman spectra can be detected over background fluorescence. However, the most important limitation of this system is its failure to discriminate between small equally sized molecules (Holtom et al., 2001).

Confocal Raman Microscopy/Raman Micro-spectroscopy

With classic RS, the entire sample area is evenly illuminated, while combining Raman spectrometry with confocal microscopy, the objective of the microscope focuses the incident laser light onto only a small point of the specimen depending on the objective used (Petry et al., 2003). The Raman scatter is collected by the objective which is coupled to a spectrometer by a pinhole to separate the light releases from the excited spot and in the same time prevents the light from the surrounded zones, similar results can be achieved by using a CCD detector (Petry et al., 2003). Since the laser is focused on a very small area of sample, it is very difficult to reduce the fluorescence in a shorter period of time, compared with the conventional RS. This system allows femtolitre volume (10^{-15} litre) and very high spatial resolution of >1 μm (Chan et al., 2006) allowing single cell analysis (Holtom et al., 2001) and three dimensions (Zhang et al., 2008). It is worth mentioning that in spite of high ability in detecting small biological molecules, which may be difficult to be detected by other methods (Lambert et al., 2006), the main limitation of this system is the long time needed to obtain a Raman image (Schafer et al., 2003).

CASRS and confocal Raman microscopy are the main systems used for biological tissue analysis.

Basic Concept and Raman System Description

Raman spectroscopy is an optical technique based on the interaction of light with matter. When incident photons from monochromatic laser light are directed towards target matter, while most will pass through unchanged, some photons will come into contact with molecules in the matter. Most of the photons that contact the molecules will interact with these molecules by exciting the particles, which are reemitted at the same frequency as the incident photon, named Rayleigh scattering or elastic scattering (Smith and Dent, 2005). However, a smaller number of these photons will undergo inelastic scattering which is called the Raman effect or Raman inelastic scattering (Swain and Stevens, 2007). The Raman effect is an inelastic light scattering process whereby a very small proportion of incident photons are scattered, comprising approximately 1 in 10^6 to 1 in 10^9 of those scattered elastically (Huang et al., 2003b; Lau et al., 2003; Bigio and Bown, 2004; Stone et al., 2004; Koljenovic et al., 2005; Ellis and Goodacre, 2006; Swinson et al., 2006; Swain and Stevens, 2007; Kudelski, 2008; Guze et al., 2009; Kendall et al., 2009). These photons are scattered at differing wavelengths to the incident photons, with the energy difference between the incoming and scattered photons referred to as the 'Raman shift' (Kendall et al., 2009). This corresponds to the vibrational modes of molecules contributing in the interaction (Swain and Stevens, 2007; Kendall et al., 2009) and corresponds to specific vibrational energies of chemical bonds of the scattering molecules (Ellis and Goodacre, 2006). The shift or position of the Raman peaks is independent of the wavelength of excitation and it is specific for particular molecular bonds responsible for the scattering (Harris et al., 2009b) enabling the chemical characterisation of molecules within a sample (Kendall et al., 2009). The scattered photon is either red-shifted by providing energy to the bond vibration and re-emitted with smaller energy than the incident photons, named Stokes-Raman scattering or blue-shifted by receiving energy from the bond and released with higher energy, the event here is referred to anti-Stokes Raman scattering (Petry et al., 2003; Lambert et al., 2006; Wachsmann-Hogiu et al., 2009). Because the anti-Stokes effect is weaker than the Stokes at room temperature, the Stokes scattering is recorded (Gobinet et al., 2009).

The exact energy required to excite a molecular vibration depends on the masses of the atoms involved in the vibration, the type of chemical bonds between these atoms and may be influenced by molecular structure, interactions and the chemical microenvironment of the

molecule (Lyng et al., 2007). Using the fact that every chemical bond in a molecule has a characteristic vibrational energy, the interaction of the photons with a substance provides detailed biochemical information about sample constituent chemical bonds (Wachsmann-Hogiu et al., 2009). Detection of these scattering photons produces a spectrum of Raman peaks. Each peak represents a specific molecular bond, together these peaks deliver an intrinsic molecular characterisation of the sample, resulting in rich information about the chemical bonds relating to nucleic acids, proteins, lipids, carbohydrates and other biological molecules present in the tissue or in a single cell (Chan et al., 2006; Wachsmann-Hogiu et al., 2009). In addition, RS provides information about the interaction between macromolecules, such as DNA-protein or protein-lipid interactions (Wachsmann-Hogiu et al., 2009).

Any physiological or pathological process associated with biochemical tissue changes would therefore lead to changes in Raman spectra (Harris et al., 2009a), where a wide range of chemical bonds and functional groups can be attributed to every single peak (Movasaghi et al., 2007). Raman spectrum is a direct function between the intensity of an individual peak and the concentration of the sum of molecular constituents of the investigated area within the tissue (Kendall et al., 2009). Therefore, it is mainly based on molecular concentrations rather than on architectural variations, hence the most important characteristic feature is the peak intensities which are positively proportionate to the concentration of molecular material within the examined tissue (Stone et al., 2004). So, one can understand that RS provides both qualitative and quantitative information through shapes and positions of the bands in a Raman spectrum and relative peak intensities respectively, all carry detailed information about the molecular composition of the sample.

RS can detect and record information from specific molecular species from very small sections and may be applied to samples over a wide size range from single cells through to intact tissue (Huang et al., 2003b; Jess et al., 2006; Swain and Stevens, 2007). Accordingly, a Raman system is able to provide a library of spectra that can be utilised for identification and analysis of molecules in a variety of biological specimens (Lambert et al., 2006).
Raman systems require a monochromatic high intensity light; typically a laser source is used for the excitation of the specimen which is delivered to the sample often using an optical microscope. Depending on the objective used, the microscope focuses the laser to a 0.5–10 μm diameter spot; the analysis region on the sample matches this spot size. The Raman signal

is collected back through the microscope objective and through the confocal optics. Raman spectrometers use edge filters to cut out the signal from a spot corresponding to the laser radiation on the sample. Coupling a Raman spectrometer with a confocal microscope allows the acquisition of full spectral information with a high spatial resolution (<1 μm), a high biochemical sensitivity (da Silva Martins et al., 2010) and in three dimensions (Zhang et al., 2008).

Figure 6.1 shows the schematic diagram of the experimental Raman system. This diagram showing the laser light which comes from the sample passes back through the microscope optics into the spectrometer. An edge filter (sharp cut-off high pass filter) is used to reject the elastically scattered light prior to entering the spectrometer allowing only Raman inelastic scattered light to pass. Consequently, the Raman shifted radiation is detected with a charge-coupled device (CCD). An entrance slit is used to block all light except that which is in the focal plane, with data acquisition performed by a computer. These factors have helped RS to become a very sensitive and accurate technique. The final Raman spectrum is a plot of the Raman intensity at each wavelength shift, recorded in wavenumbers (cm^{-1}) which are the units that represent the number of wavelengths in a centimetre (Shim, 1996).

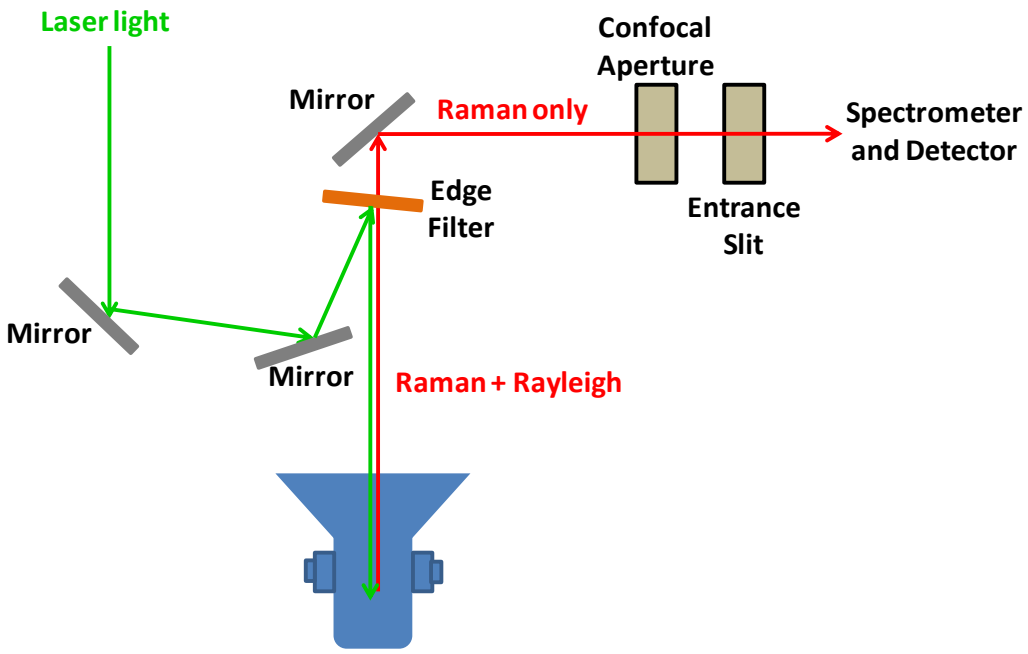

Figure 6.1: Schematic diagram of Raman experimental system set-up.

Important Practical Considerations

According to da Silva Martins and colleagues (2010) the available spectroscopic instrumentations (detectors, gratings, lasers) have better performance in the visible-light spectral region (400–700 nm). However, Raman excitation in this range may interfere with strong auto-fluorescence for nearly all human biological samples (Manoharan et al., 1996; Petry et al., 2003; Krafft, 2004; Gobinet et al., 2007; Harris et al., 2009b; da Silva Martins et al., 2010). A high noise to signal ratio compromises the accuracy of the acquired spectra and affects the spectroscopic measurement of tissue changes (Stone, 2001). Noise occurs due to the random motion of electrons which sometimes can totally obscure the Raman signal, so this ratio refers to the magnitude of measured Raman signal alone describing the quality of spectrum (Stone, 2001). To overcome this problem, many strategies have been proposed such as using different sources of excitation and more accurate tools (Lau et al., 2003). Two light excitations have been established both of them are far from the visible region: ultraviolet radiation (UV) (Manoharan et al., 1996; Lambert et al., 2006) and near-IR (NIR) excitation wavelengths (Krafft, 2004; Andrade et al., 2007; Guze et al., 2009; Kendall et al., 2011). Although UV excitation (10–400 nm) has a better avoidance of the fluorescence found with visible and NIR excitation (Ager et al., 2005), the instrumentation is limited, expensive and causes damage to biological tissues particularly affecting *in vivo* applications (da Silva Martins et al., 2010). NIR (750–1200 nm) has a relatively small extinction coefficient (a parameter related to how strongly a substance absorbs light) in human biological tissue, and therefore penetrates deeply (>1 mm), probing a relatively larger tissue volume (Manoharan et al., 1996). Also, this small absorption coefficient prevents tissue photo-degradation commonly associated with UV excitation, and reduces the fluorescence interference generated with visible-wavelength excitation. Furthermore, NIR wavelengths are simply transmitted along optical fibres for *in situ* or remote Raman spectral diagnosis (Manoharan et al., 1996).

Several techniques have been developed to overcome high fluorescent backgrounds using visible light excitation in biological tissue samples. One of these methods is photo-bleaching (Manoharan et al., 1996). Since the fluorescence and Raman signals are located in the same spectral region and the fluorescence is due to the intrinsic tissue fluorophores, elimination of the fluorescence is difficult without altering the sample composition (Mahadevan-Jansen and

Richards-Kortum, 1997). Therefore, the tissue sample is subjected to a laser light for a considerable length of time, before acquiring any spectra (pre-laser irradiation) to reduce the fluorescence background contamination (Manoharan et al., 1996). Basically, fluorescent molecules in the ground state absorbed light and fluoresce, but with photo-bleaching, laser light progressively excites these molecules from the ground state losing their ability to emit light under prolonged exposure time (Glawdel et al., 2009).

Another techniques to overcome the fluorescent background for biomedical applications is the use of computational algorithms for automated subtraction of the fluorescent background (Lieber and Mahadevan-Jansen, 2003; Zhao et al., 2007; Beier and Berger, 2009). Thus a combination of both photo-bleaching and automated fluorescent background subtraction for the treatment of the fluorescent problem using visible light excitation may be regarded as a suitable strategy to remove the intrinsic autofluorescent background signals, which are usually a few orders of magnitude stronger than those arising from Raman inelastic scattering (Zhao et al., 2007).

RS has been widely used in the biomedical sciences applied over a wide range of samples from single cells to tissues (Jess et al., 2006). One important limitation of RS is the low Raman intensity signal from biological molecules which requires long acquisition time and high laser power for tissue excitation (Notingher et al., 2003b). However, prolonged laser light illumination or increased laser power to acquire high spectral quality might lead to undesirable sample degradation and damage to living cells due to local heating on sample from long acquisition times or extended radiation (Omberg et al., 2002; Jess et al., 2006). Therefore, carefully choosing an established optical technique can be one of the important steps in a spectroscopic tissue analysis.

In spite of the large amount of biochemical information provided by RS which is regarded as the main strength, it is in the same time the greatest associated difficulty. The volume of information often makes the extraction of the required information difficult. Consequently, researchers usually select a defined spectral range for data analysis to exploit the relative and important information (Movasaghi et al., 2007). Furthermore, the main vibrational modes of most proteins that are probed due to C–C, C–N, C–H and other similar organic bonds, are very similar (Wachsmann-Hogiu et al., 2009). This fact makes it very difficult for RS to

measure differences between different isoforms of proteins or protein mutants, particularly against a background of other proteins (Wachsmann-Hogiu et al., 2009).

Components used in Raman studies are important and can contribute to Raman signal if they are Raman active. So for spectral acquisition, the choice of a substrate for specimen mounting is of particular importance (Romeo et al., 2006) and it is vital that the substrate should not have a strong Raman signature within the studied spectral range, to eliminate any spectral contamination from the mounting agent. Although many types of substrate are available for spectroscopic studies, barium fluoride is one of the commonly used and the most suitable substrate for Raman tissue measurements. This mounting material has no Raman signature within the spectral range of biological tissue components named the fingerprint region (800–1700 cm^{-1}) (Gobinet et al., 2009), with only one known Raman peak at ~ 242 cm^{-1} (Chen and Shen, 2006).

6.3.5. Comparisons between Raman and Infrared spectroscopy

In general, both statistical data analysis and information evaluation are the same for both Raman inelastic scattering and IR absorption techniques (Manoharan et al., 1996; Mourant et al., 2005). IR spectroscopy is able to provide biochemical information about the main cellular components such as proteins, lipids and nucleic acids (Mourant et al., 2003), whilst RS provides similar information in addition to much more information about specific species in the main tissue components such as phenylalanine, tyrosine and adenine, which is not available from IR (Short et al., 2005).

RS is an inelastic scattering technique of monochromatic laser excitation based on changes in polarisability (relative tendency of charge distribution), whereas IR is a light absorption of a polychromatic light source which is based on changes on dipole moment (relative tendency of the separation of positive and negative electrical charges in a system due to non-uniform distributions on the various atoms) (Smith and Dent, 2005). It has been shown that RS can provide spectra from thick sections (> 15 μm) with superior spatial resolution ~ 1 μm (Kendall et al., 2009), whereas IRS has been shown not to provide spectra from thick

sections, as these samples may lead to spectral saturation with lower spatial resolution (~ 10 μm) compared to RS (Kendall et al., 2009).

Generally, Raman spectra of biological samples often show narrow bands with a better understanding and better assignment of Raman peaks (Mahadevan-Jansen and Richards-Kortum, 1996). Whereas, IR spectra of cells and tissue often show broader spectral features (Naumann, 2001). The IR signal is greater compared to Raman scatter, but in general there are fewer spectral peaks compared to RS indicating less detailed information (Krafft and Sergo, 2006).

The most important application difference between the two systems is related to the strong absorption of water in IR which disturbs the spectral measurement (Kendall et al., 2009) and compromises the analysis of hydrated biological tissue. RS using UV, visible or NIR is more suitable for cellular based biological systems since the strong asymmetric nature of water molecules results in very weak Raman scattering bands, with very little or no interference with the Raman spectra (Notingher et al., 2003a; Short et al., 2005; Oshima et al., 2009; da Silva Martins et al., 2010). This greatly supports the suitability of *in vivo* spectral collection via fibre optic probe (Oshima et al., 2009; da Silva Martins et al., 2010).

6.3.6. Biochemical Tissue Components

Biological tissue is a heterogeneous mixture of four major components: proteins, lipids, carbohydrates and nucleic acids with the concentrations of these constituents varying within cells, between the same types and different types of the cells and in different physiopathlogical states (Malini et al., 2006; Harris et al., 2009b). Proteins are the major constituent of the human body (~ 20% of total body weight). They are responsible for structural support and are involved in all metabolic processes, with different types of proteins being constructed from 20 types of amino acids (Martini and Ober, 2001). Lipids form between 12%-24% of the total body weight, providing structural support and acting as an energy source, while carbohydrates such as sugars and starches form less than 1% of total body weight and are considered the most important source of energy (Martini and Ober, 2001). Nucleic acids which store and possess genetic information are of two types

specifically, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Both nucleic acids consist of chains of repeating units called nucleotides and each have three components: a sugar molecule (deoxyribose or ribose), a purine or pyrimidine nitrogen base and a phosphate group (Martini and Ober, 2001). RNA molecules are predominantly single stranded with the branches consisting of the nitrogen bases adenine, cytosine, guanine and uracil, while DNA molecules are double-stranded helices of the nitrogen bases adenine, cytosine, guanine and thymine (Martini and Ober, 2001).

Detecting such a wide variety of cellular components gives complex tissue spectra for tissue discrimination (Harris et al., 2009a). This complexity is mostly related to the fact that more Raman bands correspond to a functional group identifying a class of molecules rather than to one particular compound. Additionally, the spectral variations occur in a number of different Raman bands (Beljebbar et al., 2009). Morphological and/or biochemical cellular changes may result in different Raman spectra and could be identified by investigating the spectral differences acquired from normal and pathological tissues (Bakker Schut et al., 2000; Stone et al., 2000; Venkatakrishna et al., 2001; Stone et al., 2002b; Krishna et al., 2004). This may provide a disease marker(s) for tissue discrimination with the aid of multivariate statistical analysis.

Figure 6.2 demonstrates a typical Raman spectrum with the assignment of the key features labelled.

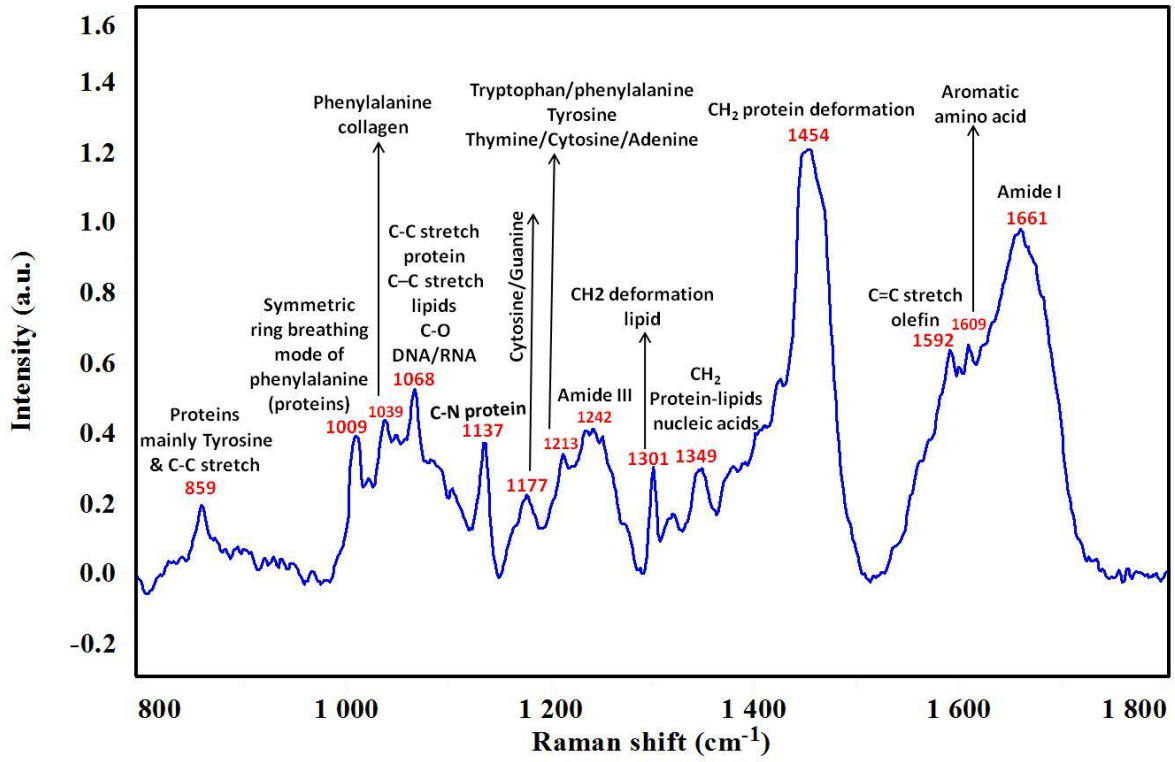

Figure 6.2: Example of a Raman spectrum of oral epithelial tissue with assignment of the key features.

Before developing a diagnostic approach for the purpose of comparison, it is important to understand both the spectral features of individual tissue components and changes in molecular species in the Raman spectra which are important for identifying and differentiating different proteins in different pathological states (Malini et al., 2006). Previous studies conducted by Venkatakrishna *et al.* (2001), Krishna *et al.* (2004) and Malini *et al.* (2006) showed that the normal tissue spectrum is similar to the lipid spectrum, while malignant and pre-malignant tissue spectra are similar to proteins spectra; amide I (N-C=O, C-N, C-C-N, N-H), amide III (N-H, C-N, CH_3-C) and phenylalanine. As a result, standardised models of normal, premalignant and malignant spectra could be used as a comparable model for classifying and then diagnosing suspicious tissues.

6.3.7. Tissue Preparation for Raman Analysis

Different types of biological tissues with different methods of sample preparation have been studied using RS. Fresh tissue section, fresh-frozen, air-dried, formalin-fixed and formalin-fixed paraffin embedded (FFPE) tissues and de-waxed FFPE. Although fresh tissue is the ideal type of tissue for optical spectroscopy (Krishna et al., 2005a) because it is very close to the *in vivo* optimal conditions, paucity, sample handling difficulties, rapid deterioration and unsuitability for retrospective analysis are the major limitations for it being widely used (Ó Faoláin et al., 2005c). However, fresh tissue specimens may be placed in saline and spectra measured within 30 minutes of biopsy (Huang et al., 2003b) or in ice-cold saline (Chowdary et al., 2009).

In general, spectroscopic analysis is commonly performed on fresh-frozen tissue sections with the advantage of lipid preservation that might be used as differentiation markers between normal and cancerous tissues (Tfayli et al., 2005). However, a freezing effect on cells has been documented in the cryogenics field. Specifically, a reduction in temperature cause depolymerisation of the cellular cytoskeleton which is formed from different types of protein fibres (Watson and Morris, 1987). This depolymerisation leads to an unravelling of protein secondary structures leading to increase amino group (NH_3^+) deformation (Ó Faoláin et al., 2004). Thus, researchers using frozen tissue should be aware of the appearance of a Raman band at (1485–1550 cm^{-1}) due to increased vibration of this molecule because of the freezing effect which should not be used for diagnostic marker purposes (Ó Faoláin et al., 2004).

Regarding formalin-fixed tissue, it is important to mention that formaldehyde, which is the most commonly used fixative agent in pathology, might cause protein cross-linking (methylene bridging) which might cause structural alterations in secondary proteins (Mason and O'Leary, 1991). However, Shim and Wilson (1996) showed no changes in the position or the intensity of the amide bands, which are mainly used to study protein changes. This was supported by a study conducted by Mason and O'Leary (1991) who showed that the IR spectra of both fixed and unfixed proteins are similar. This indicates that formaldehyde fixation results in little or no protein secondary structure changes. Huang *et al.* (2003a) investigated the effect of formalin fixation in detecting lung cancer. Raman spectra were

collected from fresh normal and tumour bronchial tissue samples from six patients, compared to formalin-fixed normal and tumour tissue spectra. Formalin fixed tissues spectra showed decrease in overall intensities of the Raman peaks in addition to spectral contaminations of formalin at peaks 1041 cm^{-1} and 1492 cm^{-1} noticed in the both tissues. They indicated that the diagnostic markers derived from fixed tissues was not applicable for *in vivo* lung cancer detection and they recommended the use of fresh tissue for Raman studies, or if not available, a thorough rinsing of tissue specimens using phosphate-buffered saline before spectral measurements may help reduce the formalin contamination. Recent studies have shown that this type of tissue provided considerable discriminant features between normal and malignant tissues in ovarian tissue (Krishna et al., 2005a) and cervical tissue (Krishna et al., 2007b).

To summarize, formalin fixed tissue samples can be used for Raman spectroscopic study after thorough washing in phosphate-buffered saline, and understanding that formalin peaks at 907, 1040, 1492 cm^{-1} region will not be used as a diagnostic markers (Shim and Wilson, 1996; Huang et al., 2003b; Krishna et al., 2004; Krishna et al., 2005a; Krishna et al., 2007b).

Microscopic examination of a tissue needs a thin section of the tissue which is usually between 3–20 μm thick to transmit light. However, preparation of such thin slices requires pre-processing steps using embedding medium to support the specimen and for easy sectioning (Ó Faoláin et al., 2005a). Paraffin wax is the most commonly used embedding medium for both normal and pathological histology, allowing not only diagnostic but also retrospective studies with good cutting qualities and long term storing without any deterioration (Ó Faoláin et al., 2005a). FFPE *ex vivo* tissue samples have been used by many spectroscopic research groups due to availability, ability to enable larger sample studies to validate a technique and to provide retrospective information on patients outcome for spectral correlation (Kendall et al., 2009). Additionally, they benefit from better morphological details and easily handling compared to the shortage, rapid decay and handling difficulties of fresh tissue (Ó Faoláin et al., 2005c; Krishna et al., 2007b). Although biochemical information might be endangered (Krishna et al., 2005a), the suitability of this type of tissue for spectroscopic studies has been investigated and found to be appropriate for tissue classification, carrying sufficient discriminant information and reasonable classification between benign and malignant after excluding paraffin bands (Krishna et al., 2005a; Tfayli et al., 2005) at 1062, 1296 and 1441 cm^{-1} (Ó Faoláin et al., 2005b).

For the FFPE tissue samples to be as close to its *in vivo* state as possible and to minimize the paraffin wax contributions in the resultant spectra that might overlap or mask the important biochemical spectral information, tissue de-waxing has been used in spectroscopic studies to remove paraffin wax (Ó Faoláin et al., 2005a). There are many types of de-waxing agents, however, the currently used de-waxing agents such as xylene, Histoclear and other types are not completely effective in wax removal. A study conducted by Ó Faoláin *et al*. (2005b) on the efficacy of the de-waxing agents, showed that hexane has a superior de-waxing properties compared to the currently used solvents and they have recommended the use of hexane for tissue de-waxing for the spectroscopic tissue analysis.

Due to the unavailability of fresh tissue for spectroscopic tissue analysis, it has been well accepted that the effect of any tissue processing method such as fixation or paraffin embedding has similar effects on both normal and diseased tissue specimens under examination. With the effects on both normal and malignant tissues more or less the same, tissue discrimination may not be greatly affected (Krishna et al., 2004), providing there is sufficient understanding of the effect of each particular processing method on the tissue specimens used.

6.3.8. Raman Peaks Assignments

Raman assignments may be considered the most difficult part of Raman data analysis because of the complicated structures of biological tissue, generating complex tissue spectra and comprising of overlapping bands that might be seen in many cases (Stone et al., 2004). In biological tissue studies, a wide range of chemical bands and functional groups can be attributed to every single peak which can provide information about the presence and the concentration of biochemical constituents. Peak assignments show many inconsistencies in the literature because they depend on the scattering of many biological molecules co-added, providing the Raman peak with variations in shape and position (Stone et al., 2004). So finding appropriate meanings for the clinical importance of these constituents is considered one of the most important steps to achieve results and to complete any spectroscopic research.

Although different techniques have been used by RS researchers, the spectral interpretation of comparable areas of the collected Raman spectra, show a noticeable similarity (Movasaghi et al., 2007). Table 6.1 lists some biological tissue Raman peak assignments from previously published works and have been identified in oral and other tissue studies.

Table 6.1: Tentative Raman peak assignments.

Peak	Assignment	Reference
824-840	Out of plane breathing Tyrosine, O-P-O stretching of nucleic acids	(Stone et al., 2002b) (Puppels et al., 1991)
856-857	Amino acid side chain vibrations of proline & hydroxyproline	(Stone et al., 2002b) (Hayashi et al., 1986) (Cheng et al., 2005)
859	Molecular vibration mode of proteins mainly Tyrosine and C-C stretch	(Oliveira et al., 2006)
861	Phosphate group	(Krafft et al., 2005)
872-879	Hydroxyproline(C-C), tryptophan	(Cheng et al., 2005) (Gniadecka et al., 1997) (Krishna et al., 2004)
881-886	Tryptophan (protein)	(Shetty et al., 2006)
890	Protein bands	(Hanlon et al., 2000) (Utzinger et al., 2001) (Dukor, 2002) (Maiti et al., 2004) (Krafft et al., 2005)
892-896	Backbone, C-C skeletal	(Chan et al., 2006)
898	Monosaccharides (β-glucose), (C-O-C) skeletal mode	(Shetty et al., 2006)
921-924	C-C stretch of proline ring/glucose/lactic acid	(Benevides et al., 1997) (Stone et al., 2002b) (Stone et al., 2004) (Krishna et al., 2004) (Kendall et al., 2011)
927-929	C-C, stretching-probably in amino acids ,proline & valine (protein band)	(Lau et al., 2003)

Continued

Peak	Assignment	Reference
934-937	C-C stretching mode of proline, valine and protein backbone (α-helix conformation) /collagen	(Stone et al., 2004) (Cheng et al., 2005)
944-946	C-C protein	(Venkatakrishna et al., 2001)
949	CH_3 rock, olefinic CCH deformation, CH_3 α-helix	(Krishna et al., 2004) (Chrit et al., 2005)
954	Hydroxyapatite /carotenoid/cholesterol CH_3 α-helix	(957) (Stone et al. 2004) (952)(Krishna et al., 2004)
961-962	Symmetric stretching vibration of PO_4-	(Silveira et al., 2002) (Cheng et al., 2005)
963-964	Unassigned in protein assignments	Huang, 2003)
967-968	Lipids	(Dukor, 2002)
972-977	CH_3, CCH olefinic (protein assignment)	(Katainen et al., 2007)
981	C-C stretching β-sheet (proteins), CH bending (lipids)	(Notingher et al., 2003b)
1003-1014	Symmetric ring breathing mode of phenylalanine (proteins)	(Puppels et al., 1991) (Mahadevan-Jansen and Richards-Kortum, 1997) (Venkatakrishna et al., 2001) (Stone et al., 2002b) (Stone et al., 2004) (Krishna et al., 2004) (Malini et al., 2006) (Chan et al., 2006) (Jess et al., 2006) (Zhang et al., 2008)
1021-1023	Glycogen	(Dukor, 2002)
1035	C-H in- plane breathing mode of phenylalanine /collagen	(Stone et al., 2002b) (Stone et al., 2004) (Cheng et al., 2005)
1037	Phenylalanine of collagen	(Cheng et al., 2005)
1048	C-C stretch In deoxyribose, C-O stretching carbohydrate	(Puppels et al. 1991) (Jess et al., 2006) (Hutchings, 2009)
	Glycogen	

Continued

Peak	Assignment	Reference
1050-1050	Glycogen	(Krishna et al., 2007b)
1066-1068	C-C stretch protein, C–C stretch lipids, C-O DNA/RNA, phospholipids, O-P-O stretch (nucleic acids), C–N stretching mode of proteins.	(Puppels et al., 1991) (Mahadevan-Jansen and Richards-Kortum, 1997) (Borchman et al., 1999) (Naumann, 2001) (Krafft et al., 2005) (Krishna et al., 2004) (Chrit et al., 2005)
1080	Typical phospholipids	(Malini et al., 2006)
1084	C-N protein Phosphodiester groups in nucleic acids, C-N stretching mode of proteins (lipids mode to lesser degree).	(Stone et al., 2002b) (Dukor, 2002) (Stone et al., 2004) (Krishna et al., 2004)
1087-1089	C–C stretch, C-C stretch lipid, O-P-O nucleic acid	(Benevides et al., 1997) (Puppels et al., 1991) (Shafer-Peltier et al., 2002b) (Stone et al., 2004) (Krishna et al., 2004) (Chrit et al., 2005) (Short et al., 2005) (Jess et al., 2006) (Taleb et al., 2006)
1099	C-N proteins	(Cheng et al., 2005)
1101-1103	Collagen C–C stretch, O-P-O-stretching DNA/RNA, C-C stretch lipid	(Benevides et al., 1997) (Puppels et al., 1991) (Shafer-Peltier et al., 2002b) (Stone et al., 2004) (Krishna et al., 2004) (Chrit et al., 2005) (Short et al., 2005) (Jess et al., 2006) (Taleb et al., 2006)
1104	Phenylalanine (proteins)	(Lakshimi et al., 2002)
1135	C-N protein (1129), CO,CH_3 (lactic acid)	(Venkatakrishna et al., 2001) (Hutchings, 2009)
1166	C-H in plane bending Tyrosine	(Krishna et al., 2004) (Cheng et al., 2005)
1174	Tyrosine, phenylalanine, C-H bend (protein)	(Chan et al., 2006)
1176	C-H bending Tyrosine (proteins)	(Stone et al., 2002b)
1177	Cytosine, Guanine	(Ruiz-Chica et al., 2004) (Jess et al., 2006)
1213-1214	Amide III, Tryptophan and phenylalanine, Tyrosine, Thymine, Cytosine, Adenine	(Puppels et al., 1991) (Stone et al., 2002b) (Huang et al., 2003b) (Stone et al., 2004) (Krishna et al., 2004) (Krafft et al., 2005) (Taleb et al., 2006) (Jess et al., 2006)

Continued

Peak	Assignment	Reference
1230-1234	Amide III (β-sheet), antisymmetric stretching of phosphate groups of the polynucleotide chain (DNA)	(Shetty et al., 2006) (Dukor 2002)
1237-1239-1250	Amide III	(Dukor, 2002) (Ó Faoláin et al., 2005c) (Shetty et al., 2006)
1243	Amide III, asymmetric phosphate stretching modes (phosphate stretching modes originated from the phosphodiester groups of nucleic acids and suggest an increase the in nucleic acids in the malignant tissues)	(Dukor, 2002) (Cheng et al., 2005)
	C-O aromatic stretch or amide III collagen (CH_2 wagging, C-N stretch /pyrimidine bases (Cytosine, Thymine)	(Stone et al., 2002b) (Stone et al., 2004)
1245	Amide III disorder structure of proteins, collagen	(Krishna et al., 2004) (Shetty et al., 2006) (Chowdary et al., 2009)
1250	Guanine, cytosine (NH_2)	(Movasaghi et al., 2007)
1235-1270	Amide III C-N stretch, N-H in plane bending unordered	(Mahadevan-Jansen et al., 1998)
1272	C=C phenylalanine, Tyrosine	(Venkatakrishna et al., 2001) (Chowdary et al., 2009)
1288	Amide III, α-helix CH bend	(Krishna et al., 2004) (Zenone et al., 2006)
	Phosphodiester groups in nucleic acids	(Dukor, 2002)
1299	CH_2 deformation (lipid)	(Stone et al., 2004) (Malini et al., 2006) (Chowdary et al., 2009) (Hartman et al., 1973)
1301	Adenine, cytosine (1300)	
1315	Guanine ring breathing modes of the DNA/RNA bases, C-H deformation	(Ruiz-Chica et al., 2004) (Chan et al., 2006) (Chowdary et al., 2009)
	Protein, Amide III, (α–helix)	
1317	Guanine ring breathing modes of DNA/RNA bases, C-H deformation (protein)	(Benevides et al., 1997) (Ruiz-Chica et al., 2004) (Chan et al., 2006)
1318		(Benevides et al., 1997) (Ruiz-Chica et al., 2004) (Chan et al., 2006)
1320	Guanine, DNA/RNA CH deformation	(Puppels et al., 1991) (Ruiz-Chica et al., 2004) (Chan et al., 2006)
1322	CH_3 CH_2 deforming modes of collagen and nucleic acids	(Huang et al., 2003b)
1323	Guanine	(Ruiz-Chica et al., 2004)

Continued

peak	Assignment	Reference
1325	CH_3 CH_2 wagging mode in purine bases of nucleic acids	(Viehoever et al., 2003)
1344	CH_3 CH_2 wagging mode protein of collagen	(Stone et al., 2004)
1345-1349	CH_2 twisting &bending (protein, lipids), nucleic acids	(Short, 2005)
1373	Thymine, Guanine, Adenine amino acids breathing modes of nucleic acids	(Chan et al., 2006)
1375-1377	Thymine, Guanine, Adenine amino acids CH_3 deformation	(Short et al., 2005) (Jess et al., 2006)
1378-1379	CH_3 glycosamineglycans/ COO, CH_3 hyaluronic acid	(Krishna et al., 2004)
	Thymine nucleic acid	(Krafft et al., 2005)
1389	CH_2 deformation	(Short et al., 2005)
1407-1411	A symmetric stretching carboxylate (IgG)	(Lakshimi et al., 2002) (Hayashi et al., 1986) (Puppels et al., 1991)
	Adenine, Guanine	
1422	Backbone CH_2	(Krafft et al. 2005)
	COO- of protein	(Chowdary et al., 2009)
1420-1450	CH_2 scissoring vibration of lipids	(Gniadecka et al., 1997)
1423-1424	Deoxyribose,	(Ruiz-Chica et al. 2004)
1442	Fatty acids, CH_2 bending mode	(Mahadevan-Jansen and Richards-Kortum, 1997) (Hanlon et al., 2000) (Dukor, 2002)
1443	CH_2 deformation (lipids and protein)	(Stone et al., 2004)
1445	CH_2 deformation (protein)	(Venkatakrishna et al., 2001)
1448	CH_2 CH_3 deformation lipids, proteins	(Cheng et al., 2005)
	Collagen	(Kaminaka et al., 2002)

Continued

Peak	Assignment	Reference
1450	Methylene deformation in biomolecules	(Dukor, 2002)
	CH_2 bending	(Malini et al., 2006)
1451	Lipids/proteins CH deformation	(Jess et al., 2006)
	CH_2 CH_3 deformation	(Lakshimi et al., 2002)
1453	Protein bands	(Hanlon et al., 2000) (Dukor, 2002)
	Proline	(Zhu et al., 2011)
	Structural protein modes of tumours	(Utzinger et al., 2001)
1454	Collagen & phospholipids	(Mahadevan-Jansen et al., 1998) (Utzinger et al., 2001) (Lau et al., 2003)
1457	Nucleic acid	(Feld et al., 1995)
1460	CH_2 CH_3 deformation of lipids & collagen	(Cheng et al., 2005)
1462	CH_2, Disaccharides, sucrose	(Shetty et al., 2006)
1469-1471	C=N stretching	(Naumann, 2001)
1507	C=N stretching	(Naumann, 1998)
	Cytosine	(Ruiz-Chica et al., 2004)
1522-1537	C=C carotenoid	(Stone et al., 2004)
1589-1592	C=C stretch olefinic	(Chrit et al., 2005)
1600	C=C stretching mode of phenylalanine and Tyrosine	(Stone et al., 2002b)
1607	Cytosine (NH_2)	(Puppels et al., 1991) (Lakshimi et al., 2002) (Ruiz-Chica et al., 2004)
1608-1609	Aromatic amino acid (protein)	(Venkatakrishna et al., 2001)

Continued

Peak	Assignment	Reference
1619	C=C bending of proteins Tryptophan	(Cheng et al. 2005)
1621	Tryptophan (Ig G)	(Lakshimi et al., 2002)
	Tryptophan (Ig G), phenylalanine, Tyrosine	(Chowdary et al., 2009)
1623	Tryptophan and /or β-sheet	(Cheng et al. 2005)
1658-1660	Amide I (protein)	(Naumann, 1998) (Jess et al., 2006)
1661	Amide I/collagen	(Krishna et al., 2004)
1673	Amide I	(Ó Faoláin et al., 2005c)

6.4. Data Pre-Processing

Following spectral data acquisition a significant spectral processing is essential because the collected Raman spectra represented a combination of intrinsic tissue fluorescence, weak tissue Raman scattering signals, cosmic ray and noise (Huang et al., 2003b; Teh et al., 2008). Pre-processing steps aimed to remove any contaminations to the real tissue spectrum (Mahadevan-Jansen et al., 1998). Data pre-processing may be generally categorised into baseline correction, calibration shifts, cosmic ray removal, averaging and normalisation, with the baseline correction and normalisation are the most important steps.

6.4.1. Baselines Correction

Baseline correction can be conducted using either fully or semi-automated techniques. Fully automated techniques although more convenient to use, tends to be less accurate than semi-automated techniques (Prakash and Wei, 2011). In many spectroscopic techniques, it is not uncommon to have baseline offsets from spectrum to spectrum; this type of effects creates an additional confounding effect on principal component analysis (PCA) models. High or broad and weak baselines may result from instrument drifting or fluorescent background signals. This is referred to a non-Raman background (Taleb et al., 2006).

Although several baseline correction techniques exist which can be performed using different software, according to Gan *et al.* (2006), it is difficult to develop a theoretically perfect method for baseline correction when an approximate estimation of baseline has been the general method. A straight line to connect the two ends of a signal peak may be used and taken as the baseline for further calculation of peak area or peak height. However, if the straight line does not fit the real baseline, a polynomial fitting approach for baseline calculation by a sequential step-wise process will be implemented (Gan et al., 2006). For each step, the information from signal peaks is reduced and the baseline information takes the control position, which finally reaches the best estimation of the baseline (Gan et al., 2006).

6.4.2. Normalisation

The absolute signal intensity is not very reproducible in RS and is dependant on many experimental factors, such as the intensity of laser light source used for sample excitation, accumulation time, sample tissue orientation, as well as constituent and temperature variations (Skoulika et al., 1999; Gemperline, 2006; Taleb et al., 2006). For the purpose of quantitative measurements, and to compensate for any fluctuations in the intensity, a normalisation procedure is used (Skoulika et al., 1999).

Depending on the needs of an application, many normalisation schemes can be used, such as normalisation to a well defend peak, total area or centred and normalised to a given variance (normally unity) (Hutchings, 2009). The last normalisation procedure which is also referred to standard normal variate (SNV) normalises the spectrum, so the standard deviation of all intensities in a spectrum is 1.

6.4.3. Smoothing

Smoothing is the elimination of noise from the signal with the lowest possible signal distortion (Vivo-Truyols and Schoenmakers, 2006). Basically, smoothing reduces the pixel to pixel variance of the signal, but does not eliminate the noise from the signal. However, it is difficult to find a method that is able to eliminate all the noise without losing any valuable information. Therefore, a balance must be found between removal of noise and signal distortion. This balance depends on both the features of the signal and on the noise (signal frequency, noise variance, or the peak-shape) which are changeable from sample to sample (Vivo-Truyols and Schoenmakers, 2006).

Several smoothing methods such as Savitzky–Golay, mean filtering, exponential smoothing, and Fourier transfer, have been used in managing noise problems (Gan et al., 2006). According to Gobinet *et al.* (2009), polynomial smoothing is the most commonly used technique which is also called Savitzky-Golay (SG) smoothing after the names of the two authors who described the technique in 1964 (Savitzky and Golay, 1964). In the SG algorithm, the smoothing can be controlled with two parameters, the window size and the polynomial degree. With small window sizes and high polynomial degrees yielding noisy

signals (light smoothing), while large window sizes and low polynomial degrees may yield distorted signals (strong smoothing) (Vivo-Truyols and Schoenmakers, 2006). The window size allows for better fine-tuning of the smoothing. Therefore, the polynomial degree is kept constant and the smoothing process is optimised by varying only the window size (Vivo-Truyols and Schoenmakers, 2006). Smoothing fits a polynomial function of a specified 'degree' through a range 'size' of adjacent pixels, and replaces those with the polynomial curve which is applied across the entire spectrum.

One can understand that smoothing of the spectra should be done with considerable caution (Smith and Dent, 2005; Gemperline, 2006). The strong smoothing programme provides better signal-to-noise ratios than weak smoothing. However, this may result in the loss of significant spectral information for interpretation (Smith and Dent, 2005). For example, sharp peaks or shoulders are smoothed in a similar way to noise (Gemperline, 2006) and that will mix the source of variation in spectral data. To improve the Raman signal, it is important to reduce the amplitude of the noise, leaving the signal unaffected (Gemperline, 2006). For that reason, heavy spectral smoothing should be avoided.

6.5. Data Analysis

Statistical data analysis following pre-processing of raw spectral data which is a very important step prior to data analysis obtains successful and adequate data classification. By data classification, a representative spectrum of each group can be recognised and then features of spatial allocation of each biochemical constituent can be examined (Lee et al., 2007a).

There are two main approaches for spectral data analysis: univariate and multivariate analyses.

6.5.1. Univariate Data Analysis

Spectroscopic data are very complex and large for visual analysis and although variations in spectral data are often visible, subtle differences may be missed and can be difficult to identify (Kelly et al., 2011). Univariate statistical techniques based on peak intensities or peak-ratios have been widely used by many research groups to identify potential biochemical markers from spectral data (Mahadevan-Jansen et al., 1998; Stone et al., 2004; Teh et al., 2008). For comparison purposes, a representative spectrum of each group has been constructed by calculating the mean spectrum of the collected spectra. Univariate statistical analysis such as, t-test and ANOVA for normally distributed and non-parametric tests for non-normally distributed data, such as Mann-Whitney U and Kruskal-Wallis tests for pair-wise and groups differences determination, respectively, were used for selected important particular peaks.

Relative peak intensity and peak ratio intensity may be considered as a simple and useful calculation for group comparisons. Many research groups have developed classification models based on these methods. However, a significant amount of spectral information is still not exploited using such methods. While a univariate approach only considers a specific region of the spectra (Hutchings, 2009), complicated biochemical changes might affect the entire spectrum rather than just a particular spectral band. Although using single peak intensity and ratios are found to be useful to identify diagnostic markers, this approach may be of limited value compared to multivariate techniques (Hutchings, 2009).

Potential Biochemical Tissue Markers in Dysplastic Epithelium

Understanding the carcinogenesis process greatly assists the improvement of diagnostic techniques (Beljebbar et al., 2009). The process of tissue transformation to cancer is associated with fundamental changes in cellular morphology and biochemistry (molecular tissue composition) which can be detected by RS at early stages even before the morphological appearance (Beljebbar et al., 2009). In general, a dysplastic tissue shows increased cellular crowding with disorganization, increased nuclear material, increased nuclear cytoplasmic ratio, increased cells proliferation (mitotic activity) and abnormal distribution of chromatin (Pindborg et al., 1997). These changes are associated with increased metabolic activity (Mahadevan-Jansen and Richards-Kortum, 1997) and lead to increased relative amounts of nucleic acids and proteins (Short et al., 2005). These features result in specific changes in nucleic acid, protein, lipid and carbohydrate quantities and/or conformations (Robbins et al., 1994).

A lipid-dominated spectrum of normal epithelium and protein-DNA dominated spectra in malignant tissue have been reported by previous studies (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006). The normal tissue lipid-like spectrum is mainly due to the fact that the epithelial surface consists of a closely packed layer of cells with their lipid bilayer cell membranes subjected to the laser beam excitation (Krishna et al., 2004). While in inflammatory, dysplasia, and malignant tissues, protein and DNA dominated spectra are largely related to the increased amounts of surface proteins such as signalling agents, receptor proteins, enzymes, antigens, antibodies, immunoglobulins and DNA from the fast growing and proliferating cells in cancer tissue (Krishna et al., 2004; Malini et al., 2006). This is also supported by DNA studies which indicate that increased cellular nucleic acid content is one of the more prominent changes in cancer and pre-cancer which can be investigated by RS (Robbins et al., 1994).

For instance, Figure 6.3 shows typical mean Raman spectra for normal and malignant oral tissue (Malini et al., 2006). The normal tissue spectrum (a) which can show sufficient differences, shows prominent peaks at the 1301 cm^{-1} (CH_2 lipids) and 1745 cm^{-1} (phospholipids) bands. While the malignant tissue spectrum (b) exhibits more distinct peaks between 951 and 1337 cm^{-1} relating to nucleic acids and proteins.

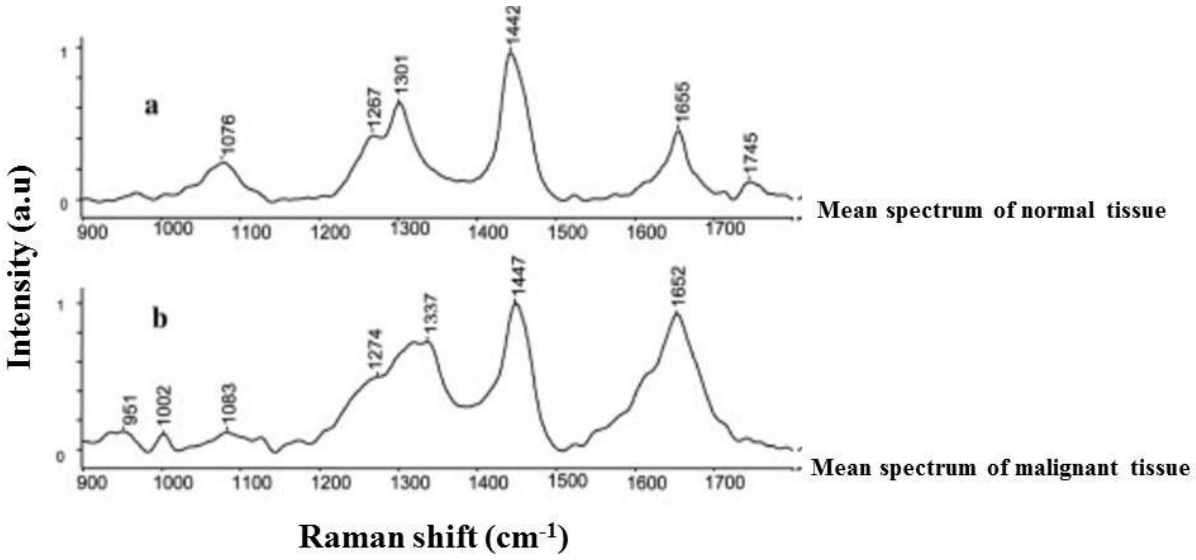

Figure 6.3: Mean Raman spectra of oral tissue: (a) normal and (b) malignant.

(Malini et al., 2006)

The morphological and biochemical tissue changes associated with cancer development are numerous and depend on the type and location of the cancer. These changes may be located in the cell membrane, cytoplasm, nucleus and extracellular space (Mahadevan-Jansen and Richards-Kortum, 1997). The progression of epithelial tissue towards cancer is associated with biochemical changes including increased nucleic acids, protein conformation and a reduction in glycogen and carotenoids (Stone et al., 2004). In epithelial tissue, such as oesophagus, colon and prostate tissue, it has been shown that benign tissue spectra exhibited increased levels of amino acids, such as tryptophan, tyrosine, proline and α-helix proteins, while neoplastic tissue spectra showed higher concentrations of nucleobases such as guanine, adenine, cytosine, uracil, O-P-O, β-sheet and unordered proteins (Stone et al., 2004). These general features have led to the development of a number of ratios between peak intensities being suggested as potential markers. For instance, Teh *et al.* (2008) reported that the differentiation between normal and dysplastic stomach tissue, could be achieved with a diagnostic sensitivity and a specificity of 86% and 80% respectively, if the intensity ratio of the hydroxyprolin peak (875 cm^{-1}) to the intensity of the CH_2 mode for protein/lipids (1450 cm^{-1}) was calculated. These features of biochemical tissue changes have, in a number of

cases, been found to agree with histological criteria used for cancer grading (Huang et al., 2003b; Mourant et al., 2005).

Studies on spectroscopic tissue markers for oral cancer are very limited and currently no specific spectroscopic marker is found for tissue diagnosis. However, a previous study on oral epithelium conducted by Isacsson and Shear (1981), found a lower concentration of glycogen in oral epithelial dysplasia including mild dysplasia compared with non-dysplastic epithelium. This decrease in glycogen content was found to be enhanced with increased severity of dysplasia. The reason for the decrease of glycogen was proposed to be due to consumption due to increased metabolic activity or decreased synthesis.

Diagnostic algorithms have been identified by Mahadevan-Jansen *et al.* (1998) which may have the potential for diagnosis of cervical pre-cancer. This was based on the decrease in the intensities of collagen at 1656 and 1070 cm^{-1} and increased intensity of peaks attributed to phospholipids, DAN and glucose 1-phosphate (a glucose molecule with a phosphate group on the 1-carbon) at 1454, 1330 and 978 cm^{-1} in squamous intraepithelial lesions of cervical tissue. These results are consistent with the histopathology of the neoplastic process, where the number of proliferating cells increases as dysplasia progresses (Mahadevan-Jansen et al., 1998). Furthermore, Mahadevan-Jansen *et al.* (1998) and Utzinger *et al.* (2001) identified similar algorithms related to the intensities at 1330, 1454 and 1656 cm^{-1} attributed to collagen, phospholipids and DNA and used to differentiate high grade squamous dysplasia from all other cervical samples with minimal classification errors. These peaks exhibited a consistent increase in intensity as disease progressed from normal to high-grade squamous dysplasia in an *in vivo* study. The intensity ratio of 1657 cm^{-1} to 1445 cm^{-1} may be used for differentiation in all gynaecologic tissue; the intensity is greater in normal than malignant in uterine and cervical samples. The intensity of 1070 cm^{-1} and 1656 cm^{-1} may be used to discriminate dysplasia from other tissues, with sensitivity and specificity of 88% and 92% (Mahadevan-Jansen and Richards-Kortum, 1997).

6.5.2. Multivariate Data Analysis Techniques

Multivariate techniques use mathematical, statistical and computer sciences to extract useful information from data set (Wang and Mizaikoff, 2008) and it is either 'unsupervised' which has no prior information about the samples or 'supervised' which requires a prior knowledge about the sample groups such as normal or pathological classification (Wang and Mizaikoff, 2008; Martin and Pollock, 2009). For both types, sufficient number of samples are required for robust model generation (Wang and Mizaikoff, 2008). For example, one third of the sample size may be taken for the test set, however, that is not always possible and may be difficult to generate such test sets due to insufficient number of spectra (Hu et al., 2009).

Unsupervised Procedures

Principal Component Analysis (PCA)

Many research groups working with different tissues have successfully used PCA for Raman data analysis (Mahadevan-Jansen et al., 1998; Krishna et al., 2004; Stone et al., 2004; Malini et al., 2006; Teh et al., 2008; Teh et al., 2010a). PCA is a widely used multivariate statistical tool for Raman data analysis to identify spectral changes that might be related to the pathological state of the biological tissue.

Usually there are many different sources of variation in a spectral data set related to a particular spectrum. Including, differences in constituents, instrument variation, sample handling error and others, and all can affect the appearance of the resulting spectrum. However, only few independent variables in the sample constitution are usually responsible for spectral differences (Nogueira et al., 2005). Raman spectra include information about peak intensity for a very large number of wavelengths. However, only a small number of variables might be responsible for variation in the data. PCA is a data compression multivariate technique (Krishna et al., 2007b) that removes irrelevant variations in spectral features, while preserving spectral features with independent variations. It is an unsupervised model with no prior knowledge of group classification.

Basically, PCA is able to extract the relevant information from the raw data and create a new set of variables named principal components (PCs) (factors) and scores (S). The PCs are related to the most important variation of the spectral data (Gemperline, 2006) and appear in order, with the first PC usually accounting for the most variation in the spectral data set, the second PC representing the next highest variance. This continues until the principal components represent only noise. Usually the first 8–10 PCs may be used (Martin and Pollock, 2009). Scores are related to the weight of each PC to reconstruct the original data (Geladi and Kowalski, 1986). PCA may be used under different conditions, entire spectra, selected regions and derivative spectra (Krishna et al., 2005a). Thus, it is important to find which principal components carry most of the information (Gemperline, 2006). The most significant principal components can then be used for group separation by plotting the scores in principal component space, each spectrum represented by a single point or score (Martin and Pollock, 2009).

PCA is the analysis of choice followed by classification, cluster analysis or other multivariate methods. It is able to reduce both the dimensionality of complex data and the influence of the measurement error by eliminating the principal component associated with noise (Gemperline, 2006). Also, PCA can identify an overall variance over the entire data set, although it is unable to detect within-groups and among-groups variations.

Cluster Analysis

Clustering techniques are unsupervised methods for grouping data into classes (Wang and Mizaikoff, 2008). Clustering may be applied when information about data classes is not available, or to see the trend of data grouping without using the data classes (Kelly et al., 2011). Many clustering techniques are used that do not need prior knowledge about the number of data groups, such as hierarchical clustering analysis (HCA), whereas other clustering techniques such as k-mean cluster analysis (KMCA) and fussy c-means cluster analysis (FCA) need such information for data input as a basic requirement. Cluster analysis has the same principle that in a measurement space, the distances between pairs of cases is inversely associated with degree of similarity (Gemperline, 2006). All these techniques group spectra according to the similarity between them, however, FCA has an additional advantage

over others by providing information about the relationship of each spectrum to each cluster (Wang and Mizaikoff, 2008). FCA is similar to KMCA, but FCA is able to determine the possibility of the fitness of each case to a specific cluster and also the association with other clusters, so one case may be assigned to more than one cluster which may provide valuable information for disease progression such as MT (Wang and Mizaikoff, 2008).

However, a major disadvantage of cluster analysis techniques which limits their practicality is that there is no validity measurement for quality of data clustering. Thus, these techniques mostly depend on the data set criteria and on the problem itself (Gemperline, 2006), not on a mathematical analysis. For that reason, it is important to have previous information about the study problem before using such methods (Wang and Mizaikoff, 2008).

Supervised Classification Procedures

The aim of the classification or discriminant analysis or supervised learning is to obtain bases that describe the separation between known groups of observations (Gemperline, 2006). A simple example of that is a binary classifier when data space is divided into two areas; with cases sharing the same properties are seen on one side of the decision line, whereas those possessing the other features are observed on the other side.

Soft Independent Modeling of Class Analogy (SIMCA)

SIMCA is a classification model capable of classifying high-dimensional data set observations (Branden and Hubert, 2005) and using separate PC models for different classes (Wang and Mizaikoff, 2008). This method is also useful when small datasets are analysed (more variables than objects) since it performs a substantial dimensionality reduction (Marengo et al., 2007). It is normally used for identifying local models for defined groups and to predict a possible class membership for new observations (Sikirzhytski et al., 2010). Hotelling's T^2 and Q statistics is used for group membership decisions (Sikirzhytski et al., 2010). The significance of the classification is assessed using the default of parameter of Quality of model fit (Q^2). The Q^2 statistic employed by SIMCA refers to: $1 - \prod(\frac{PRESS}{SS})$;

where PRESS is the prediction error sum of squares (measure of the classification error) and SS is the sum of squares. A probability used that a value of Q^2 greater than 0.097 arose by random chance is equivalent to $p < 0.05$; (SIMCA P-manual guide).

The classification in SIMCA is made by comparing the residual variance of a sample with the average residual variance distances of those samples that make up the class. This comparison provides a direct measure of the similarity of a sample to a particular class and can be considered as a measure of goodness of fit of a sample for a particular class model. Good classification of the data is greatly affected by irrelevant features (noise), thus removal of these features is essential to provide a clear separation of class structure (Gemperline, 2006).

There are several advantages of using SIMCA. Firstly, the unknown sample is not assigned to any class if its residual variance exceeds the upper limit of every class because it is either an outlier or not represented in the training set. Secondly, if the residual distance of a sample is below the statistical limit, other classification methods assign the sample to a single class forcibly leading to misclassification, while SIMCA assigns such a sample to multiple classes (overlapping between classes). Thirdly, to generate classification models, SIMCA is known to be sensitive to the data quality, so variables having low power for both modelling and discriminant are usually deleted from the analysis, because they mainly contribute to noise (Gemperline, 2006; Wang and Mizaikoff, 2008). Due to the aforementioned, SIMCA is considered the first successful approach for the discriminant analysis for datasets.

Linear Discriminant Analysis

Linear discriminant analysis (LDA) is a supervised technique which forms linear combinations of variables depending on differences between classes in the data set and determining the directions of the variable in the spectral space (German et al., 2006). An important feature in LDA is the establishment of discriminant functions which maximize the variations between groups and minimize them within group. Mahalanobis distance (MD) is the standard measure of distance between two cases when the observed data are quantitative (Bedrick et al., 2000). It is based on correlations between variables by which different patterns can be identified and analysed. Usually MD is used for classification of unknown

samples depending on the distance of the new sample to the centre of each class (Wang and Mizaikoff, 2008). For the validation of the LDA model, the leave-one-out cross-validation has been often used in which all spectra except one were used and then to classify the left out spectrum, this method is repeated so that each spectrum is predicted once (German et al., 2006). For its reliability, LDA needs the number of objects to be higher than the number of variables (Wang and Mizaikoff, 2008).

Partial Least Squares Discriminant Analysis (PLS-DA)

PLS-DA is an analytical technique that has been successfully applied to laser Raman spectral analysis derived from PLS regression models (Barker and Rayens, 2003). It is basically the inverse-least squares approach to LDA, which produces essentially the same result but with the noise reduction and variable selection advantages of partial least square (PLS) (Barker and Rayens, 2003). PLS-DA is more suitable for spectral data analysis when both data reduction and discrimination are needed (Barker and Rayens, 2003). While PCA is an unsupervised technique employed to reduce dimensionality and generate a visualisation of data based on total variances, PLS-DA is used to create 'scores' summarising the main variation among–group within the spectral data set (Taleb et al., 2006; Wang and Mizaikoff, 2008). Basically, PLS-DA constructs a set of linear combinations of the wavenumbers the same way as PCA, but uses the data classes at the same time, so it identifies and ranks of the basic signals based on their simultaneous ability to account for the variation in both group assignment and the spectral data. This means that variation between groups is emphasised and ranked higher, making the analysis more sensitive and accurate in the face of confounding variation. PLS-DA model is used successfully to group classification and it is preferred over PCA when both discrimination and dimension reduction is required (Barker and Rayens, 2003).

Artificial Neural Networks (ANNs)

It is a non-linear multivariate data analysis technique composed of several layers of connected processing units. Every processing unit transforms input data into an output by a

non-linear transferring function after assigning a weighting function for each node input (Wang and Mizaikoff, 2008). Although ANNs have the advantages of handling a very large amount of datasets and differentiate groups by subtle changes, it requires an extensive training and sufficient number of training samples to cover all the possible variations in the data set. ANNs is an inflexible technique in organising the number of units, neurons or layers, so it is time consuming, however, it is a useful application for biomedical samples with different disease stages or severities (Wang and Mizaikoff, 2008).

In conclusion, all the approaches described previously are available from commercial software packages including Matlab, Unscrambler and SIMCA. The existing multivariate methods of spectroscopic data analysis can extract useful information from the acquired complex data; however, novel solutions to the spectroscopic data analysis problem are always mandatory. Multivariate data analysis plays an increasingly important role for extraction of biomarkers, which are used for clustering and classification of samples from spectral datasets. The extraction of such markers is one of the important steps in the interpretation of group differences to detect the presence of certain diseases or identify their stages (Kelly et al., 2011).

6.5.3. Sensitivity and Specificity

In medical practice, to measure the power of a diagnostic test for correctly prediction a particular medical condition, sensitivity and specificity are commonly used (Stone et al., 2004). According to Van Belle *et al.* (2004), the sensitivity of a diagnostic test is the percentage of subjects with a disease who are classified positively with that disease. A diagnostic test is considered to be sensitive when the majority of subjects are positively classified with a disease. Specificity is the percentage of subjects having no disease, who are correctly classified as such. A highly specific test is when a small percentage of subjects are diagnosed positively with disease and most of them are without a disease (Stone et al., 2004). Using conventional histopathology as the standard for testing the ability of RS in tissue classifications, Raman assignment is classified as true positive or true negative when the is an agreement between both the diagnostic test and the spectral prediction; or Raman assignment

is classified as false positive or false negative when there is no agreement between them (Mahadevan-Jansen et al., 1998).

6.3.9. Biological Tissue Applications of Raman Spectroscopy

Since the early 1990s many research groups have used RS as a biospectroscopic diagnostic tool to differentiate normal from malignant cells in several different tissues (Kendall et al., 2009). Recently, with the development of instrumentation and analytical technologies, much progress has been made and researchers have started to consider changes at very early stages of dysplasia, moving toward early detection and classification of different stages of dysplastic features. RS may be regarded as a system of choice with a diversity of applications. This is mostly due to its high sensitivity to subtle molecular alterations, minimal sample preparations with no need for tissue fixation, labelling or staining as well as easily used for *in situ*, remote examination via fibre optic probe (Baena and Lendl, 2004). It has the ability to provide an enormous amount of information on many specific chemical bonds present in the investigated tissue (Wachsmann-Hogiu et al., 2009).

Studying biological tissues using RS is quite different from other types of samples. The reasons for that have been addressed by Manoharan and colleagues (1996) as follow:

1) Due to inhomogeneity in tissue composition and high scattering (tissue fluorphorse).
2) Because Raman signals are inherently weak and detection of early stage of disease that might be associated with low concentration of molecular constituents, requiring high intensity laser light excitation to acquire weak signals with high possibility of sample degradation.
3) The complexity of biological tissue with absorption of light throughout the visible spectrum causes auto-fluorescence emission which strongly interferes with Raman spectra.

However, such problems have been recognised and treated with new technology and instrumentations such as sensitive detectors, spectrographs, laser light sources and automated fluorescence subtraction with multivariate statistical data analysis.

Thus, RS is shown to be a promising diagnostic tool for discriminating normal from dysplastic or cancerous tissue, in both *in vivo* or *in vitro* samples analysis of cellular

constituents and metabolites of epithelial cell changes in different anatomical sites (Stone et al., 2002b) providing a great patient benefit for an early, rapid and accurate diagnosis with the most important clinical implementation being *in vivo* application via optical fibres. From this a great deal has been learned and extensive spectral library datasets now exist.

Table 6.2 summarises key Raman assignments within various biological tissues to help discriminate pathological from morphologically normal tissue spectra.

Table 6.2: Key Raman assignments of different biological tissues.

Type of tissue	Key Raman assignments	References
Skin	800–1000 cm^{-1} (amino acids proline, valine and polysaccharides) were observed with marked loss of intensities in basal cell carcinoma (BCC) compared to normal skin; 1220–1360 cm^{-1} (secondary proteins structure) showed higher intensity in BCC	(Gniadecka et al., 1997)
	1660 cm^{-1} (amide I of protein) can differentiate melanoma from the other types of tissue	(Gniadecka et al., 2004)
Lung	Amide I (~1670 cm^{-1}) and CH_2 bend mode of (1450 cm^{-1}) differentiate between normal and cancerous tissues; 1490 and 1449 cm^{-1} (CH_2 bend mode) differentiate between squamous cell carcinoma and adenocarcinoma	(Kaminaka et al., 2002)
	Higher percentage signals for nucleic acids, tryptophan, and phenylalanine, and lower percentage signals for phospholipids, proline, and valine in tumours compared to normal tissues	(Huang et al., 2003b)
Oesophagus	Increased levels of glycogen in normal tissue compared with increased DNA and protein levels in the dysplastic tissue areas	(Shetty et al., 2006)
Breast	Diseased breast tissue showed spectra similar to collagen with weaker lipid bands compared to normal	(Frank et al., 1995) (Frank et al., 1994)
	Normal breast tissue is primarily composed of lipid, while the amount of collagen increased in all abnormal breast tissues	(Haka et al., 2005)
	A strong presence of lipids in normal, collagen in benign and DNA in malignant breast tissues	(Chowdary et al., 2009)
Stomach	1200–1500 cm^{-1} (amide III and amide I of proteins) and 1600–1800 cm^{-1} (CH_3 CH_2 twisting of proteins nucleic acids, and the C=C stretching mode of phospholipids) can differentiate between normal and dysplastic tissues	(Teh et al., 2008)
	848–917, 960–1015, 1088–1133, 1206–1213, 1277–1313, 1395–1445, 1517–1549, 1607-1690, and 1714-1767 cm^{-1}, which are mainly assigned to proteins, lipids and porphyrin can differentiate between different gastric tissues	(Teh et al., 2010b)
	850-1150 cm^{-1} (proteins), 1200-1500 cm^{-1} (nucleic acids) and 1600-1750 cm^{-1} (lipids) bands identified between normal tissue and the two gastric adenocarcinoma subtypes	(Teh et al., 2010a)
Cervical/ovarian	1070 cm^{-1} (collagen) and 1656 cm^{-1} (amide I) can differentiate non-precancerous from precancerous, both were found to be greater in non-precancerous compared to precancerous; 1454 cm^{-1} (phospholipids), 1330 cm^{-1} (DNA) and 978 cm^{-1} (glucose-1 phosphate) were found to be increased in cervical precancerous	(Mahadevan-Jansen et al., 1998)
	1330, 1454 and 1650 cm^{-1} average intensities increase as tissue progressed from normal to high-grade dysplasia	(Utzinger et al., 2001)
	Normal and benign tissues dominated by proteins and lower concentrations of DNA and lipids compared to malignant tissue	(Krishna et al., 2007a)
Laryngeal	850-950 cm^{-1} and 1200-1350 cm^{-1} assigned to protein conformation and C-H bond stretch in nucleic acid bases can differentiate normal, dysplastic and squamous cell carcinoma	(Stone et al., 2000)
Oral	Normal tissue spectra originated from the lipid bi-layer cellular membrane, while inflammatory or cancerous cells dominated with protein spectra, related mainly to antigens, antibodies and DNA from the proliferating cells with large amount of surface proteins	(Malini et al., 2006) (Venkatakrishna et al., 2001) (Krishna et al., 2004)

Oral Tissue

The efficacy and potential application of RS technique in oral cancer and PMDs has been well demonstrated using various statistical approaches to exploit the subtle differences for diagnosis (Ghanate et al., 2011). Malini *et al.* (2006) applied RS to discriminate among 216 oral tissue spectra from 10 premalignant, 90 malignant, 37 inflammatory and 79 normal tissue samples. Specimens were placed in ice-cold saline and stored at -80 ºC. Spectra were collected after samples were passively thawed. Noticeable differences in spectral profiles were observed; normal tissue spectra originated from the lipid bi-layer cellular membrane, while inflammatory or cancerous cells dominated with protein spectra which are mainly related to several proteins on the cell surface. Although PCA scores discriminated normal from all other pathologies, poor discrimination was observed among the three disease sates. While combining PCA with multiparameter tests, all four tissue groups could be differentiated and diagnosed. Venkatakrishana *et al.* (2001) analysed oral tissue samples obtained from biopsy or surgical resection. Tissue from normal and squamous cell carcinomas were kept in saline and spectra were measured within half an hour of tissue removal. More than 140 spectra were acquired from different tissue sites and classified into normal and malignant sets and then subjected to PCA. The results showed that spectra could be classified into normal and malignant groups, with sensitivity and specificity of 90 test spectra were greater than 85% and 90%, respectively. Further spectroscopic work on oral tissue has been performed by Krishna and co-workers (2004). Oral tissues from normal and carcinoma were taken from biopsies fixed in formalin and stored at room temperature for two months. Spectra were collected from epithelial and subepithelial regions. The results showed significant differences between normal and malignant epithelial tissues, with no subepithelium spectral differences. The major differences between normal and malignant tissue spectra related to conformational changes in proteins that related mainly to contributions of antigens, antibodies and DNA from the proliferating cells with large amount of surface proteins. PCA showed very good discrimination between malignant and normal tissue spectra indicating the suitability of formalin-fixed tissues for optical pathology in oral oncology.

6.3.10. Fibre Optic Probe

As the Raman spectrum is not affected by water in biological tissue, it is considered as an appropriate possible future tool for portable equipment for *in situ* optical diagnosis (Santos et al., 2005; Beljebbar et al., 2009). Raman optical fibre is quite useful to measure the spectra in an optimal natural tissue condition providing valuable diagnostic information in a very short time (seconds) (Bakker Schut et al., 2000), not only from the surface, but also several microns deep (Zenone et al., 2006). The oral cavity is easily accessible for clinical examination and may be considered as ideal for the use of a Raman fibre optic probe for the purpose of early detection and diagnosis of oral pathologies.

With technical improvement, such as efficient lasers, detectors and advanced statistical multivariate data analysis, RS combined with data algorithms can be used routinely and objectively for optical diagnosis of oral pre-cancer and cancer through application of small portable optical fibre probes which can accurately collect tissue spectra *in situ* (Motz et al., 2005; Guze et al., 2009). The first promising *in situ* application was conducted on rat oral palatal mucosa by Bakker Schut *et al.* (2000) who reported a sensitivity of 93% and a specificity of 78% in detection of low grade dysplasia and sensitivity and specificity of 100% for detection of high grade-dysplasia and CIS.

Since fibre optic probe materials have high Raman signal in the fingerprint region of Raman (800-1800 cm^{-1}), in which many molecular vibrations scatter and produce Raman spectra, it may hinder the fingerprint spectra causing difficulties in differentiating Raman tissue spectra from background spectra (Utzinger et al., 2001; Koljenovic et al., 2005; Santos et al., 2005). To overcome such a problem, separate excitation and collection paths were designed (Santos et al., 2005). Also, materials used for optical probe construction should be considered for their fluorescence and optical properties (Utzinger and Richards-Kortum, 2003). Many optical fibres have been tested commercially for the best suitability for *in situ* use considering the time and irradiation powers for safety and practicality (Utzinger et al., 2001). Due to low hydroxyl groups (OH) in its core material (Santos et al., 2005), silica core-silica clad fibres, an acrylate coating and a black nylon jacket probe is found to be the most applicable for *in vivo* application. Although the noise problem persists in the fingerprint area, researchers tried to use high frequency region 2400–3800 cm^{-1} where there is only small background signal

contribution (Santos et al., 2005). This region has less Raman spectra; however, a significant achievement has been obtained utilizing C-H stretching bands close to 3000 cm^{-1} to differentiate between various tissue types (Guze et al., 2009). Different optical probes have been used with the Raman spectrometer using filters placed at or near the distal end of the optical fibre to reduce the contribution of the background signals (Koljenovic et al., 2005). Regarding the laser excitation used and according to Hutchings (2009), with NIR wavelengths thermal damage is possible, however, UV irradiation should be avoided because it breaks DNA strands.

Fibre optic probe can serve effectively as an adjunct to clinical examination may be of great benefit in clinic as a non-invasive, objective real-time diagnostic tool and for patient follow-up. This may include biopsy targeting by identifying the high risk area to eliminate random biopsies and reducing sampling errors (Beljebbar et al., 2009) as well as assessment of surgical margins during surgery.

6.6. Materials and Methods

6.6.1. Preparation of Oral Tissue Samples

All of the samples used in this study (n=32) were taken from the FOM; the most common oral sub-site affected by PMDs. The samples were selected from previously FFPE laser excised samples housed in the Royal Victoria Infirmary (RVI) histopathology archive. These blocks had been stored in the archive between 1999 and 2009.

Using haematoxylin and eosin (H and E) stained sections taken at diagnosis of samples that showed epithelial dysplasia with adjacent normal epithelium were selected. The FFPE blocks were then retrieved and 10 μm thick specimens were cut using a microtome (Olympus microtome) in the pathology department of the RVI. For every case, two adjacent sections were cut. The first section, to be used for the Raman analysis, was mounted onto a 20 mm x 20 mm x 0.5 mm barium fluoride (BaF_2, Photox Optical Systems Ltd, Sheffield, UK) window which was then dried overnight. The second section was mounted onto a glass slide and stained with H and E. Typical examples of the two sections are shown in Figure 6.4.

The purpose of this second H and E stained section was two-fold. Firstly, at least two oral pathologists independently assessed the tissue to grade the epithelial dysplasia using two separate classification systems; namely the WHO classification system (Gale et al., 2005) (mild, moderate, severe dysplasia and CIS) and a novel binary grading system; (high and low grade dysplasia) (Kujan et al., 2006); Figure 6.5. Where disparity between classifications was found between the pathologists, both pathologists reviewed the sections together a second time and a consensus grade agreed. Once this grade had been decided for all sections, the areas on the H and E slides relating to epithelial dysplasia and morphologically normal epithelium were marked by one of the pathologists, so that the areas for Raman analysis could be identified on the parallel BaF_2-mounted section.

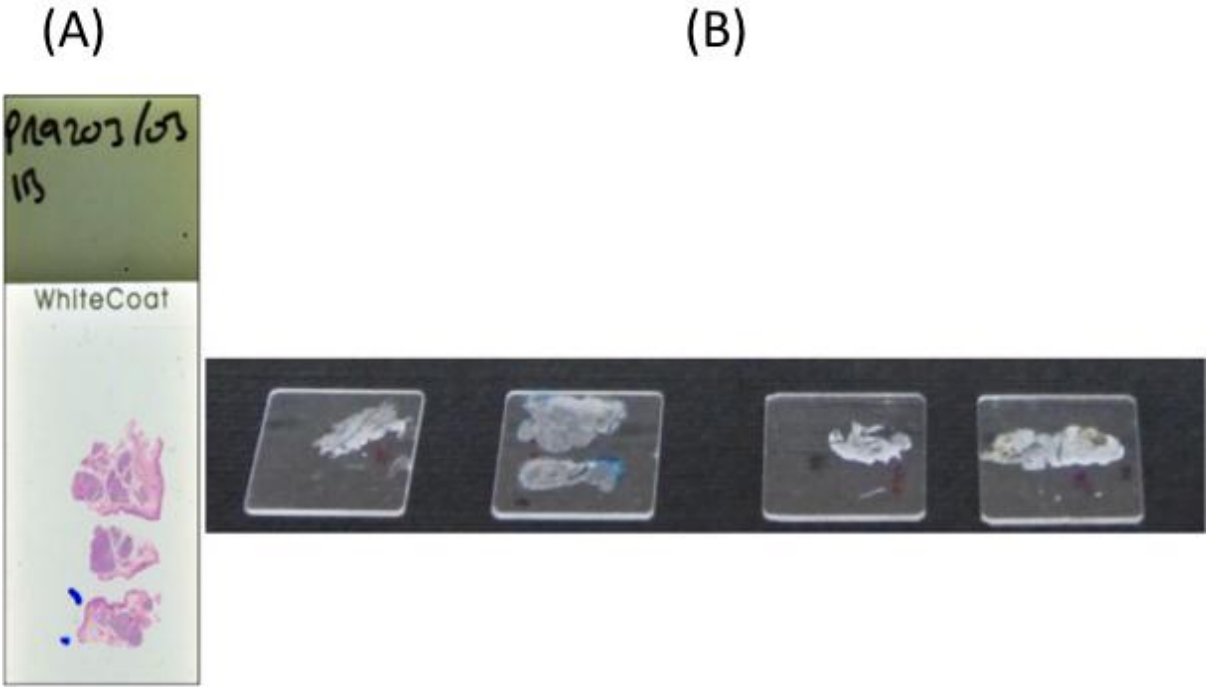

Figure 6.4: Typical examples of (A) a H & E stained section and (B) BaF_2 mounted specimens as used in this study.

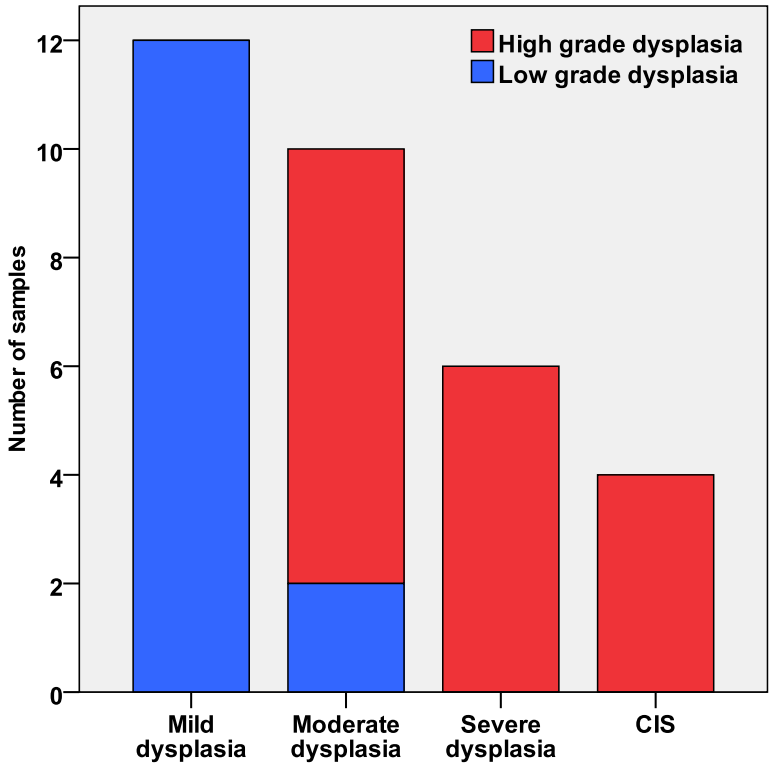

Figure 6.5: Tissue samples details.

The paraffin used to mount the archived samples has a characteristic Raman spectrum, which has been shown to affect the spectra of tissue samples (Manoharan et al., 1996; Glawdel et al., 2009). Consequently, before Raman analysis all tissue sections on the BaF_2 windows were de-waxed using the following protocol:

1. Immersed in a sealed glass bath containing 60 ml of n-hexane (3 pg/ml impurities, Merck, Germany) for 5 minutes.
2. Immersed in a sealed glass bath containing 60 ml ethanol (99.8%, Fisher Scientific, UK) for 5 minutes.
3. Placed overnight in an oven (25 $^{\circ}$C) to allow to fully dry.
4. Stored in a sealed desiccator containing silica gel beads until analysis.

6.6.2. Spectroscopic Instrumentation

All of the Raman analyses were conducted using a LabRam HR Raman spectrometer (HORIBA Scientific, France), shown in Figure 6.6. The spectrometer was equipped with a coherent diode pumped solid-state continuous wave-frequency doubled Nd:YAG laser (neodymium-doped yttrium aluminium garnet; $Nd:Y_3Al_5O_{12}$) operating at 532 nm. The intensity of the Raman signal with respect to the frequency (wavenumber) was detected with a CCD detector. A diffraction grating of 600 grooves per mm and confocal-hole diameter of 250 µm was selected as measuring parameters for the experiments. The Raman microscope was equipped with a motorized (xyz) sample stage for automatic scanning of tissue mapping points. The instrument was controlled using Lab Spec 5 control software (HORIBA Scientific, France).

The wavelength calibration of the Raman spectrometer was performed using the centre frequency of the silicon band from silicon wafer sample 520.7 cm^{-1} with a signal accumulation of 1 second.

With the H and E stained specimen placed in one light microscope (VWRI, ML 200) used to identify the correct experimental, each unstained BaF_2-mounted specimen was mounted in a specifically manufactured holder (Figure 6.7) and positioned in the Raman spectrometer, using a 10x lens (Olympus) so that the area of interest was in the centre of the visual field.

Once in place, a series of locations on the basal epithelium were selected, using the attached software, for each area (10-12 per location) as the locations from which spectra would be measured. Once this was complete, the lens was swapped for a 50x lens (Leica with numerical aperture (NA) of 0.55) to focus the laser onto the specimen (spot size approximately 1.5 μm in diameter, with an approximate laser power at the sample surface of 2.5 mW).

Preliminary investigation revealed that some specimens exhibited strong fluorescence background signals. As has been previously shown (Mahadevan-Jansen and Richards-Kortum, 1996; Petry et al., 2003; Krafft, 2004; Gobinet et al., 2007; Harris et al., 2009b) these can result in a reduced spectral quality. Consequently, a standard protocol, to be used for each specimen, was developed to reduce this fluorescence contamination by exposing the specimen to the laser light before acquiring spectra; a process previously termed photo-bleaching (Mahadevan-Jansen and Richards-Kortum, 1996). For the present study, an exposure time of 120 seconds was found, in a preliminary study, to reduce this fluorescence signal sufficiently to allow no obvious contribution in the spectrum. Consequently, this photo-bleaching procedure was used for all specimens in the study.

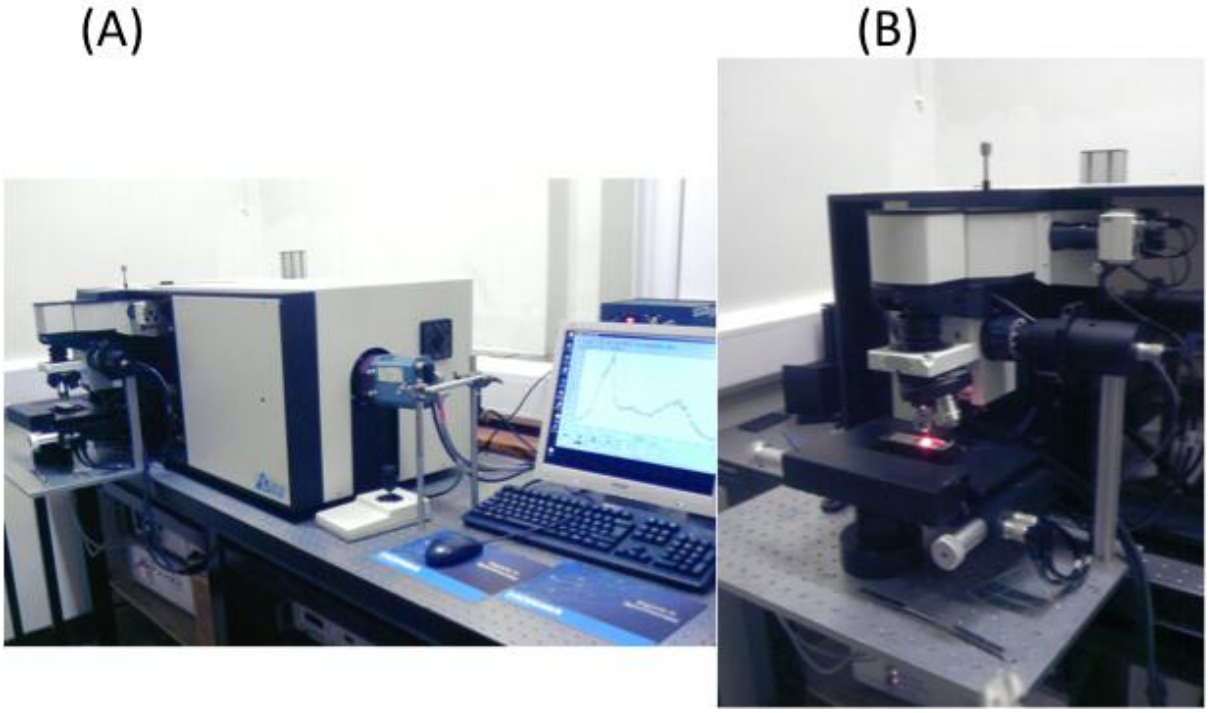

Figure 6.6: (A) The Raman system set up used for all experiments, (B) the confocal microscope unite with a close-up of a specimen in place with the laser focussed on the specimen.

Figure 6.7: Specifically designed holder for BaF_2 windows.

6.6.3. Collection of Spectra

Upon completion of the bleaching period, the specimen was orientated under the optical microscope so that the relevant region (either dysplastic or morphologically normal) could be identified. Next, a series of points ($10 \leq n \leq 30$) along the basal epithelial layer, within the boundaries indicated by the pathologists for each region, was selected using the spectrometer software (Lab Spec), a typical example of which is shown in Figure 6.8. These points marked the locations from which spectra were obtained. For dysplastic regions, the spectra were recorded from the central region within the boundaries indicated by the pathologists in the relevant adjacent section for dysplasia. For the morphologically normal epithelium, spectra were recorded at the excision margins.

Once the points had been selected, spectral acquisition was commenced with each point analysed automatically using the software. All spectra were measured over the spectral range of 800 to 1800 cm^{-1}. At each point two co-added spectra were measured, each collected over 30 seconds.

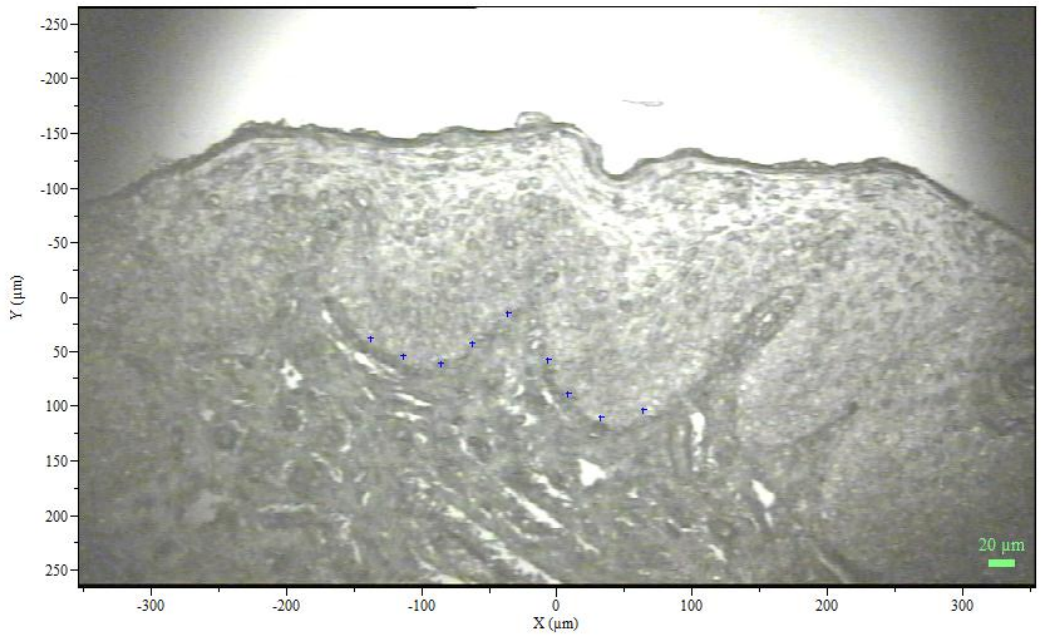

Figure 6.8: Typical optical image with points for measuring spectra marked by blue triangles.

Table 6.3 and Table 6.4 summarise the tissue samples classified according to WHO and binary grading systems, number of patient in each group, demography and number of Raman spectra used in the analysis.

Table 6.3: Histopathological diagnosis according to WHO (2005) with number of spectra used in the data analysis.

Histopathology group	N	Sex		Mean age/range (years)	Number of spectra		Total
		M	F		Dysplastic	Morphologically normal	
Mild	12	3	9	51 (33-71)	134	110	244
Moderate	10	5	5	58 (40-77)	79	93	172
Severe	6	4	2	57 (39-76)	83	107	190
CIS	4	1	3	58 (52-63)	90	82	172
Total	32	13	19	55 (33-77)	386	392	778

Table 6.4: Histopathological diagnosis according to the binary system with number of spectra used in the data analysis.

Histopathology group	N	Sex		Mean age in years (range)	Number of spectra		Total
		M	F		Dysplastic	Morphologically normal	
High grade	18	11	7	58 (39-77)	253	255	508
Low grade	14	4	10	51 (33-71)	147	123	270
Total	32	15	17	55 (33-77)	400	378	778

6.6.4. Data Processing

Once the spectra had been collected it was necessary to process them to remove a series of environmental components common to all Raman spectra but not related directly to the real tissue spectra. Consequently, a number of procedures were used after acquisition before the spectra could be analysed, summarised in Figure 6.9 and detailed below.

First, all spectra were assessed visually for detector saturation. Those spectra found to have a flat line in all, or part of, the spectral range were excluded from the data set. A typical example of a spectrum with and a spectrum without detector saturation are shown in Figure 6.10. Next all the remaining spectra were loaded into one file (Excel 2007, Microsoft) and the mean spectrum calculated. This mean spectrum was then subtracted from each individual spectrum to eliminate the majority of the variation between the spectra.

Careful observation of the spectra after mean centring revealed that a number of spectra exhibited systematic shifts in the locations of all of the peaks, a typical example of spectra exhibiting this are shown in Figure 6.11. Using the CH_2 scissoring peak at 1450 cm^{-1} as a datum, the spectra were edited in Excel so that the peaks lined up with the correct wavenumber. Those spectra that were corrected in this manner were noted down in case this process was subsequently found to affect the data.

All Raman spectra measured from biological tissue are affected by a background spectrum, mostly due to tissue fluorescence and the laboratory environment, which can be several orders of magnitude more intense than the actual tissue spectrum. Consequently, after the spectra were aligned the data file was loaded in MatLab (v R2008a, Mathworks, Cambridge, UK) to allow the removal of this background. The correction was done by selecting local minima in the spectra by eye and then conducting linear interpolation between these points. Figure 6.12 (A) shows the original spectrum collected from the resection margins of mild dysplasia (morphologically normal) and (B) baseline corrected.

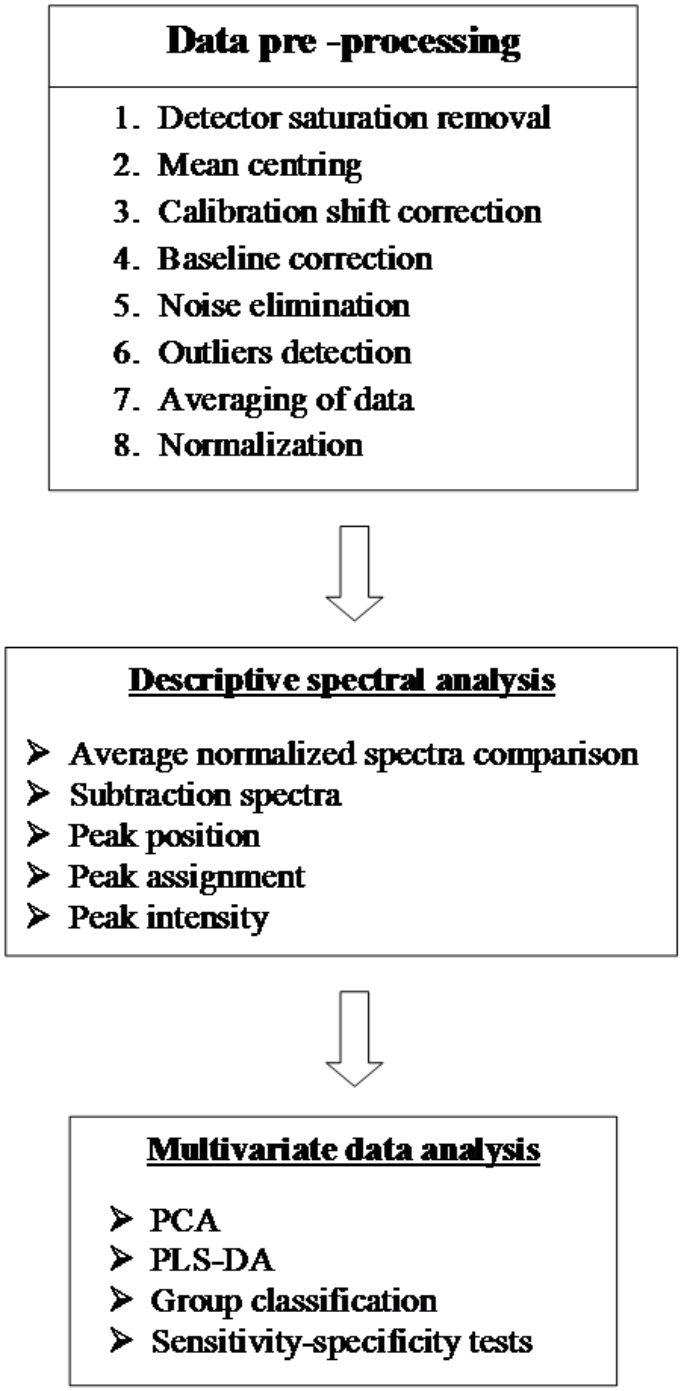

Figure 6.9: Data processing.

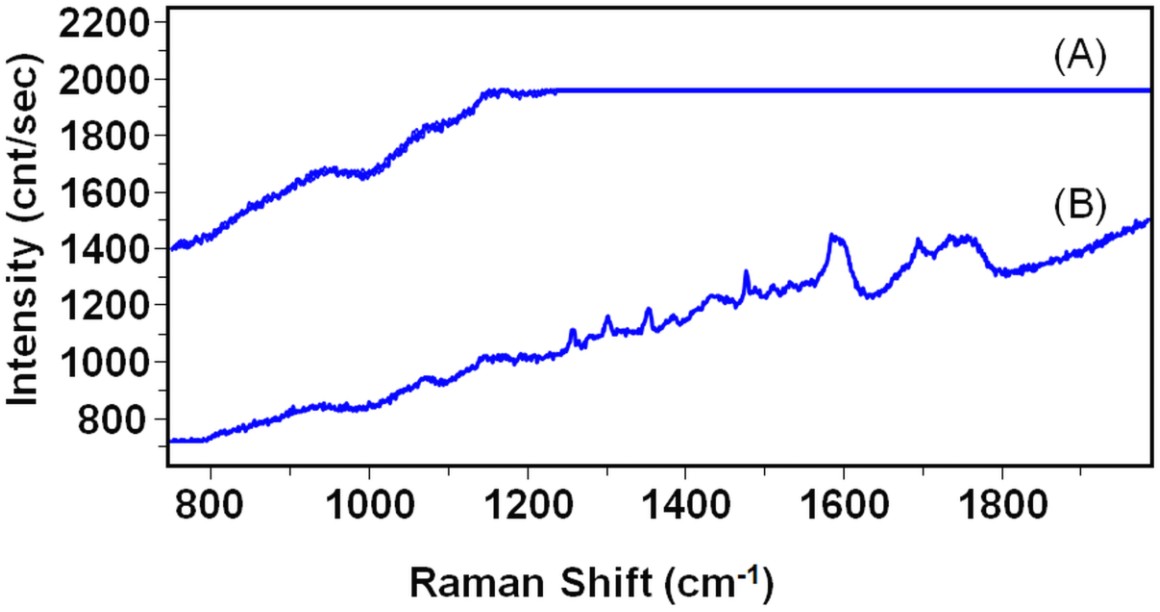

Figure 6.10: Example of unprocessed spectra showing (A) saturation and (B) without saturation.

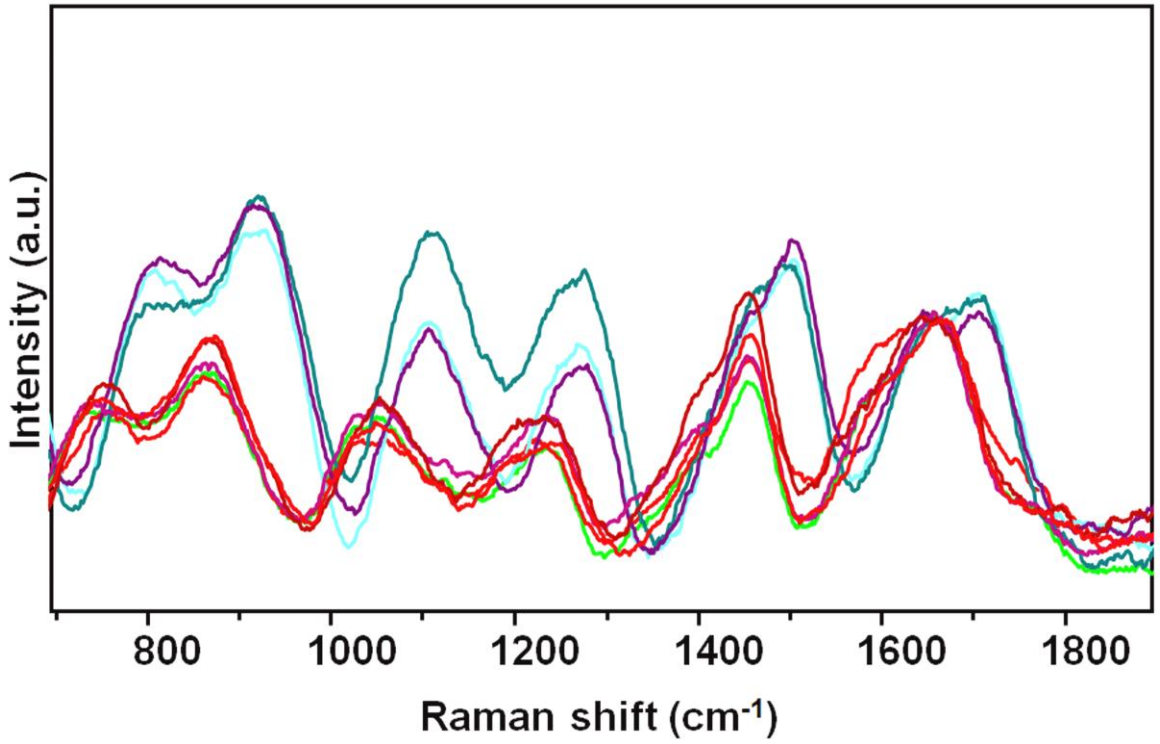

Figure 6.11: Example of calibration shift in spectra; as determined by a consistent x-axis offset compared to majority of the spectra.

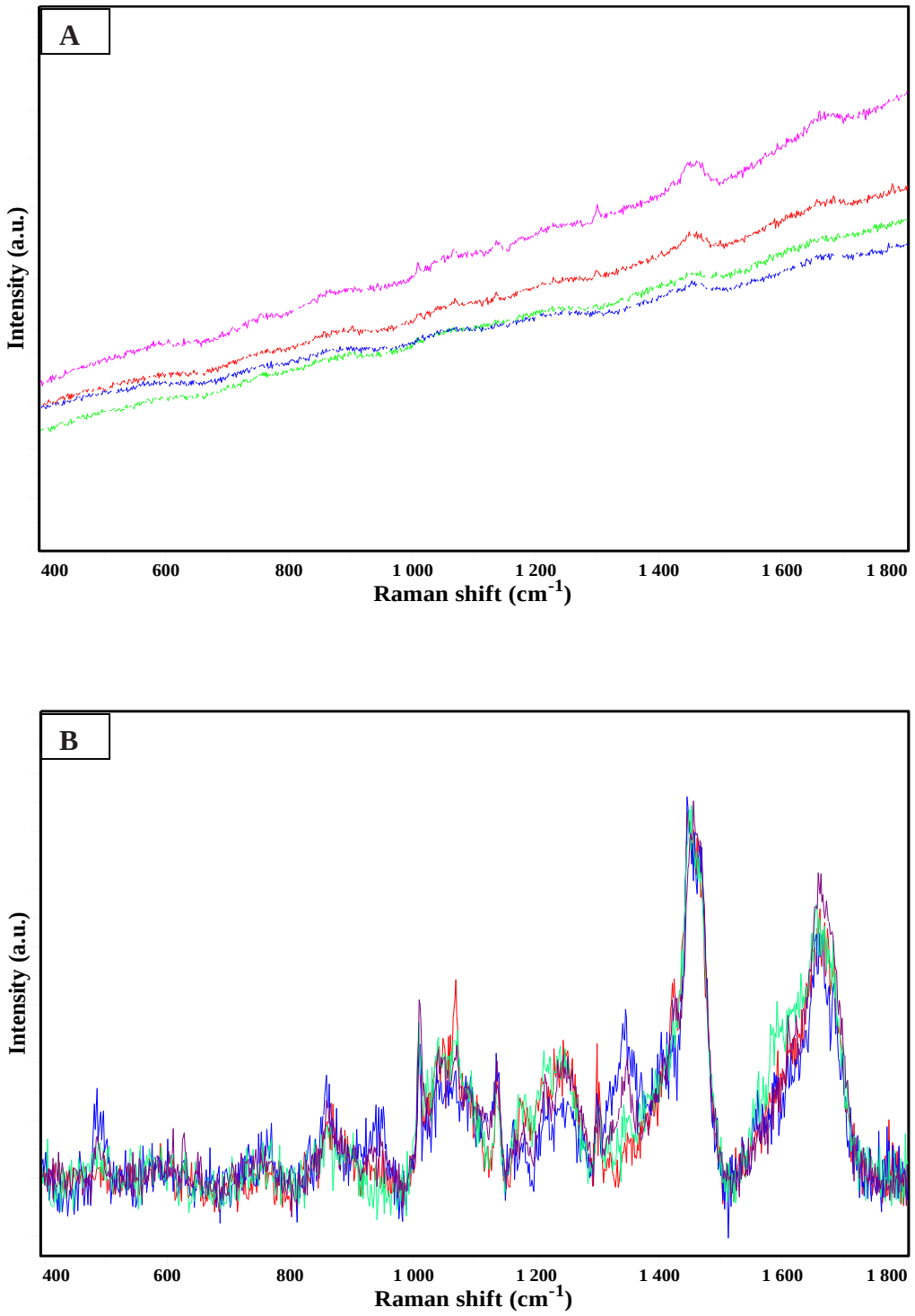

Figure 6.12: Raman spectra acquired from morphologically normal tissue at the resection margins of mild dysplasia sample; (A) original spectra (B) background corrected spectra.

After baseline correction it was clear that there were spectra in which the signal to noise ratio was low, with many of the spectral features obscured by noise. Using PCA, the average intensity of the CH_2 scissor mode (1450 cm^{-1}) was used to define three threshold intensity values: 250, 500, and 750 counts; Figure 6.13 (A), (B) and (C).

Only data above the threshold of 750 counts which eliminates all the noisiest signals that affect the performance of the data set (masking the features of the real Raman spectra) were included in further data analysis.

After that the data were assessed for outliers, using Hotelling's T^2 test in PCA (Unscrambler, v 9.7 Camo, Oslo, Norway) to identify potential strong outliers (outliers that are the most different from the rest of the data set) and the use of sample leverage (distance from model centre) and residual variance (distance to model plane) to identify weak outliers (outliers that having the biggest undue influence on the model generation).

These data were then normalised against the mean intensity, between 1435 cm^{-1} and 1480 cm^{-1}, corresponding to the full width at half maximum for the CH_2 scissoring mode, centred at approximately 1450 cm^{-1}. The next step in data processing was to average the spectra by patient and by tissue type, as dysplastic and morphologically normal tissue groups.

The spectra were reviewed blind to the case so that no bias would be caused during these selection procedures. This was true of all processing steps and that is why the full raw data set was inserted into one file for the processing. After all spectral exclusion, the total number of spectra was reduced to 778 spectra, from an initial collected 1045 spectra.

Regarding tissue classification, once the data processing was complete, the fully processed data were analysed with PLS-DA using SIMCA (SIMCA P 8.0, Umetrics, Umea, Sweden) and a series of models were generated to discriminate between different groupings. Partial least square analysis components (PLSCs) were calculated to maximise the co-variance between the data and the groupings, so that the models simultaneously described information contained in both the spectra and the group assignments. The significance of the classification by each PLS component was assessed using "Quality of model fit" (Q^2).

To highlight the differences between spectra, a representative average spectrum of each group of dysplastic and morphologically normal tissue with their subtraction spectrum was plotted using Lab Spec spectroscopy suite (HORIBA Scientific, France). To improve the spectral clarity and for presentation purposes only, spectra were lightly smoothed by fitting a fourth order polynomial through adjacent 9 pixel segments of the spectra; care was taken to ensure that no spectral information was lost during this processing.

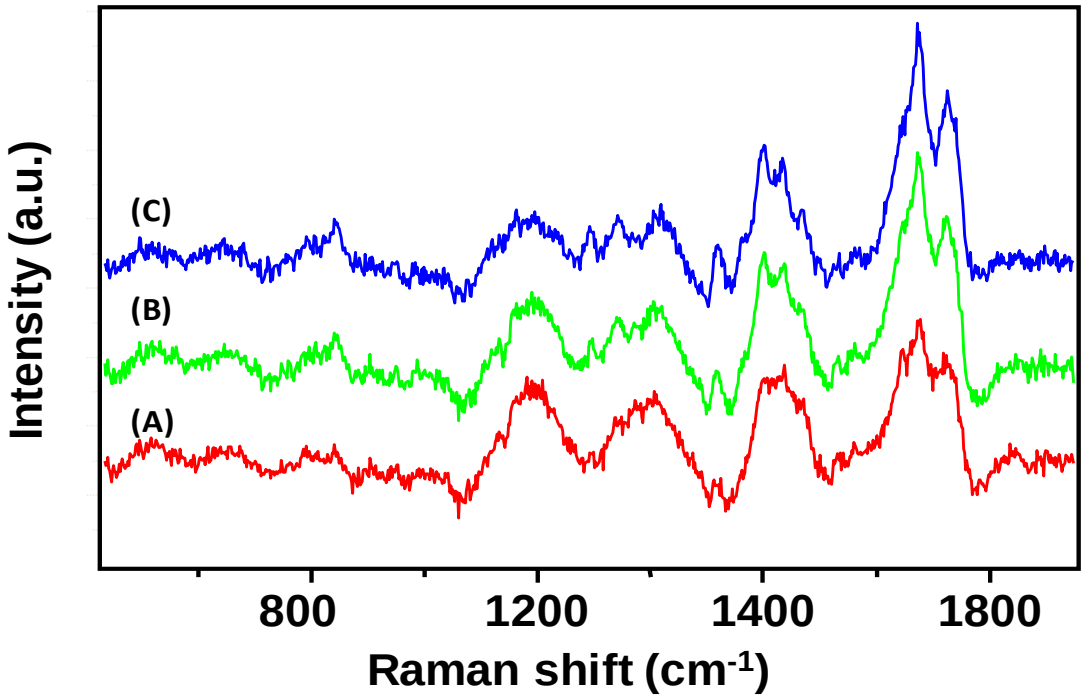

Figure 6.13: Three threshold intensity values overlaid from PCA loading (PC1): (A) 250 counts, (B) 500 counts, (C) 750 counts which eliminates all the noisiest signals.

6.6.5. Raman Data Analysis

A representative spectrum of each patient and each group of patient was obtained by calculating the mean spectrum of the collected spectra. To compare between the groups, peak relative intensity, peak position and other spectral features were considered.

Normality tests were performed using the Shapiro-Wilk test to check the distribution of spectral data. Spectral data were found to be not normally distributed, as the Shapiro-Wilk *p*-value was less than the chosen alpha level (0.05). Therefore, a nonparametric test, which does not assume that the data are normally distributed was used. The Mann-Whitney U test; to determine the statistical significance of differences on the mean value in relative peak intensities for each pair of groups (pairs-wise comparisons) and Kruskal-Wallis test to test for significance of groups separation were used.

To quantify the intensity differences between the study groups at the identified peaks, univariate statistical techniques based on single-peak intensities were used in this study. SPSS (Statistical Package for Social Sciences) version 19.0 software package (SPSS Inc.; Chicago, IL, US) was used in this study. The probability (*p*-value) of less 0.05 was used to represent real significant structural differences.

Regarding Raman tissue classification, the performance was quantified using a sensitivity and specificity test (Mahadevan-Jansen et al., 1998). Using histopathological diagnosis as a gold standard, Raman assignment was classified as true positive or true negative when there is an agreement between both the diagnostic test and the spectral prediction or classified as false positive or false negative when there is no agreement (Mahadevan-Jansen et al., 1998; Stone et al., 2004). These can be calculated as below:

$$Sensitivity = \frac{TP}{TP + FN}$$

TP is the number of true positives
FN is the number of false negatives

$$Specificity = \frac{TN}{TN + FP}$$

TN is the number of true negatives
FP is the number of false positives

6.7. Raman Results

Two grading systems were used to group the tissue specimens, the WHO classification system (Gale et al., 2005) of mild, moderate, severe dysplasia and CIS, and the binary scoring system of high/low grade dysplasia (Kujan et al., 2006).

No spectral contamination from formalin was observed, confirmed by the absence of very strong formalin bands at 907, 1041 and 1492, cm^{-1} (Shim and Wilson, 1996; Huang et al., 2003a; Krishna et al., 2004; Krishna et al., 2007a). Also, no strong paraffin contribution was observed in Raman spectra at 1062, 1296 and 1441 cm^{-1} (Ó Faoláin et al., 2005b), indicating an effective de-waxing protocol used in this study. Mean spectra were calculated for each group to allow the identification of the important Raman peaks for each tissue type.

6.7.1. Practical Considerations for Sample Mounting

Two potential specimen substrates were considered, conventional silicate glass and BaF_2. The former routinely used in histopathology labs, the latter are routinely used by IR and Raman spectroscopists for tissue analysis. Tissue specimens mounted on glass slides were not suitable for Raman study due to the relatively strong Raman signal of glass which masked any contribution from tissue components; Figure 6.14. Consequently, BaF_2 was chosen for use as a substrate for the Raman tissue measurements. This material has no Raman signature within spectral range of the biological tissue samples (800-1700 cm^{-1}) (Gobinet et al., 2009) and has only one Raman peak at approximately 242 cm^{-1} (Chen and Shen, 2006) minimising the contamination of the tissue spectrum by the slide; Figure 6.15.

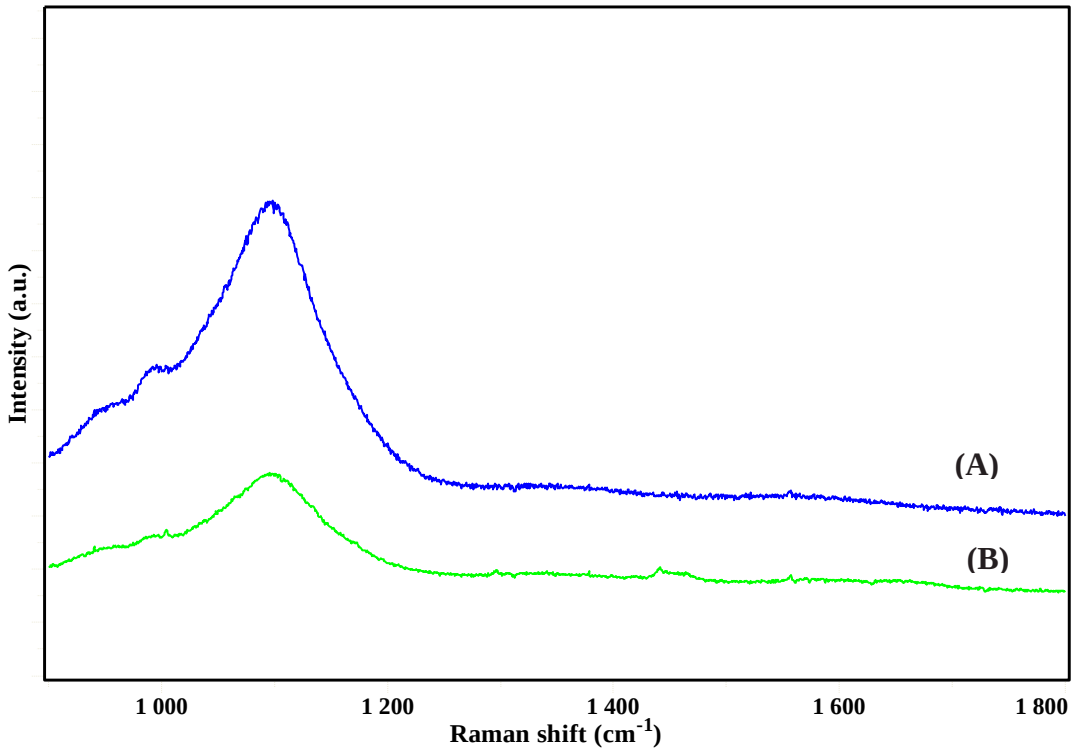

Figure 6.14: Typical spectrum for (A) bare glass and (B) an oral tissue spectrum mounted on a glass slide.

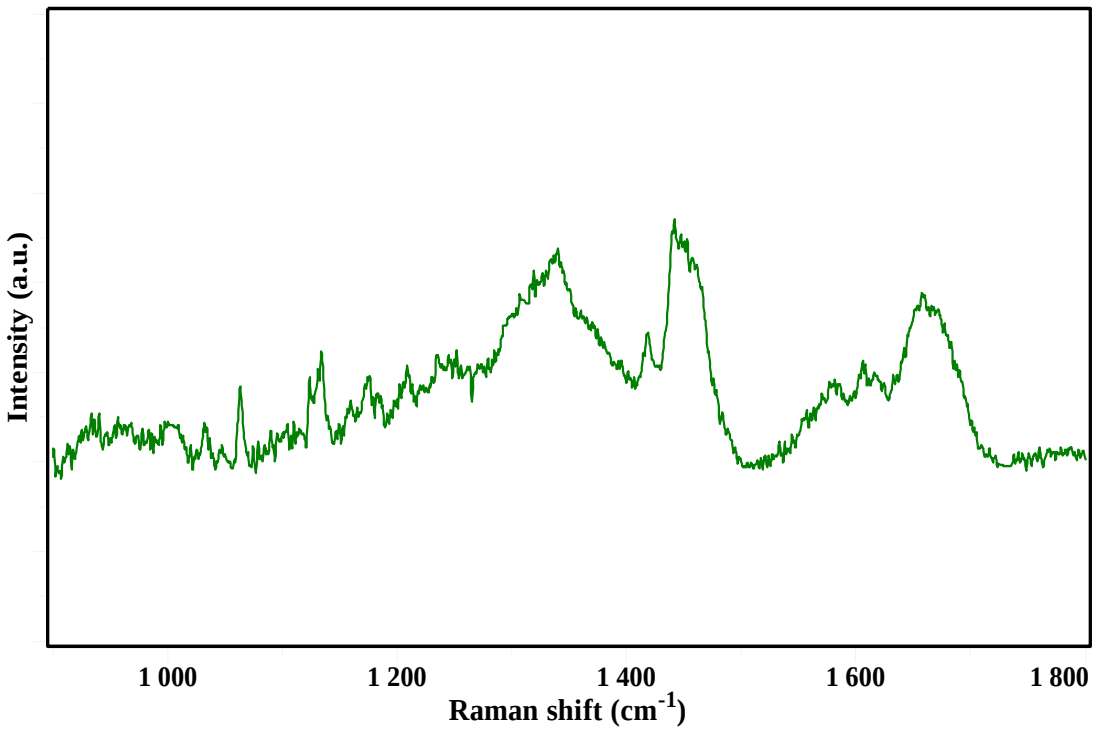

Figure 6.15: Typical oral tissue spectrum mounted on barium fluoride substrate.

6.7.2. Raman Spectra

Mild Dysplasia Tissue Specimens

The mean spectra for morphologically normal and mild dysplastic tissue specimens are shown in Figure 6.16 and Figure 6.17, respectively. In general, the spectra for the two tissue types exhibited the same shape, with the key peaks for each tissue type with their relative intensities summarised in Table 6.5 using the peak identification routine in Lab Spec.

Careful analysis of the spectra showed clear intensity differences in some regions indicating a biochemical tissue changes between the two types of tissue. To better show these key differences, spectra overlaid and the difference spectrum was constructed, where the morphological normal mean spectrum was subtracted from the mild dysplastic mean spectrum; Figure 6.18. The morphologically normal tissue spectrum exhibited more intense peaks in the region at 859-967 cm^{-1} (amino acids), 1022-1105 cm^{-1} (glycogen, proteins and nucleic acids), 1301 cm^{-1} (CH_2 deformation lipids) and 1423 cm^{-1} (nucleic acids). Whereas, the mild dysplastic tissue specimens showed a higher intensity of phenylalanine protein at 1009 cm^{-1} and in the peaks of the region at 1135-1661 cm^{-1}. In this region, differences between the two tissue types were seen at 1317 and 1347 cm^{-1} (nucleic acids bases and C-H deformation proteins), 1377 cm^{-1} (amino acids CH_3 deformation), 1591 cm^{-1} (C=C olefinic stretch of lipids), 1609 cm^{-1} (aromatic amino acid of proteins) and 1661 cm^{-1} (amide I of protein). However, only the difference in intensity of the symmetric ring breathing mode of phenylalanine at 1009 cm^{-1} was found to be significantly more intense in mild dysplastic tissue specimens compared to morphologically normal tissue (0.642 vs. 0.557) (p=0.001; Mann-Whiney U test); Table 6.6. This indicates a major contribution from proteins in dysplastic tissue spectra.

Peaks such as 1251 cm^{-1} and 1272 cm^{-1} (amide III of protein), 1288 cm^{-1} (phosphodiester groups in nucleic acids) and 1600 cm^{-1} (proteins assignment of phenylalanine and tyrosine) occurred in morphologically normal tissue, but were absent or very weak in the mild dysplastic tissue specimens indicating a degradation or disordering of these cellular components in mild dysplasia compared to normal tissue specimens.

An average spectrum of morphologically normal and mildly dysplastic tissue from each patient in this group (n=12) can be seen in the Appendix (2-A).

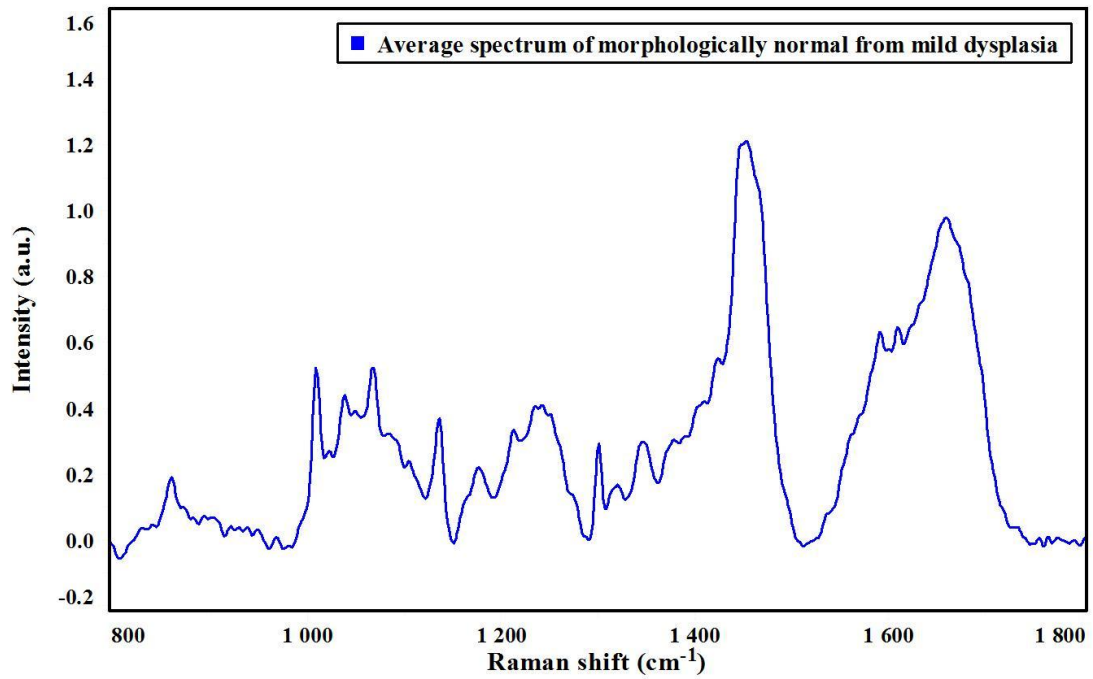

Figure 6.16: Mean Raman spectrum of morphologically normal tissue of mild dysplasia specimens (n=110 spectra).

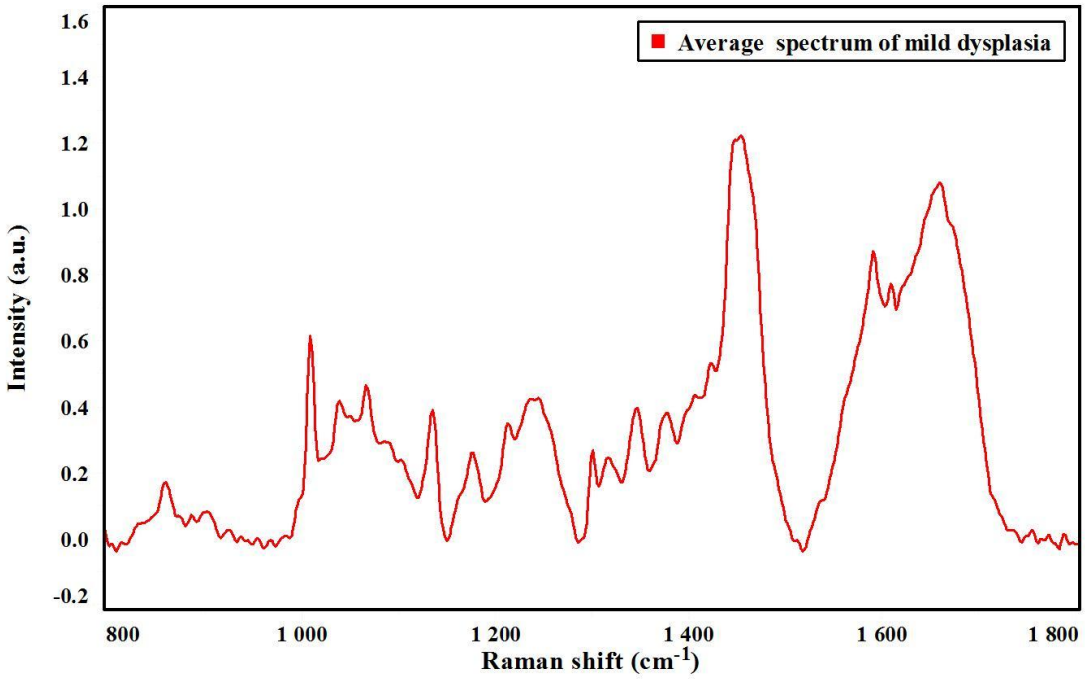

Figure 6.17: Mean Raman spectrum of mild dysplastic tissue (n=134).

Table 6.5: Peak position and relative intensity for mild dysplasia and morphologically normal tissue mean spectra.

Morphologically normal tissue spectra (Mn)		Mild dysplastic tissue spectra (Md)		Spectral features
Peak position (cm^{-1})	Intensity (a.u)	Peak position (cm^{-1})	Intensity (a.u)	
839	0.040	**834**	**0.047**	Higher intensity in Md
859	**0.215**	859	0.180	Higher intensity in Mn
872	**0.100**	874	0.088	Higher intensity in Mn
882	0.082	**886**	**0.087**	Higher intensity in Md
901	0.069	**902**	**0.076**	Higher intensity in Md
920	**0.029**	925	0.019	Higher intensity in Mn
937	**0.044**	938	0.026	Higher intensity in Mn
948	**0.029**	944	0.010	Higher intensity in Mn
967	**0.030**	968	0.013	Higher intensity in Mn
1009	0.557	**1009**	**0.642**	Higher intensity in Md
1022	**0.279**	1020	0.269	Higher intensity in Mn
1039	**0.446**	1039	0.417	Higher intensity in Mn
1048	**0.393**	1050	0.365	Higher intensity in Mn
1068	**0.530**	1066	0.472	Higher intensity in Mn
1084	**0.337**	1086	0.310	Higher intensity in Mn
1105	**0.244**	1103	0.216	Higher intensity in Mn
1137	0.375	**1135**	**0.381**	Higher intensity in Md
1177	0.202	**1177**	**0.263**	Higher intensity in Md
1213	0.338	**1214**	**0.364**	Higher intensity in Md
1235	0.409	**1237**	**0.443**	Higher intensity in Md
1243	0.429	**1245**	**0.447**	Higher intensity in Md
1251	**0.379**	**-**	**-**	Missing in Md
1272	**0.148**	**-**	**-**	Missing in Md
1288	**0.021**	**-**	**-**	Very weak in Md
1301	**0.294**	1301	0.267	Higher intensity in Mn
1320	0.180	**1317**	**0.243**	Higher intensity in Md
1347	0.292	**1347**	**0.399**	Higher intensity in Md
1378	0.323	**1377**	**0.375**	Higher intensity in Md
1407	0.425	**1407**	**0.443**	Higher intensity in Md
1423	**0.554**	1423	0.552	Higher intensity in Mn
1454	1.220	**1454**	**1.231**	Higher intensity in Md
1536	0.070	**1536**	**0.098**	Higher intensity in Md
1592	0.654	**1591**	**0.883**	Higher intensity in Md
1600	**0.654**	**-**	**-**	Missing in Md
1609	0.663	**1609**	**0.766**	Higher intensity in Md
1660	0.988	**1661**	**1.080**	Higher intensity in Md

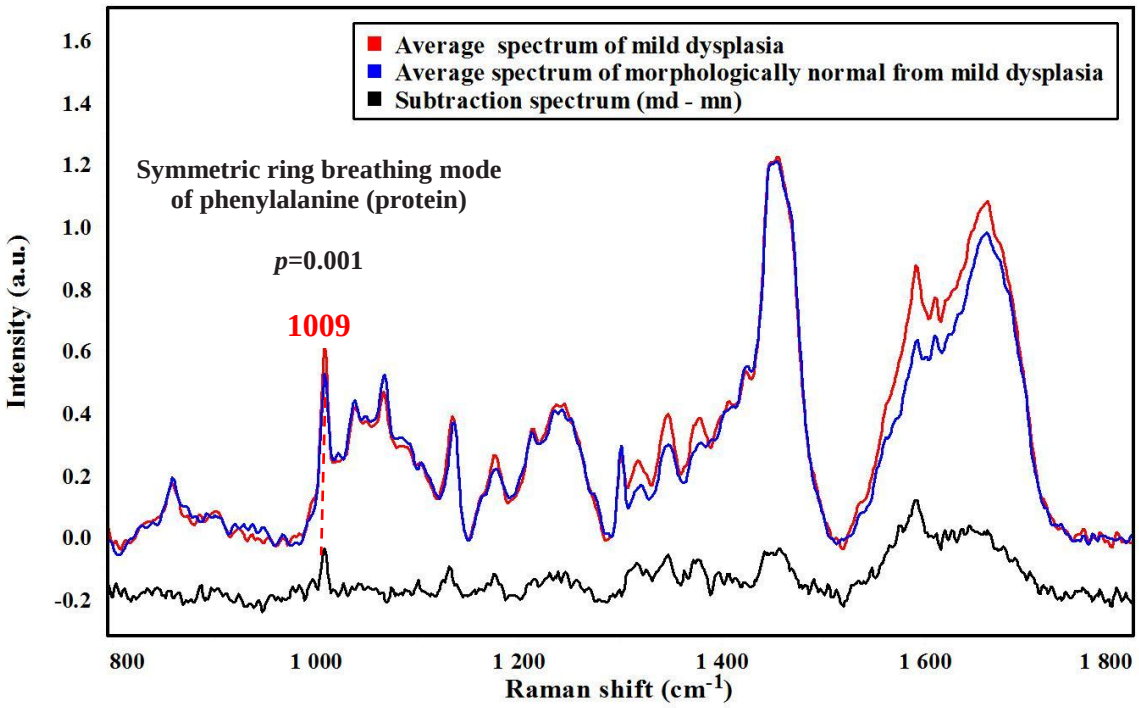

Figure 6.18: Overlaid normalised mean spectra for morphologically normal and mild dysplastic tissue with the difference spectrum beneath.

Table 6.6: Main Raman bands and assignments in mild dysplasia and morphologically normal tissue with *p*-value resulting from Mann-Whitney test comparison of the relative peak intensity between the two tissue types.

Peak position (cm^{-1})	Raman Assignment	*p*-value
834- 839	Out of plane breathing Tyrosine, O-P-O stretching of nucleic acids	*0.974*
859	Molecular vibration mode of proteins mainly Tyrosine and C-C stretch	*0.224*
872-874	Hydroxyproline, tryptophan	*0.158*
882-886	Tryptophan (protein)	*0.375*
920-925	C-C stretch of proline ring, glucose, lactic acid	*0.375*
937-938	C-C stretching mode of proline, valine, protein backbone (α-helix conformation), collagen	*0.491*
944-948	CH_3 rock, olefinic CCH deformation, CH_3 α-helix	*0.491*
967-968	Lipids	*0.158*
1009	Symmetric ring breathing mode of phenylalanine (proteins)	***0.001***
1020-1022	Glycogen	*0.818*
1039	Phenylalanine of collagen	*0.412*
1048-1050	Glycogen	*0.450*
1066-1068	C-C, C–N stretching mode of proteins; C–C stretch lipids; O-P-O stretch nucleic acids; C-O DNA/RNA	*0.491*
1103-1105	Collagen C–C stretch, O-P-O-stretching DNA/RNA, C-C stretch lipid	*0.974*
1135-1137	C-N protein CO, CH_3 (lactic acid)	*0.818*
1177	Cytosine, Guanine	*0.082*
1213-1114	Amide III, Tryptophan and phenylalanine, Tyrosine, Thymine, Cytosine, Adenine.	*0.768*
1243-1245	Amide III, disorder structure of proteins, collagen	*0.922*
1301	CH_2 deformation (lipids)	*0.818*
1317-1320	Guanine ring breathing modes of DNA/RNA bases, protein C-H deformation	*0.450*
1347	CH_2 twisting and bending in protein, lipids, nucleic acids	*0.533*
1377-1378	Thymine, Guanine, Adenine amino acids CH_3 deformation	*0.974*
1407	A symmetric stretching carboxylate (IgG)	*0.922*
1423	Adenine, Guanine, backbone CH_2	*0.974*
1454	Collagen & phospholipids	*0.922*
1536	C=C carotenoid	*0.200*
1591-1592	C=C stretch olefinic of lipids	*0.341*
1609	Aromatic amino acid (proteins)	*0.450*
1660-1661	Amide I protein	*0.577*

Moderate Dysplasia Tissue Specimens

The mean spectra for morphologically normal and moderate dysplastic tissue specimens are shown in Figure 6.19 and Figure 6.20. Overall, the spectra for the two tissue types were quite similar, however relative intensity differences between the morphologically normal and moderate dysplastic tissue were observed, with the key peaks for each type of tissue summarised in Table 6.7.

To clearly demonstrate the key differences between the two types of tissue, the spectra were overlaid and the difference spectrum was plotted in Figure 6.21. Although the majority of Raman peaks were more intense in morphologically normal tissue spectrum, the differences were only statistically significant at 1087 cm^{-1}, which can be attributed to C-C stretch lipids (0.289 vs. 0.252) (p=0.001; Mann-Whitney U test); Table 6.8.

The moderate dysplastic tissue specimens exhibited more prominent peaks particularly at 1137 cm^{-1} (C-N stretching modes of protein), 1301 cm^{-1} (CH_2 deformation lipids), 1322 cm^{-1} (CH_3CH_2 deforming modes of collagen and nucleic acids), 1345 cm^{-1} (CH_2 twisting and bending modes of proteins), 1448 cm^{-1} (lipids and nucleic acids) and 1658 cm^{-1} (CH_2 deformation proteins and amide I of protein). Notably, peaks such as 1315 cm^{-1} and 1457 cm^{-1} (nucleic acids modes), 1623 cm^{-1} (tryptophan, phenylalanine) and 1673 cm^{-1} (tyrosine and amide I of protein) which are present in morphologically normal tissue were absent or very weak in moderate dysplastic tissue.

An average spectrum of morphologically normal and moderate dysplastic tissue from each patient in this group (n=10) can be found in the Appendix (2-B).

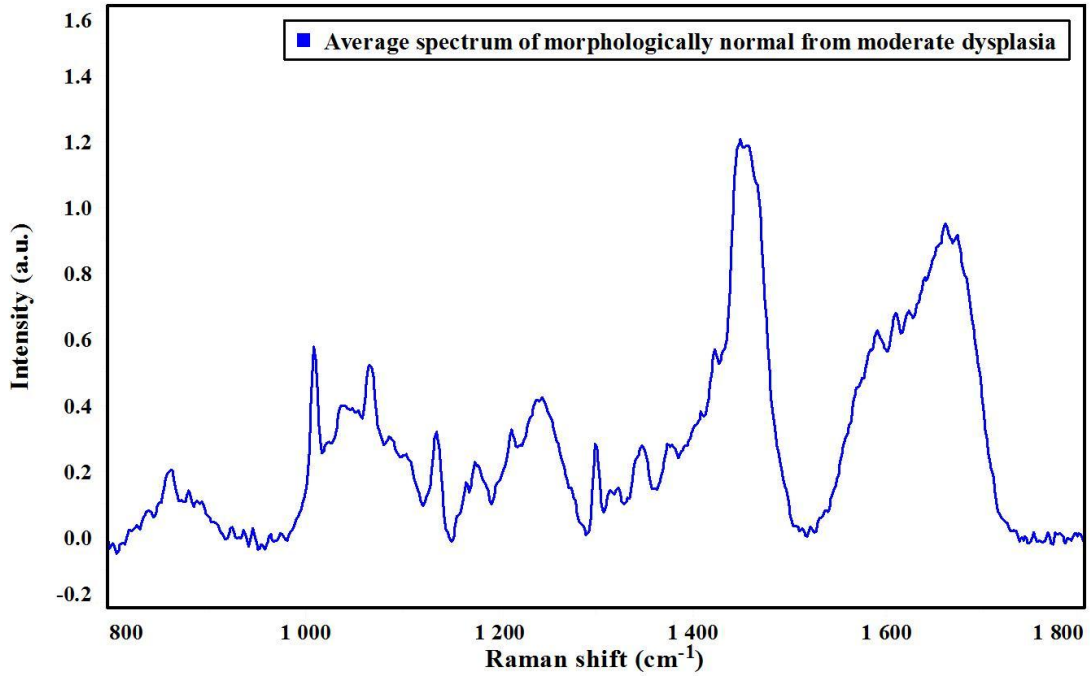

Figure 6.19: Mean Raman spectrum of morphologically normal tissue of moderate dysplasia specimens (n=93).

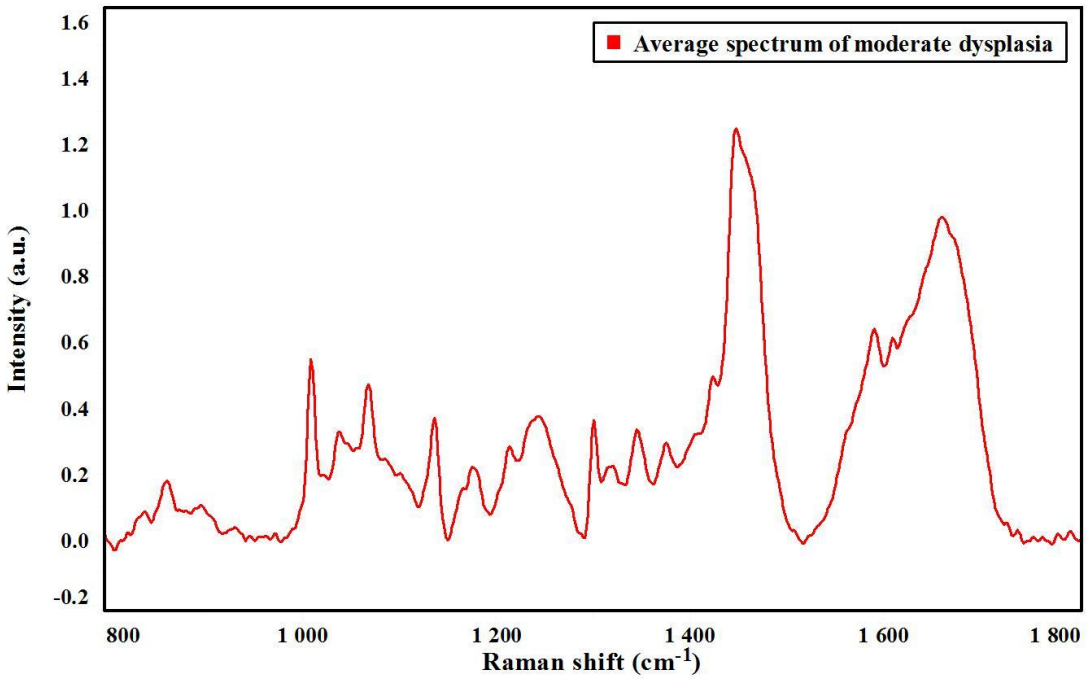

Figure 6.20: Mean Raman spectrum of moderate dysplastic tissue (n=79).

Table 6.7: Peak position and relative intensity of moderate dysplasia and morphologically normal tissue mean spectra.

Morphologically normal tissue spectra (Modn)		Moderate dysplastic tissue spectra (Modd)		Spectral features
Peak position (cm^{-1})	**Intensity (a.u)**	**Peak position (cm^{-1})**	**Intensity (a.u)**	
835	0.065	**835**	**0.082**	Higher intensity in Modd
862	**0.207**	859	0.181	Higher intensity in Modn
879	**0.156**	-	-	Missing or very weak in Modd
890	0.091	**896**	**0.106**	higher intensity in Modd
924	0.028	**929**	**0.052**	Higher intensity in Modd
936	0.0156	**931**	**0.0317**	Higher intensity in Modd
946	**0.0317**	945	0.0009	Higher intensity in Modn
974	0.007	**971**	**0.024**	Higher intensity in Modd
1009	**0.583**	1009	0.552	Higher intensity in Modn
1024	**0.287**	1023	0.189	Higher intensity in Modn
1039	**0.404**	1037	0.326	Higher intensity in Modn
1066	**0.521**	1068	0.480	Higher intensity in Modn
1087	**0.289**	1084	0.252	Higher intensity in Modn
1104	**0.264**	1101	0.214	Higher intensity in Modn
1135	0.306	**1137**	**0.378**	Higher intensity in Modd
1166	**0.163**	1166	0.158	Higher intensity in Modn
1176	**0.225**	1174	0.223	Higher intensity in Modn
1213	**0.332**	1214	0.283	Higher intensity in Modn
1245	**0.412**	1243	0.393	Higher intensity in Modn
1299	0.277	**1301**	**0.364**	Higher intensity in Modd
1315	**0.143**	-	-	Missing in Modd
1323	0.143	**1322**	**0.220**	Higher intensity in Modd
1349	0.261	**1345**	**0.336**	Higher intensity in Modd
1374	**0.287**	1375	0.282	Higher intensity in Modn
1408	**0.373**	1403	0.314	Higher intensity in Modn
1422	**0.556**	1424	0.499	Higher intensity in Modn
1449	1.192	**1448**	**1.244**	Higher intensity in Modd
1457	**1.165**	-	-	Missing in Modd
1591	0.618	**1591**	**0.656**	Higher intensity in Modd
1609	**0.691**	1609	0.628	Higher intensity in Modn
1623	**0.684**	-	-	Missing in Modd
1661	0.971	**1658**	**0.977**	Higher intensity in Modd
1673	**0.922**	-	-	Missing in Modd

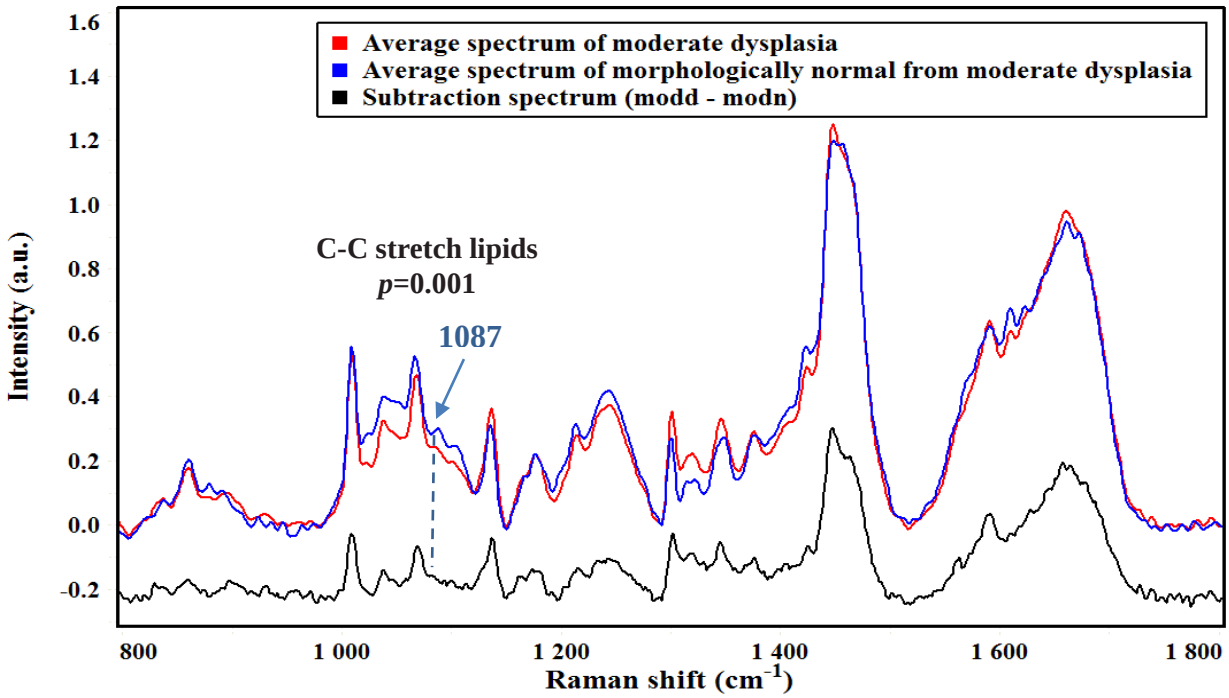

Figure 6.21: Overlaid normalised mean spectra for morphologically normal and moderate dysplastic tissue specimens with the difference spectrum beneath.

Table 6.8: Main Raman bands and assignments in moderate dysplasia and morphologically normal tissue with *p*-value resulting from Mann-Whitney test comparison of the relative peak intensity between the two tissue types.

Peak position (cm^{-1})	Raman Assignment	*p*-value
835	Out of plane breathing Tyrosine, O-P-O stretching of nucleic acids	*0.974*
859-861	Molecular vibration mode of proteins mainly Tyrosine and C-C stretch	*0.224*
890-896	Backbone, C-C skeletal	*0.158*
924-929	C-C stretch probably in amino acids, proline and valine protein band	*0.375*
971-974	CH_3, CCH olefinic (protein assignment)	*0.375*
1009	Symmetric ring breathing mode of phenylalanine (proteins)	*0.491*
1023-1024	Glycogen	*0.491*
1037-1039	Phenylalanine of collagen	*0.158*
1066-1068	C-C and C–N stretching mode of proteins; C–C stretch lipids, O-P-O stretch (nucleic acids) C-O (nucleic acids)	*0.922*
1084-1086	C–C stretch, C-C stretch lipid, O-P-O nucleic acid	***0.001***
1101-1104	Collagen C–C stretch, O-P-O-stretching (nucleic acids), C-C stretch lipid	*0.818*
1135-1137	C-N protein, CO, CH_3 (lactic acid)	*0.412*
1174-1176	Cytosine, Guanine	*0.450*
1213-1214	Amide III, Tryptophan, phenylalanine, Tyrosine, Thymine, Cytosine, Adenine.	*0.491*
1243-1245	Amide III, disorder structure of proteins, collagen	*0.972*
1299-1301	CH_2 deformation (lipids)	*0.818*
1322-1323	CH_3 CH_2 deforming modes of collagen, Guanine and nucleic acids	*0.082*
1345-1349	CH_2 twisting-bending (protein, lipids) and nucleic acids	*0.768*
1374-1375	Thymine, Guanine, Adenine amino acids CH_3 deformation	*0.922*
1422-1423	Adenine, Guanine, backbone CH_2,, deoxyribose	*0.818*
1448-1449	CH_2 CH_3 deformation	*0.450*
1591	C=C stretch olefinic	*0.922*
1609	Aromatic amino acid (proteins)	*0.974*
1661	Amide I protein	*0.922*

Severe Dysplasia

Figure 6.22 and Figure 6.23 show the mean spectra of morphologically normal and severe dysplastic tissue specimens. In general, the two types of tissue showed the same shape with the identified key peaks for each type of tissue with their relative intensities summarised in Table 6.9.

To carefully identify the differences between the two types of tissue, overlaid and the difference spectra were constructed; Figure 6.24. Although severe dysplastic tissue specimens exhibited higher relative intensity in the region between 1299 cm^{-1} and 1658 cm^{-1}, which is mainly assigned to proteins and nucleic acids, the differences were only significant at 1344 cm^{-1} (CH_2 twisting/bending modes of proteins and nucleic acids) (0.333 vs.0.236) (p=0.011; Mann-Whitney U test); Table 6.10. In the same spectral region, the severe dysplastic tissue spectrum showed a lower intensity at 1421 cm^{-1} which is mainly attributed to CH_2 scissoring vibration of lipids. The morphologically normal tissue specimens showed higher relative intensity in the majority of peaks between 859 cm^{-1} and 1243 cm^{-1}, which can mainly give information about amino acids, proteins and nucleic acids; however, Mann-Whitney U test showed significant differences in relative peak intensity only at 1243 cm^{-1} (amide III β-sheet) (0.449 vs. 0.384) (p=0.018); Table 6.10.
In the same spectral region, the morphologically normal tissue spectrum showed a lower intensity at 824, 963, 1009, and 1135 cm^{-1} mainly corresponding to proteins and amino acids.

Further, peaks in morphologically normal tissue spectrum such as 1102 cm^{-1} (C-C stretching modes of lipids) and 1326 cm^{-1} (nucleic acids) were either absent or very weak in the severe dysplastic tissue spectrum.

An average spectrum of each pair of morphologically normal and severe dysplastic tissue spectra of each patient in this group can be seen in the Appendix (2-C).

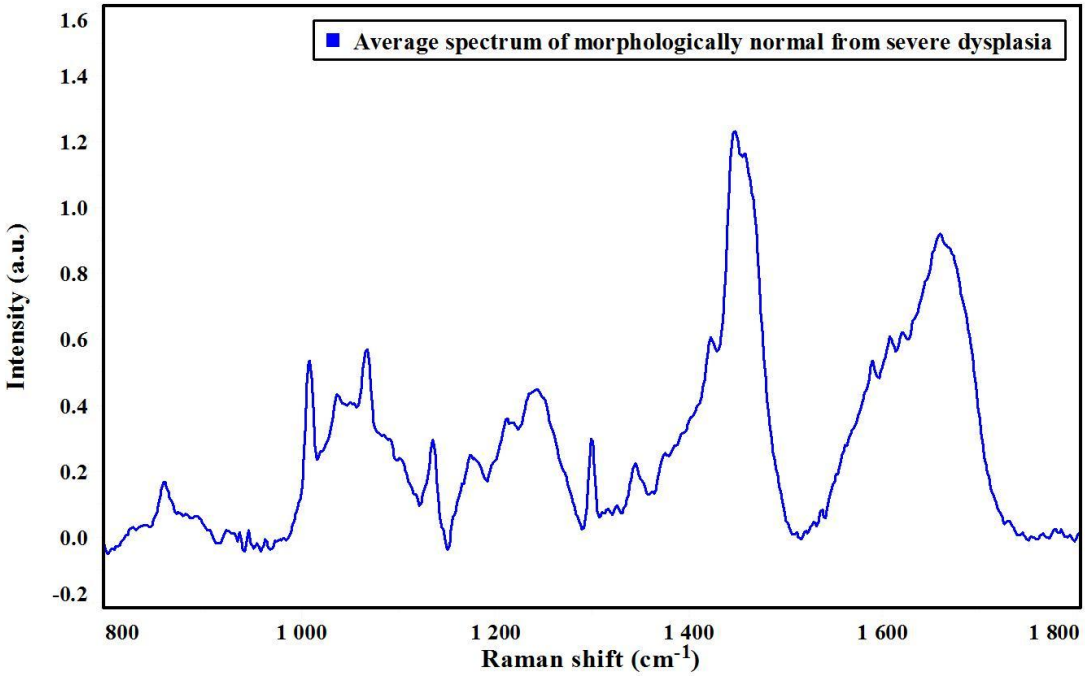

Figure 6.22: Mean Raman spectrum of morphologically normal tissue of severe dysplasia specimens (n=107 spectra).

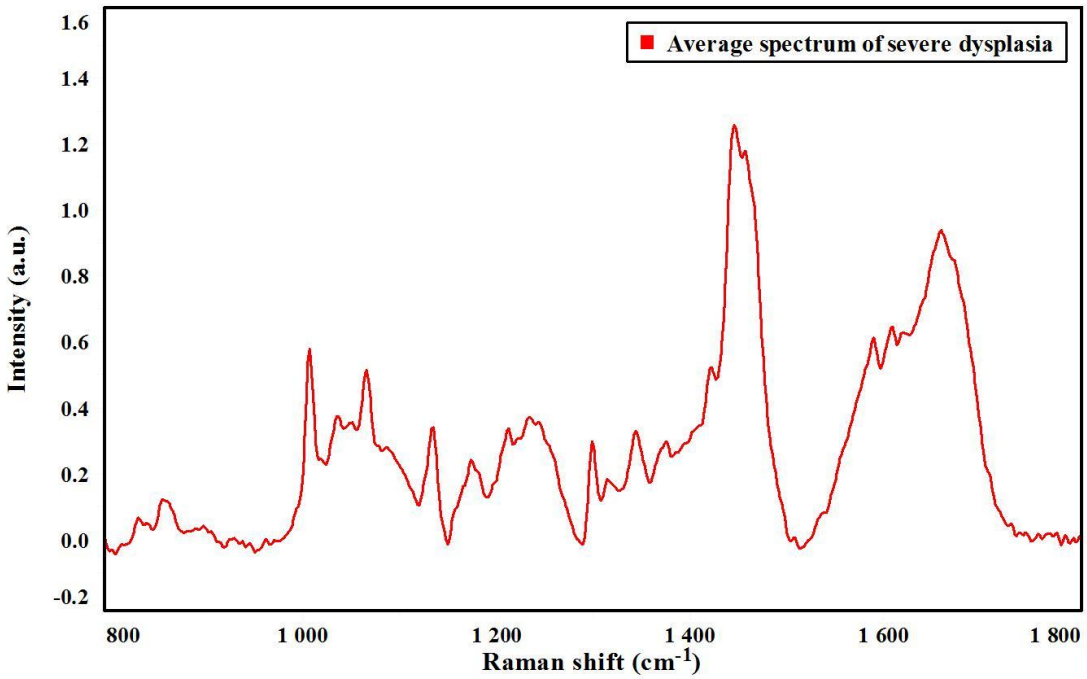

Figure 6.23: Mean Raman spectrum of severe dysplasia tissue specimens (n=83 spectra).

Table 6.9: Peak position and relative intensity of morphologically normal and severe dysplastic tissue mean spectra.

Morphologically normal tissue spectra (Sn)		Severe dysplastic tissue spectra (Sd)		Spectral features
Peak position (cm^{-1})	**Intensity (a.u)**	**Peak position (cm^{-1})**	**Intensity (a.u)**	
824	0.021	**829**	**0.048**	Higher intensity in Sd
859	**0.177**	859	0.124	Higher intensity in Sn
963	0.001	**961**	**0.016**	Higher intensity in Sd
1009	0.551	**1007**	**0.594**	Higher intensity in Sd
1037	**0.455**	1035	0.376	Higher intensity in Sn
-	-	**1045**	**0.337**	Missing in Sn
1052	**0.415**	1052	0.378	Higher intensity in Sn
1066	**0.560**	1065	0.526	Higher intensity in Sn
-	-	**1078**	**0.302**	Missing in Sn
1091	**0.299**	1085	0.280	Higher intensity in Sn
1102	**0.246**	-	-	Missing in Sd
1135	0.282	**1135**	**0.348**	Higher intensity in Sd
1174	0.252	**1174**	**0.255**	Higher intensity in Sd
-	-	**1190**	**0.128**	Missing in Sn
1213	**0.356**	1213	0.342	Higher intensity in Sn
1243	**0.449**	1235	0.384	Higher intensity in Sn
-	-	**1245**	**0.350**	Missing in Sn
1299	0.286	**1299**	**0.293**	Higher intensity in Sd
1317	0.072	**1315**	**0.195**	Higher intensity in Sd
1326	**0.082**	-	-	Missing in Sd
1345	0.236	**1344**	**0.333**	Higher intensity in Sd
1375	0.243	**1375**	**0.312**	Higher intensity in Sd
1423	**0.597**	1421	0.499	Higher intensity in Sn
1448	1.250	**1446**	**1.260**	Higher intensity in Sd
1457	1.177	**1458**	**1.201**	Higher intensity in Sd
1589	0.548	**1589**	**0.617**	Higher intensity in Sd
1608	0.616	**1609**	**0.636**	Higher intensity in Sd
1619	0.605	**1620**	**0.611**	Higher intensity in Sd
1660	0.920	**1658**	**0.925**	Higher intensity in Sd

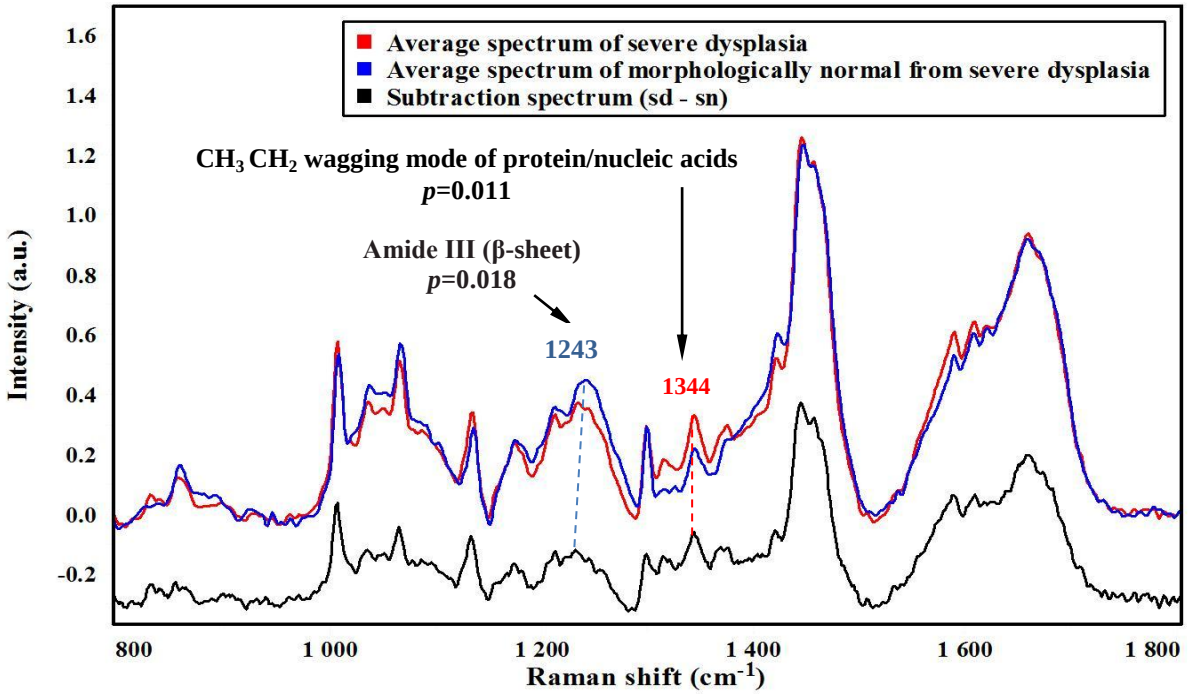

Figure 6.24: Overlaid normalised mean spectra for morphologically normal and severe dysplastic tissue with the difference spectrum beneath.

Table 6.10: Main Raman bands and assignments in severe dysplasia and morphologically normal tissue with *p*-value resulting from Mann-Whitney test comparison of the relative peak intensity between the two tissue types.

Peak position (cm^{-1})	Raman Assignment	p-value
824-829	Out of plane breathing Tyrosine/ O-P-O stretching of nucleic acids	*0.100*
859	Molecular vibration mode of proteins mainly Tyrosine and C-C stretch	*0.144*
892-898	Backbone, C-C skeletal	*0.100*
923-927	C-C stretch probably in amino acids ,proline & valine protein band	*0.361*
945-946	C-C protein assignment	*0.715*
961-963	Symmetric stretching vibration of PO_4-	*0.855*
1007-1009	Symmetric ring breathing mode of phenylalanine (proteins)	*0.273*
1037-1035	Phenylalanine of collagen	*0.201*
1052	Glycogen	*0.273*
1065-1066	C-C and C–N stretching mode of proteins; C–C stretch lipids, O-P-O stretch (nucleic acids) C-O (nucleic acids)	*0.715*
1085-1091	C–C stretch proteins, C-C stretch lipid, O-P-O nucleic acids	*0.855*
1135	C-N protein	*0.361*
1174	Tyrosine, phenylalanine, C-H bend (protein)	*0.584*
1213	Amide III, Tryptophan, phenylalanine, Tyrosine, Thymine, Cytosine, Adenine.	*0.855*
1243-1245	Amide III, disorder structure of proteins, collagen	***0.018***
1299-1301	CH_2 deformation (lipids)	*0.201*
1315-1317	Guanine ring breathing modes of DNA/RNA bases, C-H deformation protein	*0.068*
1344-1345	CH_2 twisting-bending (protein, lipids) and nucleic acids	***0.011***
1375	Thymine, Guanine, Adenine amino acids CH_3 deformation	*0.068*
1421-1423	CH_2 scissoring vibration of lipids	*0.584*
1446-1448	CH_2 CH_3 deformation	*0.465*
1457-1458	Collagen and phospholipids	*1.000*
1589	C=C olefinic stretch of lipids	*0.361*
1608-1609	Aromatic amino acid (proteins)	*0.201*
1619-1620	C=C bending of proteins Tryptophan (Ig G), phenylalanine, Tyrosine	*0.465*
1658-1661	Amide I protein	*0.465*

Carcinoma *in situ* (CIS)

Figure 6.25 and Figure 6.26 display the mean spectra of morphologically normal and CIS tissue specimens. The spectral shape of the two types of tissue is very similar with key Raman peaks for each tissue type with their relative intensities summarised in Table 6.11.

To clearly observe the variations between the two types of tissue; overlaid and the difference spectra were plotted in Figure 6.27. The intensity differences between the two types of tissue were small, with the morphologically normal tissue demonstrating higher peak intensity mainly at 1374 cm^{-1} (amino acids breathing modes of nucleic acids), 1589 cm^{-1} (C=C olefinic stretch of lipids), 1609 cm^{-1} (aromatic amino acid of proteins) and 1621 cm^{-1} (C=C bending modes tryptophan of proteins); however, the differences were not significant.
Although the differences between the two types of tissue is subtle, CIS tissue specimens showed a significantly higher relative peak intensity at 1007 cm^{-1} (symmetric ring breathing mode of phenylalanine proteins) (0.654 vs. 0.627) (p=0.021) and at 1091 cm^{-1} (O-P-O stretching mode of nucleic acids, C–C stretching modes of proteins) (0.267 vs. 0.250) (p=0.043). Also, the peak at 1037 cm^{-1} corresponding to O-P-O stretching mode of nucleic acids was more intense in CIS tissue spectra, but was non-significant; Table 6.12.

An average spectrum of each pair of morphologically normal and CIS of each patient in this group can be seen in the Appendix (2-D).

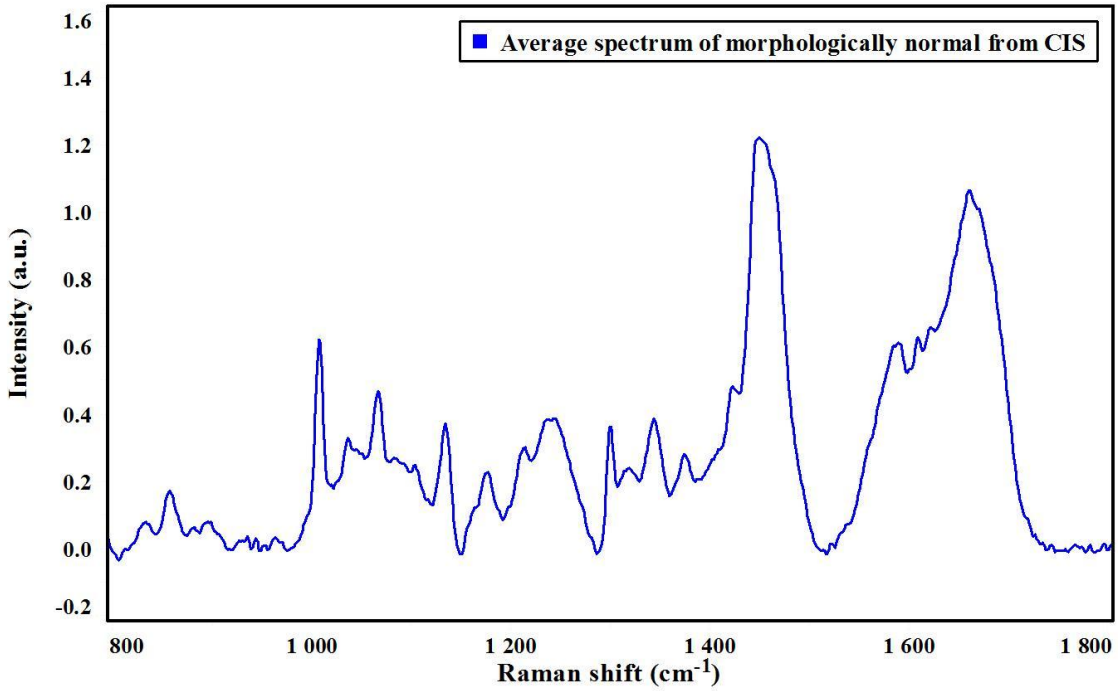

Figure 6.25: Mean spectrum of morphologically normal tissue of carcinoma *in situ* (CIS) tissue specimen (n=82 spectra).

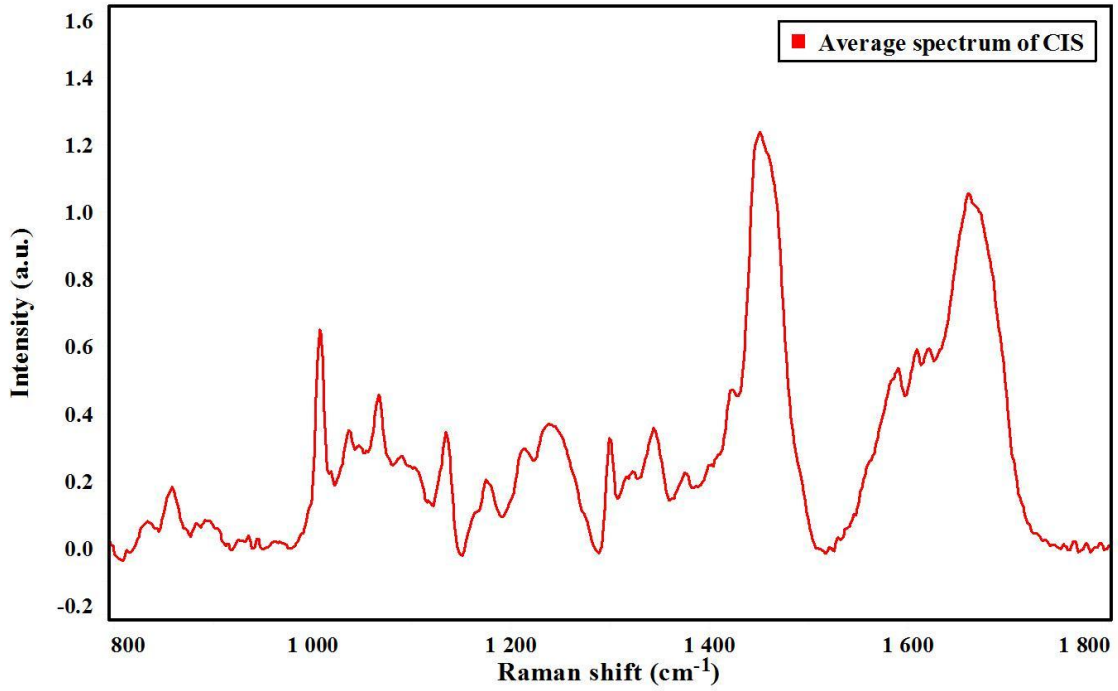

Figure 6.26: Mean spectrum of CIS tissue specimen (n=90 spectra).

Table 6.11: Peak position and relative intensity of CIS and morphologically normal tissue mean spectra.

Morphologically normal tissue spectrum (CISn)		Carcinoma *in situ* tissue spectrum (CIS)		Spectral features
Peak position (cm^{-1})	**Intensity (a.u)**	**Peak position (cm^{-1})**	**Intensity (a.u)**	
832	**0.092**	833	0.069	Higher intensity in CISn
857	0.157	**859**	**0.173**	Higher intensity in CIS
880	0.063	**882**	**0.067**	Higher intensity in CIS
898	0.066	**892**	**0.075**	Higher intensity in CIS
925	0.017	**926**	**0.023**	Higher intensity in CIS
936	0.031	**936**	**0.039**	Higher intensity in CIS
944	**0.040**	945	0.008	Higher intensity in CISn
952	**0.007**	-	-	Missing or very weak in CIS
964	**0.035**	961	0.019	Higher intensity in CISn
1007	0.627	**1007**	**0.654**	Higher intensity in CIS
1037	0.336	**1037**	**0.345**	Higher intensity in CIS
-	-	**1047**	**0.312**	Very weak in CISn
1066	**0.442**	1066	0.425	Higher intensity in CISn
1082	**0.265**	-	-	Missing or very weak in CIS
1091	0.250	**1091**	**0.267**	Higher intensity in CIS
1104	**0.244**	1103	0.238	Higher intensity in CISn
1135	**0.372**	1135	0.358	Higher intensity in CISn
1177	**0.221**	1176	0.186	Higher intensity in CISn
1214	**0.294**	1214	0.293	Higher intensity in CISn
1243	**0.379**	1238	0.375	Higher intensity in CISn
1301	**0.361**	1299	0.310	Higher intensity in CISn
1318	**0.233**	1317	0.208	Higher intensity in CISn
-	-	**1323**	**0.233**	Missing or very weak in CISn
1344	**0.385**	1344	0.337	Higher intensity in CISn
1374	**0.270**	1375	0.216	Higher intensity in CISn
1422	**0.477**	1423	0.475	Higher intensity in CISn
1449	1.215	**1451**	**1.238**	Higher intensity in CIS
1589	**0.617**	1591	0.549	Higher intensity in CISn
1609	**0.629**	1609	0.602	Higher intensity in CISn
1621	**0.668**	1621	0.603	Higher intensity in CISn
1660	**1.063**	1661	1.059	Higher intensity in CISn

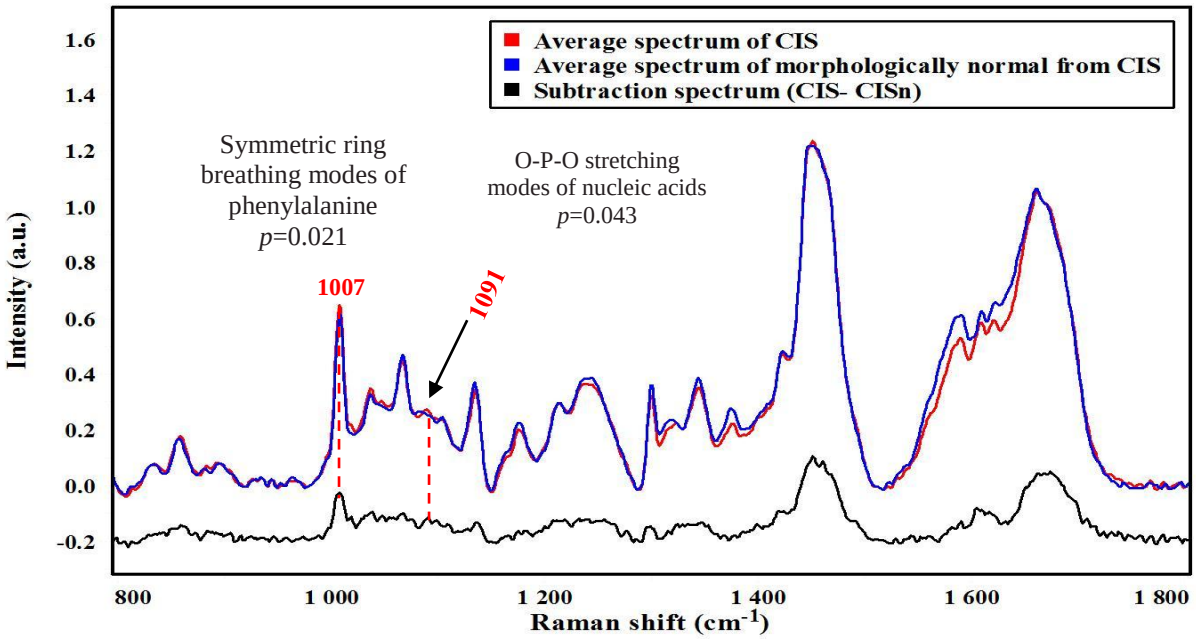

Figure 6.27: Overlaid normalised mean spectra of morphologically normal and CIS tissue with the subtraction spectrum beneath.

Table 6.12: Main Raman bands and assignments in CIS and morphologically normal tissue with p-value resulted from Mann-Whitney test comparison of the relative peak intensity between the two tissue types.

Peak position (cm^{-1})	Raman Assignment	p-value
832-833	Out of plane breathing Tyrosine/ O-P-O stretching of nucleic acids	0.083
857-859	Molecular vibration mode of proteins mainly Tyrosine and C-C stretch	0.386
880-882	Tryptophan (proteins)	0.564
892-898	Backbone, C-C skeletal	0.564
936	C-C stretching mode of proline, valine and protein backbone, (α-helix conformation) /collagen	0.248
944-945	C-C protein	0.564
961-964	Symmetric stretching vibration of PO_4-	0.564
1007	Symmetric ring breathing mode of phenylalanine (proteins)	**0.021**
1037	Phenylalanine of collagen	0.149
1066	C-C and C–N stretching mode of proteins; C–C stretch lipids, O-P-O stretch (nucleic acids), C-O (nucleic acids)	0.248
1091	C–C stretch proteins, C-C stretch lipid, O-P-O nucleic acid	**0.043**
1135	C-N protein	0.083
1176-1177	Cytosine, Guanine	0.248
1214	Amide III, Tryptophan, phenylalanine, Tyrosine, Thymine, Cytosine, Adenine.	0.564
1238-1243	Amide III, disorder structure of proteins, collagen	1.000
1299-1301	CH_2 deformation (lipids)	0.773
1317-1318	Guanine ring breathing modes of DNA/RNA bases, C-H deformation protein	0.083
1344	CH_3 CH_2 wagging mode protein of collagen	0.773
1374-1375	Thymine, Guanine, Adenine amino acids CH_3 deformation	0.564
1422-1423	Adenine, Guanine, backbone CH_2,, deoxyribose	0.564
1449-1451	CH_2 CH_3 deformation	0.564
1589-1591	C=C stretch olefinic of lipids	0.248
1609	Aromatic amino acid (proteins)	0.564
1621	C=C bending of proteins Tryptophan (Ig G), phenylalanine, Tyrosine	0.564
1660-1661	Amide I protein	0.386

Low Grade Dysplasia

The mean spectra for morphologically normal tissue and low grade dysplastic tissue specimens are presented in Figure 6.28 and Figure 6.29, respectively. Overall, the two types of tissue specimens exhibited similar spectral shape, with the main peaks for each tissue type with their relative intensities summarised in Table 6.13.

Careful analysis of the two types of tissue revealed intensity differences and to better show these differences, overlaid and difference spectra were constructed in Figure 6.30.

Low grade dysplastic tissue compared with morphologically normal tissue exhibited more intense peaks in the spectral region at 1591-1661 cm^{-1}, (lipids, proteins, amino acids), 1301-1379 cm^{-1} (proteins, amino acids, CH_3 hyaluronic acid), 1135 cm^{-1}, 1177 cm^{-1} and 1009 cm^{-1} which mainly corresponds to amino acids and proteins. The morphologically normal tissue spectrum showed more intense peaks in the regions at 1039-1104 cm^{-1} (proteins, amino acids, nucleic acids) and at 859-967 cm^{-1} (amino acids); however, the differences were not significant. The most significant intensity differences demonstrated higher levels of phenylalanine proteins at 1009 cm^{-1} in low grade dysplasia tissue specimens compared with morphologically normal tissue (0.600 vs. 0.579) and at 1661 cm^{-1} (amide I of protein) (1.056 vs. 1.028) (p=0.010, p=0.0001; Mann-Whitney U test, respectively); Table 6.14.

Further, contribution of new peaks such as 886 cm^{-1} (tryptophan-protein) and 984 cm^{-1} (C-C stretching β-sheet proteins) were identified in low grade dysplastic tissue specimens that were absent in morphologically normal tissue. Similarly, peaks in the morphologically normal tissue spectrum at 1624 and 1632 cm^{-1} which may be assigned to proteins, were absent or very weak in low grade dysplastic tissue specimens.

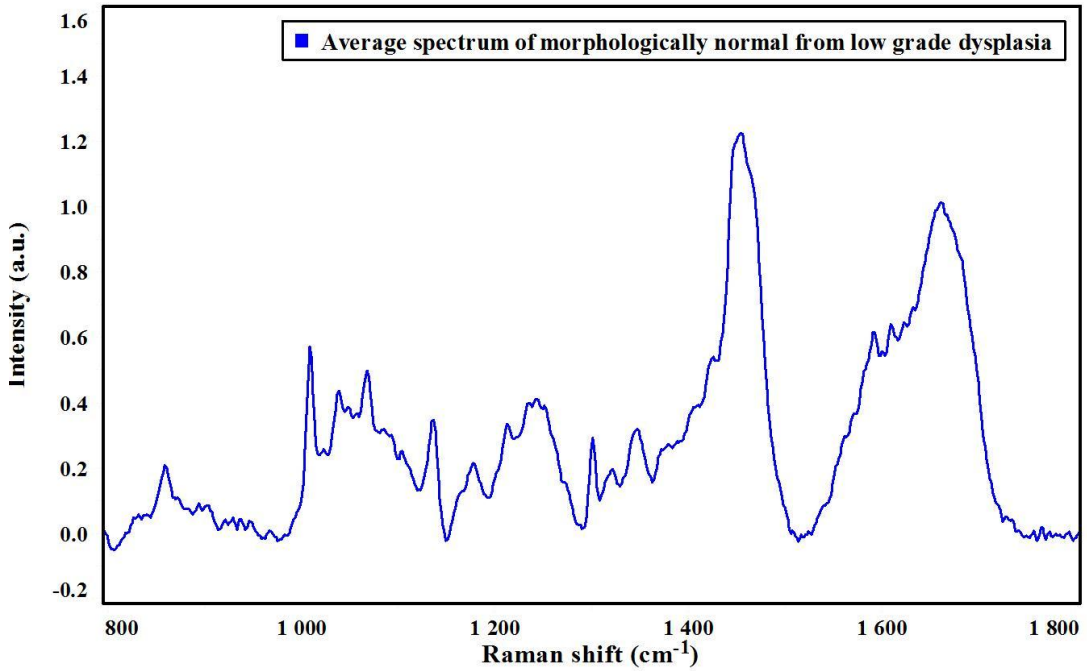

Figure 6.28: Mean spectrum of morphologically normal tissue of low grade dysplasia specimens (n=123 spectra).

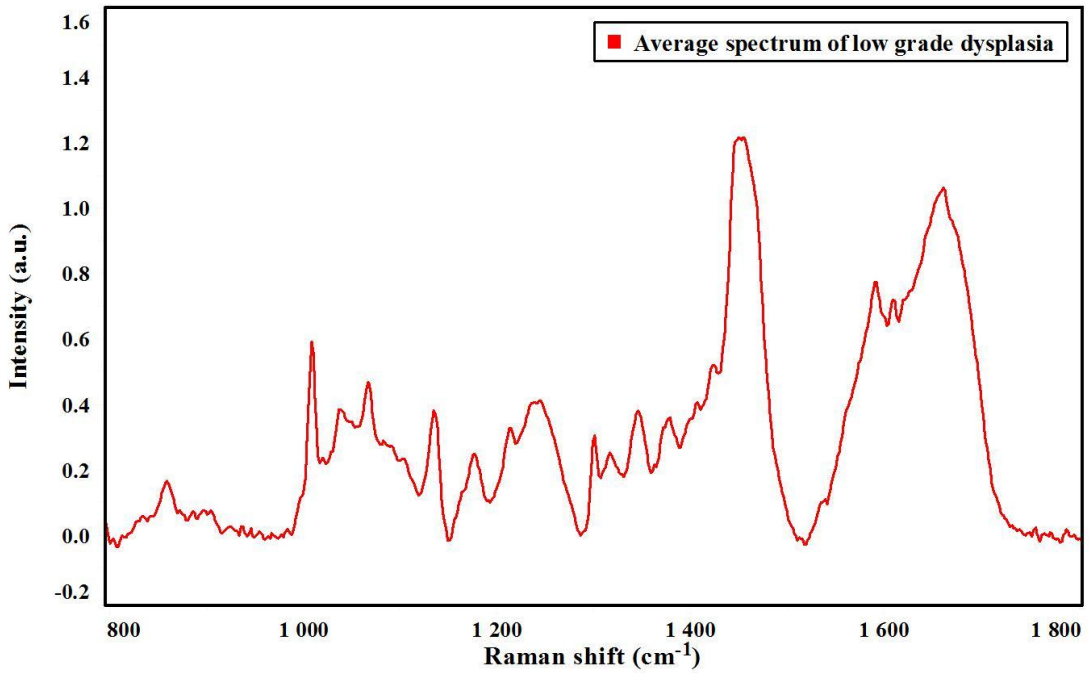

Figure 6.29: Mean spectrum of low grade dysplastic tissue specimens (n=147 spectra).

Table 6.13: Peak position and relative intensity of the average spectra of low grade dysplasia and morphologically normal tissue specimens.

Morphologically normal tissue spectrum (LGN)		Low grade dysplastic tissue spectrum (LGD)		Spectral features
Peak position (cm^{-1})	**Intensity (a.u)**	**Peak position (cm^{-1})**	**Intensity (a.u)**	
830	0.063	834	0.063	
859	**0.192**	859	0.157	Higher intensity in LGN
-	-	**886**	**0.048**	Missing in LGN
894	**0.084**	896	0.072	Higher intensity in LGN
903	**0.085**	904	0.072	Higher intensity in LGN
920	**0.056**	924	0.022	Higher intensity in LGN
938	**0.070**	936	0.027	Higher intensity in LGN
946	**0.015**	946	0.002	Higher intensity in LGN
967	**0.025**	967	0.008	Higher intensity in LGN
-	-	**984**	**0.019**	Missing in LGN
1009	0.579	**1009**	**0.600**	Higher intensity in LGD
1024	**0.252**	1023	0.206	Higher intensity in LGN
1039	**0.435**	1039	0.384	Higher intensity in LGN
1048	**0.387**	1048	0.358	Higher intensity in LGN
1068	**0.499**	1066	0.463	Higher intensity in LGN
1086	**0.328**	1083	0.278	Higher intensity in LGN
1104	**0.262**	1104	0.231	Higher intensity in LGN
1137	0.344	**1135**	**0.361**	Higher intensity in LGD
1177	0.194	**1177**	**0.242**	Higher intensity in LGD
1213	**0.338**	1213	0.318	Higher intensity in LGN
1243	**0.424**	1245	0.420	Higher intensity in LGD
1301	0.285	**1301**	**0.305**	Higher intensity in LGD
1321	0.199	**1317**	**0.259**	Higher intensity in LGD
1347	0.316	**1345**	**0.367**	Higher intensity in LGD
1379	0.289	**1379**	**0.373**	Higher intensity in LGD
1408	0.389	**1408**	**0.409**	Higher intensity in LGD
1426	**0.536**	1423	0.532	Higher intensity in LGN
1454	**0.233**	1449	1.211	Higher intensity in LGN
1592	0.611	**1591**	**0.773**	Higher intensity in LGD
1609	0.617	**1609**	**0.689**	Higher intensity in LGD
1624	**0.642**	**-**	**-**	Missing or very weak in LGD
1632	**0.710**	-	-	Missing or very weak in LGD
1660	1.028	**1661**	**1.056**	Higher intensity in LGD

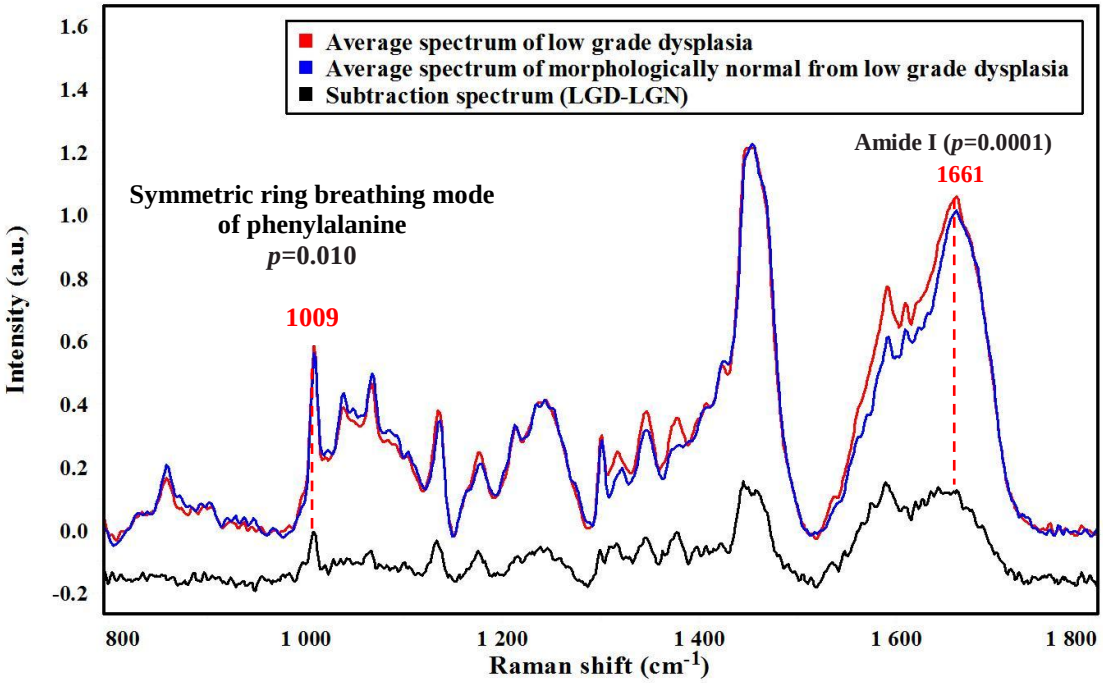

Figure 6.30: Overlaid normalised mean spectra for morphologically normal and low grade dysplastic tissue specimens with the difference spectrum beneath.

Table 6.14: Main Raman bands and assignments of low grade dysplasia and morphologically normal tissue with *p*-value resulted from Mann-Whitney test comparison on the relative peak intensity between the two tissue types.

Peak position (cm^{-1})	Raman Assignment	*p*-value
830-834	Out of plane breathing Tyrosine, O-P-O stretch of nucleic acids	0.951
859	Molecular vibration mode of proteins mainly Tyrosine and C-C stretch	0.157
894-896	Backbone, C-C skeletal	0.902
920-924	C-C stretch of proline ring/glucose/lactic acid	0.424
936-938	C-C stretching mode of proline, valine and protein backbone, (α-helix conformation) /collagen	0.268
946	C-C protein	0.460
967	Lipid	0.242
1009	Symmetric ring breathing mode of phenylalanine (proteins)	**0.010**
1039	Phenylalanine of collagen	0.295
1066-1068	C-C and C–N stretching mode of proteins, C–C stretch lipids, O-P-O stretch nucleic acids, C-O nucleic acids	0.667
1083-1086	C–C stretch proteins, C-C stretch lipid, O-P-O nucleic acid	0.622
1104	Phenylalanine proteins	0.712
1135-1137	C-N protein	0.951
1177	Cytosine, Guanine	0.074
1213	Amide III, Tryptophan, phenylalanine, Tyrosine, Thymine, Cytosine, Adenine.	1.000
1243-1245	Amide III, disorder structure of proteins, collagen	0.712
1301	CH_2 deformation lipids	0.580
1317-1321	Guanine ring breathing modes nucleic acids bases, C-H deformation protein	0.295
1345-1347	CH_3 CH_2 twisting and bending mode of protein lipids, nucleic acids	0.667
1379	Thymine nucleic acid	0.854
1408	A symmetric stretching carboxylate (IgG)	1.000
1423-1426	Adenine, Guanine, backbone CH_2 deoxyribose	0.806
1449-1454	CH_2 CH_3 deformation	0.758
1591-592	C=C stretch olefinic of lipids	0.424
1609	Aromatic amino acid (proteins)	0.622
1660-1661	Amide I protein	**0.0001**

High Grade Dysplasia

The mean spectra of morphologically normal (HGN) and high grade dysplastic tissue specimens (HGD) are shown in Figure 6.31 and Figure 6.32, respectively. At first glance, the spectra of the two tissue types appear similar, with the identified key peaks and their intensities for each type of tissue summarised in Table 6.15.

For a closer study of the spectral differences, the two types of tissue spectra were overlaid and their difference spectrum constructed (Figure 6.33) in which the morphologically normal tissue mean spectrum was subtracted from the high grade dysplastic mean spectrum. Variations in the relative peak intensities between the two types of tissue exist; high grade dysplasia specimens showed more intense peaks in three main spectral regions: at 837-971 cm^{-1} which is mainly attributed to amino acids and proteins; 1301-1377 cm^{-1} (protein, lipid and nucleic acids) and 1591-1661 cm^{-1} corresponding mainly to proteins and to lesser extent lipids. The morphologically normal tissue specimens exhibited more intense peaks in the region between 1037-1104 cm^{-1} which can give information on proteins, lipids and nucleic acids, and 1176-1243 cm^{-1} which may be assigned to proteins and amino acids.

To test the significant differences in relative intensities between the two types of tissue, Mann-Whitney U test was used. As can be seen in Table 6.16, the most significant intensity differences demonstrated higher levels in high grade dysplasia tissue specimens at 934 cm^{-1} (C-C stretching mode of proline, valine and protein backbone), 1137 cm^{-1} (C-N protein), 1301 cm^{-1} (CH_2 deformation lipids), 1322 cm^{-1} (CH_3 CH_2 deformation modes of the nucleic acids), 1345 cm^{-1} (CH_3 CH_2 twisting and bending mode of protein, lipids, nucleic acids), 1377 cm^{-1} (amino acids CH_2 deformation), 1591 cm^{-1} (C=C olefinic stretch of lipids) and 1622 cm^{-1} (C=C bending modes of tryptophan, phenylalanine, tyrosine, β-sheet of proteins). In morphologically normal tissue specimens, the most significant intensity variations revealed higher intensity levels at 1037 cm^{-1} (phenylalanine proteins), 1081 cm^{-1} (phospholipids), 1104 cm^{-1} (phenylalanine proteins), 1176 cm^{-1} (C-H bending amino acids proteins), 1213 cm^{-1} (amino acids proteins) and amide III of protein at 1243 cm^{-1}.

In high grade dysplastic tissue specimens, an additional contribution of a new peak corresponding to protein assignment was observed at 971 cm^{-1} which was not seen in the

morphologically normal tissue specimens. While the peak attributed to O-P-O-stretching in nucleic acids at 1099 cm^{-1} was very weak in morphologically normal tissue specimens, peaks such as 880 cm^{-1} (tryptophan-proteins) and 1326 cm^{-1} (purine bases of nucleic acids) were very weak or absent in high grade dysplastic tissue specimens.

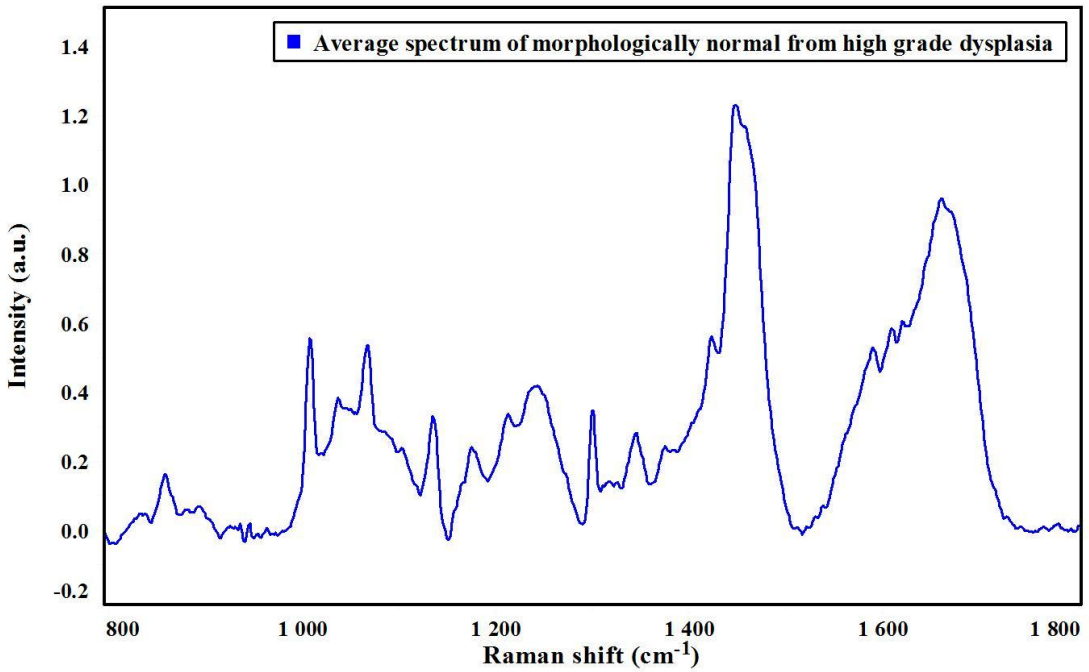

Figure 6.31: Normalized mean spectrum of morphologically normal tissue from high grade dysplasia (n=255).

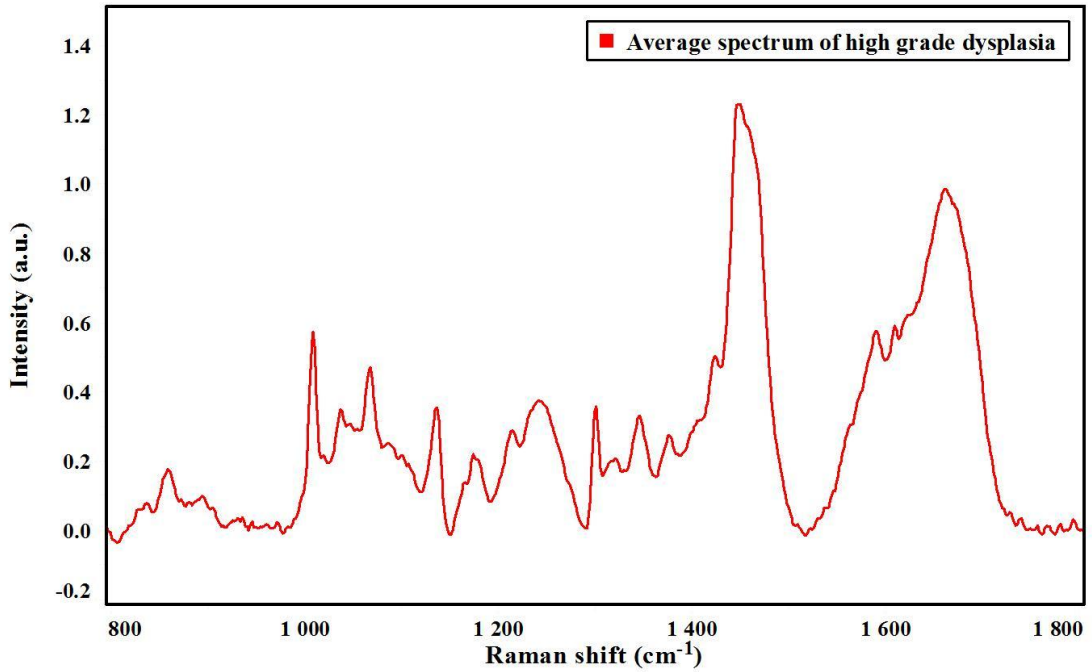

Figure 6.32: Normalized mean spectrum of high grade dysplastic tissue specimens (n=253).

Table 6.15: Peak position and relative intensity of the average spectra of high grade dysplasia and morphologically normal tissue specimens.

Morphologically normal tissue spectrum (HGN)		High grade dysplastic tissue spectrum (HGD)		Spectral features
Peak position (cm^{-1})	**Intensity (a.u)**	**Peak position (cm^{-1})**	**Intensity (a.u)**	
832	0.040	**837**	**0.065**	Higher intensity in HGD
859	0.174	**859**	**0.177**	Higher intensity in HGD
880	**0.050**	-	-	Weak in HGD
893	0.065	**894**	**0.094**	Higher intensity in HGD
936	0.007	**934**	**0.016**	Higher intensity in HGD
946	0.009	**946**	**0.001**	Higher intensity in HGD
964	0.007	**961**	**0.009**	Higher intensity in HGD
-	-	**971**	**0.019**	Missing in HGN
1007	0.561	**1009**	**0.577**	Higher intensity in HGD
1037	**0.364**	1037	0.344	Higher intensity in HGN
1068	**0.537**	1068	0.470	Higher intensity in HGN
1081	**0.290**	1086	0.251	Higher intensity in HGN
-	**-**	**1099**	**0.149**	Weak in HGN
1104	**0.229**	1104	0.182	Higher intensity in HGN
1135	0.319	**1137**	**0.326**	Higher intensity in HGD
1176	**0.241**	1174	0.211	Higher intensity in HGN
1213	**0.331**	1214	0.285	Higher intensity in HGN
1243	**0.412**	1242	0.378	Higher intensity in HGN
1299	0.347	**1301**	**0.366**	Higher intensity in HGD
1317	0.150	**1322**	**0.211**	Higher intensity in HGD
1326	**0.144**	-	-	Missing or very weak in HGD
1345	0.289	**1345**	**0.340**	Higher intensity in HGD
1375	0.228	**1377**	**0.271**	Higher intensity in HGD
1423	**0.554**	1422	0.494	Higher intensity in HGN
1448	**1.238**	1448	1.132	Higher intensity in HGN
1589	0.510	**1591**	**0.553**	Higher intensity in HGD
1608	0.574	**1609**	**0.584**	Higher intensity in HGD
1620	0.596	**1622**	**0.623**	Higher intensity in HGD
1661	0.955	**1661**	**0.964**	Higher intensity in HGD

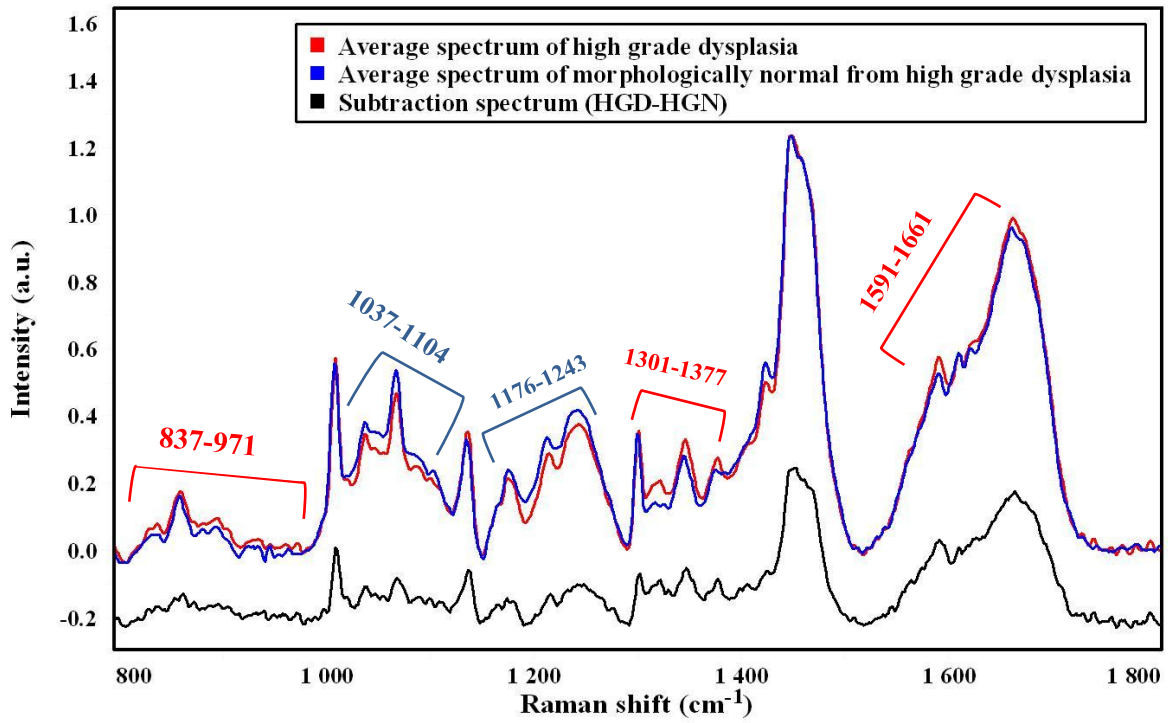

Figure 6.33: Overlaid normalised mean spectra for morphologically normal and high grade dysplastic tissue with the difference spectrum beneath.

Table 6.16: Main Raman bands and assignments in high grade dysplasia and morphologically normal tissue with *p*-value resulting from Mann-Whitney test comparison of the relative peak intensity between the two tissue types.

Peak position (cm^{-1})	Raman Assignment	p-value
832-837	Out of plane breathing Tyrosine, O-P-O stretching of nucleic acids	0.254
857-859	Molecular vibration mode of proteins mainly Tyrosine and C-C stretch	0.419
893-894	Backbone, C-C skeletal	0.373
934-936	C-C stretching mode of proline, valine and protein backbone, (α-helix conformation) /collagen	**0.007**
946	C-C protein	0.443
961-964	Symmetric stretching vibration of PO_4-	0.885
1007-1009	Symmetric ring breathing mode of phenylalanine (proteins)	0.950
1037	Phenylalanine of collagen	**0.0001**
1068	C-C and C–N stretching mode of proteins, C–C stretch lipids, O-P-O stretch (nucleic acids)	0.520
1081-1086	C-O (nucleic acids) C–C stretch proteins, C-C stretch lipid, O-P-O nucleic acid	**0.007**
1104	Phenylalanine (proteins)	**0.0001**
1135-1137	C-N protein	**0.001**
1174-1176	C-H bending Cytosine, Guanine, Tyrosine, phenylalanine proteins	**0.0001**
1213-1214	Amide III, Tryptophan, phenylalanine, Tyrosine, Thymine, Cytosine, Adenine.	**0.0001**
1242-1243	Amide III, disorder structure of proteins, collagen	**0.004**
1299-1301	CH_2 deformation (lipids)	**0.049**
1317-1322	CH_3 CH_2 deforming modes of collagen and nucleic acids, C-H deformation protein	**0.003**
1345	CH_3 CH_2 twisting and bending mode of protein lipids, nucleic acids	**0.001**
1375-1377	Thymine, Guanine, Adenine amino acids CH_3 deformation	**0.0001**
1422-1423	Adenine, Guanine, backbone CH_2, deoxyribose	0.604
1448	CH_2 CH_3 deformation	0.290
1589-1591	C=C stretch olefinic	**0.0001**
1609	Aromatic amino acid (proteins)	0.290
1621-1622	C=C bending modes of Tryptophan, phenylalanine, Tyrosine, β-sheet of proteins	**0.0001**
1660-1661	Amide I protein	0.694

6.7.3. Dysplasia Severity in Relation to the Peak Relative Intensity

To study the association between relative peak intensity and the severity of epithelial dysplasia, the intensities of the identified peaks were measured from the mean spectrum of each grade of dysplasia (mild, moderate, severe dysplasia and CIS) along the spectral range from 800-1800 cm^{-1}; Table 6.17. To clearly demonstrate the relation between peak intensities and dysplasia severity, a histogram in Figure 6.34 was used. In general, no clear relation was found for the majority of the identified Raman peaks. However, for two peaks an apparent relationship was seen, at 1235-1243 cm^{-1} (amide III of protein) and 1589-1591 cm^{-1} (C=C olefinic stretch of lipids). These two Raman bands showed a decreased intensity with increased dysplasia severity from the mild through moderate and severe dysplasia to the CIS.

Five spectral regions 1007-1009 cm^{-1}, 1084-1091 cm^{-1}, 1235-1243 cm^{-1}, 1344-1347 cm^{-1} and 1589-1591 cm^{-1} were selected to investigate the relation between their relative peak intensities and the severity of dysplasia. This selection was based on the significant differences found between morphologically normal and dysplastic tissue groups (pair-wise comparison) in these spectral regions and also on the consistent relation found; Figure 6.35 and Table 6.18. In general, an increased dysplasia severity from mild through to CIS was associated with a decrease in relative intensity at 1235-1243 cm^{-1} and 1589-1591 cm^{-1}, although this trend was not found to be significant for any of the 5 selected peaks (p=0.329, 0.657, 0.640, 0.532, 0.736; Kruskal-Wallis test), respectively.

Table 6.17: Mean relative intensity of the identified Raman peaks in dysplastic groups.

Spectral Range (cm^{-1})	Mean Relative Intensity (a.u)			
	Md	Modd	Sd	CIS
829-835	0.047	0.082	0.048	0.069
859	0.18	0.181	0.124	0.173
1007-1009	0.642	0.552	0.594	0.654
1035-1039	0.417	0.326	0.376	0.345
1065-1068	0.472	0.158	0.526	0.425
1084-1091	0.31	0.252	0.28	0.267
1135-1137	0.381	0.378	0.348	0.358
1174-1177	0.263	0.223	0.255	0.186
1213-1214	0.364	0.283	0.342	0.293
1235-1243	0.443	0.393	0.384	0.375
1299-1301	0.267	0.364	0.293	0.31
1315-1322	0.243	0.22	0.195	0.208
1344-1347	0.399	0.336	0.333	0.337
1375-1377	0.375	0.282	0.312	0.216
1403	0.443	0.314	0.318	0.25
1421-1424	0.552	0.499	0.499	0.475
1446-1454	1.22	1.244	1.26	1.238
1589-1591	0.883	0.656	0.617	0.549
1609	0.766	0.628	0.636	0.602
1658-1661	1.08	0.977	0.925	1.059

Md=mild dysplasia, Modd=moderate dysplasia, Sd=severe dysplasia, CIS=carcinoma *in situ*, a.u =arbitrary unit.

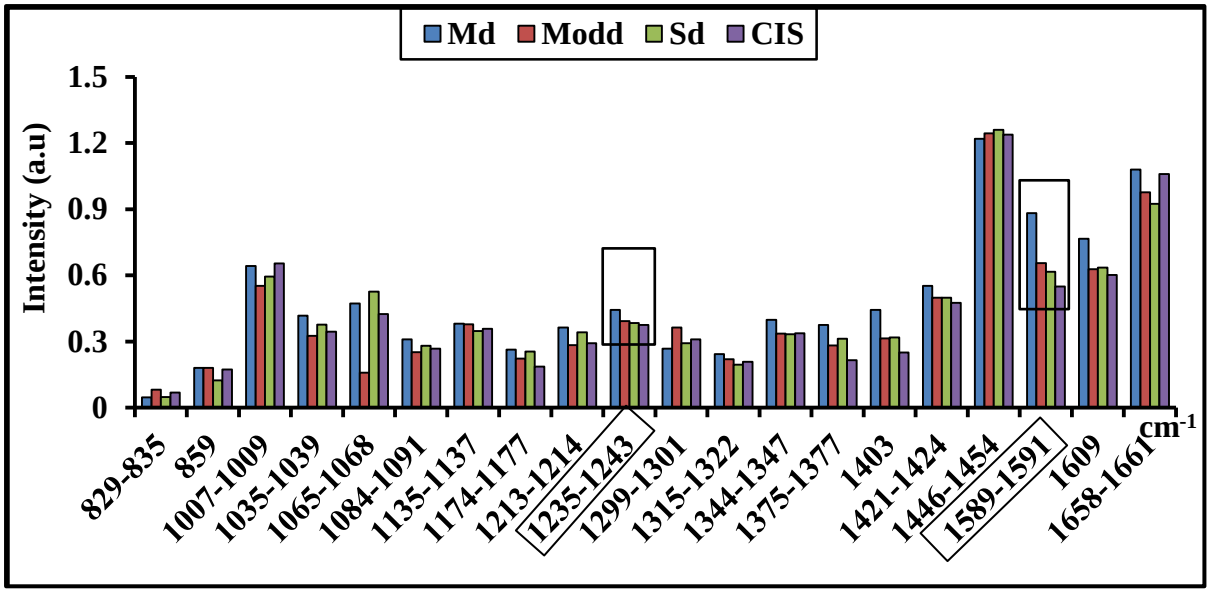

Figure 6.34: The association between the relative peaks intensities and the severity of oral epithelial dysplasia.

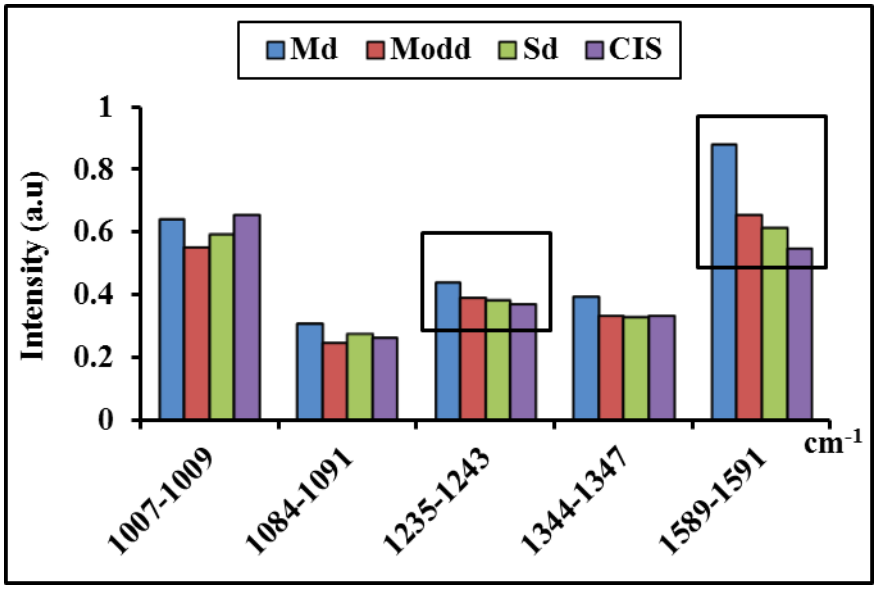

Figure 6.35: The relation between peaks relative intensities and degree of oral epithelial dysplasia at 5 selected Raman peaks.

Table 6.18: Mean relative intensity of the dysplastic tissue groups of the 5 selected Raman bands.

Spectral range (cm^{-1})	Mean relative intensity (a.u)			
	Md	Modd	Sd	CIS
1007-1009	0.642	0.552	0.594	0.654
1084-1091	0.31	0.252	0.28	0.267
1235-1243	0.443	0.393	0.384	0.375
1344-1347	0.399	0.336	0.333	0.337
1589-1591	0.883	0.656	0.617	0.549

Considering high/low grade dysplasia groups, Figure 6.36 compares the relative intensities of the identified peaks in both types of tissue. Overall, the differences were small between the two types of tissue. High grade dysplasia tissue specimens showed more intense peaks at 1066-1068 cm^{-1} (proteins and nucleic acids), 1301 cm^{-1} (CH_2 deformation lipids) and 1448-1449 cm^{-1} (CH_2 CH_3 deformation proteins). Low grade dysplasia exhibited more intense peaks in the majority of the identified peaks, with a more pronounced difference was seen at 1591 cm^{-1}. No significant differences between low and high grade dysplasia tissue spectra in the relative peak intensity for almost all the identified peaks; however, one peak at 1242-1245 cm^{-1} (amide III of proteins) approaching the significance difference (p=0.051; Mann Whitney U test); Table 6.19.

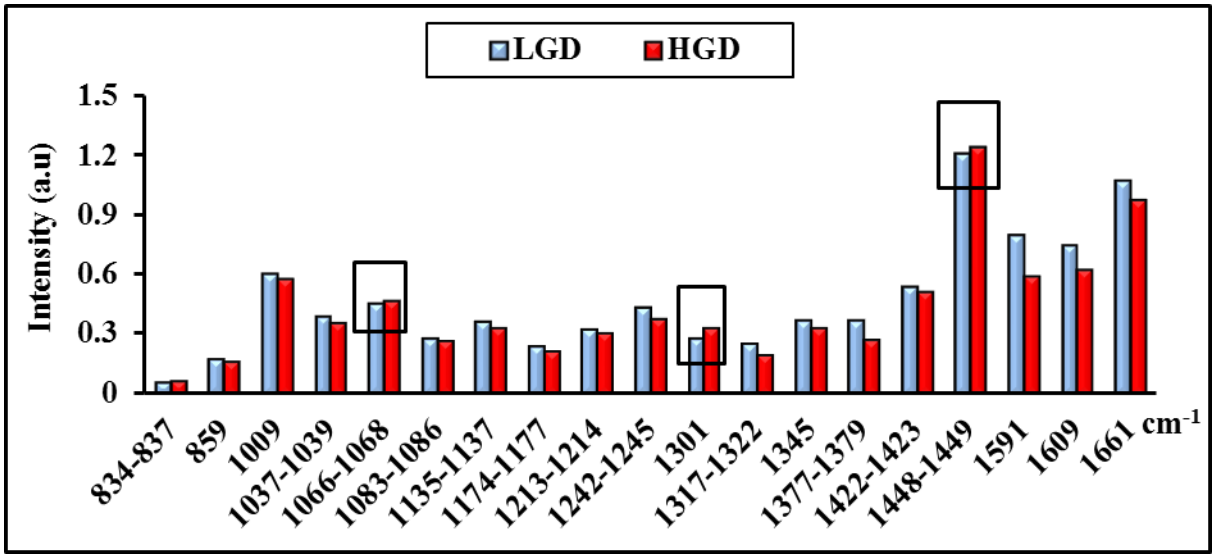

Figure 6.36: The relation between peak relative intensities and severity of oral epithelial dysplasia between low and high grade dysplasia.

Table 6.19: Mann-Whitney U test on relative peak intensity between low and high grade dysplastic tissue groups.

Peak position (cm^{-1})	*p*-value
834-837	*0.884*
859	*0.435*
1009	*0.354*
1037-1039	*0.435*
1066-1068	*0.696*
1083-1086	*0.961*
1135-1137	*0.922*
1174-1177	*0.222*
1213-1214	*0.494*
1242-1245	***0.051***
1301	*0.241*
1317-1322	*0.188*
1345	*0.223*
1377-1379	*0.495*
1422-1423	*0.407*
1448-1449	*0.143*
1591	*0.380*
1609	*0.329*
1661	*0.071*

6.7.4. Spectral Features of Dysplastic Tissue

To carefully investigate the spectral features among the dysplastic groups labelled as mild, moderate, severe dysplasia and CIS, the mean spectra of the four dysplastic groups were plotted and overlaid in Figure 6.37. Also, for clarity the four dysplastic tissue spectra were offset in Figure 6.38 to show the differences in the main spectral features among the dysplastic groups. The areas showing the greatest visual spectral distinctions were enclosed in boxes. The area enclosed in the box A at 829–838 cm^{-1} corresponding to the out of plane breathing mode of tyrosine protein assignments/O-P-O stretching mode of nucleic acids, is broad in mild dysplastic tissue specimens and starts to develop and become more prominent with increased degree of dysplasia indicating more tissue activity with increased dysplasia severity and greater biochemical changes in proteins and nucleic acids.

Box B encloses the area attributed to the symmetric ring breathing mode of phenylalanine proteins at 1007–1009 cm^{-1} which is prominent in all grades of dysplasia, becoming more intense and sharper with increased dysplasia severity, reflecting the progressive increase in proteins tissue components with higher degrees of dysplasia. The peak at 1315–1322 cm^{-1} enclosed in box C is assigned to the guanine ring breathing modes of the nucleic acids bases, C-H deformation protein. It is prominent in mild dysplasia but starts to weaken and appears less prominent with increased grades of dysplasia. Similarly, the box labelled D encloses the band at 1375–1377 cm^{-1} (thymine, guanine, adenine amino acids), is more prominent in mild dysplasia and becomes weaker and less prominent with increased dysplasia severity. The Raman peaks broaden as the molecular complexity increases.

Box E shows that there is a small shoulder seen in the spectrum for severe dysplastic tissue specimens at 1458 cm^{-1} corresponding to nucleic acid, but absent in the spectra of mild, moderate dysplasia and CIS tissue specimens, indicating increased cellular nucleic acid content (nuclear material) in dysplastic tissue from the proliferation cells.

At 1589–1591 cm^{-1} (C=C stretch olefinic of lipids), a mild dysplastic tissue spectrum exhibited a sharp peak which starts to broaden gradually with increased dysplasia grade severity (Box F). This suggests disturbances in the lipids content with increased dysplasia severity during the carcinogenesis process.

The shoulder enclosed in box G at 1620–1624 cm^{-1} that can be assigned to C=C bending modes of tryptophan, phenylalanine, tyrosine, β-sheet of proteins is very weak or absent in mild dysplastic tissue specimens starts to develop in moderate and become more prominent in severe and CIS. This indicates the substantial changes in spectral profiles from normal to CIS tissue arising from expression of new and different proteins from progressive cell proliferation and cellular response to disease.

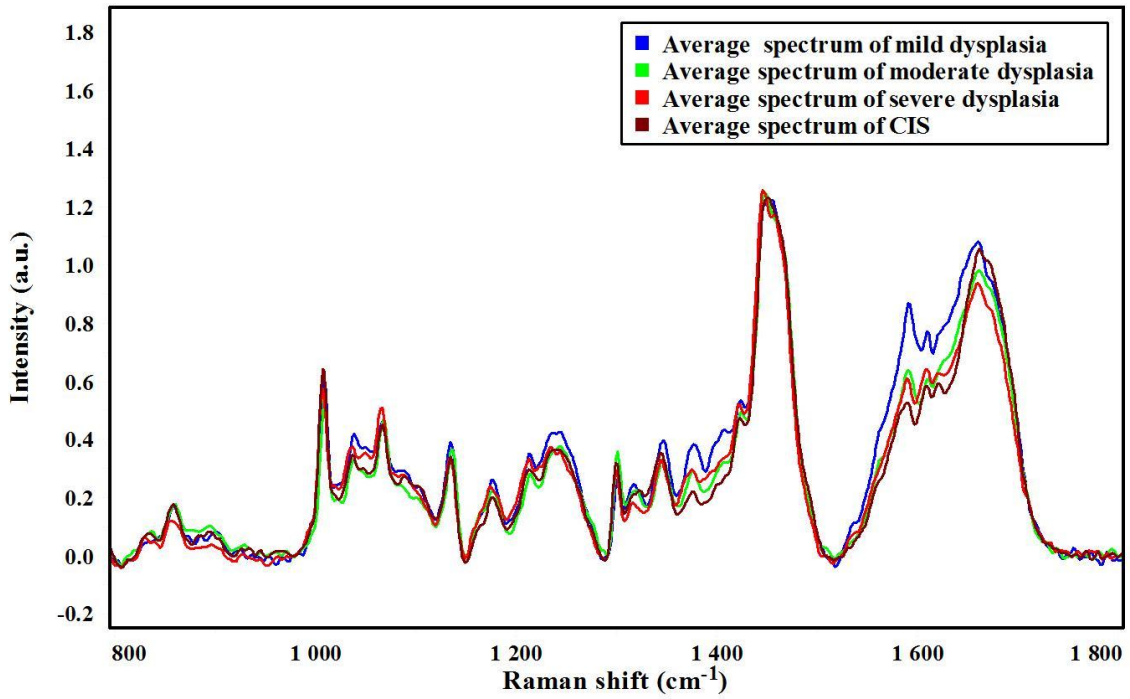

Figure 6.37: Mean spectra of the four dysplastic tissue groups; mild (n=134) moderate (n=79), severe dysplasia (n=83) and CIS (n=90).

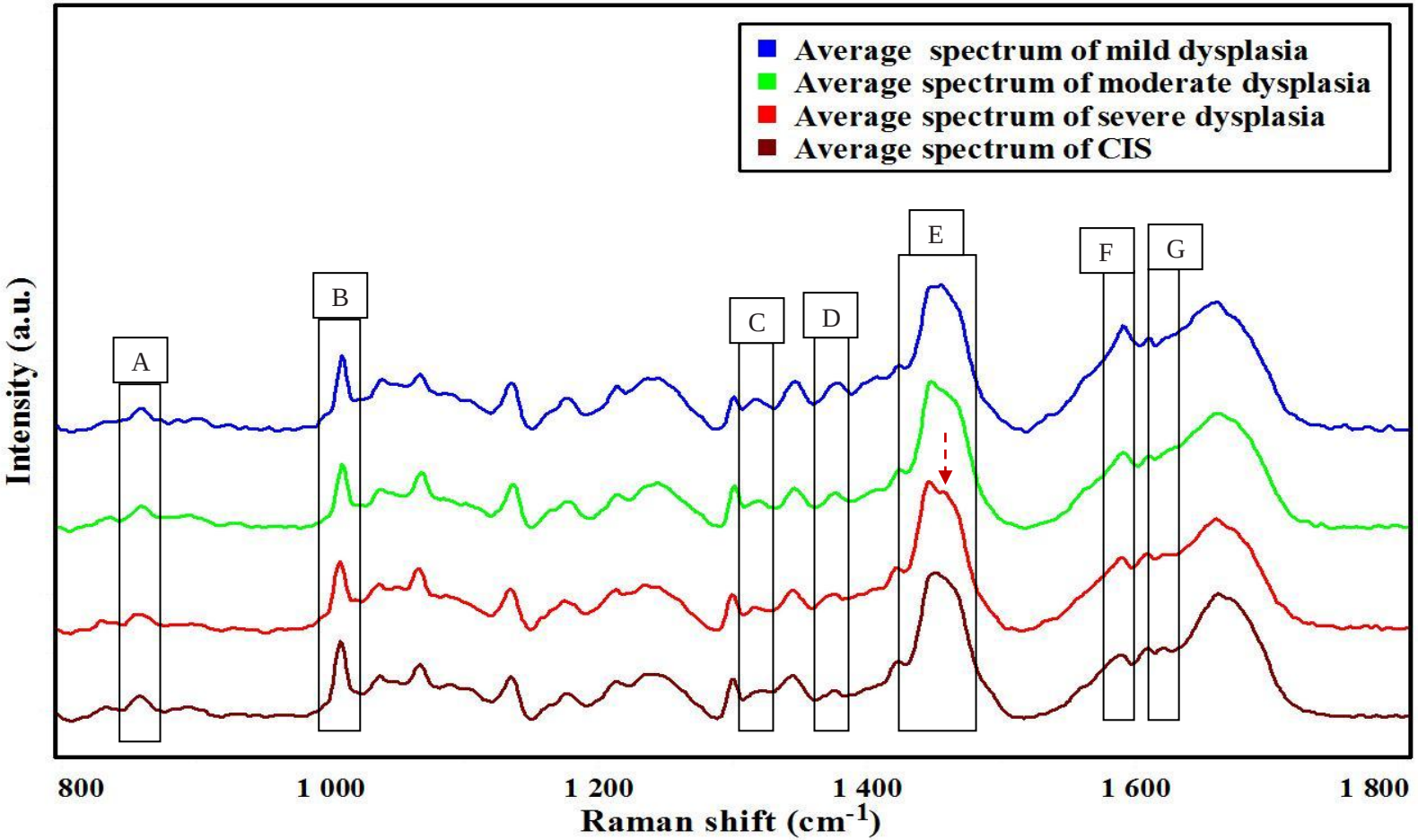

Figure 6.38: The main spectral characterizations in the four grades of oral epithelial dysplasia. A= (829-838), B= (1007-1009), C= (1315-1322), D= (1375-1377), E=1458, F= (1589-1591) and G= (1620-1624)

Considering the spectral features in high and low grade dysplastic tissue specimens, the overlaid mean spectra of both types of tissue specimens can be seen in Figure 6.39. To better clarify the spectral differences between the two groups of tissue, the spectra were offset and the main spectral features were enclosed in boxes; Figure 6.40. In the plot of the high grade dysplasia, the shoulder enclosed in box A at 1166 cm^{-1} (C–H in-plane bending modes of tyrosine proteins) appears very weak in low grade dysplastic tissue specimens, indicates the formation of more proteins from the proliferated cells.

The peak labelled B at 1317–1322 cm^{-1} (guanine ring breathing modes of the nucleic acid base, C-H deformation proteins) for low grade dysplastic tissue showed sharp peak which becomes broader in the high grade dysplasia. This broad peak in high grade dysplasia reflects increased cellular proteins and nucleic acid content.

The small peak at 1407 cm^{-1} corresponding to a symmetric stretching carboxylate IgG, labelled C, is more prominent in low grade dysplasia, appearing broader in high grade dysplasia, indicating high levels of immunoglobulins in the disease condition which increases with high grade dysplasia.

Box D enclosed the shoulder at 1623 cm^{-1} for the high grade dysplastic tissue specimens corresponding to C=C bending modes of tryptophan, phenylalanine, tyrosine, β-sheet of proteins, appears very weak or absent in low grade dysplastic tissue spectrum. This represents the formation of new proteins from progressive cell proliferation and cellular response to disease.

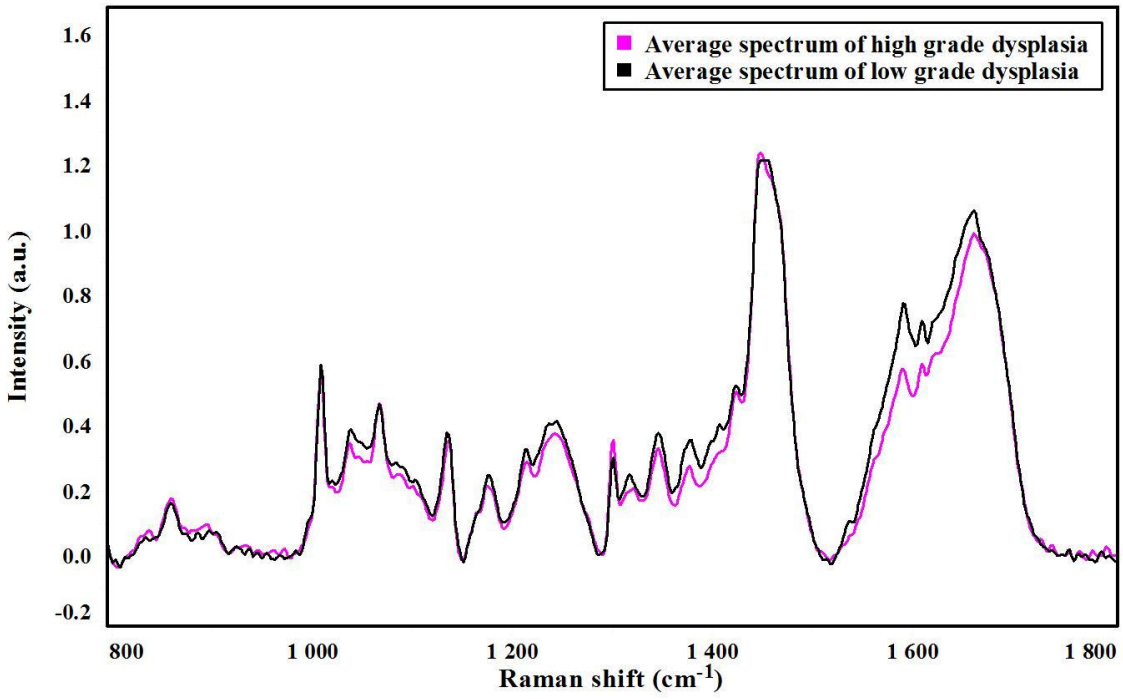

Figure 6.39: Mean spectra of high grade dysplasia (n=253 spectra) and low grade dysplasia (n=147 spectra).

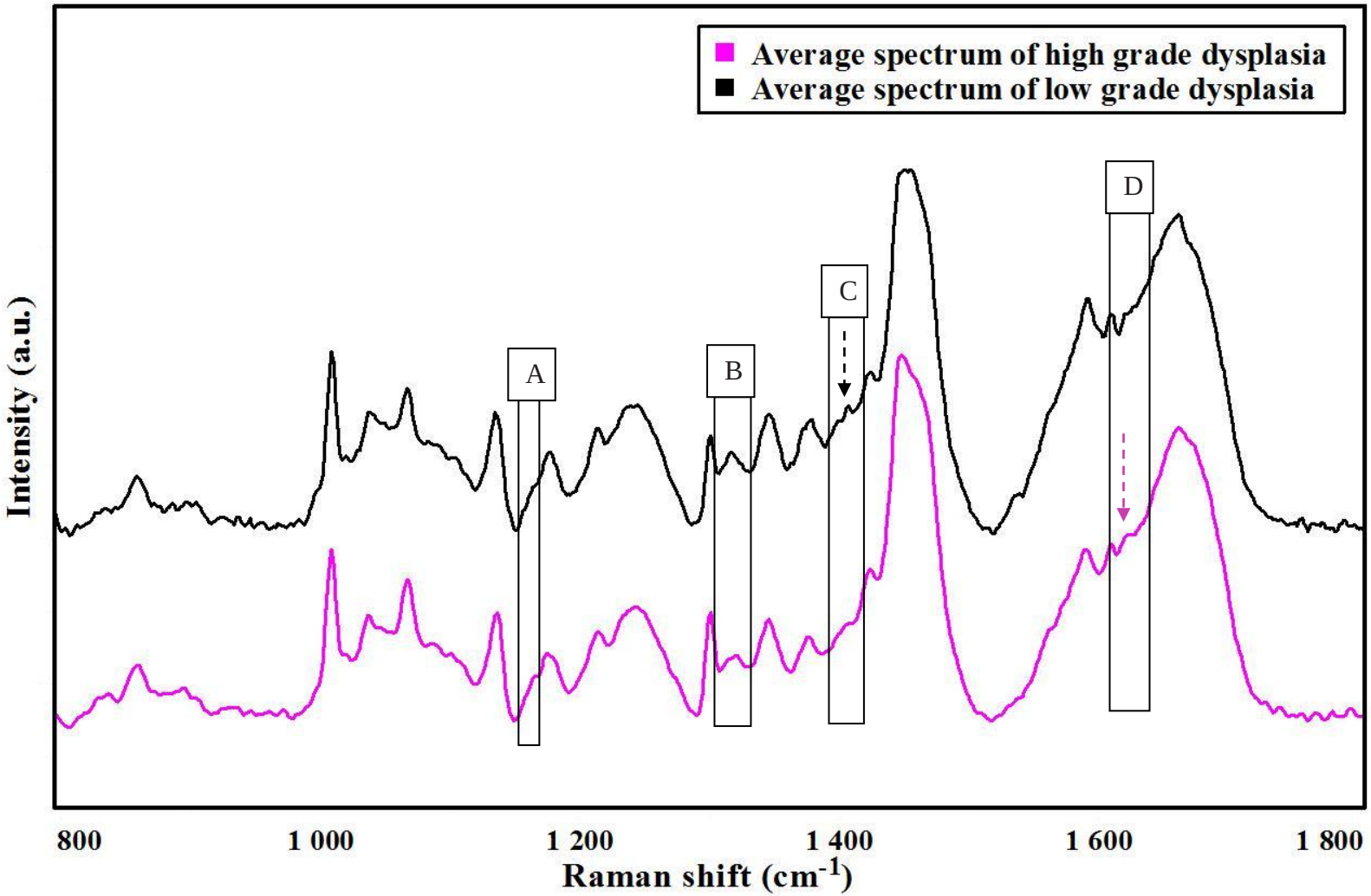

Figure 6.40: The main spectral characterizations of high grade and low grade dysplastic tissue specimens. A=1166, B= (1317-1322), C= (1405-1407) and D=1623

6.7.5. Spectral Features of Morphologically Normal Tissue

Figure 6.41 shows the mean spectra of morphologically normal epithelial tissue from the resection margins of mild, moderate, severe dysplasia and CIS tissue specimens. To better understand the differences among morphologically normal tissue groups, the spectra were offset and the greatest visual differences were enclosed in boxes shown in Figure 6.42.

The peak enclosed in box A at 1009 cm^{-1} corresponds to the symmetric ring breathing mode of phenylalanine protein and appears in all cases of morphologically normal tissue specimens. It showed intensity increases as dysplasia severity increased from mild dysplasia through to CIS indicating the similarity between morphologically normal and dysplastic tissue with more proteins formation with increased dysplasia severity from the proliferation cells.

The morphologically normal tissue from the resection margins of mild and moderate dysplasia exhibited a more prominent feature at 1022 cm^{-1} corresponding to glycogen (box A, arrows), but absent in severe dysplasia and CIS tissue spectra. This reflects a reduction in the glycogen content in morphologically normal tissue from severe-CIS tissue specimens, indicating close similarity of these morphologically normal with dysplastic tissue with high metabolic activity.

The peak labelled B at 1347 cm^{-1} (CH_2 twisting and bending modes of lipids) appears in all morphologically normal tissues, with notably increased sharpness, respectively, in the morphologically normal tissue from mild, moderate, severe dysplasia and CIS. This represents disturbances in the lipid content of morphologically normal tissue adjacent to severe dysplasia and CIS during the carcinogenesis.

At box C, the region for morphologically normal epithelium from moderate, severe dysplasia and CIS show prominent shoulders at 1619–1623 cm^{-1} (C=C bending modes of tryptophan, phenylalanine, tyrosine, β-sheet of proteins) which may be absent or very weak in the morphologically normal from mild dysplastic tissue. This indicates the formation of new proteins and reflects the similarity of the morphologically normal tissue from moderate, severe dysplasia and CIS to their dysplastic tissues.

In morphologically normal tissue from moderate dysplasia labelled D, there is a shoulder at 1673 cm^{-1} corresponding to unordered protein secondary structure, but absent in all other morphologically normal tissue from mild, severe dysplasia and CIS, indicating the formation of new proteins in morphologically normal looking cells from moderate dysplasia and supports the concept of field change of malignancy-associated changes throughout the entire oral mucosa.

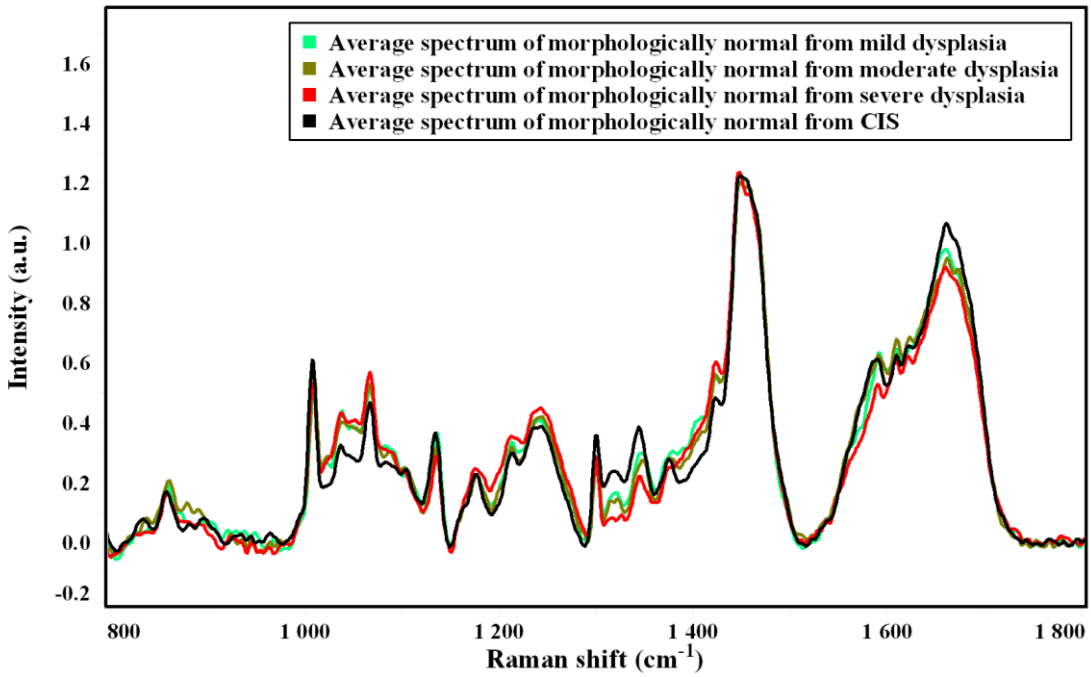

Figure 6.41: Mean spectra of the four morphologically normal tissue groups from the resection margins of mild (n=110), moderate (n=93), severe dysplasia (n=107) and CIS (n=82).

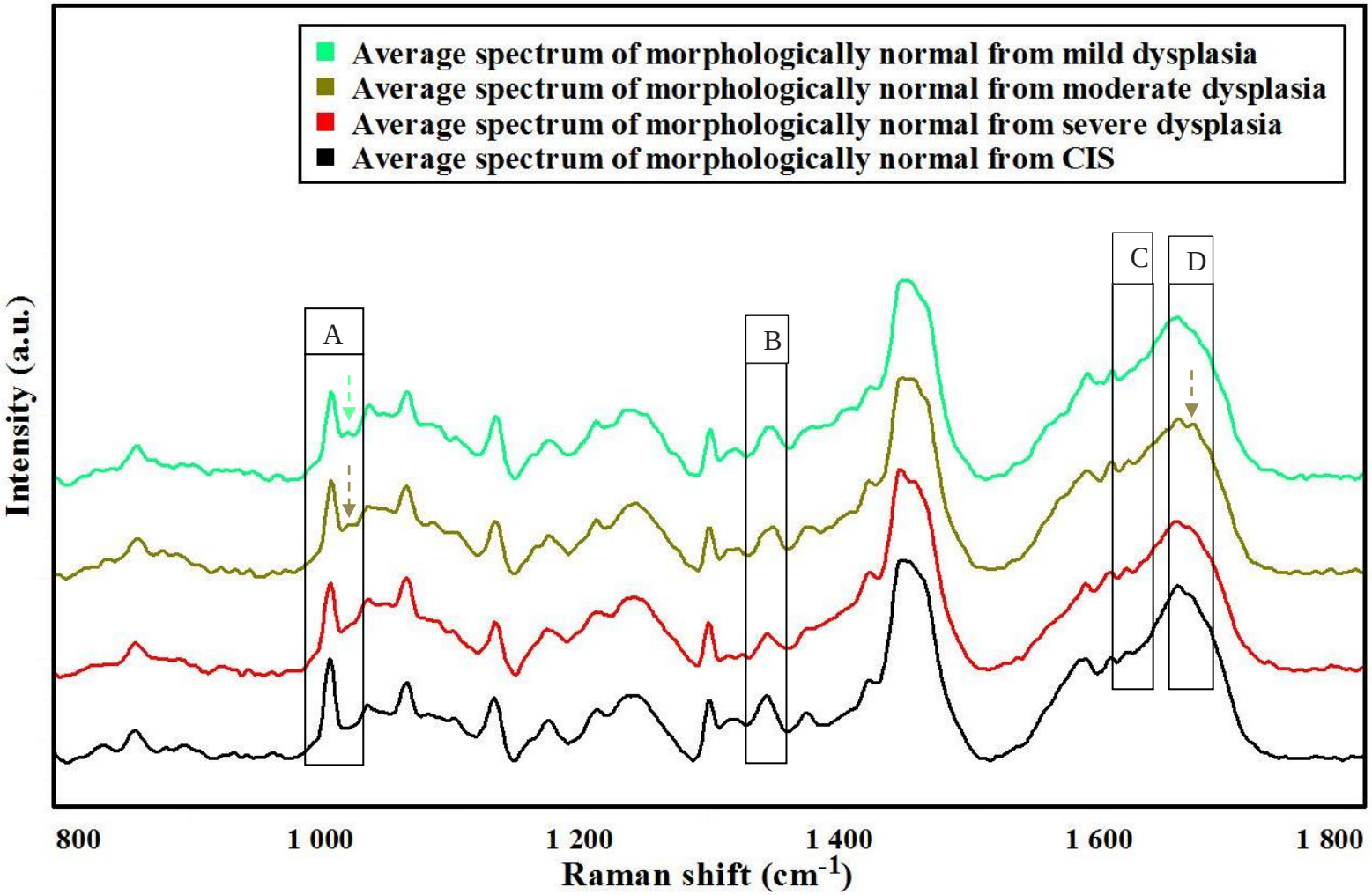

Figure 6.42: The main spectral characterizations of the 4 morphologically normal tissue groups. A= (1009, 1022), B=1347, C= (1619-1623) and D=1673

With respect to the binary grading system, Figure 6.43 shows the mean spectra of the morphologically normal tissue from the resection margins of both high and low grade dysplasia overlaid. To better compare the two tissue types, the spectra were offset and the main distinction features were enclosed in boxes; Figure 6.44.

The peak enclosed in box A at 1048 cm^{-1} (glycogen) appears prominent in morphologically normal from low grade dysplastic tissue, but very weak or may be absent in morphologically normal from high grade dysplasia. This reflects a reduction in the glycogen content in tissues of morphologically normal from high grade dysplasia indicating close similarity with the dysplastic tissue in lower glycogen content due to increase metabolic activity.

The morphologically normal tissue from low grade dysplastic tissue specimens labelled B, shows two small shoulders at 1235 and 1251 cm^{-1} corresponding to amide III C-N stretch, N-H in plane bending unordered proteins, it is absent in morphologically normal samples from high grade dysplasia. This represents degradation or disordering of protein components in morphologically normal tissue from high grade which becomes similar to dysplastic tissue by the phenomenon of field change cancerization.

The peak at 1322 cm^{-1} (guanine ring breathing modes of the nucleic acid base, C-H deformation proteins) enclosed in box C, appears to be more prominent in morphologically normal from low grade dysplastic tissue specimens becoming weaker and broader in morphologically normal tissue from high grade dysplasia. These features represent degradation or disordering of protein components in morphologically normal tissue from high grade which becomes similar to dysplastic tissue by the phenomenon of field cancerization.

The area labelled D at 1423 cm^{-1} (adenine, guanine, backbone nucleic acids) is more prominent in morphologically normal tissue from high grade dysplasia specimens, appearing broader in morphologically normal from low grade dysplasia. This reflects increased nucleic acid content in morphologically normal from high grade dysplasia indicating a similarity with high grade dysplastic tissue features.

In the morphologically normal tissue from high grade dysplasia tissue specimens labelled E, the small peak at 1620 cm^{-1} (C=C bending modes of tryptophan, phenylalanine, tyrosine, β-

sheet of proteins) appears more prominent compared to that in the morphologically normal tissue from low grade dysplasia. This represents additional protein formation in morphologically normal from high grade dysplasia, a tissue which becomes similar to high grade dysplastic tissue.

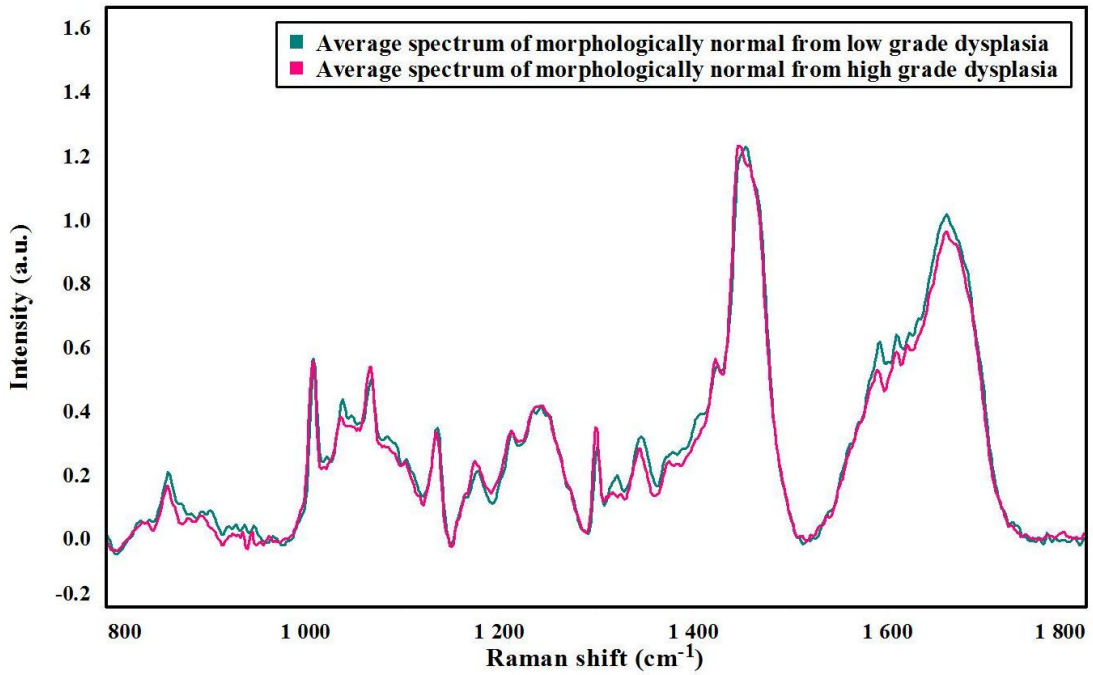

Figure 6.43: Mean spectra of morphologically normal tissue from the resection margins of high grade dysplasia (n=255) and low grade dysplasia (n=123).

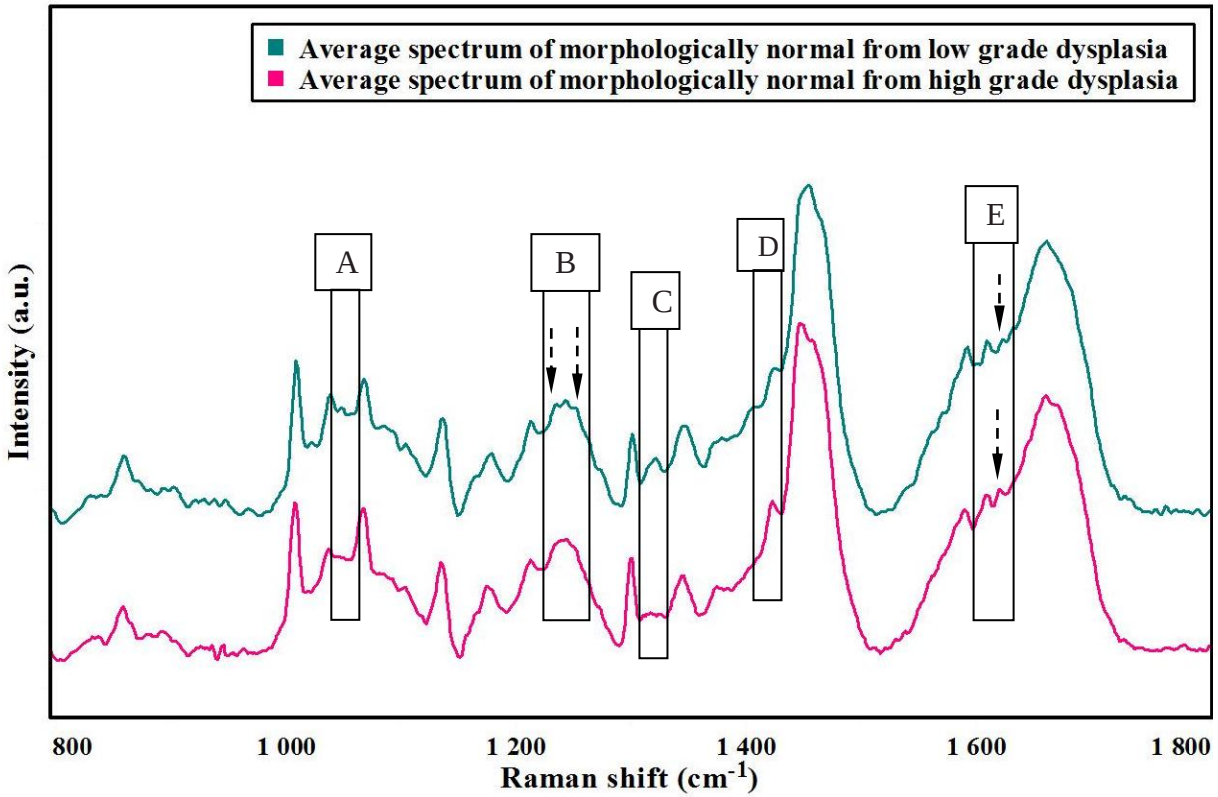

Figure 6.44: The main spectral characterizations of morphologically normal tissue from high and low grade dysplastic tissue. A= (1048) B= (1235, 1251), C= 1322, D= 1423 and E= 1620

6.7.6. Tissue Classifications

Using histopathology diagnosis as a standard of tissue diagnosis, the potential of RS for tissue classification to provide diagnostic information for the early detection of dysplastic changes (Raman classification/prediction) was investigated. PLS-DA was employed on the data matrix consisting of the fully processed data of tissue Raman spectra. 12 significant partial least squares components (PLSCs) were identified, which best described the original variance in the data between groups.

The histopathological diagnosis was compared to the Raman tissue prediction. Different classification models which showed important clinical implementations were generated using both WHO and binary classification systems of epithelial dysplasia. A diagnostic model combining all dysplastic against all morphologically normal tissue samples was generated. Pair-wise comparisons between dysplasia and morphologically normal tissue cases for each grade of dysplasia were used: Mn vs. Md, Modn vs. Modd, Sn vs. Sd and CISn vs. CIS. Further, discrimination of high grade and low grade dysplasia from their morphologically normal tissue samples and a model to discriminate low grade dysplasia from high grade dysplasia cases were also performed.

The significant loadings obtained from PLS-DA for each group of tissues were plotted (Appendix 2-E), in which Q^2 is higher than 0.097 (equivalent to the *p*-value ≤ 0.05). These significant loadings were used to plot the scatter plots of the discriminant functions. Considering the discrimination of all dysplastic from all morphologically normal tissue, Figure 6.45 shows the scatter plots that discriminate all dysplastic from all morphologically normal tissues, in which three morphologically normal were misclassified as dysplastic tissue, while five dysplasia cases were misclassified as morphologically normal tissues. Figure 6.46 shows the details of these misclassified cases; two of them were Modd, one Md, one Sd, one CIS, one Mn and two Sn. To quantify the performance of this model, sensitivity and specificity tests were performed and showed 80.77% sensitivity and 81.48% specificity; Table 6.20.

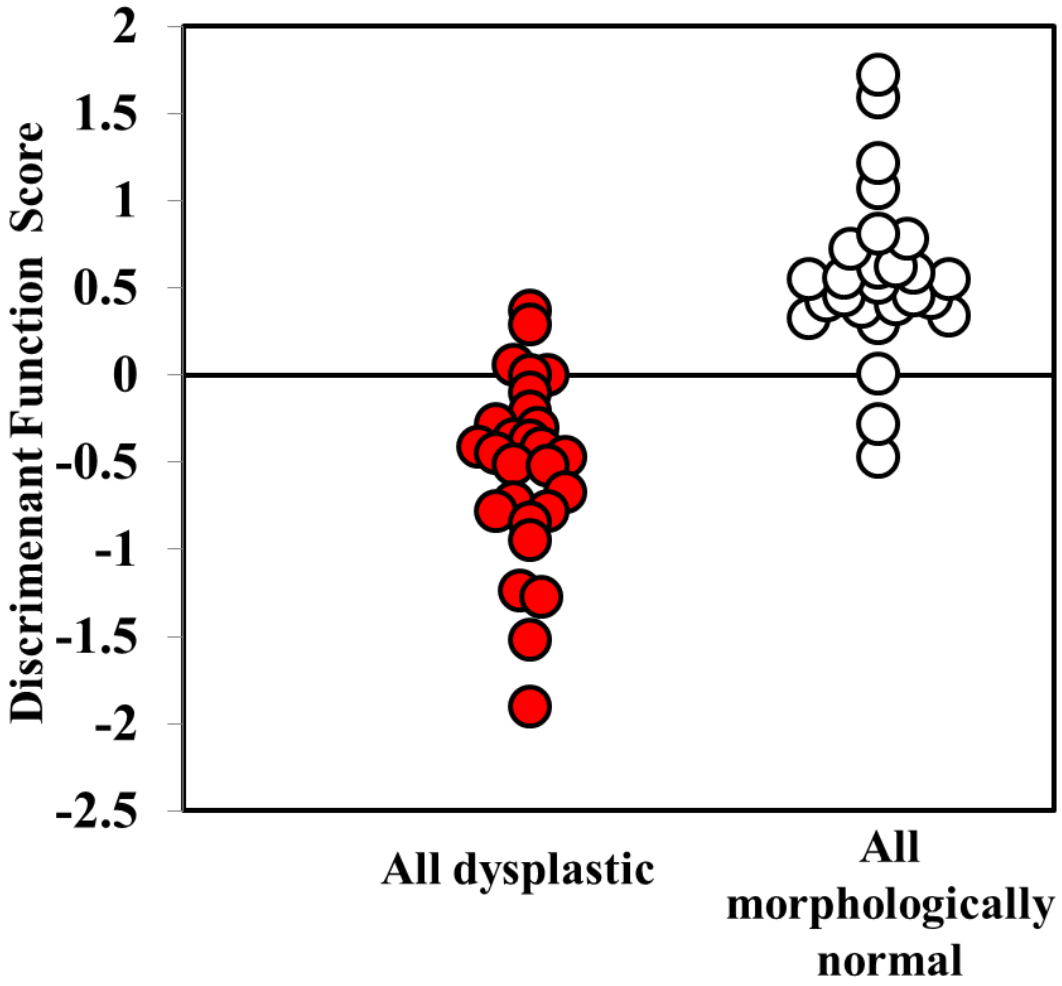

Figure 6.45: Scatter plots of the significant discriminant scores between all dysplastic and all morphologically normal tissue samples.

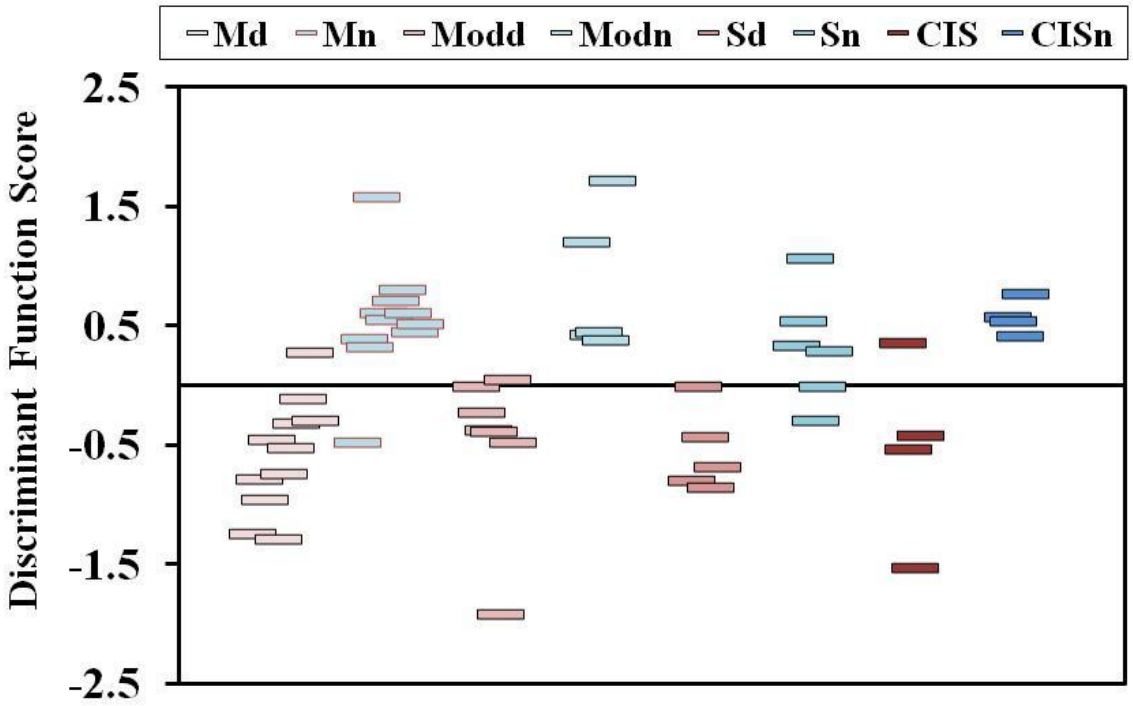

Figure 6.46: Samples details of the scatter plots of the significant discriminant scores between all dysplastic and all morphologically normal tissue samples.

Md= mild dysplasia; Mn=morphologically normal from mild dysplasia; Modn=morphologically normal from moderate dysplasia; Modd=moderate dysplasia; Sn=morphologically normal from severe dysplasia, Sd=severe dysplasia; CIS=carcinoma in *situ*; CISn=morphologically normal from CIS.

Table 6.20: Sensitivity and specificity of Raman classification for all dysplastic tissue samples and all morphologically normal.

Pathology Diagnosis (groups)		Raman prediction		Total
		Ds	Ns	
All dysplastic tissues (Ds)	26	21	5	26
All morphologically normal tissues (Ns)	27	3	24	27
Sensitivity	80.77%			
Specificity	81.48%			

Based on the pathological diagnosis of WHO system and spectroscopic Raman predictions, Figure 6.47 displays the scatter plots of the discriminant functions of each pair-wise grade of dysplasia comparisons. The two groups' classification was found to be promising. Looking to the decision line (zero-line) of the scatter plot, there is complete agreement between pathologist and Raman prediction regarding the discrimination of Sd from Sn (no misclassification). While there is just one Mn was misclassified as Md and one Md misclassified as Mn, two Modd were misclassified as Modn and one Modn was misclassified as Modd, two CIS samples were misclassified as CISn.

The performances of these classification models were tested using sensitivity and specificity tests. Using histopathology diagnosis, Raman prediction achieved a sensitivity and specificity of 100% in distinguishing Sd from Sn and 90.9% in differentiating Md from Mn. For distinguishing Modd from Modn a sensitivity of 71.4% and specificity of 80% were achieved. While CIS separation from CISn showed a sensitivity of 60% and specificity of 100%; Table 6.21.

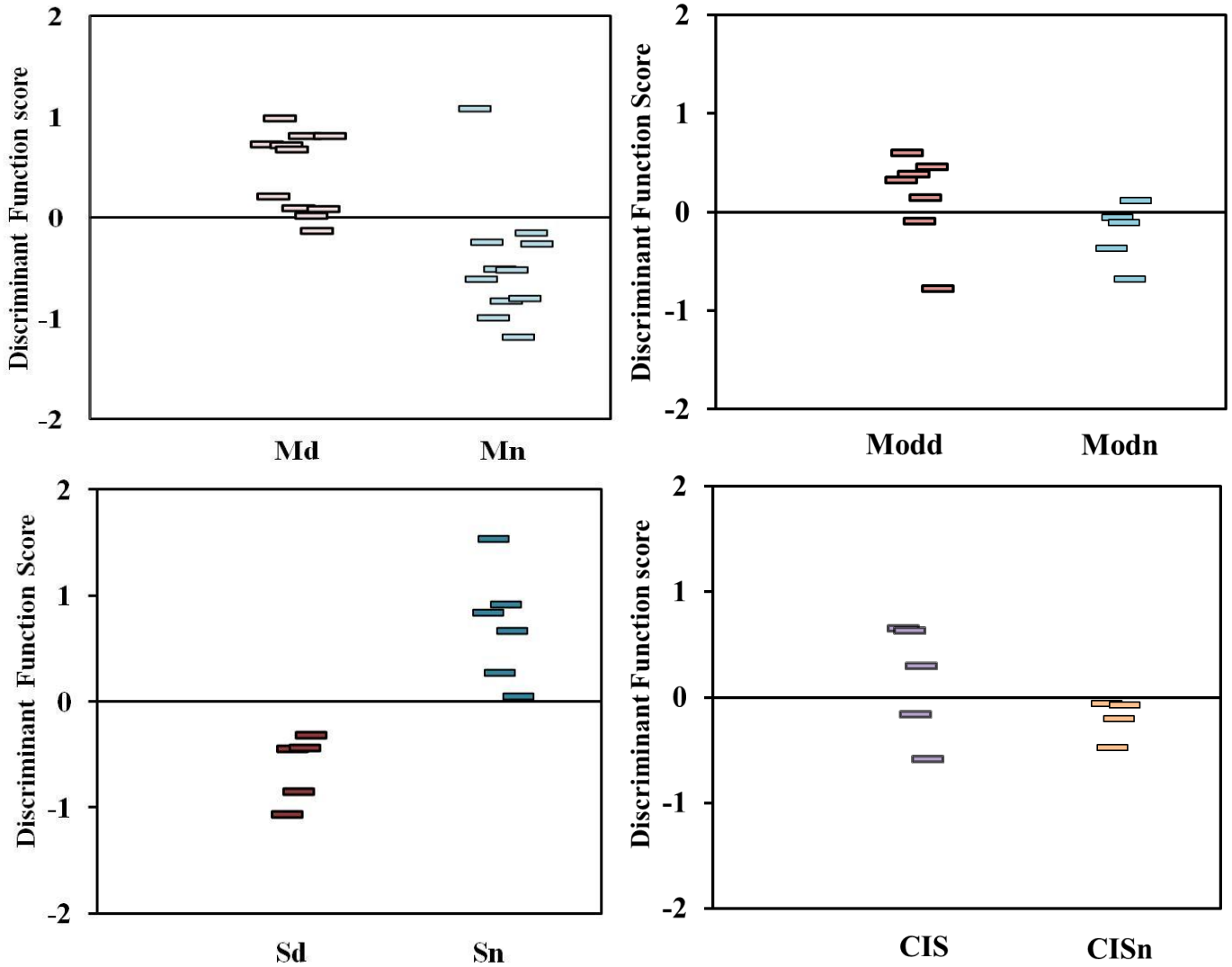

Figure 6.47: Scatter plot of the discriminant scores for the morphologically normal and dysplastic tissue of each grade of dysplasia.

Table 6.21: Sensitivity and specificity of the Raman classifications of the pair-wise groups (WHO classification system).

Pathology Diagnosis		Raman Classifications								Total
		Md	Mn	Modd	Modn	Sd	Sn	CIS	CISn	
Mild dysplasia (Md)	11	10	1							11
Morphologically normal from mild dysplasia (Mn)	11	1	10							11
Moderate dysplasia (Modd)	7			5	2					7
Morphologically normal from moderate dysplasia (Modn)	5			1	4					5
Severe dysplasia (Sd)	5					5				5
Morphologically normal from severe dysplasia (Sn)	6						6			6
Carcinoma *in situ*	5							3	2	5
Morphologically normal from CIS (CISn)	4								4	4
Sensitivity		90.9%		71.4%		100%		60%		
Specificity		90.9%		80%		100%		100%		

Regarding the binary classification system models, Figure 6.48 shows scatter plot of the discriminant functions of high grade dysplasia (HGD) and morphologically normal from high grade dysplasia (HGN), in which two HGD were misclassified spectroscopically as HGN, while one HGN was misclassified as HGD.

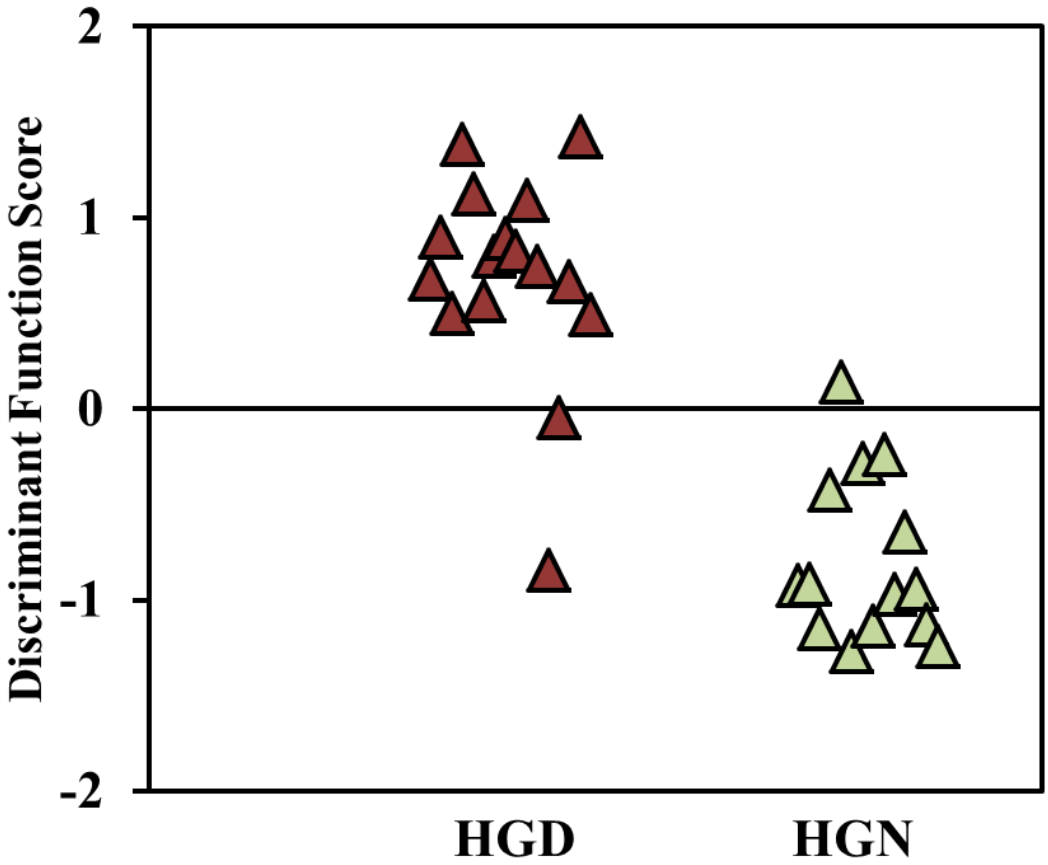

Figure 6.48: Scatter plots of the discriminant scores of pair-wise of high grade dysplasia (HGD) and morphologically normal tissue (HGN).

Considering low grade dysplasia, Figure 6.49 displays scatter plot of the discriminant functions of low grade dysplasia (LGD) and morphologically normal from low grade dysplasia (LGN). Looking to the decision line of the scatter plot, similarly two LGD cases were misclassified as LGN, whereas one LGN was misclassified as LGD.

The performance of these models was tested by sensitivity and specificity tests which are shown in Table 6.22. A sensitivity and specificity of 86.7% and 93.3% respectively, was achieved to distinguish HGD from HGN; whilst a sensitivity of 83.3% and specificity of 90.9% to discriminate between LGD from LGN were recorded.

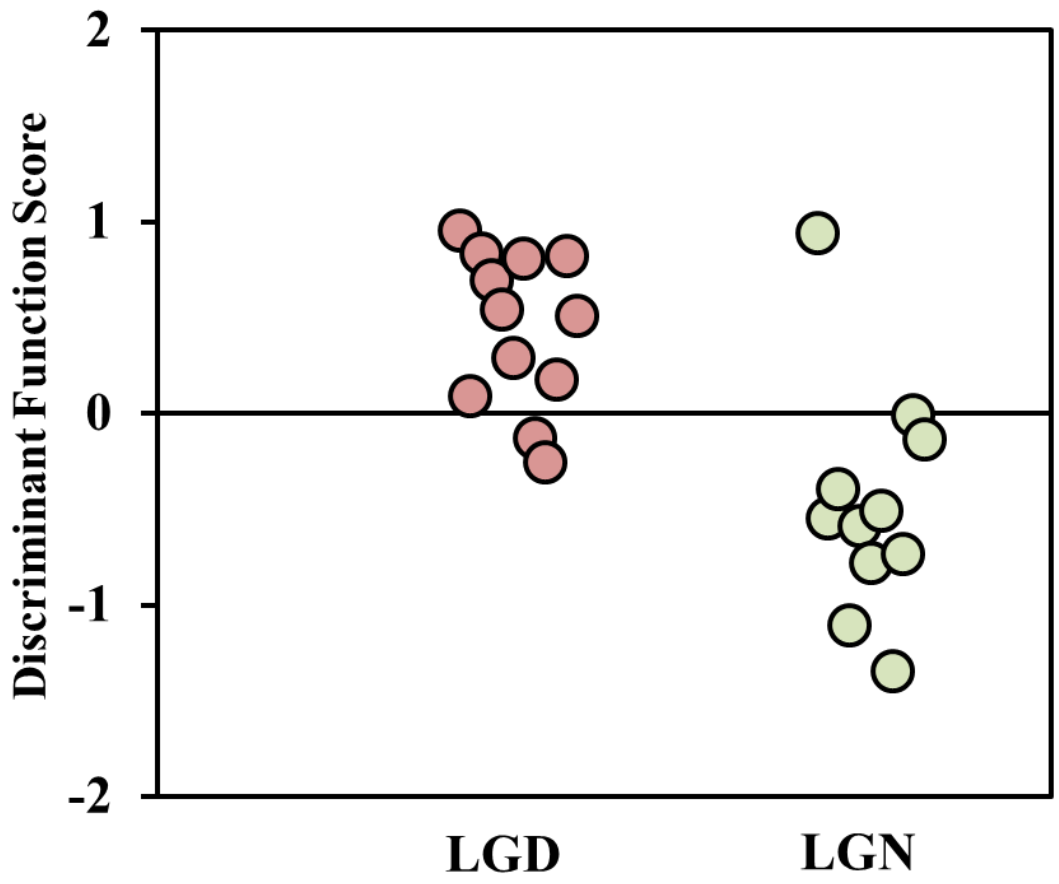

Figure 6.49: Scatter plots of the discriminant scores of pair-wise of low grade dysplasia (LGD) and morphologically normal tissue group (LGN).

Table 6.22: Sensitivity and specificity of Raman classifications of pair-wise of high and low grade dysplasia.

Pathology Diagnosis		Raman Assignment				Total
		LGD	LGN	HGD	HGN	
Low grade dysplasia (LGD)	12	10	2			12
Low grade morphologically normal (LGN)	11	1	10			11
High grade dysplasia (HGD)	15			13	2	15
High grade morphologically normal (HGN)	15			1	14	15
Sensitivity		83.3%		86.7%		
Specificity		90.9%		93.3%		

Discriminating between dysplastic tissue as low grade and high grade dysplasia, the scatter plots that discriminates the two types of tissue samples are shown in Figure 6.50, in which five cases of LGD were misclassified as HGD, whilst four HGD samples were misclassified as LGD. This indicats a poor performance of this model associated with lower levels of sensitivity (58.3%) and specificity (73.3%); Table 6.23.

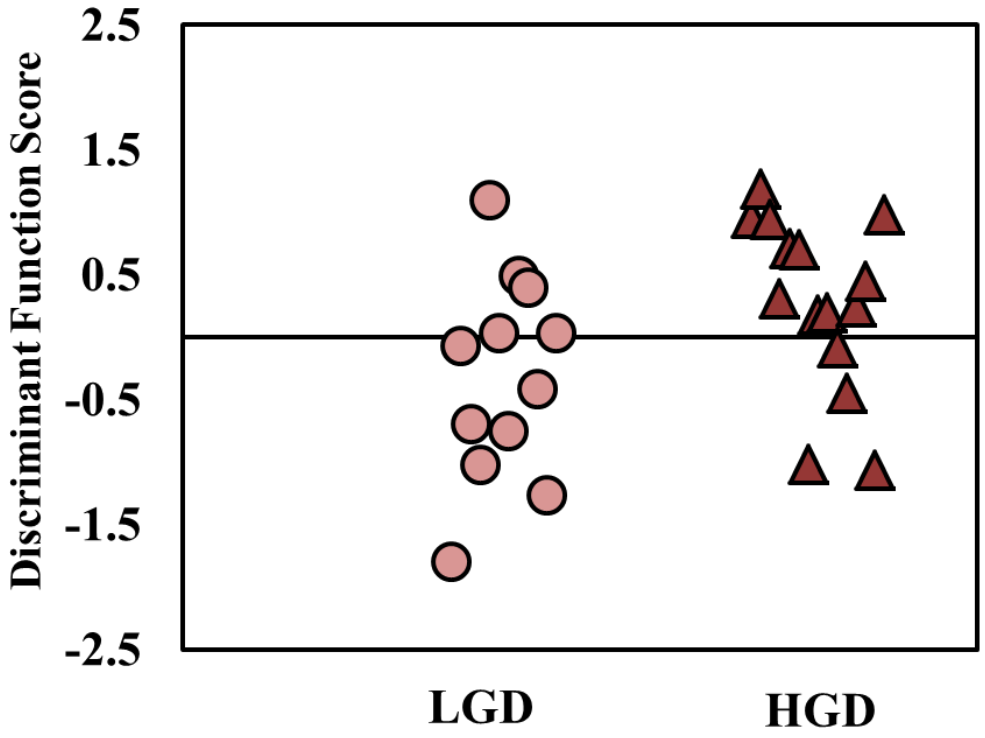

Figure 6.50: Scatter plots of the significant discriminant scores between low grade dysplasia (LGD) and high grade dysplasia (HGD).

Table 6.23: Sensitivity and specificity test of Raman classification between low and high grade dysplasia.

Pathology Diagnosis		Raman Assignment		Total
		LGD	HGD	
Low grade dysplasia (LGD)	12	7	5	12
High grade dysplasia (HGD)	15	4	11	15
Sensitivity		58.3%		
Specificity		73.3%		

6.7.7. Clinical Relations of the Raman Misclassified Cases

Table 6.24 presents the main clinical features of cases used for the present Raman study. Tissue specimens used for current spectroscopic analysis were from 32 patients affected with different grades of the FOM epithelial dysplasia. Those patients were of an age range from 33–77 years (mean, 55.28 years) and all patients were treated with laser surgery and followed up from 11 to 122 months (mean 48.5 months). Males and females were equally distributed in the Raman investigated cases (16 males and 16 females).

In general, the majority of Raman misclassified cases were presented primarily as homogenous leukoplakias (7/9), whilst the remaining 2/9 were non-homogenous speckled. Also, all the misclassified cases were tobacco users and alcohol drinkers and the majority being males. The details of the clinical characterisations of the Raman misclassified cases, dysplastic and morphologically normal tissue samples were summarised in Table 6.25. Overall, five dysplastic cases were spectroscopy misclassified as morphologically normal tissues indicating the presence of normal constituents within the dysplastic tissue components. While four morphologically normal tissues were misclassified as dysplastic tissues representing the presence of dysplasia in the resection margins, since the morphologically normal tissue controls were taken from the resection margins. However, out of the four morphologically normal misclassified cases only one was with dysplastic margins and the remaining three cases were with clear excision margins. This indicates that these morphologically normal tissues with clear margins were subjected to field cancerization phenomenon and they were no longer considered as normal. Also, all the misclassified cases were disease-free, except the one with dysplastic margins, and underwent new site dysplasia formation at the most recent follow-up.

Table 6.24: The main clinical characterisations of all Raman cases.

No	Age years	Sex	Clinical types	Smoking	Drinking	Hpath WHO	Raman prediction	Hpath Binary	Raman prediction	Follow-up months	Clinical outcome
1	36	F	Non-homogenous/Speckled	Yes	Yes	Md	Md	LGD	LGD	15	DF
2	64	F	Homogenous leukoplakia	Yes	Yes	Md	Md	LGD	LGD	14	DF
3	48	M	Homogenous leukoplakia	Yes	Yes	Md	Md	LGD	LGD	122	DF
4	54	F	Homogenous leukoplakia	Yes	No	Md	Md	LGD	LGD	15	DF
5	48	F	Homogenous leukoplakia	Yes	Yes	Md	Md	LGD	LGD	68	DF
6	45	F	Homogenous leukoplakia	Yes	Yes	Md	Md	LGD	LGD	30	DF
7	56	F	Homogenous leukoplakia	Yes	No	Md	Md	LGD	LGD	89	DF
8	64	M	Homogenous leukoplakia	Yes	Yes	Md	Md	LGD	LGN	26	DF
9	71	F	Homogenous leukoplakia	Yes	No	Md	Md	LGD	LGD	56	DF
10	33	F	Homogenous leukoplakia	Yes	Yes	Md	Mn	LGD	LGN	12	DF
11	59	F	Homogenous leukoplakia	Yes	Yes	Md	Md	LGD	LGD	16	DF
12	35	M	Homogenous leukoplakia	Yes	Yes	Md	Md	LGD	LGD	81	DF
13	62	M	Homogenous leukoplakia	Yes	Yes	Modd	Modn	HGD	HGD	74	DF
14	67	M	Non-homogenous/Speckled	Yes	Yes	Modd	Modd	HGD	HGD	11	DF
15	42	M	Homogenous leukoplakia	Yes	Yes	Modd	Modd	HGD	HGD	116	DF
16	40	F	Homogenous leukoplakia	Yes	Yes	Modd	Modd	HGD	HGD	35	R
17	77	M	Homogenous leukoplakia	Yes	Yes	Modd	Modd	HGD	HGD	12	DF
18	56	F	Homogenous leukoplakia	Yes	Yes	Modd	Modn	HGD	HGD	26	DF
19	54	F	Homogenous leukoplakia	Yes	Yes	Mod d	Modd	LGD	LGD	72	DF
20	65	M	Homogenous leukoplakia	Yes	Yes	Modd	Modd	LGD	LGD	60	DF
21	58	F	Homogenous leukoplakia	Yes	Yes	Modd	Modd	HGD	HGN	73	DF
22	58	F	Homogenous leukoplakia	Yes	No	Modd	Modd	HGD	HGN	24	DF
23	66	F	Homogenous leukoplakia	Yes	Yes	Sd	Sd	HGD	HGD	21	DF
24	39	M	Non-homogenous/Speckled	Yes	Yes	Sd	Sd	HGD	HGD	119	NSD
25	76	F	Homogenous leukoplakia	Yes	No	Sd	Sd	HGD	HGD	27	NSD
26	58	M	Non-homogenous/Speckled	Yes	Yes	Sd	Sd	HGD	HGD	38	DF
27	47	M	Erythroplakia	Yes	Yes	Sd	Sd	HGD	HGD	110	R
28	58	M	Non-homogenous/Speckled	Yes	Yes	Sd	Sd	HGD	HGD	24	MT
29	52	M	Homogenous leukoplakia	Yes	Yes	CIS	CISn	HGD	HGD	48	DF
30	59	M	Non-homogenous/Speckled	Yes	Yes	CIS	CIS	HGD	HGD	35	MT
31	59	M	Homogenous leukoplakia	Yes	Yes	CIS	CISn	HGD	HGD	12	DF
32	63	M	Non-homogenous/Exophytic	Yes	Yes	CIS	CIS	HGD	HGD	70	R

F=female; M=male; Md=mild dysplasia;Mn=morphologically normal from Md; Modd=moderate dysplasia; Modn=morphologically normal from Modd; Sd=severe dysplasia; Sn=morphologically normal from Sd; CIS=carcinoma in *situ*; CISn=morphologically normal from CIS; HGD=high grade dysplasia; HGN=morphologically normal from HGD; LGD=low grade dysplasia; LGN=morphologically normal from LGD; DF=disease-free; R=recurrence; MT=malignant transformation; NSD=new–site dysplasia.

Table 6.25: Details of the Raman misclassified cases based on dysplastic tissue against morphologically normal tissue.

Age (years)	Sex	Clinical types	Smoking behaviour	Drinking behaviour	Hpath	Raman prediction	Resection Margin	Follow-up (months)	Clinical outcome
59	F	Homogenous leukoplakia	Intermediate 20 cig./day	Mild 1 u/w	Dysplasia	MorphN	Clear	16	DF
62	M	Homogenous leukoplakia	Heavy 30 cig/day	Heavy 42 u/w	Dysplasia	MorphN	Modd	74	DF
65	M	Homogenous leukoplakia	Intermediate 15 cig/day	Intermediate 25 u/w	Dysplasia	MorphN	Md	60	DF
59	M	Homogenous leukoplakia	Intermediate 15 cig/day	Heavy 50 u/w	Dysplasia	MorphN	Md	10	DF
52	M	Homogenous leukoplakia	Heavy 25 cig/day	Heavy 40 u/w	Dysplasia	MorphN	Clear	48	DF
48	M	Homogenous leukoplakia	Intermediate 20 cig/day	Mild 14 u/w	MorphN	Dysplasia	Clear	121	DF
66	F	Homogenous leukoplakia	Heavy 25 cig/day	Mild 10 u/w	MorphN	Dysplasia	Clear	20	DF
39	M	Non-homogenous Speckled	Heavy 40 cig/day	Heavy 70 u/w	MorphN	Dysplasia	CIS	119	NSD
58	M	Non-homogenous Speckled	Intermediate 20 cig/day	Heavy 50 u/w	MorphN	Dysplasia	Clear	38	DF

F=female; M=male; Hpath=histopathology; morphN=morphologically normal; Modd=moderate dysplasia; Md=mild dysplasia; CIS=carcinoma *in situ*; DF=disease-free; cig=cigarettes; u/w=units/week; NSD=new–site dysplasia.

6.8. Discussion

6.8.1. Practical Considerations

The present study was conducted to investigate the suitability of confocal Raman microspectroscopy to differentiate between morphologically normal and dysplastic tissue spectra, and also to examine the ability of this technique to detect dysplastic changes at early stages. This work differs from that previously published in that the present study used a single oral subsite, with different grades of dysplasia and morphologically normal tissue from their resection margins, using two grading systems (WHO and binary).

Only laser excision biopsy samples were used in this study, as they provided sufficient tissue material, optimal orientation, adjacent to morphologically normal epithelium at the excision margins, representative of the clinical lesion and these biopsies were the standard approach for the final diagnosis. Sixty-four tissue locations within 32 FOM tissue specimens (the most common affected oral site) from 32 patients were studied. Based on consensus histopathological diagnosis of the two efficient oral pathologists, tissues were classified according to the current WHO classification systems: mild, moderate, severe dysplasia and CIS (Gale et al., 2005) and novel binary system: high and low grade dysplasia (Kujan et al., 2006). The current study used only one oral site (FOM) to reduce the variations that might be related to different anatomical sites. This study was different from Malini *et al.'s* (2006) study which used mixed oral tissue (gingiva and FOM). In addition, all patients in this study were from one ethnic group (caucasian) recruited from the North-East of the England at Newcastle General Hospital.

Recently, it has been demonstrated by Guze *et al.* (2009) that Raman spectra were consistent among individuals of different ethnicity and sex and they depend largely on the type of oral mucosa being investigated. Raman signal varied between gingival tissue and cheek mucosa and similarly between dorsal and ventral tongue surfaces. They explained that under the basis of differences or similarities in the molecular composition of lipids, proteins, carbohydrates of different oral subsites quantitatively and/or qualitatively (Guze et al., 2009). This was also supported by a study conducted by Krishna *et al.* (2004) who reported that Raman spectrum

is site dependent and the location of spectral measurement should be mentioned for the purpose of differentiation between normal and pathological tissue spectra.

The current gold standard of diagnosis of oral epithelial dysplasia is through clinical examination, biopsy taking and histopathological evaluation of cytological and architectural abnormalities (Muller et al., 2003). However, histopathological assessment of tissue is based mainly on cellular and nuclear morphology, heavily relying on the pathologist's experience with unsatisfactory levels of inter- and intra-observer variability. It remains a time consuming technique requiring a preparatory process and is unable to provide immediate diagnostic feedback (Bird et al., 2008). Therefore, there is a real need for an objective real time diagnostic system able to overcome the observer variability and independent of morphological changes (Papamarkakis et al., 2010), requiring no tissue preparation (staining and labelling) and amenable to multivariate statistical techniques.

The choice of substrate is a vital issue and important step in experimental design of a microspectroscopic study (Romeo et al., 2006). In a preliminary validation study, ordinary glass slides that were routinely used in histopathology were used for FFPE tissue mounting with subsequent de-waxing and spectral measurements. However, no Raman signal was identified from the investigated area and that might be due to strong absorbance property of glass slides as reported previously by Romeo *et al.* (2006). In the current study, tissue samples were mounted on BaF_2 substrate (10 µm thick sections). BaF_2 window has been chosen based on the fact that it has no Raman signature within the selected spectral range (800–1800 cm^{-1}) of the biological samples (Gobinet et al., 2009), with only one Raman peak at ~242 cm^{-1} (Chen and Shen, 2006) eliminating any spectral tissue contamination from the mounting materials, and also has been successfully used in similar studies.

Fresh tissue in saline may be an ideal tissue for optical spectroscopy because biochemically, it is the best simulation for *in vivo* conditions. However, quick decay, handling difficulties and unavailability (Krishna et al., 2005a) necessitated the evaluation of suitability of other types of tissue for Raman study, such as formalin fixed (Krishna et al., 2004) and FFPE tissue samples (Krishna et al., 2005a). FFPE tissues were used in this study due to the availability from the pathology archive for larger sample study (Krishna et al., 2007b) and suitability for retrospective studies providing valuable information about patients outcomes which can be

correlated with Raman spectral data (Kendall et al., 2009) for a robust spectroscopic diagnostic technique.

As with any microscopic tissue examination, Raman microspectroscopic tissue analysis requires a thin homogenous tissue section (similar thickness) which transmits light. This necessitates a preparatory process of embedding medium for supporting and easily sectioning of the specimen (Ó Faoláin et al., 2005b), with the advantage of easily dissolving away from tissue. Therefore, the investigation of tissue that has been chemically treated is unavoidable in this situation, and it is likely to have small affects upon the biochemistry of the tissue, with these modifications affecting the comparable tissues (normal and dysplastic) more or less similarly (Krishna et al., 2004). For example, during the paraffin-wax embedding procedure, which is an efficient procedure for conserving tissue biopsies for several years, tissue sections were subjected to a series of solvents that may dissolve lipids and consequently remove them from the tissue sections. Therefore, such tissue preparation is not recommended for the investigation of adipose rich tissues (Shim and Wilson, 1996; Bird et al., 2008) and fresh or fresh frozen tissues samples that can preserve lipids, was recommended to discriminate normal from diseased tissues (Tfayli et al., 2005). Additionally, using FFPE sections directly are very restricting due to highly intense paraffin signals which are able to mask Raman spectra (Haka et al., 2002; Bird et al., 2008; Tfayli et al., 2009). Therefore, de-paraffinisation and rehydration of tissue is recommended for spectroscopic tissue analyses.

In this study after tissue mounting and prior to tissue analysis, all samples were deparaffinised using n-hexane as a de-waxing agent followed by alcohol wash. Previously, it has been assumed that after formalin fixation there will be no further alteration of the secondary protein structures in the tissue due to the de-waxing process (Mason and O'Leary, 1991). The same group also indicated that formalin fixation does not cause noticeable changes in proteins and they supported that by similarity of both fixed and unfixed tissue spectra acquired. However, previous work in ovarian tissue conducted by Krishna *et al.* (2005a) showed severe tissue alterations following tissue de-waxing spatially to the protein structures.

In light of the current study, some of the previously reported problems associated with de-waxing procedures, such as the remnant of a residual layer of paraffin wax have been shown

to be related to the choice of inefficient routine de-waxing agents (solvents). In the present study, n-hexane followed by ethanol was used to remove the wax. This type of solvent has been shown to be an effective and a superior de-waxing agent in the removal of paraffin and eliminating the contamination of Raman tissue spectra (Ó Faoláin et al., 2005b). Since all specimens used in this study were processed using an identical protocol, the type of alterations that might have occurred to the proteins after processing is consistent amongst all specimens and was unlikely to cause any major effect to the tissues under investigation, and leading to no overall reduction in the precision of this study.

The present study is the first spectroscopic study, to the best of our knowledge, that used de-paraffinised FFPE oral tissues; however, similar studies were conducted using the same processing but on different types of tissue, such as breast (Haka et al., 2002), ovarian (Krishna et al., 2005a) and cervical tissue (Ó Faoláin et al., 2005a; Lyng et al., 2007).

In spite of sufficient discriminant information for spectroscopic pathology to discriminate normal from abnormal tissue, obtained from chemically de-waxed tissues (Ó Faoláin et al., 2005a), another study conducted by Tfayli *et al.* (2009) reported that the de-waxing process is both time and reagent consuming and causes alteration in the protein tissue structure, in addition to the remnants of some residual paraffin.

Recently, attempts have been made to remove the paraffin-wax contamination to the Raman spectrum; a process termed digital de-waxing (Vrabie et al., 2007; Tfayli et al., 2009). In these studies and as part of the pre-processing steps, the spectrum of paraffin is subtracted from the spectrum taken from the tissue sample and from the mounting substrate. However, this technique relies on complicated mathematical processing of the spectra, with consequential increases in processing time. It also is not clear if the digital removal procedure actual removes only the contribution from the paraffin or may affect other information, or if it is possible that extra information, not actually present in the spectrum, may be added to the processed data.

In pathological diagnosis, since a particular disease state is the same for all patients' age group and sex, the same should be for the spectroscopic tissue diagnosis (Viehoever et al., 2003), with an ultimate aim of developing an accurate and objective optical tissue diagnosis

regardless of inherent patients' diversity in both demography and genetic state. Furthermore, the similarity between histopathological and Raman measurements is that the histopathology examines several tissue sites under the microscope, some of these sites may not show any abnormality; however, if one site is found to be abnormal the diagnosis will be abnormal (dysplasia or cancer). Similarly, Raman spectra can be recorded at several sites and spectra from each site subjected to PCA to check if any site is abnormal (Krishna et al., 2005a). Since histopathological changes involve morphological and biochemical alterations within cells/tissue (Huang et al., 2003b; Lau et al., 2005), RS has the ability to provide highly specific detailed information about these biochemical changes qualitatively and quantitatively. This technique may be a promising objective diagnostic tool for discriminating dysplastic from morphologically normal tissue by early detection of molecular dysplastic changes at a cellular level.

In this study, Raman spectra acquisition along the selected wavelength 800–1800 cm^{-1} which is well known to encode the most important information of biochemical tissue changes (Li et al., 2010), the so-called "fingerprint" (Teh et al., 2008) commonly used by research groups working within oral tissues (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006). Spectra were collected from multiple points per mapping area along the basal cell layer. The advantages were to cover most of the constituents in the area for the benefit of searching for the largest differences and to minimise the intra-sample variability by avoiding mispositioning of the points in the investigated area if point-by-point scanning was used (de Veld et al., 2005b). Also, the mapping technique allows time-efficient automated spectral acquisition from a defined area of a tissue (Venkatakrishna et al., 2001). Further, in epithelial cancer, the abnormal cells proliferation start near the basal lamina and then advance into the epithelial cell layers (Malini et al., 2006). Thus, in this study, the spectral measurements were taken along the basement membrane.

The Raman spectra of biological tissue specimens are very complex and they are usually combination of Raman spectra with noise signals, and known to be affected by a broad fluorescence background (Huang et al., 2003b; Gobinet et al., 2007; Teh et al., 2008). The CCD detector in Raman system records a noise signal, named dark current even without sample and laser light, so there is unfortunately unavoidable contamination to Raman spectrum. The fluorescence background which is mainly due to intrinsic tissue fluorophors

(Mahadevan-Jansen and Richards-Kortum, 1997; Gobinet et al., 2007), is often several orders of magnitude more intense than the Raman spectrum, and if untreated, can dominate the Raman spectra and make data analysis impractical.

In the current study, in order to eliminate the influence of the aforementioned contaminations on Raman tissue spectra and other sources of variation that may be related to instrumentation imperfection (calibration shift) (Gemperline, 2006); data pre-processing prior to data analysis has been accomplished through sequential steps to achieve a successful data analysis (Lieber and Mahadevan-Jansen, 2003; Taleb et al., 2006; Lee et al., 2007a). Thus, in this study altering sample composition (Mahadevan-Jansen and Richards-Kortum, 1997) through a photo-physical bleaching of 120 seconds was used to excite the fluorescent molecules within the tissue from ground state to higher energy levels to eliminate their fluorescent properties. Under the foregoing conditions, no sample degradation was observed during the measurements, as confirmed by the morphological appearance of the white light image, indicating the temporary effect of the photo-bleaching on tissue rather than permanent damage.

In the current study, similar to a previous study on skin cell line conducted by Donfack *et al.* (2010), the majority of the excluded bad spectra were found to be from the morphologically normal tissue compared to dysplastic tissue spectra. This may be due to simple physical distortions, bending or twisting of the normal tissue which is usually located at the distal end of the resection margins of the dysplastic tissue mounted on the barium fluoride near the substrate margins. Also, the absolute signal intensity is not very reproducible in the measured Raman spectra and has different intensity ranges depending on many experimental conditions (laser intensity, accumulation time, temperature variations and tissue constituents) (Skoulika et al., 1999). In the present study, for the purpose of groups comparison and to compensate for such variations in spectral shape and peak intensities (Huang et al., 2003b; Teh et al., 2008), Raman spectra were normalised against the mean intensity between 1435 and 1480 cm^{-1} band corresponding to the full width at half maximum for CH_2 scissor mode centred at ~1450 cm^{-1} to minimise the impact of variation of peak position and reduce the effect of noise. The choice of CH_2 band for normalisation is based on both commonly used and the most stable in all biochemical constituents of the cells, for example Le Bihan *et al.* (1996), Notingher *et al.* (2003b) and Notingher *et al.* (2004).

There are hundreds, to more than thousands, of Raman spectra with each spectrum ranging from 800–1800 cm^{-1} and containing 629 intensity values. Manual investigation of such a high dimensional data sets proves difficult to achieve efficient data analysis (Lee et al., 2007a), hence multivariate data analysis was used to reduce the high dimensional data set by condensing the spectral information into completely new variables, describing the key information from the original data for groups classification, using the established techniques, such as PCA fed to PLS-DA. However, a univariate approach of data analysis was also used to investigate the diagnostic capability of Raman by using an individual spectral feature, such as peak relative intensity and position, which was also found to be useful in identifying spectral diagnostic markers in previous studies (Mahadevan-Jansen et al., 1998; Krishna et al., 2004; Stone et al., 2004; Lyng et al., 2007; Huang et al., 2010; Teh et al., 2010a).

For diagnostic purposes and to compare spectral differences between groups of tissue, all the scanning parameters, such as time, accumulation number, laser power and wavelength were kept constant during the experiment for all samples. Changing any of these conditions may result in intensity differences not related to the disease state, but to the experimental environment.

In this study, a small pilot study was designed to investigate the effect of different scanning times and accumulate numbers on spectral quality, 30 second acquisition times with 2 accumulations were found to be suitable and providing a good spectral quality. That was supported by a study conducted by Lyng *et al.* (2007) who used the same accumulation time on cervical tissue spectral analysis. Also, it has been reported earlier that spectral intensity differences may be related to variations in tissue section densities (Ó Faoláin et al., 2005c). However, that is not applicable in this study, because all tissue sections were with the same thickness (10 μm) and the differences in the relative peak intensity observed between groups are due to biochemical changes related to disease state and not to the effect of specimen size.

6.8.2. Spectral Feature and Peak Assignments

The primary aim of this work was to develop a spectroscopic tissue diagnosis based on overall spectral differences. Thus, it was necessary to obtain an average spectrum from different points in the tissue to be used as a representative spectrum of the investigated site (Krishna et al., 2004; Tfayli et al., 2005). These mean spectra from several sites in both dysplastic and morphologically normal tissue were used for spectroscopic data analysis to eliminate the variation of single spectrum and maintain commonality (Li et al., 2010).

A set of Raman peaks of each representative spectrum of dysplastic and morphologically normal tissues were identified and a direct visual comparison was carried out. The observed differences between spectra are generally based on the associated changes in chemical bonds vibrations identified at particular wavenumbers (Wang and Mizaikoff, 2008) and reflect the biochemical tissue changes related to the disease state. Increases or decreases in the concentration of a certain type of biomolecule in dysplastic tissue is reflected by an intensity difference from morphologically normal tissue. In this study, a difference spectrum was also calculated by subtracting one spectrum from another to emphasise the main differences between the two spectra for comparison purposes (Hutchings, 2009).

A similar biochemical tissue changes between the dysplastic and the malignant tissues were reported and confirmed by the presence of the same spectral features at some wavelengths in both carcinoma and dysplasia in cervical tissue spectra (Lyng et al., 2007). Shim *et al.* (2000) reported the same differences between normal and dysplasia in oesophageal epithelial tissue in the same bands which was observed between normal and cancer tissues in the nasopharynx that was found by Lau *et al.* (2003).

Mild Dysplasia

In general, this has shown that the dysplastic spectrum showed a predominant protein pattern with contributions from nucleic acids, amino acids, such as phenylalanine, tryptophan and tyrosine. Although no significant intensity differences between mild dysplasia (Md) and morphologically normal tissue from mild dysplasia (Mn) for the majority of the identified peaks, a significantly higher relative intensity of phenylalanine protein at 1009 cm^{-1} was

observed in Md. This reflects increased protein content associated with dysplastic changes. This is an expected finding due to the fact that mild dysplasia is a first step in the progression from normal, with most of the components of normal tissue still found in this early stage of dysplasia and the changes in biochemical tissue constituents may be in low or undetectable concentrations (Kendall et al., 2011). The significantly higher intensity of phenylalanine protein in mild dysplasia reflects the ability of the Raman system to detect early biochemical tissue changes at early stages. In addition, the relative intensity of the small bands at 834, 886 and 902 cm^{-1} in Md, are more intense than in Mn. These bands may be attributed to nucleic acids, proteins and amino acids, respectively, which is in agreement with the concept of protein predominant patterns in dysplastic tissue with contributions from amino acids and nucleic acids from the proliferating cells and from surface proteins, signalling, antibody, antigens and enzymes associated with transformational changes (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006).

The wavenumber at 1039 cm^{-1} corresponding to phenylalanine (protein) showed a lower intensity in Md compared to Mn with no obvious reason. This is consistent with a study conducted by Teh *et al.* (2008) who reported a lower intensity of phenylalanine proteins in dysplasia of gastric mucosa compared to normal tissue, with no clear explanation. Also, a higher intensity of glycogen at 1022 and 1048 cm^{-1} in Mn compared with Md was found. The decreased intensity of glycogen in dysplastic oral tissue even in mild dysplasia has been reported previously by Isacsson and Shear (1981) who studied the content and distribution of glycogen in oral epithelial dysplasia and found to be decreased in dysplasia. This has been explained under the basis of either increases in metabolic activity, for more energy consumption from cellular proliferation during the transitional process from normal to dysplastic (Stone et al., 2002b), or may be due to a decrease in glycogen synthesis in oral epithelial dysplasia; however, the difference was not statistically significant in this study.

The band at 1301 cm^{-1} (CH_2 deformation lipids) is more intense in Mn, which is in agreement with the fact of lipids dominated the spectra of normal epithelial tissue. This is mainly due to the fact that normal tissue consists of a closely packed layer of surface epithelial cells, with their lipid bilayer membranes expose to the laser excitation, resulting in the membrane lipid dominated spectrum for normal tissue (Krishna et al., 2004). In addition to their small nuclei with less contribution from nucleic acids of non-proliferating cell (Krishna et al., 2004).

Similarly, Mn showed a higher relative intensity at 1423 cm^{-1} (nucleic acids), but for no clear reason.

Raman peaks only seen in the spectrum of Mn at 1251 cm^{-1} (amino acids), 1272 cm^{-1} (amide III of protein) and 1600 cm^{-1} (phenylalanine, tyrosine). This may be due to the degradation of these constituents in Md as a part of the disease process and no longer can be detected because of low concentrations (Kendall et al., 2011).

A noticeably higher relative intensity at 1317 cm^{-1} (guanine ring breathing mode of nucleic acids bases), 1347 cm^{-1} (CH_2 protein, nucleic acids) and 1377 cm^{-1} (thymine, guanine, adenine amino acids) in Md was found. This suggests an increased amount of nucleic acids and proteins in proliferating cells (Short et al., 2005) and also increased nuclear/cytoplasmic ratio as a part of cellular transformation in dysplastic changes (Stone et al., 2002b). This agrees with the knowledge of histopathology in grading of epithelial dysplasia depending upon nucleic acid to cytoplasmic ratio (Huang et al., 2003b). The results of the spectroscopic study are in agreement with histopathological study of biopsy as a typical standard for the biospectroscopic identification.

Also, in Md, a higher intensity at 1591 cm^{-1} assigned to olefinic stretch of lipids was seen, but the difference was not statistically significant. This is an unexpected finding, since lipids mainly dominate the spectra of normal tissue and this may be due to the presence of normal constituents within the Md spectrum. Similarly, Md showed a higher relative intensity at 1609 cm^{-1} (aromatic amino acids) which is consistent with the development of new surface proteins, amino acids formation, and contribution from the proliferating epithelial cells in dysplasia (Krishna et al., 2004). Also, Md showed a higher relative intensity of amide I of proteins at 1661 cm^{-1} which suggests an increase in the relative amount of protein in dysplastic tissue (Mahadevan-Jansen and Richards-Kortum, 1996).

Moderate Dysplasia

A significant higher intensity at 1087 cm^{-1} (C-C stretching mode of lipids) in morphologically normal tissue from moderate dysplasia (Modn) was observed, compared with moderate dysplasia (Modd). This is supported by the observations of lipid dominated spectra of normal epithelium previously reported (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006).

Peaks or shoulders seen in Modn were either very weak or absent in Modd including 879 cm^{-1} (hydroxyproline, tryptophan), 1315 cm^{-1} and 1457 cm^{-1} (guanine ring breathing mode of nucleic acids base), 1623 cm^{-1} (tryptophan, phenylalanine protein) and 1673 cm^{-1} (tyrosine and amide I of protein). Similar to mild dysplasia, this may suggest degradation or disordering in the related tissue components associated with the dysplastic transformation process. These biochemical changes are more likely reflective of the progression from normal to moderate dysplasia supported by the histopathological base of knowledge.

In this study, in Modd, higher relative intensity at 896 cm^{-1} (backbone of nucleic acids), 929–931 cm^{-1} (amino acids proline, valine), 971 cm^{-1} and 1137 cm^{-1} assigned to proteins, 1322 cm^{-1} (guanine of nucleic acids), 1345 cm^{-1} (CH_2 proteins thymine, guanine, adenine amino acids) and amide I of proteins at 1658 cm^{-1}. This suggests the progressive increase in proteins, amino acids and nucleic acid components reflecting the progress of the disease from normal towards dysplasia. It is in agreement with what was reported by Krishna *et al.* (2004) and Stone *et al.* (2002b).

The band at 1301 cm^{-1}, 1448 cm^{-1} (CH_2 deformation lipids) and 1591 cm^{-1} attributed to C=C olefinic stretch of lipids were unexpectedly more intense in Modd, similar to Md, although this was not significant. This may be due to the presence of normal tissue constituents within the dysplastic region of Modd (Krishna et al., 2004), which may lead subsequently to misclassification.

Severe Dysplasia

Two significant peaks were identified: one at 1243 cm^{-1} assigned to amide III of protein which was more intense in morphologically normal from severe dysplasia (Sn) compared to severe dysplasia (Sd). This unexpected decreased in amide III of protein intensity in Sd suggests degradation and/or disordering of proteins accompanying the disease process and is supported by the study conducted by Donfack *et al.* (2010) who reported disordered and degraded protein structures in neoplastic skin cells compared to normal cells. This finding is in contrast with the earlier finding of a laryngeal tissue study conducted by Stone *et al.* (2000) who found a higher intensity of amide III based on protein secondary structures with progression from normal to precancerous to carcinoma, indicating an increased protein cellular contents in precancerous lesions compared to normal tissue.

The second significant peak was found in severe dysplastic tissue specimens at 1344 cm^{-1} (CH_2 CH_3 proteins), which suggests increased protein content in Sd from the proliferating cells, surface proteins, antigens, antibody, and enzymes associated with transformation process from normal to dysplastic (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006), or may be due to the synthesis of new protein contributions in Sd from the proliferated cells (Notingher et al., 2003b).

In Sn and similar to Mn and Modn, a higher relative intensity of phenylalanine at 1037 cm^{-1} was observed; however, there is no direct cause for the higher phenylalanine level in Sn tissue sample compared to Sd, but probably, as previously mentioned, is due to degradation and disorientation of proteins in severe dysplastic tissue.

The wavenumber corresponding to 1052 cm^{-1} attributed to glycogen was more intense in Sn compared to Sd, this is in agreement with the finding of Isacsson and Shear (1981) who reported a decrease in glycogen content in oral epithelial dysplasia due to increased metabolic activity that needs the glycogen source of energy or may be due to a decrease in glycogen synthesis in dysplastic tissue.

The bands at 1052 cm^{-1} (glycogen), 1066 cm^{-1} and 1091 cm^{-1} (lipids) were more intense in Sn. This is supported by both higher level of glycogen (Isacsson and Shear, 1981) and lipid

dominated spectra (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006) in normal tissue spectra.

Another unexpected finding in Sn is the higher relative intensity of 1213, 1243 and 1423 cm^{-1} attributed to proteins and nucleic acid backbone. Although there is no clear reason for more intense levels of these components in Sn, this may reflect the ability of RS for early detection of dysplastic features at a molecular level before their morphological appearance (Keller et al., 2008), or may be due to the presence of dysplasia in the resection margin, which was considered as histologically normal.

Severe dysplasia showed higher relative intensities at 829 cm^{-1} (tyrosine of nucleic acids), 961 cm^{-1} (symmetric stretching of PO_4^-), 1007 cm^{-1} (phenylalanine), 1135 cm^{-1} (C-N proteins), 1174 cm^{-1} (tyrosine, phenylalanine, proteins), 1315 cm^{-1} (guanine, nucleic acids), 1344 cm^{-1} (CH_2 protein), 1375 cm^{-1} (thymine, guanine, adenine amino acids), 1609 cm^{-1} (aromatic amino acids), 1620 cm^{-1} (tryptophan, proteins) and 1658 cm^{-1} (amide I of proteins). These suggest higher content of proteins, amino acids and nucleic acids in dysplastic tissue spectra which was also reported previously (Stone et al., 2002b).

Peaks such as 1299 cm^{-1} and 1446–1458 cm^{-1} attributed to CH_2 deformation lipids, and peak at 1589 cm^{-1} assigned to oleifinc stretching mode of lipid showed higher intensity in Sd compared to Sn. These unexpected findings may further support the concept of normal tissue constituents within the dysplastic tissue components and is in agreement with a previous study conducted by Krishna *et al.* (2004) who indicated that most of the normal tissue components are still found in the malignant tissue.

Furthermore, in Sn, a peak such as at 1102 cm^{-1} assigned to lipids was either absent or very weak in Sd. This agrees with lipid-dominated spectra of normal tissue compared with protein-dominated spectra in dysplastic tissue. Also, the peak assigned to nucleic acids at 1326 cm^{-1} was seen in Sn, while absent or weak in Sd. This is an unexpected finding and may be due again to degradation of proteins as cells respond to the disease (Papamarkakis et al., 2010) with subsequent lower concentration for detection (Kendall et al., 2011).

Carcinoma *in situ*

Although the differences in the majority of Raman peaks between CIS and morphologically normal from CIS (CISn) were subtle, there were two peaks in CIS tissue specimens with significantly higher intensity compared to CISn spectrum: 1007 cm^{-1} and 1091 cm^{-1} corresponding to phenylalanine proteins and O-P-O stretching mode of nucleic acids, C-C proteins, respectively. The peak at 1037 cm^{-1} attributed to phenylalanine proteins was more intense in CIS, but was not significant. As reported previously by Krishna *et al.* (2004), in CIS, cells in all layers of stratified epithelium are proliferating and are more likely to express many surface proteins from signalling agents, antigens, antibodies and enzymes. This may explain the higher proteins and nucleic acid content in this type of tissue, which is in complete agreement with histopathology.

Clearly, a decrease in relative intensities was observed in CIS at 1135 cm^{-1} (C-N protein), 1176 cm^{-1} (tyrosine, phenylalanine, proteins), 1238 cm^{-1} (amide III of proteins), 1299 cm^{-1} (CH_2 deformation lipids), 1344 cm^{-1} (CH_2 protein), 1375 cm^{-1} (thymine, guanine, adenine amino acids), 1589 cm^{-1} (C=C stretch olefinic of lipid), 1608 cm^{-1} (aromatic amino acids), 1621 cm^{-1} (tryptophan proteins) and 1661 cm^{-1} (amide I of proteins). The lower intensity of amide III of proteins and amino acids in CIS tissue specimens compared to CISn was similar to what were seen in severe and moderate dysplasia, indicating disturbances of protein structures from ordered to unordered proteins with progression of dysplasia severity compared to normal (Gniadecka et al., 1997). Or may be due to degradation of proteins as cells respond to the disease (Papamarkakis et al., 2010), with subsequent lower concentration for detection (Kendall et al., 2011).

Contrary, in this study, mild dysplasia demonstrates higher intensity of amide III of protein compared to normal tissue, suggesting increased relative amount of proteins (Mahadevan-Jansen and Richards-Kortum, 1996). Similar to amide III of protein, the intensity of amide I is less intense in CIS compared to CISn, which is more likely due to lower proteins contents in CIS tissue reflecting protein degradation and/or disordering, or may be due to highly ordered protein content in CISn. This was confirmed by a prominent or strong peak in CISn compared to degraded unordered protein conformations in CIS confirmed by a lower intensity, weak or missing peaks.

Considering the lower intensity of 1299 cm^{-1} and 1589 cm^{-1} band in CIS compared to CISn which suggests lower contents of lipid in CIS. This agrees with the concept of biological transformation process from normal to CIS in which lower contents of lipid and higher content of proteins/nucleic acids. However, the same wavelengths (1299 cm^{-1} and 1589 cm^{-1}) were found to be more intense in Md, Modd and Sd, compared to CIS tissue spectra.

In addition, small band such as 859, 882, 892, 926 and 936 cm^{-1} which were generally attributed to amino acids proteins and nucleic acids, were seen more intense in CIS. This was in agreement with the histopathological findings of dysplastic tissue changes of more proteins and nucleic acids with dysplastic transformation process from cellular proliferation (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006).

Low Grade Dysplasia (LGD)

The significant intensity differences between LGD and morphologically normal from LGD (LGN) were identified at two Raman peaks. The first one is the higher relative intensity of amide I of proteins at 1660 cm^{-1} in LGD, suggesting an increase in protein content from proliferating cells, compared to LGN. This is in agreement with the concept of protein-dominated spectra in malignant tissue and supported by the findings of Malini *et al.* (2006) who reported a broad and strong amide I of proteins (±5 cm^{-1}) in oral tissue spectra of inflamed, premalignant and malignant tissues. Also, this is consistent with the findings of Lyng *et al.* (2007) who reported an increased intensity of amide I of proteins (±5 cm^{-1}) in spectra of cervical carcinoma compared to normal tissue. However, this finding is inconsistent with the findings of Hu *et al.* (2009) who found a decreased relative intensity of amide I of proteins at 1660 cm^{-1} in oral cancer compared to normal tissue.

The second significant peak corresponding to higher phenylalanine proteins level at 1009 cm^{-1} in LGD compared to LGN. This may be due to more protein formation in dysplastic tissue. This is inconsistent with Teh *et al.* (2008) who reported lower intensity of phenylalanine proteins in dysplasia of gastric mucosa compared to normal tissue, but with no clear explanation.

In this study, a noticeably lower relative intensity was observed in LGD at 859 cm^{-1} (tyrosine protein), 1039 cm^{-1} (phenylalanine proteins), 1066 cm^{-1}, 1083 cm^{-1} (C-C stretch lipids) and 1104 cm^{-1} (phenylalanine proteins). The lower proteins contents in LGD suggests again degradation or disturbing of these constituents in dysplastic cells, while lower lipids content in LGD is consistent with the concept of lower lipids/higher protein content in dysplasia compared to morphologically normal. Generally, these spectroscopic changes further support protein-dominated spectra in malignant tissue and lipid-dominated spectra in normal tissue.

The current study demonstrated more intense peaks in LGD, compared to LGN at 1135 cm^{-1} (C-N protein), 1177 cm^{-1} (tyrosine, phenylalanine proteins), 1317 cm^{-1} (guanine ring breathing mode of nucleic acids bases), 1345 cm^{-1} (CH_2 protein), 1379 cm^{-1} (CH_3 hyaluronic acids), 1591 cm^{-1} (C=C stretch olefinic of lipids) and 1609 cm^{-1} (aromatic amino acids). This suggests more proteins, amino acids and nucleic acids associated with transformation process from normal to dysplastic and is supported by previous spectroscopic studies on oral tissues (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006). While higher intensity of hyaluronic acids at 1379 cm^{-1} contributes significantly to cell proliferation and progression to malignancy (Krishna et al., 2004). A higher intensity of lipids at 1591 cm^{-1} in LGD is inconsistent with the knowledge of protein-dominated spectra in malignancy and lipid-dominated spectra in normal tissue. However, this may further support the idea of normal tissue constituents still found within the dysplastic tissue components and it is in agreement with previous study indicated that most of the normal tissue components are still found in the malignant tissue (Krishna et al., 2004).

New peaks in LGD were observed at 886 cm^{-1} and 983 cm^{-1} assigned to tryptophan and phenylalanine proteins respectively, suggesting new protein formation as disease progresses from normal to dysplasia, which is consistent with knowledge of disease process.

In this study, in LGN, bands such as 1624 cm^{-1} and 1632 cm^{-1} assigned to proteins were very weak or absent in LGD. This again suggests degradation and/or disordering of these proteins associated with tissue transformation and reflected by weak or absent peaks in LGD, with subsequent lower or undetectable concentrations for spectroscopic detection (Kendall et al., 2011).

High Grade Dysplasia (HGD)

Significant differences in relative peak intensities were identified between HGD and morphologically normal from HGD (HGN) in 15 wavenumbers. In HGD, a significant higher relative intensity was found at 934 cm^{-1} (amino acids proline, valine), 1137 cm^{-1} (C-N proteins), 1322 cm^{-1} (guanine of nucleic acids), 1345 cm^{-1} (CH_2 protein), 1377 cm^{-1} (thymine, guanine, adenine amino acids), and 1622 cm^{-1} (tryptophan, proteins). This suggests higher amount of proteins, amino acids and nucleic acids in HGD specimens associated with biological tissue transformation and seems to be consistent with other studies (Stone et al., 2002b; Krishna et al., 2004; Lyng et al., 2007). In addition, the peaks at 1301 cm^{-1} and 1591 cm^{-1} corresponding to CH_2 deformation lipid and C=C olefinic stretch of lipid, respectively were also significantly more intense in HGD. These unexpected findings are not supported by the concept of protein-dominated spectra in dysplasia and cancer tissue. However, the higher intensity of 1301 cm^{-1} in HGD was similarly reported in dysplasia of gastric mucosa in a study conducted by Teh *et al.* (2008). The higher intensity of olefinic/lipid at 1591 cm^{-1} in HGD compared to HGN suggests the presence of normal tissue constituents within the dysplastic tissue spectra; however, it is inconsistent with the finding of Hu *et al.* (2009) who reported more intense C=C stretch olefinic of lipids (±4 cm^{-1}) in normal oral tissue than in malignant tissue which is in agreement with the concept of lipid-dominated spectra in normal tissue.

In this study, other significant bands were seen with lower relative intensity in HGD compared to HGN, at 1037 cm^{-1} (phenylalanine proteins), 1081 cm^{-1} (C-C stretch lipids), 1104 cm^{-1} (phenylalanine proteins), 1176 cm^{-1} (tyrosine, phenylalanine, proteins), 1213–1243 cm^{-1} (amide III of proteins) and 1448 cm^{-1} (CH_2 deformation lipids). The lower intensity of phenylalanine protein in HGD may suggest degradation or disordering of proteins in dysplasia compared to morphologically normal tissue. This is consistent with Teh *et al.* (2008) who reported a lower intensity of phenylalanine at 1004 cm^{-1} in dysplasia of gastric mucosa, compared to normal tissue. They explained that in light of higher or lower amounts of a certain types of biomolecules in relation to the total Raman active component in dysplastic tissue. Also, a decreased intensity of 1081 cm^{-1} (phospholipids) in HGD is more likely supported by the previously mentioned concept of protein-dominated spectra in malignancy and lipid-dominated spectra in normal tissue. The lower relative intensity of

proteins and amino acids bands in HGD due to degradation and/or disturbances of theses structures, compared to HGN with subsequent low concentration and lower spectral intensity (Papamarkakis et al., 2010).

In addition, the contribution of new peaks in the spectrum of HGD at 971 cm^{-1} and 1099 cm^{-1} suggests new protein formation from cellular proliferation and other associated transformation processes. In HGN, the bands at 880 cm^{-1} and 1326 cm^{-1} corresponding to tryptophan proteins and purine bases of nucleic acids, respectively were absent in HGD. This may suggest degradation or disturbances of these components in dysplastic tissue, compared to normal tissue associated with disease progression. This is in agreement with the findings of Papamarkakis *et al.* (2010) who reported that with disease progression, degradation of common proteins and expression of different proteins as cells respond to the disease.

6.8.3. Spectral Intensity and Dysplasia Severity

By investigating the relationship between relative peak intensity and degree of dysplasia severity from the mild through moderate and severe to CIS, only two peaks were found with a consistent relation. The first one was attributed to amide III of protein at 1235–1243 cm^{-1} in which the intensity decreased with increased grade of dysplasia. This suggests lower protein content in relation to the total Raman active components, which disagrees with knowledge of the disease process that should reflect the progression from mild through moderate to severe and CIS. Also, this was unable to support the previous studies which indicated progressive increases of proteins with transformation from normal to dysplasia and malignancy due to more surface proteins, signalling, antibodies, antigens, and enzymes from the increased number of proliferating cells (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006).

The second consistent band corresponding to C=C olefinic stretching of lipids at 1589–1591cm^{-1} where the relative intensity decreased with increased dysplasia severity. This is strongly supported by the concept of protein-dominated spectra in malignant tissue (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006). Since the number of proliferating cells increases progressively with higher grades of dysplasia and with more

energy consumption for their biological transformation process, this may results in low lipid content and be associated with more protein formation as the disease progresses.

In this study, the potential diagnostic ability of a Raman peak(s) to differentiate between different grades of dysplasia was not investigated. Since the identification of a significant potential diagnostic peak (marker) that can differentiate between different grades of dysplasia needs larger number of samples to overcome the large inter-sample variations of the individual peak intensities between different grades, which may mask the group differences. Thus, further work using the identified peaks with significant relation to try to find diagnostic markers among groups of dysplasia is required.

6.8.4. Spectral Features of Dysplastic Tissue Groups

The bands at 829–838 cm^{-1} assigned to tyrosine, O-P-O nucleic acids and the shoulder at 1621 cm^{-1} attributed to tryptophan, phenylalanine and tyrosine amino acids are broad and weak in the spectrum of mild dysplasia, but became more prominent with progression of dysplasia from mild through moderate and severe dysplasia to CIS. This suggests higher percentages of nucleic acids and proteins relative to total Raman active components and supported by the histopathological criteria used for biopsy diagnosis, such as nuclear cytoplasmic ratio (Huang et al., 2003b) and also formation of new proteins from the proliferating cells and other transformation process. Similarly, for the phenylalanine proteins at 1007–1009 cm^{-1} which appears to be more pronounced with higher grades of dysplasia.

The wavenumbers 1315–1322 cm^{-1} and 1375–1377 cm^{-1} corresponding to nucleic acids and amino acids, became broad, less prominent and weak with progression of dysplasia from mild to CIS. A possible explanation is more degradation or disturbing of tissue proteins accompanying the disease progression which was no longer detected because of low concentrations (Kendall et al., 2011). This is confirmed by lower intensity, weak or absent in high grade dysplasia compared to highly ordered proteins contents in normal and low grade dysplasia which confirmed by a prominent peak. This is supported by a study conducted by Donfack *et al.* (2010) who reported disordered and degraded proteins structures in neoplastic skin cells compared to normal cells.

The band of C=C olefinic stretching of lipids at (1589–1591) cm^{-1} became broad and weak with increased severity of dysplasia, suggesting lower concentration of lipids with higher grades dysplasia which is in line of lipid-dominated spectra in normal tissue (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006).

A small peak at 1458 cm^{-1} which corresponds to CH_2 deformation lipids was observed only in severe dysplasia, which is more likely due to the presence of normal constituents within dysplastic tissue reported by (Krishna et al., 2004).

Considering the spectral features of high and low grade dysplasia, in HGD the bands at 1166 cm^{-1} (C-H in plan tyrosine modes of amino acids) and 1623 cm^{-1} (tryptophan, phenylalanine proteins) appeared very weak or absent in LGD. This suggests a progressively larger contribution of amino acids and proteins from the proliferating cells in HGD, compared to LGD and is supported by knowledge of the histopathology of disease progression and has been previously reported (Venkatakrishna et al., 2001; Krishna et al., 2004; Malini et al., 2006).

The band at 1317 cm^{-1} (guanine ring breathing mode of nucleic acids bases) was sharp in LGD, but became broad in HGD. Similarly, the small peak at 1407 cm^{-1} (a symmetric stretching carboxylate, IgG) was more prominent in LGD, but became broader in HGD. This may suggest the synthesis of more unordered proteins in HGD compared to LGD with disease progression, which can be identified at different peak positions and consequently overlapped to provide a broad peak compared to LGD.

Corroborating the basic concept of vibration spectroscopy; vibration of bonds in biological Raman spectrum is very complex with different environment and energy (Smith and Dent, 2005). Proteins structure found with different types of bonds in molecules which may give different peak positions overlapped to appear as a broad peak.

6.8.5. Spectral Features of Morphologically Normal Tissue Groups

In the current study, the histologically normal tissues from the laser resection margins were used as control group. This is more likely to control the influence of individual variations on spectral data analysis such as patients' demography and risk factors. This is consistent with pervious studies conducted by Malini *et al.* (2006) who used the uninvolved areas from the same subjects as a control and Li *et al.* (2010) who used the sample of normal oral mucosa from the surgical margin of patients with oral cancer.

Interestingly, phenylalanine proteins peak at 1007–1009 cm^{-1} appear in all groups of morphologically normal tissue groups, became more prominent with increased grades of dysplasia. This suggests that normal tissue from mild dysplasia as a first step in progression process may be considered as a real normal tissue with no spectroscopic changes, with the histologically normal tissue from moderate, severe dysplasia and CIS start to change biochemically by the effect of field change cancerization (Slaughter et al., 1953). This is supported by the findings of a small study conducted by Keller *et al.* (2008) who compared the spectra from true normal cervical tissue taken from healthy subjects to the spectra of histologically normal from cervical dysplasia specimens. They reported that histologically both tissues were similar, but the differences between them can be identified spectroscopically, reflecting the biochemical tissue changes at early stage and even before the appearance of dysplasia clinically. Similarly, the shoulder at 1619–1623 cm^{-1} (tryptophan, phenylalanine proteins) was very weak or absent in morphologically normal from mild dysplasia, becoming prominent in morphologically normal from moderate, severe dysplasia and CIS. It is expected that as a tissue starts to become more active, with more biochemical changes, more protein from cellular proliferation is seen in dysplasia. These changes seem to affect on the surrounding normal tissue evidencing spectroscopically, but not histopathologically based on field change cancerization (Keller et al., 2008). Also, the morphologically normal from mild and moderate dysplasia exhibited a more prominent peak at 1022 cm^{-1} (glycogen), which was absent in both morphologically normal from severe dysplasia and CIS. This further support the field change concept on the surrounding normal tissue and suggesting less glycogen content in morphologically normal from severe dysplasia and CIS similar to that in dysplastic tissue which reflect the need for more energy consumption by the proliferating cells.

The peak at 1347 cm^{-1} (CH_2 protein, nucleic acids) was seen in all morphologically normal from all grades of dysplasia. It became more prominent with increased dysplasia severity reflecting the progressive increase in proteins and nucleic acid contents in these tissues of the dysplastic tissue specimens.

Considering the binary grading system, LGN exhibited a more prominent peak at 1048 cm^{-1} (glycogen) which was very weak or absent in HGN. This further supports the field change concept on the surrounding normal tissue with less glycogen content in high grade dysplasia suggesting more energy consumption by the proliferating cells in this tissue.

LGN showed a small peak at 1235–1251 cm^{-1} (amide III of proteins), which was absent in HGN suggesting unordered or degradation of proteins in HGN simulating HGD. Similarly, the peak at 1322 cm^{-1} (guanine of nucleic acids) appeared more prominent in LGN, but became weaker and broader in HGN, suggesting lower nucleic acids in HGN compared to LGN, but for no clear reason.

In HGN, the peaks at 1423 cm^{-1} (nucleic acids), and 1620 cm^{-1} (tryptophan, proteins) demonstrated the contribution of nucleic acids and proteins; however, these peaks became broad and very weak compared to LGN. This suggests higher contributions from these constituents simulating to HGD and reflects the effect of field changes cancerization in morphologically normal adjacent to dysplastic tissue.

In this study, the biochemical changes identified by RS reflecting the ability of Raman system to detect subtle molecular changes in certain tissue constituent at an early stage and even before the morphological/histological changes. This further supports the true normal control of morphologically normal form mild dysplasia or LGD, but not from the morphologically normal from moderate, severe dysplasia and CIS or HGD.

6.8.6. Tissue Classification

Based on the success of previous studies on discriminating morphologically normal from different grades of dysplasia on non-oral tissues, such as cervical (Mahadevan-Jansen et al., 1998; Lyng et al., 2007), oesophagus (Kendall et al., 2003), stomach (Teh et al., 2008), larynx (Stone et al., 2000) or mixed epithelial tissues; larynx, oesophagus, bladder, colon, breast, and prostate (Stone et al., 2002b; Stone et al., 2004) and oral tissue (Malini et al., 2006). In general, the majority of earlier spectroscopic studies on human oral tissues were based on the discrimination between normal and malignant oral tissues (Venkatakrishna et al., 2001; Krishna et al., 2004; Hu et al., 2009). However, one study conducted by Malini *et al.* (2006) extended further and evaluated the efficacy of the technique to discriminate between normal, inflammatory, premalignant, and malignant conditions. They showed that PCA discriminated between normal and all pathological tissue groups, but with poor separation ability among the pathological conditions. While PCA combined with multiparameter limit tests allowing match/mismatch criteria to be applied, all tissue types were shown to be discriminated with sensitivity and specificity of 100% for the diagnosis of malignancy.

Conventional histopathology (consensus diagnosis) was used as standard for testing the ability of RS for tissue classification. PCA fed to PLS-DA was used and was able to emphasis the variation between different groups of dysplasia from their morphologically normal tissue making the analysis more accurate (Wang and Mizaikoff, 2008). PLS-DA specifically looks for common patterns of variations in both histopathological groups and the spectra, identifying the spectral features that can correlate most strongly with group distribution.

In the present study, the Raman system was able to separate dysplastic from morphologically normal tissue with more than 80% specificity and sensitivity rates. This demonstrated the ability of Raman to classify dysplastic tissue from morphologically normal tissue efficiently. Raman prediction was found to be promising with small misclassification and larger percentage of correctly classified pair-wise cases. For example, Raman was able to identify severe dysplasia from the corresponding morphologically normal tissue with 100% sensitivity and specificity. Also, RS assigned mild dysplasia from morphologically normal tissue with

more than 90% specificity and sensitivity. However, a drop in the classification rate of moderate dysplasia from morphologically normal tissue with more than 71% sensitivity and 80% specificity was reported in this study. This suggests more false negative and false positive cases, compared to the ability of Raman in discriminating mild and severe dysplasia from their morphologically normal tissues. Although RS showed a low sensitivity rate of 60% in separation of CIS from CISn suggesting higher rate of false negative, RS was able to discriminate CISn from CIS with 100% specificity indicating the ability of Raman to identify CISn without any false positive.

With respect to the binary grading system, Raman classification of HGD from HGN and LGD from LGN was also promising, with sensitivity of more than 83% and specificity of more than 90%. Raman classification seems to be better in cases of high grade dysplasia (HGD vs. HGN), compared to low grade dysplasia (LGD vs. LGN) by more than 3%. This was supported by the univariate statistical analysis that identified multiple significant peaks between HGD from HGN, compared to only two peaks in case of LGD vs. LGN.

In this study, the misclassification can be explained under the basis of the consensus histopathological diagnosis. If dysplastic tissues were misclassified as morphological normal, a possible explanation might be the presence of normal tissue constituents within the dysplastic area under examination, or spectra were measured from a clear resection margin, not from the dysplastic region (points mispositioning). However, this option was not applicable in this study because of two reasons: firstly, normal and dysplastic regions were selected and marked under pathological guidance and secondly, the spectral measurements were performed within the boundaries of the pathologist selection using mapping technique, not point-by-point measurement. Further, the dysplastic tissue changes undergo gradual biochemical changes from normal to dysplastic and no one can expect to see a line between dysplasia and normal tissue (Stone et al., 2004). While if morphologically normal tissues were spectroscopically misclassified as dysplastic tissues, Raman spectra might be collected from margins which were regarded as histologically normal. However, in this case they were probably not normal and had residual dysplastic components which subsequently lead to the misclassifications. Another possible explanation is that if the resection margins were free from dysplasia (clear-margins), the measurement may be taken from the dysplastic area and not from the morphologically normal tissue; however, this was not applicable in this study

because of the pathological guidance. Furthermore, Raman misclassification of morphologically normal tissue as a dysplastic tissue, may be related to the presence of malignancy associated changes or field changes cancerization phenomenon (Slaughter et al., 1953), in which oral epithelium within the area of field change (unstable oral mucosa) could appear clinically normal, but carry biochemical tissue changes identified spectroscopically (Keller et al., 2008).

6.9. Strength of the Study

- The current study was the first spectroscopic study that used de-paraffinised FFPE oral tissue samples from different grades of dysplasia to validate an objective-automated technique for dysplastic oral tissue diagnosis, toward more valuable *in vivo* Raman probe for clinical application.

- This study is the first to demonstrate the feasibility of using RS to investigate and differentiate different grades of oral epithelial dysplasia from their counterpart morphologically normal tissues, using both WHO and binary grading systems.

- The current study used only laser excision specimens with consensus histopathological diagnosis of two expert oral pathologists, which was at least minimising the histopathological discrepancies.

- Morphologically normal tissue from an unaffected area at the resection margins from the same patient was used as control to minimise the individual source of variation that might relate to patient demography, risk factors, medical history and medications used, which may cause other source of variations not related to the disease itself.

- All samples used in this spectroscopic study were taking from patients affected with PMDs in one oral subsite, the FOM, eliminating the effect of anatomical site variation.

- All patients were selected from a cohort of patients diagnosed, treated and followed up by the same team with a similar management protocol, with known outcomes for further advantage correlated with spectroscopic data.

6.10. Limitation of the Study

The need for specific types of substrates for tissue mounting was one of the main limitations in this study, which are usually small in size, expensive and brittle requiring careful handing. However, the poor quality spectra obtained when normal glass slides were used, suggests that either these BaF_2 slides must be used or further analysis of a range of potential substrates is needed.

Although the number of the collected spectra (1045) was sufficient for data analysis, Raman tissue spectra contaminated with detector saturation, noise and high fluorescent background reduced the number of spectra by excluding these contaminated spectra (267). Subsequently, this limits the employment of a test subset prior the application of multivariate data analysis. Therefore, the measurement of more points per sample area are required to facilitate both data testing and also to decrease the sampling errors related to spectral variations not related to the disease state, which may affect the group separation. Thus, increased the number of sample will enhance the information upon which the classification models can be built and should provide significantly more robust performance.

6.11. Conclusion

2. In this study, the consensus histopathological diagnosis of two grading systems were used to group the tissue specimens; the WHO classification system of mild, moderate, severe dysplasia and CIS, and the binary system of high/low grade dysplasia.
3. All of the 32 samples used in this study were from the most common affected oral subsite (FOM).
4. Out of 1045 collected spectra, a total of 778 spectra (386 dysplastic, 392 morphologically normal) were used for spectroscopic data analysis.
5. Practical consideration revealed the unsuitability of glass slides and recommended the used of BaF_2 substrate for tissue mounting.
6. This study recommended the use of a standard protocol for photo-bleaching of 120 seconds to reduce the strong fluorescence background signals contamination to improve Raman spectral quality.
7. Spectroscopic tissue analysis confirmed the ability Raman system to identify biochemical tissue changes associated with tissue progression from normal to dysplastic.
8. The mean spectrum of Md significantly exhibited a higher phenylalanine protein concentration reflected by a higher intensity peak at 1009 cm^{-1} indicating a major contribution of proteins in Md compared to Mn.
9. The mean spectrum of Modd exhibited mainly a higher intensity of proteins and nucleic acids, although the difference was not significant.
10. The Modn showed a significantly higher intensity level of lipid at 1087 cm^{-1} compared to Modd spectrum.
11. The Sd spectrum showed a significantly higher concentration of proteins and nucleic acids reflected by higher relative intensity at 1344 cm^{-1}, compared to Sn.
12. The Sn exhibited a significantly higher relative intensity of amide III of protein at 1243 cm^{-1}.
13. The mean spectrum of CIS which was similar to Md, showed a significantly higher concentration of phenylalanine protein at 1007 cm^{-1}, in addition to a significantly higher relative intensity of 1091 cm^{-1} (proteins and nucleic acids), compared to CISn.

14. The mean spectrum of HGD compared to HGN showed a number of significant peaks corresponding to amino acids and protein back bone at 934 cm^{-1}, C-N proteins at 1137 cm^{-1}, nucleic acids at 1322 $cm^{-1}$and protein with nucleic acids at 1345 cm^{-1}. Based on our understanding of the molecular origin of the Raman bands, the study findings suggested that the relative intensity of these Raman bands increased with progression of dysplasia, indicating increased cellular proteins and nuclear content in dysplastic compared to normal.
15. The HGN exhibited a significantly higher concentration of lipids reflected by higher relative peak intensity at 1081 cm^{-1}, phenylalanine protein at 1037 cm^{-1} and 1104 cm^{-1}, amide III of protein at 1243 cm^{-1}, amino acids at 1176 cm^{-1} and 1213 cm^{-1}.
16. The mean spectrum of HGD exhibited a significantly higher concentration of phenylalanine protein and amide I of protein demonstrated by higher relative intensities at 1009 cm^{-1} and 1661 cm^{-1}, compared to HGN.
17. The current study supported the ability of RS to provide a single peak for diagnostic purposes; however, further studies with larger sample size to test the validity of the identified significant peaks for diagnostic purposes are required.
18. The relative intensity of olefinic stretch of lipids at 1589-1591 cm^{-1} was consistently decreased with increased dysplasia severity supporting the lower lipids content in dysplastic tissues (disease progressing). This peak may be used to differentiate between different grades of dysplasia; however, further studies are required to confirm and validate this finding.
19. The higher concentration of nucleic acids and proteins relative to the total Raman active components and formation of new proteins from the proliferating cells were supported by the histopathological criteria used for biopsy diagnosis. Whilst degradation and/or disturbing of tissue proteins accompany the dysplastic tissue progression with lower concentrations, may be no longer detected spectroscopically.
20. This study supported that Mn is a true normal tissue, but higher grades dysplasia (Modd, Sd and CIS) that associated with more biochemical changes affected on the surrounding normal tissue by the field change, evidencing spectroscopically but not histologically, Modn, Sn and CISn were no longer considered as normal.

21. The study supported less glycogen content in morphologically normal tissue associated with increased grades of dysplasia, apart from morphologically normal from mild dysplasia, reflecting the need for more energy consumption by the proliferating cells and simulating the dysplastic tissues changes.
22. The absence of shoulder corresponding to proteins in Mn, which was prominent in Modn, Sn and CISn. Similarly, the peaks corresponding to nucleic acids and proteins in HGN were broad and very weak compared to LGN, all these suggesting higher contributions from these constituents simulating to dysplasia and reflecting the effect of field change in morphologically normal tissue adjacent to dysplastic tissue. These findings showed that RS has the ability to identify neoplastic change in these tissues before their histological appearance. The clinical implementation of this finding is the use of Raman system to assess biopsy targeting during surveillance and to assess resection margins in the operating theatre. In many cases early cellular changes are invisible and the detection will rely upon a random biopsy procedure, with a chance of missing abnormal tissue with large numbers of unnecessary biopsy samples.
23. This study demonstrated the potential ability of RS, combined with multivariate statistical techniques, as an objective tissue classifier to successfully distinguish between morphologically normal and dysplastic tissue samples.
24. An excellent separation between severe dysplasia and their morphologically normal tissue, with 100% sensitivity and specificity was achieved.
25. The Md was differentiated from normal tissue with more than 90% sensitivity and specificity, which further supporting the ability of Raman to detect early dysplastic changes at a cellular level.
26. The Modd was differentiated from normal tissue with more than 71% specificity and 80% specificity.
27. The CIS was differentiated from morphologically normal tissue with 60% sensitivity and 100% specificity.
28. Raman classification was promising for separating high and low grade dysplasia from their morphologically normal tissue achieving a sensitivity of more than 83% and specificity of more than 91%.

6.12. Recommendations for Future Raman Work

It is necessary to identify and control as many sources of irrelevant variation as possible in order to inherently maximise the desired variation by carrying out a controlled experiments including specific form of variation to detect the subtle biochemical tissue changes. In the biomedical application and according the wide physiological ranges, cell characteristics may vary markedly, so larger numbers of samples are required to overcome these variations for a robust study.

To assess the desired variations and to improve the groups' discrimination, more measurement points per sample area are recommended to cover the whole area under investigation; otherwise spectral variations not related to the disease state may contribute to, and affect tissue classification.

It may be desirable to select representative samples from a spectroscopic data set as a completely independent test subset. This technique was previously used by using approximately one-third of the total data set (Hu et al., 2009) and found to be useful for the validation of the model (Shaw et al., 2000).

In this study, regarding the noise contamination, three datasets of spectral intensity were identified, a signal accumulated to 750 counts across the CH_2 scissoring mode gave the best results by showing an adequate signal to noise ratio. Thus, it is highly recommended to use such intensity threshold for similar type of tissue analysis for high spectral quality and subsequent efficient data analysis.

Further studies are required to confirm these preliminary results and also a multi-centric research to validate these findings. The work in this area may address experimental issues, such as exploring the use of red lasers to minimise fluorescence background for better spectral quality.

Finally, it is recommended to develop standard protocols for tissue sampling, spectral collection and data analysis for diagnostic spectral purposes to achieve a standard set of spectra transferable between the research groups and subsequently from clinic to clinic for great patient benefit.

Chapter Seven: Conclusions and Suggestions for Future Studies

7.1. Introduction

The present study was designed to identify and follow a cohort of patients affected with new, single PMDs in the North-East of England from initial presentation, through laser interventional treatment to long-term follow-up and confirmed clinical outcome. Profiling of associated risk factors and the factors affecting clinical outcome following laser treatment were also investigated. In addition, the efficacy of Raman spectroscopy as an effective and objective tool for discriminating morphologically normal from dysplastic tissue, the early detection of dysplastic tissue changes and tissue classification were also studied.

The findings of the current study emphasise the importance of both clinical and pathological characteristics of dysplastic PMDs, in addition to patients' lifestyle risk factors, in identifying high risk patients, predicting treatment outcome and directing clinical management protocols.

The study has shown that Raman spectroscopy is able to identify biochemical tissue changes associated with early dysplastic changes seen in oral PMDs and can detect field change before morphological (histopathological) appearance. Also, the Raman system is able to differentiate between dysplastic and morphologically normal tissue using de-waxed formalin fixed paraffin embedded oral tissue samples.

7.1.1. Study One:

Potentially Malignant Disorders: Demographic, clinical and histopathological features of a cohort of 100 patients, who were diagnosed, treated and reviewed at Newcastle Upon Tyne Hospitals.

100 patients with single dysplastic PMD (66 males, 34 females) with a mean age of 57.72 years (30-94 years) participated in this study. The FOM, followed by lateral and ventral surfaces of the tongue, were the most commonly affected anatomical sites. The majority of PMDs presented as leukoplakia (92%), with only 8% erythroplakia; erythroplakia was mainly seen in patients over 40 years old, with 88% (7/8) identified in males. Mild dysplasia (43%) was the main histopathological diagnosis of laser excision specimens, followed by moderate (24%) severe dysplasia (23%) and CIS 10%. The majority of homogenous leukoplakias

(58%) were diagnosed as low grade dysplasia, whilst most non-homogenous lesions (71%) were high grade; erythroplakia often showed severe dysplasia 37% (3/8). Clear surgical margins were primarily seen in mild dysplasia cases (60%), whilst dysplastic margins were more frequently associated with higher degrees of dysplasia. Higher dysplastic features were associated with larger sized PMDs which were both also associated with advancing age. Male patients exhibited greater excision margin involvement with higher grades of dysplasia in the affected margins compared to females' resection margins.

7.1.2. Study Two:

Risk factors of patients with PMDs in the North-East of England

At initial presentation, the majority of PMD patients were current smokers 63% (46 males and 17 females); with 22% (15 males and 7 females) ex-smokers and 15% (5 males and 10 females) non-smokers. Females were primarily non-smokers (67%), whilst males were mainly smokers (73%) who also started smoking earlier than females, and had significantly longer smoking histories (37.11 vs. 28.13 years). The FOM was the main affected site in smokers (60%), whilst lateral and ventral tongue surfaces were the principal sites in non-smokers (93%). All patients ≤ 40 years were tobacco users mainly presenting as FOM PMDs, whereas patients older than 41 years were a mixture of tobacco users and non-users, with both FOM and tongue affected. The tongue was the only affected site in female non-smokers and in 80% of male non-smokers. Erythroplakias were only seen in heavy smokers. Severe dysplastic features were associated with both heavy smoking and a longer smoking history.

Patients in this study were mainly current drinkers (83%), followed by non-drinkers (14%) and ex-drinkers (3%). All patients ≤ 40 years were regular drinkers. The majority of males were regular drinkers (71%), whilst the majority of females were non-drinkers (71%). Fifty-eight percent of males and 9% of females exceeded maximum recommended consumption levels (21 units/week for male, 14 units/week for female). The FOM was the main affected site in heavy drinkers. Fifty-two percent of homogenous leukoplakias affected light drinkers, 50% of non-homogenous leukoplakias were observed in heavy drinkers, whilst erythroplakias presented equally in heavy and light drinkers. Mild dysplasia was frequently diagnosed in

light drinkers, whilst CIS was usually seen in heavy drinkers. Ninety-two percent of current drinkers were also current smokers, whilst 50% of non-drinkers were also reported as ex-smokers. Eighty-eight percent of our study cohort reported a history of a systemic disorder, with hypertension the most common followed by diabetes mellitus, which was mainly seen in females. Hypertensive patients mainly presented with severe dysplasia-CIS, which may have a link with anti-hypertensive medication-induced lichenoid reactions with subsequent dysplastic change. Large sized dysplastic PMDs were frequently associated with denture wearers in FOM cases and with crowns and bridges in tongue lesions suggesting repeated mechanical irritation from intraoral appliances may play a role on pathogenesis.

7.1.3. Study Three:
Clinical Outcome

This study showed that 62% of patients were DF (39 males and 23 females), 17% had recurrent disease (13 males, 4 females), 14% new site dysplasia (10 males, 4 females), 5% underwent MT (3 males, 2 females), and 2% developed oral cancer at a site distant from the primary dysplasia (1 male, 1 female). Univariate logistic regression analysis revealed that high grade dysplasia (severe dysplasia-CIS), the ventro-lateral tongue, non-homogenous leukoplakia, the presence of dysplasia in resection margins, dysplasia size exceeding 600 mm^2 and an active systemic medical condition were all significant predictors of a disease active state. However, prediction of an individual patient's clinical course remains difficult and all patients with dysplastic PMDs should be kept under long-term surveillance regardless of clinicopathological features and method of treatment.

Recurrent (same site) Disease

Recurrent disease usually presents within the first two years following laser treatment. Regression analysis showed that the presence of severe dysplasia-CIS in surgical margins increased the risk of recurrence by 4 times. Although females exhibited a shorter mean time to recurrence, males were at increased risk. The FOM and tongue were the main anatomical

sites affected. Non-homogenous leukoplakia exhibited both increased risk and a shorter mean time to development. Lesion size exceeding 600 mm^2 increased recurrence risk by 2 times. Recurrence risk was increased in higher grades of dysplasia, with CIS 12 times, severe dysplasia 6 times and moderate dysplasia 5 times higher.

Both heavy smokers and drinkers were at increased risk of recurrent disease by 2 and 3 times, respectively; more than half of the patients continued to smoke and 98% continued to drink following laser treatment. The majority of patients who underwent recurrent disease (94%) had an active systemic medical disorder, which was found to increase the risk of recurrence by 2 times. Intraoral dental prosthesis wear was also found to increase the risk of recurrence by 2.5 times.

Further Disease (new site PMDs)

Patients who developed new site dysplasia exhibited a complex clinical course characterised by multiple laser interventions and follow-up biopsies, with most disease episodes reported by 3 years post-treatment. Although males exhibited a higher rate for new-site dysplasia, females showed a shorter mean time for this to develop. The FOM and ventral tongue were the main anatomical sites for new-site dysplasia to develop, and non-homogenous leukoplakias showed a 3 times increased risk. Lesions sized between 200-600 mm^2 showed the highest rate of new site disease (64%).

Patients with high grade dysplasia exhibited a higher risk of new-site disease, with heavy smokers and drinkers at a 3 times increased risk.
Whilst males were more likely to develop recurrent disease, females were affected equally by recurrent and new-site dysplasia. Patients with severe dysplasia exhibited an equal rate of recurrent and further disease, whereas those with mild dysplasia primarily showed new-site dysplasia.

Malignant Transformation and OSCC Development

No MT was reported in patients younger than 40 years. Males with MT were younger than females. The FOM and lateral tongue were affected equally by MT, whilst new site OSCC only affected the tongue. Non-homogenous leukoplakia showed a 3 times increased risk of oral cancer, whilst erythroplakia was found to increase the risk of oral cancer by 19 times. Lesion size exceeding 425 mm^2 increased the risk of MT by 2 times. Severe dysplasia-CIS was found to have a greater tendency for MT, whilst no MT was seen in moderate dysplasia cases. Non-users of tobacco and alcohol had higher rates of MT and patients with new site OSCC were non-smokers and light drinkers.

Resection Margin Status

Disease-free patients were significantly more likely to have had clear surgical margins, whilst the majority of disease active cases showed dysplasia in excision margins. Severe dysplasia-CIS and moderate dysplasia were found to increase the risk of dysplastic margins by 2 and 1.5 times. Lesion size exceeding 600 mm^2 significantly increased the risk for dysplastic margins by 12 times and those between 200-600 mm^2 6 times. Moderate-severe dysplasia in the margins was associated with a higher recurrence rate, whilst recurrence-free patients usually had clear margins.

7.1.4. Study Four:

Raman Spectroscopy

The Raman system, combined with confocal micro-spectroscopy, is a promising tool for oral tissue diagnosis and has the ability to identify biochemical tissue changes associated with progression from normal to dysplastic tissue.
The study showed a higher relative intensity of proteins and nucleic acids in dysplastic tissue indicating increased protein and nuclear content in proliferating cells.

Raman, combined with multivariate statistical techniques, has the ability to objectively distinguish between morphologically normal and dysplastic tissue using de-waxed FFPE

samples. These particular findings are encouraging since these tissue specimens are easily available and carry enough discriminant information to be used in optical oral pathology techniques.

This study adds to the growing body of evidence that Raman spectroscopy can detect subtle or undetectable histopathological changes in tissue and may make a significant contribution to our understanding of the mechanisms of neoplastic transformation. This study demonstrated that morphologically normal tissue from mild dysplasia excision margins is true normal tissue, but in higher grades of dysplasia, biochemical changes affect surrounding tissue with the result that morphologically normal tissue taken from moderate, severe dysplasia and CIS samples could no longer be considered normal. These spatial effects have an impact on choosing the type of tissue to use as a normal reference in classification studies, and may thus have an impact on the development of screening techniques to benefit patients in clinical practice.

7.2. Suggestions for Future Studies

This project investigated in depth the clinical and pathological presentations of dysplastic PMDs; however, further research might explore the site preference for specific clinical types in particular non-homogenous subtypes, providing more information on their biological behaviours which will help in treatment protocols.

More information on the relation between lesion size and anatomical site of PMD, in particular those affecting the tongue and the FOM, would help us to establish a greater degree of understanding of their biological behaviours.

An important finding in our study is that the lateral tongue was identified as a non-smoking-related site, whilst the soft palate was a specific smoking-related site; further investigations should concentrate on risk assessment in relation to specific anatomical sites.

To avoid the substantial underestimation of risk in alcohol and tobacco users, further work needs to be done using more accurate and objective methods of assessment in addition to self-reporting.

The presence of systemic disease is one of the important predictors for high grade dysplasia to develop; future research should be undertaken to understand potential inter relationships in these patients.

Investigating potential interactions between systemic medications and the behaviour of PMDs in patients with chronic medical conditions may be a useful future study.

A further study is required to establish whether intra-oral dental prostheses wear may influence the characterisation of dysplastic PMDs in relation to particular anatomical sites and their impact on subsequent clinical outcome.

Large size dysplastic PMDs as significant predictors of high risk disease should be further investigated with more focus on the biomolecular basis.

Non-homogenous leukoplakias are often associated with large size, high grade dysplasias and a higher tendency for recurrence after removal; further investigations are required to support these findings for the benefit of patients' classification and treatment planning for this high risk group.

The presence of residual dysplasia in an excision margin, and the extent and grade of dysplasia are important parameters for patient prognosis, associated with both recurrence and MT; therefore, a further detailed study with a larger number of cases is required.

Excised FOM lesions displayed higher rates of residual dysplasia in resection margin, contrary to the tongue for example and further investigations are needed to explore the reasons behind this and to identify associated factors.

The susceptibility of oral mucosa to develop further disease and new site oral cancer development, especially in the absence of known carcinogens, require further studies using molecular and biochemical approaches to elucidate biomarker(s) to aid in early detection of field change.

Further Raman studies using the same experimental set up with more sample numbers are required to confirm these study findings for clinical use in the future. Also, a group of patients who developed oral cancer may be involved to provide a complete spectroscopic data set for oral optical tissue diagnosis.

From a practical point of view and for future studies, it is recommended to use the signal accumulated to 750 counts across the CH_2 scissoring mode for oral tissue analysis. Such intensity gave the best results by showing an adequate signal to noise ratio for high spectral quality and subsequent efficient data analysis.

Also, to improve tissue classification and group discrimination, more measurement points per sample area are recommended to cover the whole area under investigation and to reduce spectral variations not related to the disease state.

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Appendices:

1. Clinical Studies Appendices

1.1. Appendix (1-A): Ethical Approval

County Durham & Tees Valley 1 Research Ethics Committee
The Tatchell Centre
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Telephone: 01642 624279
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09 July 2009

Professor Peter Thomson
Professor of Oral & Maxillofacial Surgery
Newcastle University
School of Dental Sciences
Framlington Place
Newcastle Upon Tyne
NE2 4BW

Dear Professor Thomson

Study Title:	**Studies in Diagnosis and Management of Unstable Mouth Lining**
REC reference number:	**09/H0905/31**
Protocol number:	**V:1**

Thank you for your letter of 07 May 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the alternate Vice-Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research

governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
CV Student		10 March 2009
CV - 2nd Supervisor		10 March 2009
CV - Educational Supervisor		01 March 2009
Protocol	V:1	02 March 2009
Investigator CV		01 October 2008
Application	V:5.6	10 March 2009
Response to Request for Further Information		
Participant Consent Form	V:2	07 May 2009
Participant Information Sheet	V:2	07 May 2009
GP/Consultant Information Sheets	V:2	07 May 2009
Covering Letter		07 May 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H0905/31	Please quote this number on all correspondence

Yours sincerely

Carol

Dr Richard Bellamy
Alternate Vice-Chair

Email: carol.cheesebrough@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: Amanda Tortice, R&D Dept.,
Newcastle Upon Tyne Hospitals NHS Foundation Trust

Dr Ameena Diajill – Researcher
c/o Professor P.J. Thomson's Secretary
Email: ameena.diajil1@newcastle.ac.uk

1.2. Appendix (1-B): Patient Information Sheet

Patient Information sheet

Studies in the diagnosis and management of unstable mouth lining

You are being invited to take part in a research study. This information sheet explains why the research study is being undertaken and what it will involve. Please read it carefully and ask questions about anything you do not understand. ***This form is for you to keep and refer to as required.***

Professor Peter Thomson is the head of the team in this study.

Why I have been chosen?

In the recent past you were diagnosed as suffering from a patch of "unstable" mouth lining. These patches contain "unstable" cells which if left untreated or unmonitored can change to precancer or cancer cells. We are conducting a study to try to improve our understanding of the behaviour of these cells and are inviting you to take part and help us with the study. This investigation will not require you to undergo any additional treatment and will not influence your current care. At all times you will remain under the care of Professor P.J.Thomson (Consultant in Oral Maxillofacial Surgery).
In this study we wish to find new and better ways to predict outcomes for patients with unstable patches in their mouths. In order to do this we plan to review the progress of patients we have previously treated to try to identify the reasons why some patients do well and others have more problems with their mouth lining.

Study details

In this study we will review the details about your mouth lining already recorded in your medical records to confidentially build a database of unstable mouth lining conditions affecting people in the North East of England. We will also re-examine your biopsy tissue specimens, previously taken for diagnosis and routinely stored in the pathology department at the RVI, using new and additional laboratory techniques to try to learn more about your condition.

This re-examination will not affect your treatment and normal planned clinic follow-up in any way, but will hopefully provide new information and better understanding of the nature of unstable mouth lining to help future patients.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen if I take part in this study?

If you agree to be in this study, you will be asked to sign a consent form to allow us to use medical information held in your normal medical records along with a re-examination of tissue biopsy specimens already held in the pathology department following your treatment.

Will my taking part in this study be kept confidential?

Information collected during the research will be kept strictly confidential, and no one outside the research team will have access to your information. Any information about your name, date of birth and address will be removed so that you cannot be recognized. With your permission, we will inform your GP that you are taking part in this study.

Who is organising and funding the research?

This study is being organised by the School of Dental Sciences /Newcastle University.

Who has reviewed the study?

This study has been reviewed by NHS Research Ethics Committee. The Ethics Committee is made up of doctors, nurses, scientists, non-scientists and people from the community.

What if there is a problem?

If you have any concerns about any aspect of this study, you should speak to the researcher who will do her best to answer your questions.

Contact for further information

If you have questions about this study, ***please contact Professor Peter Thomson,*** or the study doctor (researcher) Dr. Ameena Diajil.

Professor Peter James Thomson
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Email address: ameena.diajil1@ncl.ac.uk

1.3. Appendix (1-C): Patient Informed Consent

Studies in Diagnosis and Management of Unstable Mouth Lining

Patient Consent Form

1. I confirm that I have read and understood the information sheet for the above study and have had the opportunity to ask questions and have had these answered satisfactorily. I have been given a copy of the patient's information sheet to keep.

2. I understand that my participation is voluntary and that I am free to withdraw at any time.

3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals and I give permission for these individuals to have access to my records.

4. I understand that my personal data will be processed and stored securely in agreement with the 1998 Data Protection Act.

5. I give permission for the researchers to inform my GP of my taking part in this study.

6. I freely agree to take part in the above study.

Patient's name.....

Patient's signature.....

Date.....

Members of the research team:
Professor PJ Thomson
Dr Max Robinson
Dr Matthew German

Researcher Dr Ameena Diajil	Professor Peter Thomson
Signature.....	Signature.....
Date.....	Date.....

1.4. Appendix (1-D): General Practice Information Letter

GP Information Letter

Dear Dr

Research Project

Studies in Diagnosis and Management of Unstable Mouth Lining

I am writing to inform you that your patient …… d.o.b ……..

of …….. ……...

has kindly agreed to take part in the above study, which is designed to build up a data base for potentially malignant disorders of oral mucosal tissue.

Your patient is currently under the care of Professor PJ Thomson (Professor of Oral & Maxillofacial Surgery) at Newcastle General Hospital.

As part of this study your patients have agreed that their medical records can be reviewed to build up a database and to find methods to help predict lesion behaviour and patient outcomes in the future. We will also review biopsy material (archived in the Department of Cellular Pathology, RVI) to try to find biological markers in oral epithelial tissue which may predict clinical outcome**.**

I hope this information is helpful.

Please do not hesitate to contact me if you require any further information.

Kind regards,

Yours sincerely

Professor PJ Thomson

Professor of Oral & Maxillofacial Surgery

1.5. Appendix (1-E): Standardized Case Sheet

Data Collection Sheet	
Patient hospital NO.	
Patient study NO.	
First presentation time	/ /
Sex Female Male	
Date of Birth	
Occupation	
Civil status Married Single Divorced Widowed	
Medical history	
1) Immunodeficiency 2) Diabetes 3) Hypertension 4) Anaemia 5) Candidal infection	
Other medical conditions	
Risk factors	
Tobacco smoking 1) Current smoking 2) Ex-smoking 3) Non-smoker History of smoking (years) Cigarettes per day	
Alcohol Drinking 1) Current drinker 2) Ex-drinker 3) Non-drinker History of drinking Units per week	
Diet 1) Prepared food 2) Fresh food/vegetables	

Familial cancer history
Father ☐
Mother ☐
1st relative ☐
2nd relative ☐

Oral hygiene
Good ☐
Bad ☐

Mouth wash use
User ☐
Non-user ☐

Oral prosthesis
None ☐
Full denture ☐
Crown and bridge ☐
Upper or lower denture ☐

PMD clinical appearance

- Leukoplakia ☐

Homogenous leukoplakia ☐
Non-Homogenous leukoplakia ☐
- Speckled ☐
- Nodular ☐
- Exophytic ☐
- Ulcer ☐

- Erythroplakia ☐

PMD anatomical site

1) Floor of the mouth ☐
2) Ventral tongue ☐
3) Lateral tongue ☐
4) Dorsal tongue ☐
5) Buccal mucosa ☐
6) Soft palate ☐
7) Fauces ☐
8) Hard palate ☐
9) Retromolar area ☐
10) Alveolar mucosa ☐
11) Lower lip ☐
12) Upper lip ☐

Histopathology Diagnosis

Incisional biopsy

1) Mild dysplasia ☐
2) Moderate dysplasia ☐
3) Severe dysplasia ☐
4) Carcinoma *in situ* ☐

Date Lab No.

Excision biopsy
1) Mild dysplasia ☐ 2) Moderate dysplasia ☐ 3) Severe dysplasia ☐ 4) Carcinoma *in situ* ☐ Date Lab No.
Size mm^2
Surgical margin status
• Free margins ☐ • Dysplastic margins ☐ o Mild dysplasia ☐ o Moderate dysplasia ☐ o Severe dysplasia ☐ o Carcinoma in situ ☐
Follow-up biopsy
1) Mild dysplasia ☐ 2) Moderate dysplasia ☐ 3) Severe dysplasia ☐ 4) Carcinoma *in situ* ☐ Date Lab No.
Number of observational biopsies ☐ Number of laser interventions ☐ Number of follow-up biopsies ☐ Number of follow-up visits ☐
Treatment
Laser surgery ☐ Observation ☐
Clinical outcome at the most recent clinic follow-up
1. Clinical resolution (Disease-free) ☐
2. Persistent (same site, same disorder) ☐
3. Recurrence after treatment (same site of primary disease) ☐ o Time of recurrence ☐ o Number of recurrences ☐
4. Development of new disease (new site, further disease) ☐ o Time ☐ o New site ☐
5. Malignant transformation (same site OSCC) ☐ o Time ☐
6. OSCC development(new site, new disease) ☐ o Time ☐ o location ☐
7. Malignant events outside the oral cavity ☐ o Time ☐ o location ☐

1.6. Appendix (1-F): International Standard classification of Occupations (ISCO-08) (http://www.ilo.org/public/english/bureau/stat/isco/press1.htm)

1. Manager
2. Professional
3. Technicians and associate professionals
4. Clerical support workers
5. Service and sales worker
6. Skilled agricultural, forestry and fishery workers
7. Craft and related trades workers
8. Plant and machine operators and assemblers
9. Elementary occupations (simple)
10. Armed forces

1.7 Appendix (1-G): PhD Related Poster Presentations

1) Presented at 4th European Conference on Head and Neck Oncology-Athens 4-6th March 2010 Oto-Rhino-Laryngology and Head and Neck; Volume 267.Supp. 1 March 2010

PP054

Risk Factors and Oral Precancer

Development of a High/ Low Risk Profiling System

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Newcastle University

Introduction

Oral squamous cell carcinoma (OSCC) remains a lethal and deforming disease, with significant mortality and a rising incidence in younger and female patients. Recent studies suggest up to 50% of OSCC cases may not have been exposed to the major identifiable carcinogens tobacco and alcohol (1). It is thus imperative to determine the significance of other potential risk factors for oral carcinogenesis.

Aims

- ❑ To identify potential risk factors for oral squamous cell carcinoma and oral precancer
- ❑ To design an accurate data collection tool to try to identify patients at high risk of OSCC development

Methods

Using Pub med, Scopus and Medline, more than 200 papers published between the period of 1982 and late 2009 on risk factors for oral cancer (Figure 1) and precancer (Figure 2), from 35 countries, mainly from USA, UK and other European countries were comprehensively reviewed. The identifiable factors were classified into high and low risk categories.

Figure 1: lateral tongue SCC

Figure 2: lateral tongue leukoplakia

Results & Discussion

14 factors associated with the pathogenesis of OSCC and oral potentially malignant disorders were identified. 8 high risk (table 1) and 6 low risk (table 2) were stratified according to severity of risk, associated carcinogenicity and clinicopathological effects, using evidence obtained from the International Agency for Research on Cancer (IARC) and other epidemiological studies linking the degree of evidence to risk of cancer development.

Table 1: 8 high risk factors

	Reference
1. Tobacco 2. Alcohol	51
3. Betel quid	12
4. Genetic factors / family history/ individual susceptibility	14
5. Immunodeficiency	17
6. Dietary factor	17
7. Old age	11
8. Marijuana	18

Table 2: 6 low risk factors

	Reference
1. Socioeconomic status	12
2. Oral health	9
3. Shammah/Toombak	11
4. Human papillomavirus	11
5. Candida albicans	8
6. Diabetes mellitus	12

Conclusions

- ❑ Understanding the significance of various risk factors in oral carcinogenesis helps stratify patients, especially those with potentially malignant disorders, into high and low risk groups.
- ❑ Early recognition of disease susceptibility helps to direct interventional treatments and greatly improves prognosis of patients.

References

1. Campo-Trapero *et al* (2008) Update on molecular pathology in oral cancer and precancer. *Anticancer Res* 28: 2B

2) Presented at 15th IAOP Biannual Meeting 2010 August 16-20-Seoul, Korea

11

Smoking Behaviour and Oral Potentially Malignant Disorders- Bad News for Non-Smokers?

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Introduction

Potentially malignant disorders (PMDs) are group of disorders with variable malignant transformation rate. Whilst interventional treatment is increasingly advocated to remove discrete PMDs, attention to risk factor behaviour such as tobacco and alcohol use is crucial to comprehensive patient management.

Aims

The aim of this study was to investigate the relevance of smoking behaviour on the presentation of oral potentially malignant disorders (PMDs) in a North East England population.

Methods

100 consecutive patients presenting with single dysplastic PMDs were enrolled in the study. Age, gender, smoking status (non-smoker, current or ex-smoker), number of cigarettes per day, and history of smoking (years) were recorded. All patients underwent interventional laser surgery to excise lesions. PMD size was estimated in mm^2 by multiplying length x width of excised pathological specimens.

Results

63% of patients were current smokers, 22% ex-smokers and 15% non-smokers; **Figure 1**.

PMD size ranged from 21 to 1,800 mm^2 (mean 299.97 mm^2 ± 279.166), classified into 3 categories: minor (< 200 mm^2), intermediate (200-600 mm^2) and major (> 600 mm^2). Although mean PMD size was higher in males (344.50 mm^2) than females (216.15 mm^2) and increased with advancing age, these results were not statistically significant (p=0.089, Mann-Whitney and r=0.151, n=98, p>0.01, Spearman) respectively. A significant difference was found, however, between mean size and smoking status with non-smokers presenting with highest mean PMD size, followed by ex-smokers and then current smokers (p=0.0126, Kruskal-Wallis test); **Figure 2.**

A significant positive correlation was seen between degree of oral epithelial dysplasia and PMD size (r=0.417; n=97; p= 0.001 Spearman), with increasing dysplasia seen in larger sized PMDs; **Figure 3.**

Non-smokers also displayed more severely dysplastic lesions, 43% compared with current smokers (23%) and ex-smokers (18%); **Figure 4.**

Smallest mean PMD size was seen in heavy smokers (>20cig./day), compared with light smokers (<10cig./day), 247.48 mm^2 and 316 mm^2, respectively, although only a weak non-significant correlation was seen between number of cigarettes/day and PMD size (r=- 0.025, n=63, p>0.01 Pearson). Patients with longer smoking histories exhibited smaller PMDs, although this was a non-significant correlation (r= - 0.050; n=98; p>0.01, Spearman).

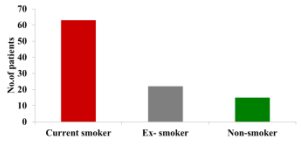


Figure 1: Smoking status of 100 PMD patients

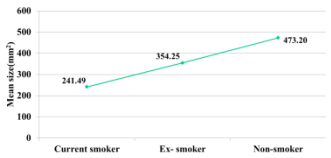


Figure 2: Mean size of PMD according to smoking status

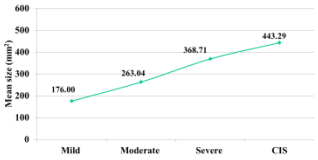


Figure 3: Mean size according to oral epithelial dysplasia

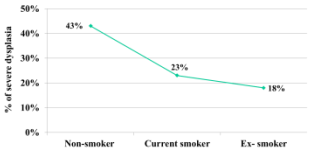
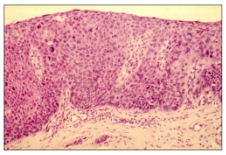


Figure 4: Severe dysplasia according to smoking status



Figures 5 & 6 : Erythroplakia (PMD) Floor of Mouth exhibiting Carcinoma-in-Situ (CIS)

Conclusions

Whilst tobacco smoking remains the principal aetiological agent in oral carcinogenesis, this study reinforces the increased risk faced by non-smokers who develop oral PMDs. Development of dysplasia in the absence of identifiable carcinogens may indicate an inherently unstable oral mucosa at high risk of malignant transformation.

3) Presented at UAE International Dental Conference & Arab Dental exhibition AEEDC Dubai 2011 1st-3rd February 2011

Smoking Behaviour Influences Outcome in Oral Potentially Malignant Disorder Patients

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Introduction

Tobacco use has been identified as the commonest risk factor for oral precancer (Dietrich et al. 2004) in a strong dose- response relationship (Reichart 2001). Smoking tobacco is associated with an increased risk of oral epithelial dysplasia (Morse et al. 2007), an important key stage in oral carcinogenesis, preceding malignant transformation (Morse et al. 2007). It has been found that one-third of oral PMDs progress to cancer (Saran et al. 2008), thus it would be of importance to consider risk factors critically and to identify patients at high risk which is essential for treatment protocol.

Objectives

To investigate the trend of smoking in a cohort of smokers with a single oral potentially malignant disorder (PMD) in the North-east of England during diagnosis, treatment and follow-up.

Methods

63 patients with single dysplastic PMD were enrolled in the study. Affected site, cigarettes smoked per day at initial presentation, at each follow-up point and treatment outcome (disease-free or further disease) were obtained. All patients underwent similar management protocol including risk-factor assessment, initial diagnostic biopsy and laser intervention

Results

Patients were followed up from 3-163 months (mean: 56; SD: 41.437 months). 73% were males and 27% were females. Age range was 30-77 years (mean: 52.27 years, SD: 9.737 years).
Floor of the mouth (FOM) was the main affected site by PMDs (60%), followed by tongue 16% (Lateral 6% & ventral 10%), soft palate 11% and equal percentage of buccal mucosa and fauces (6%); was seen ; Figure 1.
Number of cigarettes per day was divided into 3 groups: < 10 cigarettes /day for light smoking; 2 (3%), 10-20 cigarettes / day for intermediate; 38(60%) and >20 cigarettes/day for heavy smoking; 23(37%); Figure 2.Smoking intensity did not influence the PMDs site distribution, Chi-square test showed no significant association (p= 0.654).
79% of intermediate smokers and 78% of heavy smokers developed PMDs in the floor of the mouth, whilst 21% of intermediate and 22% of heavy smokers exhibited tongue; lesions ; Figure 3.

At 1st presentation, although FOM patients smoked higher average number of cigarettes per day compared with affected tongue smokers (25 vs. 20), they were more liable to reduce their smoking habit during the 1st year follow-up compared to patients with tongue lesions, who found to actually increase their tobacco consuming one year after the laser treatment.
At the most recent follow-up, a significant association was found between PMDs site (FOM and tongue) and clinical outcome as disease– free and further disease (p=0.017, fisher Exact Test), the majority of FOM cases were identified as disease- free (76%), while 52% of the affected tongue patients were diagnosed with further- disease; Figure 4.

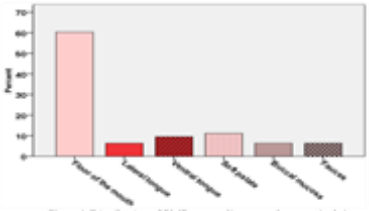
Figure1:Distribution of PMDs according to oral anatomical sites

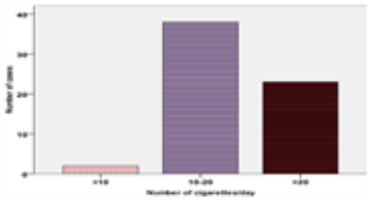
Figure 2:Amount of tobacco smoked cigarettes per day

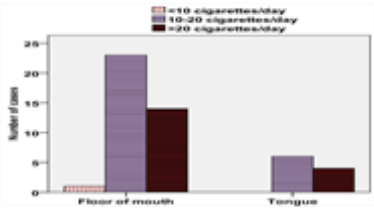

Figure 3: Cigarettes/day smoked by patients with affected FOM and tongue.

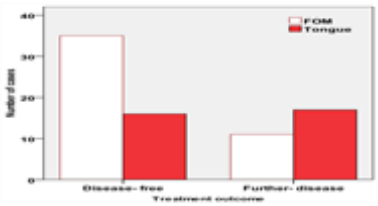

Figure 4:Clinical outcome according to the main oral sites


Conclusions

FOM and tongue patients showed different smoking behaviour. Changes in smoking behaviour appeared to influence the clinical outcomes in PMDs group of patients, accordingly disease-free was mainly seen in FOM patients 76% (35/46), while tongue patients showed mostly further disease 52% (17/33). These observations inform clinical follow up of PMD patients, suggesting that tongue lesion patients are 'high risk' and may benefit from targeted screening.

4) Presented at IADR 89th General Session and Exhibition San Diego-California-USA 16-19th March 2011.

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Smoking Behaviour and Oral Potentially Malignant Disorders

Oral & Maxillofacial Surgery, Newcastle University, United Kingdom
Ameena Diajil and Peter Thomson
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Newcastle University

Introduction

Tobacco use has been identified as the commonest risk factor for oral Precancer (1) in a strong dose-response relationship (2).Smoking tobacco is associated with an increased risk of oral epithelial dysplasia, an important key stage in oral carcinogenesis, preceding malignant transformation (3). It has been found that one-third of oral potentially malignant disorders (PMDs) progress to cancer (4), thus it is important to consider risk factors critically and to identify patients at high risk which is essential for treatment protocol.

Aims

To investigate the relevance of smoking behaviour and histopathological features of PMDs in a North East England population

Methods

100 consecutive patients presenting with single dysplastic PMDs were enrolled in the study. Age, gender, smoking status, number of cigarettes per day, history of smoking (years), pack-year score and histopathological diagnosis were recorded

Results

63% of patients were current smokers; the majority (60%) smoked intermediate amounts of tobacco (10-20 cigarettes /day). PMDs were diagnosed as mild, moderate, severe dysplasia and CIS according to WHO grading system.

No significant relation between age and degree of oral dysplasia was found (p=0.643, Chi-Square test), although most patients were seen in middle aged (41-62 yrs); Figure 1.

Although males consumed larger amounts of tobacco and showed higher mean pack-years (43.23) compared to females (32.18), 29% of severe dysplasia was seen in females compared to 22% in males; Figure 2and Table 1. Smokers with severe dysplasia smoked higher amounts of cigarettes per day (26) compared to those with mild dysplasia (23) however, Kruskal- Wallis test and Spearman correlation was not significant (p=0.183) (r=0.116; n=63; p=0.366), respectively.

Further, a positive, non-significant correlation between pack-years and degree of dysplasia was seen(r=0.140, n=36, p=0.417); the more the severity, the higher pack-year score (mild 39, moderate 42 and severe 48).

Higher dysplastic features were identified in smokers with relatively long smoking history (31-50 years) (21/36) compared to those with lower smoking history (10-30 years) (14/36); table 2.

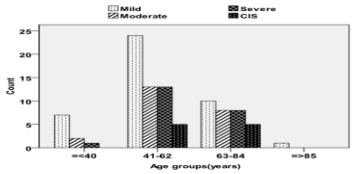

Fig. :1 Degree of oral epithelial dysplasia and age groups

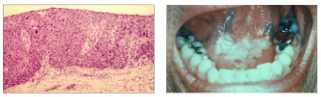

Fig 2: .Histopathological picture of CIS

Fig 3:.Oral leukoplakia

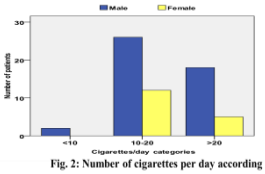

Fig. 2: Number of cigarettes per day according to gender

Table: 1 Degree of oral dysplasia and gender

Gender	Oral epithelial dysplasia(WHO grading system)				Total
	Mild	Moderate	Severe	CIS	
Male	19	12	10	5	46
	41%	26%	22%	11%	100%
Female	8	4	5	0	17
	47%	24%	29%	0%	100%
Total	27	16	15	5	63
	43%	25%	24%	8%	100%

Table : 2 Oral epithelial dysplasia according to the smoking history

Smoking history (years)	Oral epithelial dysplasia (WHO system)				Total
	Mild	Moderate	Severe	CIS	
(10-30)	7	4	3	0	14
	50%	29%	21%	0%	100%
(31-50)	9	6	3	3	21
	43%	29%	14%	14%	100%
>50	0	1	0	0	1
	0%	100%	0%	0%	100%
Total	16	11	6	3	36
	44%	31%	17%	8%	100%

Conclusions

Increasing severity of oral dysplasia seen in heavy smokers, in patients with long smoking history and with higher pack-year scores confirmed the principal aetiological effect of tobacco smoking in oral carcinogenesis. These "high risk" patients are requiring interventional management.

References

Dietrich et al (2004). "Clinical risk factors of oral leukoplakia in a representative sample of the US population." Oral Oncol 40(2): 158-63.
Reichart PA (2001). "Identification of risk groups for oral precancer and cancer and preventive measures." Clin Oral Investig 5(4): 207-13.
Morse et al (2007). "Smoking and drinking in relation to oral cancer and oral epithelial dysplasia." Cancer Causes Control 18(9): 919-29.
Saran et al (2008). "Risk assessment of oral cancer in patients with pre-cancerous states of the oral cavity using micronucleus test and challenge assay." Oral Oncology 44(4): 354-360.

5) Presented at BAHNO Scientific Meeting April 2012 - Joint Scientific Meeting With The British Society For Oral & Maxillofacial Pathology. Royal College of Physicians, London. 26th – 27th April 2012.

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Clinical Outcomes Following Oral Potentially Malignant Disorder Treatment

The Newcastle 100 Patient Study

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Introduction

Potentially malignant disorders (PMD) may present as clinically distinct oral precursor lesions **(Fig. 1)** indicative of an unpredictable and ill-defined risk of squamous cell carcinoma (SCC) development **(Fig. 2)**. There remains controversy and uncertainty over optimal treatment regimes, although most authorities recommend attention to risk factor behaviour and surgical excision of PMDs for definitive diagnosis and management.

Methods

Following ethical approval and informed consent, 100 PMD patients undergoing interventional laser surgery to excise single dysplastic oral lesions **(Figs. 3 & 4)** using a standardised treatment protocol **(1)** were followed for up to 10 years post surgery. Clinical outcome was defined using the following specific criteria: disease free, lesion recurrence (same site), further disease (new site), malignant transformation (same site) or SCC development (new site).

Results

The majority of treated PMDs were leukoplakias arising in the floor of mouth and ventro-lateral tongue **(Tables 1 & 2),** with the majority exhibiting moderate and severe dysplasia or carcinoma-in-situ histologically **(Table 3)**. 62 patients were disease free following laser, 17 developed recurrence and 14 further disease, whilst 5 underwent same site malignant transformation and 2 developed SCC at new and distinct oral sites **(Fig.5)**. A trend to increasing recurrence and further disease with increased length of follow up was observed **(Fig.6)**, with recurrent disease most common within the first 2 years and further disease within 3 years of laser surgery **(Figs. 7 & 8)**. Whilst patients who continued to smoke and drink risked developing recurrent or further dysplastic lesions, the highest rates of malignant transformation and SCC development were seen in non-smokers and non-drinkers particularly elderly females.

Conclusions

Whilst laser surgical excision facilitates PMD resolution in the majority of cases, re-appearance of disease becomes more likely with prolonged follow up. This emphasises the importance of regular and coordinated follow up for all PMD patients.

Reference

(1) Thomson PJ, Wylie J. Interventional laser surgery: an effective surgical & diagnostic tool in oral precancer management. *Int J Oral Maxfac Surg* 2002 31 : 145-153

Fig. 1 : Floor of Mouth Leukoplakia

Fig. 2 : Floor of Mouth SCC

Fig. 3 : Laser Excision of Labial Commissure Leukoplakia

Fig. 4 : Laser Excision of Lateral Tongue PMD

Clinical Appearance	Number of PMD Lesions
Homogeneous Leukoplakia	67
Non-Homogeneous Leukoplakia	9
Erythroplakia	8
Erythroleukoplakia	16

Table 1 : Clinical Appearance of PMDs

Anatomical Site	Number of PMD Lesions
Floor of Mouth	46
Lateral Tongue	19
Ventral Tongue	14
Soft Palate	9
Buccal Mucosa	5
Fauces	4
Alveolus	2
Retromolar	1

Table 2 : Anatomical Site of PMDs

Dysplasia Grading	Number of PMD Lesions
Mild	42
Moderate	26
Severe	21
Carcinoma-in-Situ	11

Table 3 : Histopathological Grading of PMDs

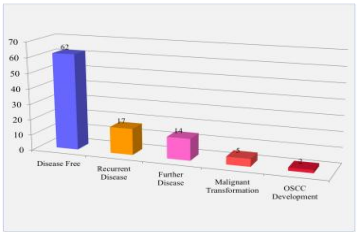

Fig. 5 : Clinical Outcomes for the 100 PMD Patients

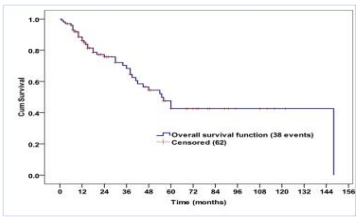

Fig. 6 : 'Disease Free Survival ' over Time (months)

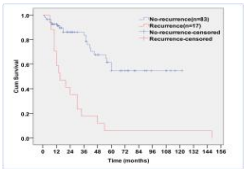

Fig. 7 : Kaplan -Meier Plot showing Recurrent (Same Site) Disease development over Time (months)

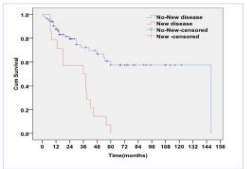

Fig. 8 : Kaplan-Meier Plot showing Further (New Site) Disease development over Time (months)

6) Presented at BAHNO Scientific Meeting April 2012 - Joint Scientific Meeting With The British Society For Oral & Maxillofacial Pathology. Royal College of Physicians, London. 26th – 27th April 2012.

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Time to Treat

Does Time to Treatment Influence Clinical Outcome following Laser Excision of Oral Potentially Malignant Disorders?

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Introduction

Modern management of potentially malignant disorders (PMD) is based upon initial incisional biopsy of clinically recognisable lesions **(Fig.1)** followed by complete lesion excision for definitive histological diagnosis **(Fig.2)** and treatment. The aim of this paper was to review the influence of time between first clinical presentation, incisional biopsy and subsequent excisional treatment **(Figs. 3 & 4)** upon overall clinical outcome.

Methods

Following ethical approval, the clinical records of 100 PMD patients presenting with new, single dysplastic oral lesions undergoing standardised interventional laser surgery **(1)** were reviewed and their clinical outcomes determined as: disease free, recurrent disease (same site), further disease (new site), malignant transformation (same site) or SCC development (new site). Overall clinical management time **(CMT)** was divided into provisional diagnostic time **(PDT)** which was the time between 1st presentation and incisional biopsy, definitive diagnosis time **(DDT)** which was the time between incisional biopsy and laser excision and follow up time **(FT)** which was the time between laser surgery and most recent clinic review.

Results

Overall mean **PDT** was 5 months, with the majority of biopsies carried out within 3 months **(Fig.5)** whereas mean **DDT** reached 13 months with 50% of laser excisions carried out within 3 months of incision biopsy **(Fig.6)**. Mean **FT** was 55 months. Mean times for **PDT** were shortest for lesions exhibiting severe dysplasia and carcinoma-in-situ and longer for mild/moderate dysplasia **(Fig.7)**, but there were no significant effects on clinical outcome (p=0.026; Kruskall-Wallis). Mean **DDT** scores exhibited a similar relationship with the severest dysplasias waiting the shortest time for laser excision ; p=0.001 **(Fig.8)**. A significant negative correlation was seen between **DDT** and degree of dysplasia, with shorter waiting times for laser treatment seen with increasing dysplasia ; r=0.546, p=0.0001 **(Fig.9)**. Overall, there was little significant relationship seen with clinical outcome except for patients who developed further (new site) disease who were actually those treated most rapidly (p=0.006; Mann-Whitney).

Conclusions

Experienced clinical judgement regarding the need for urgent incisional biopsy is supported by these results which confirm shortest **PDTs** in lesions subsequently confirmed as the most dysplastic. Patients developing new site dysplastic lesions were similarly identified as 'high risk' receiving earlier laser intervention. Only 7 patients underwent malignant transformation or SCC development during FT and there were no significant time to treatment influences on this small patient group.

Reference

(1) Thomson PJ, Wylie J. Interventional laser surgery: an effective surgical & diagnostic tool in oral precancer management. *Int J Oral Maxfac Surg* 2002 31 : 145-153

Fig.1 : Non-homogeneous ventro-lateral tongue leukoplakia

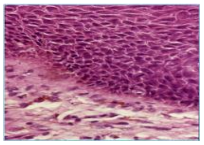


Fig.2 : Moderate Dysplasia

Fig.3 : Laser Excision of Floor of Mouth PMD

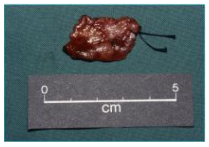


Fig.4 : Laser Excision specimen provides definitive diagnosis

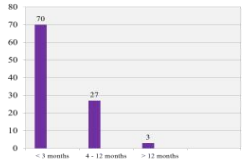


Fig. 5 : PDT (months) vs Number of PMD Incisional Biopsies

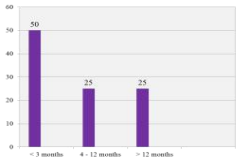


Fig. 6 : DDT (months) vs Number of PMD Laser Excisions

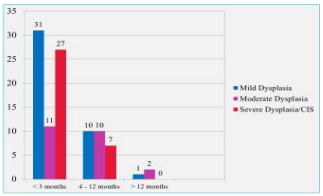


Fig. 7 : PDT (months) vs Dysplasia Grading of PMD Incisional Biopsies

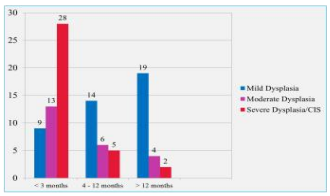


Fig. 8 : DDT (months) vs Dysplasia Grading of PMD Laser Excisions

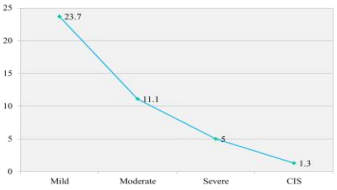
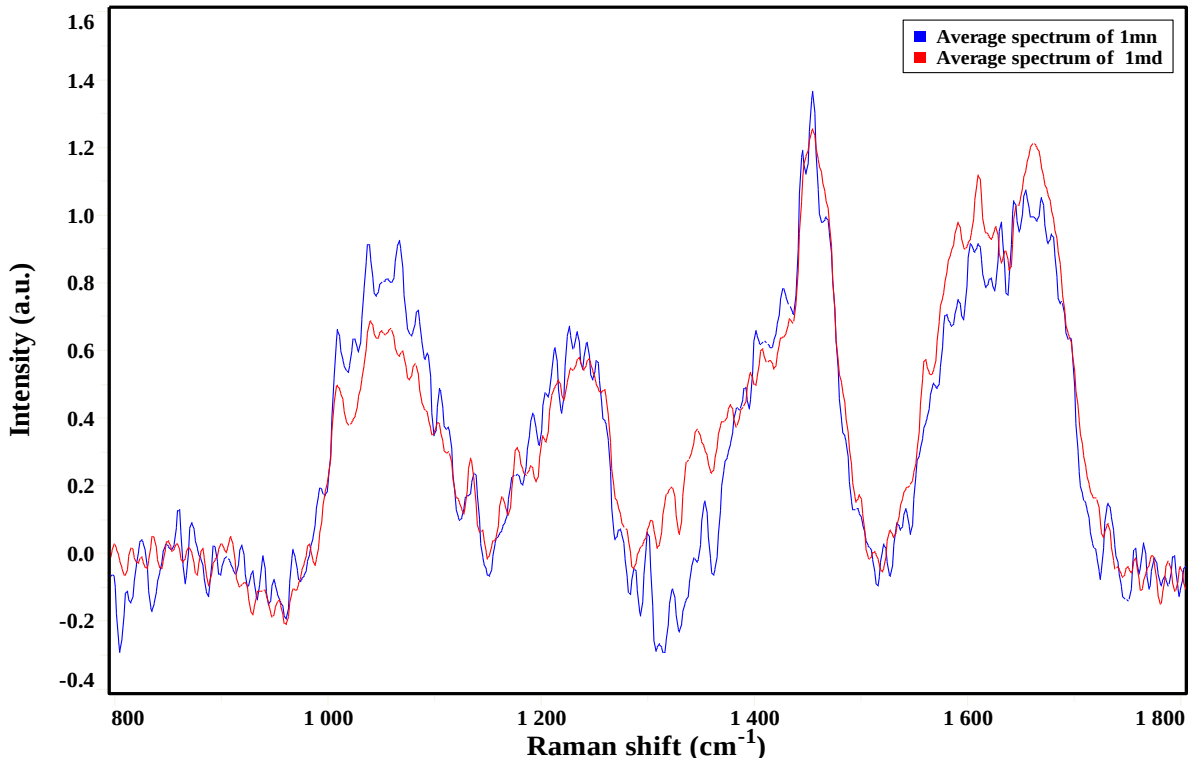


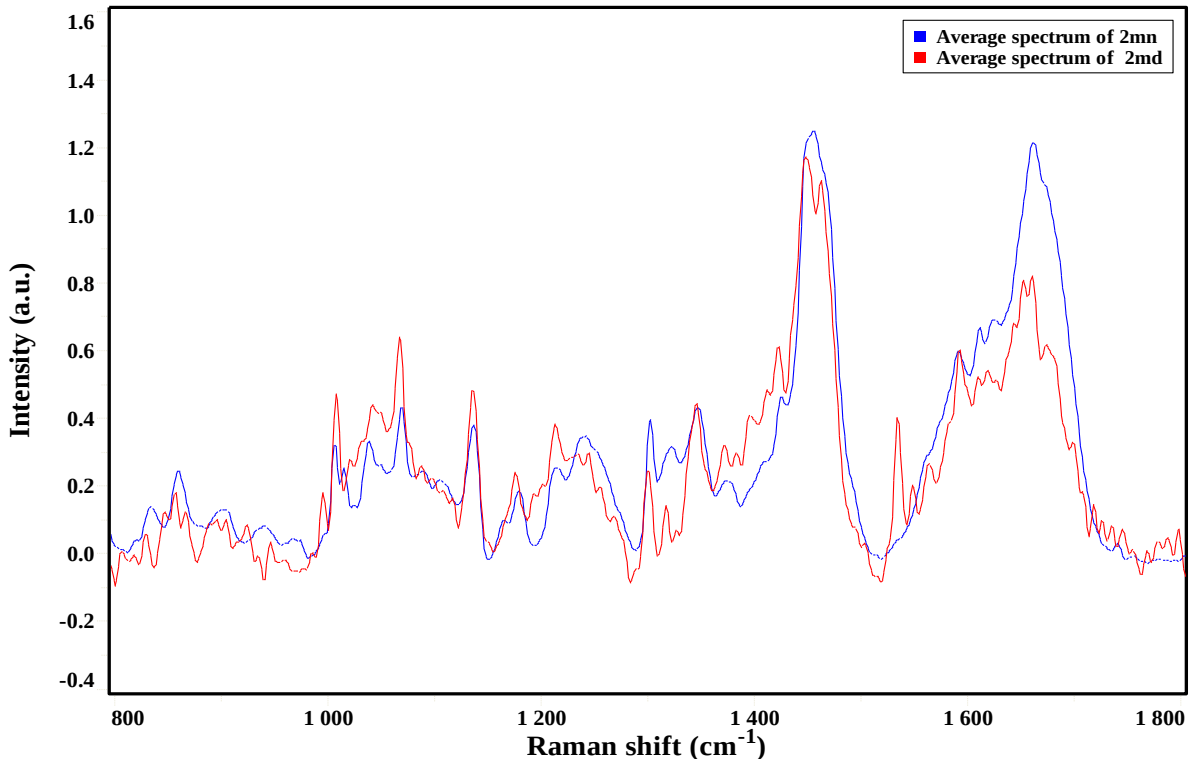
Fig. 9 : Dysplasia Grading vs Mean DDT (months)

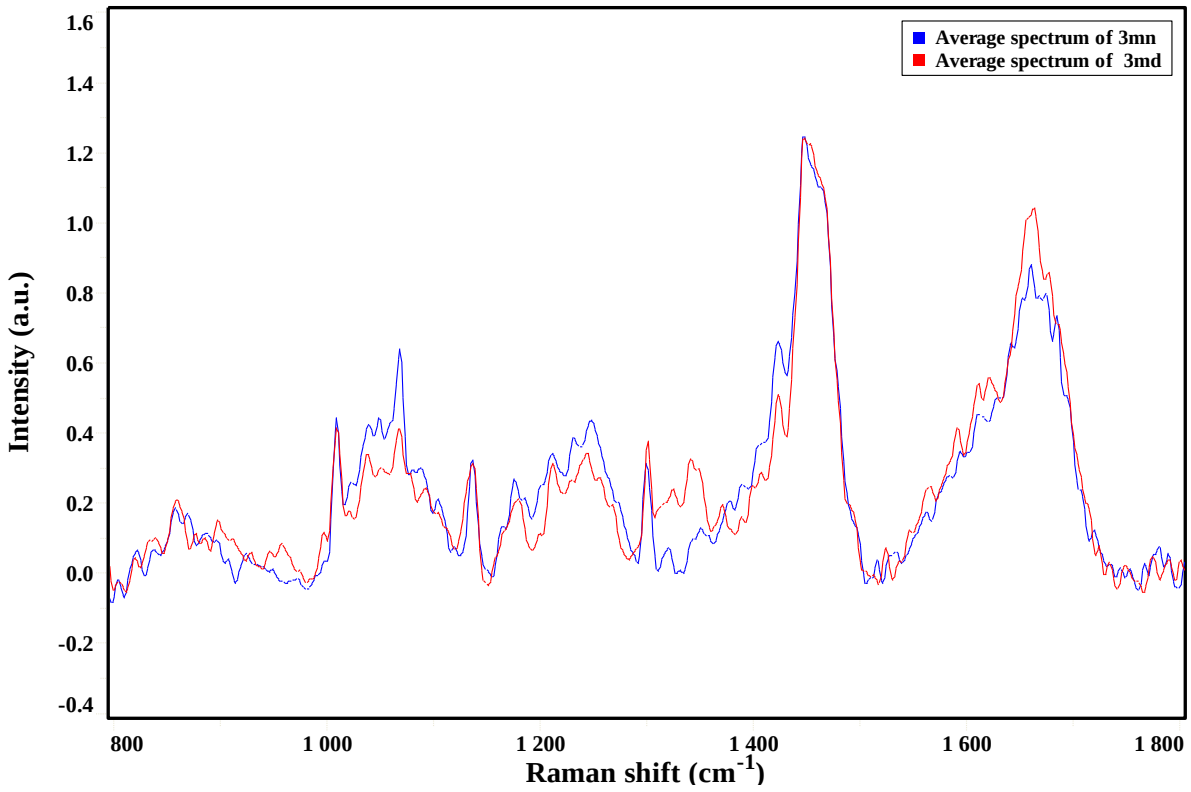
2. Raman Study Appendices

2.1. Appendix (2-A): Pair-wise average spectra of mild dysplasia tissue group.

(mn=morphologically normal tissue, md= mild dysplastic tissue)



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Average spectrum of 3md
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1.4
1.2
1.0
0.8
0.6
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0.0
-0.2
-0.4
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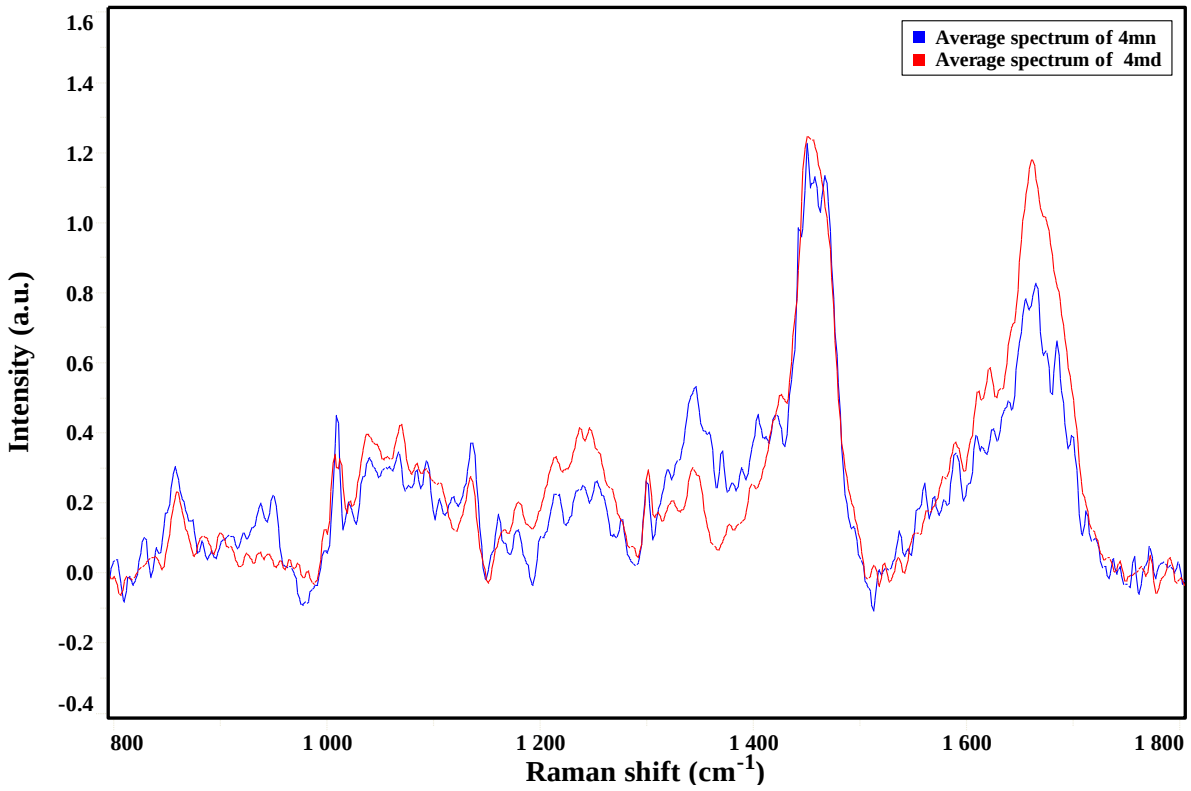
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)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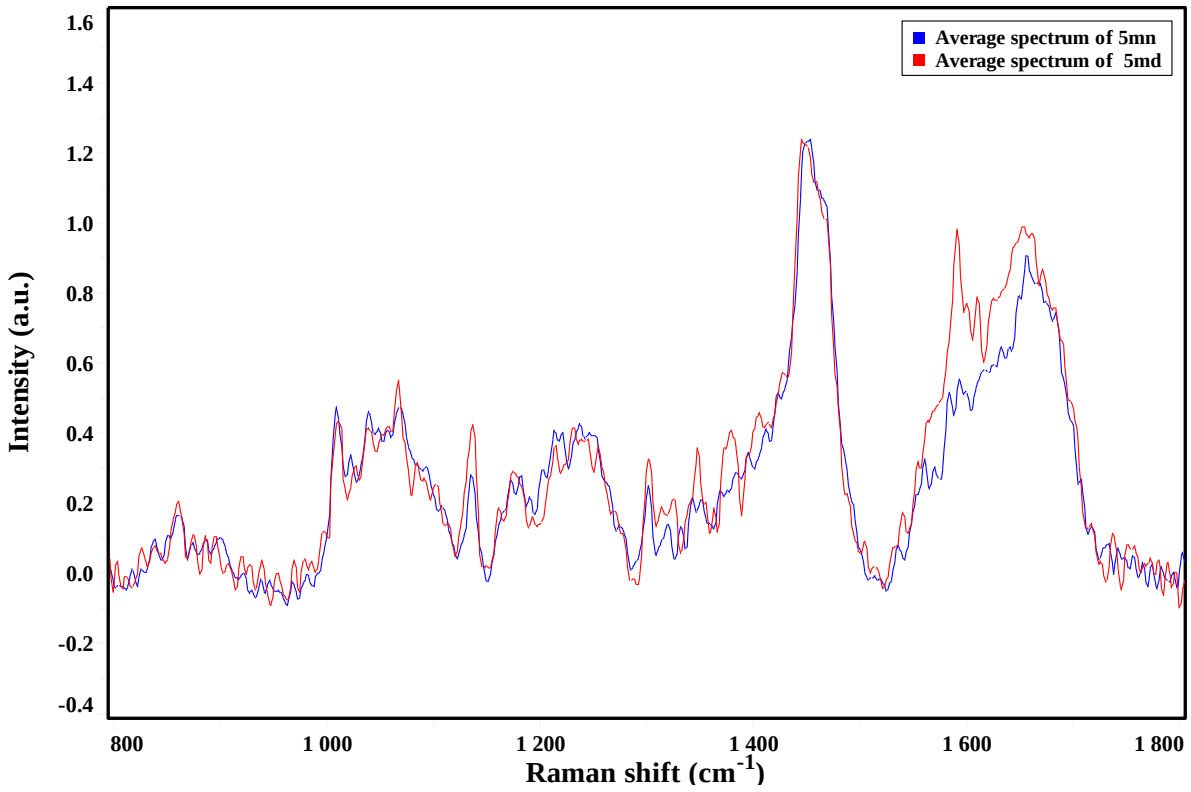
1 600
1 800
Raman shift (cm-1)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Intensity (a.u.)
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1.2
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0.8
0.6
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1 200
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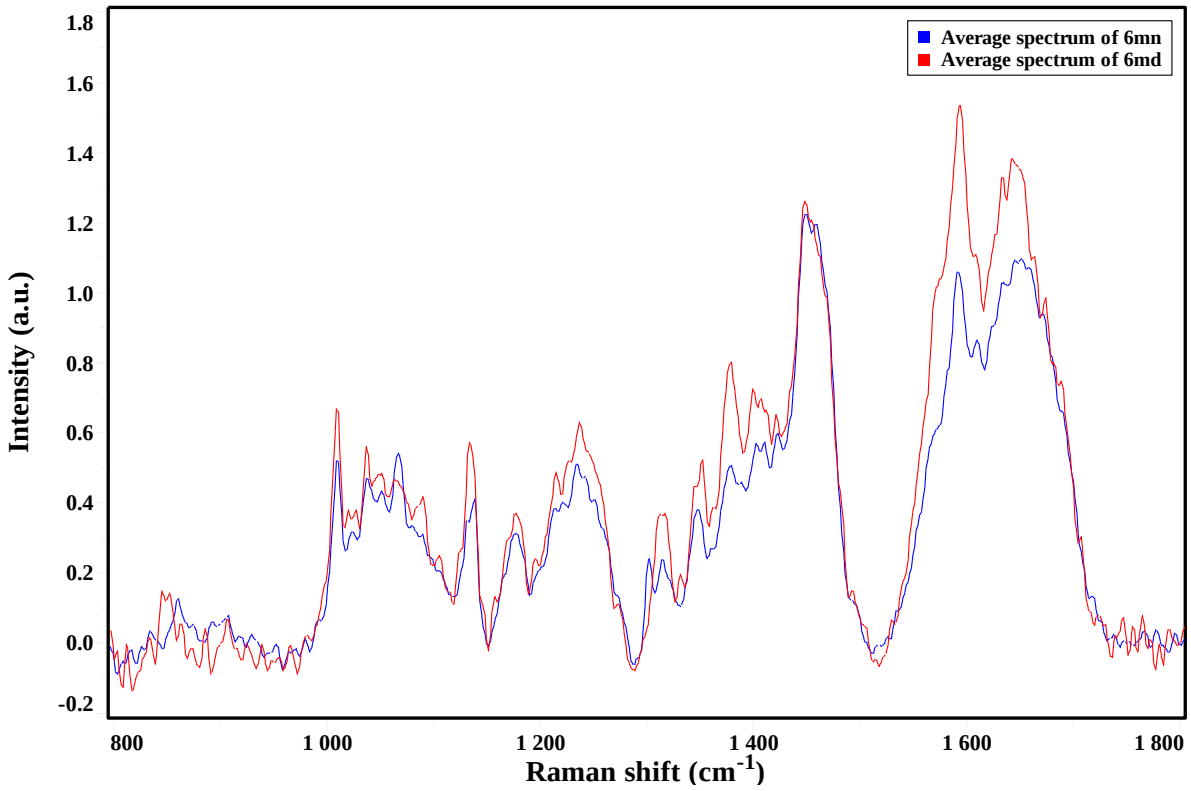
1 600
1 800
Raman shift (cm-1)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Average spectrum of 6md
Intensity (a.u.)
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1.4
1.2
1.0
0.8
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0.2
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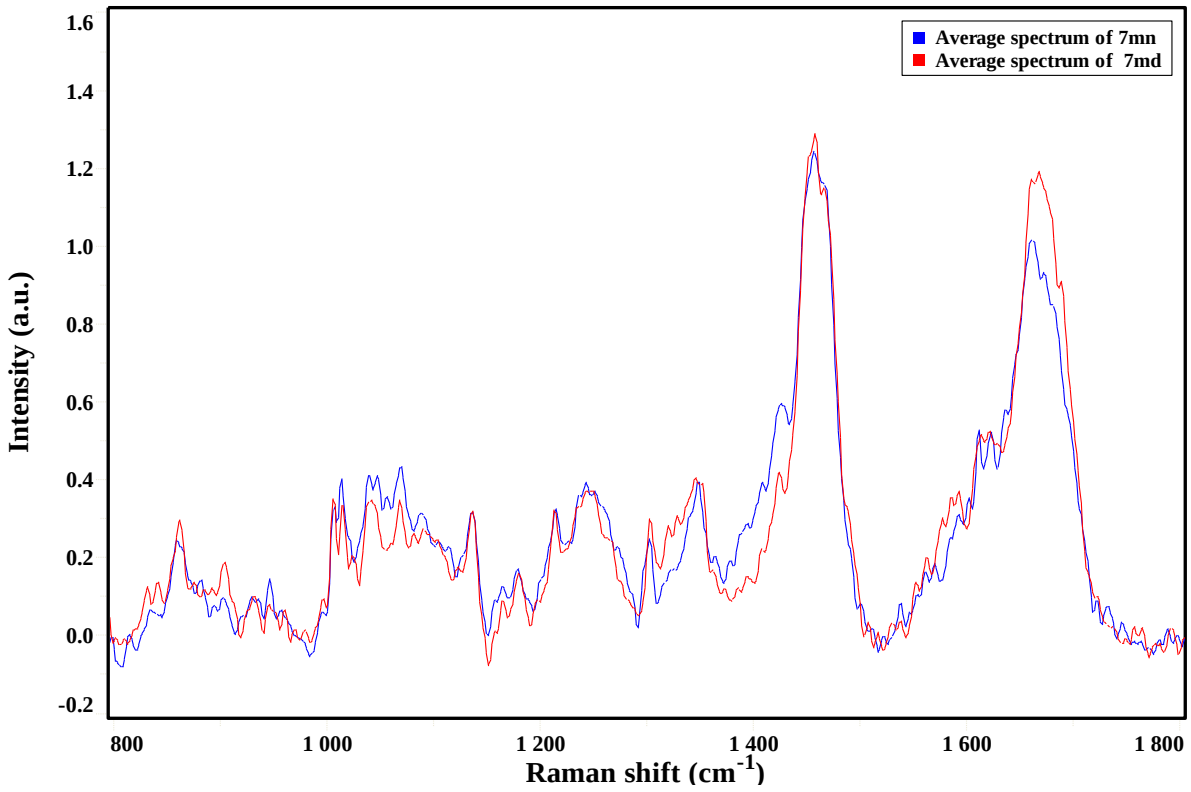
800
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)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Average spectrum of 7md
Intensity (a.u.)
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1.4
1.2
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0.8
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1 200
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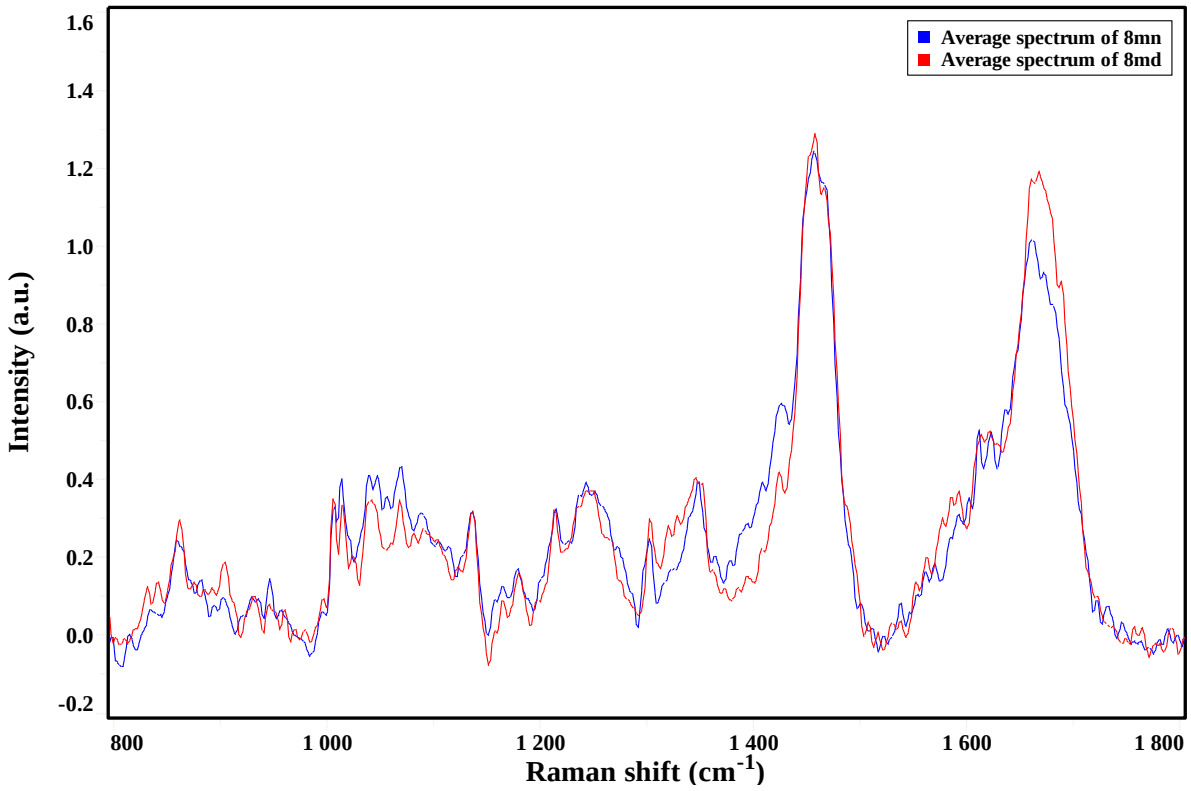
1 600
1 800
Raman shift (cm-1)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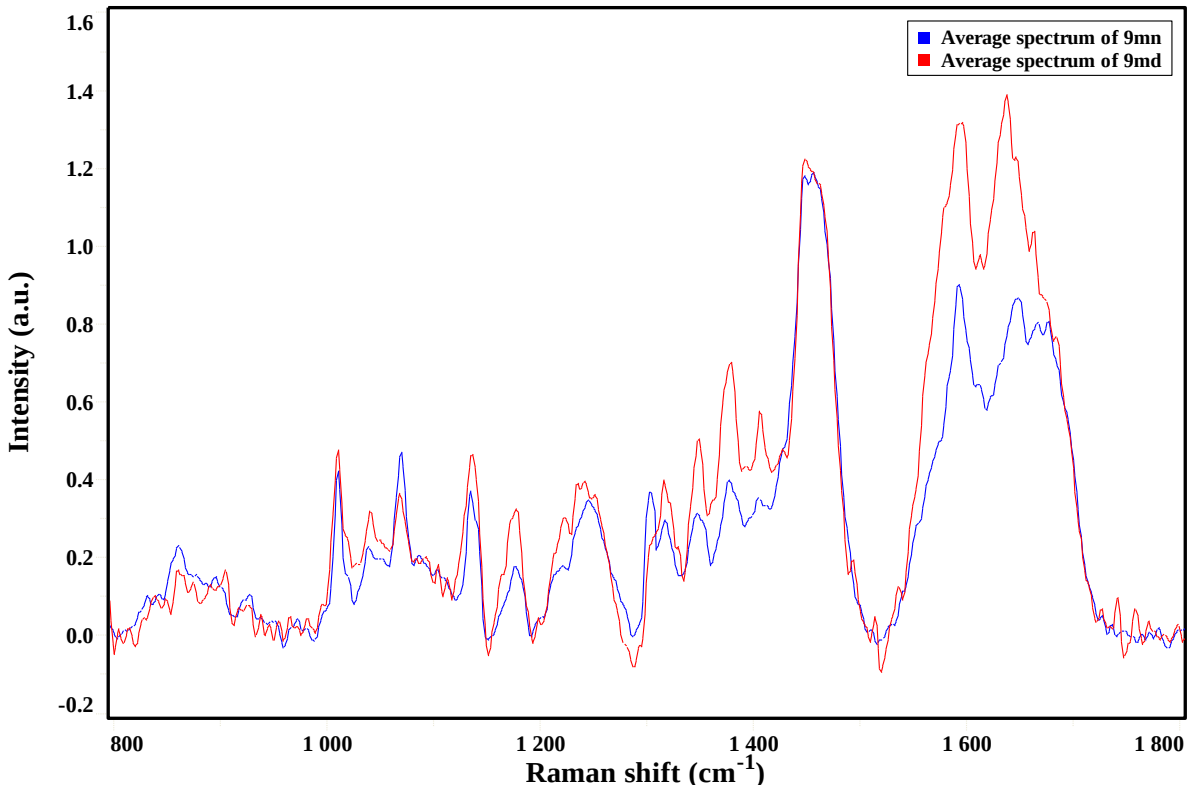
1 800
Raman shift (cm-1)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1 200
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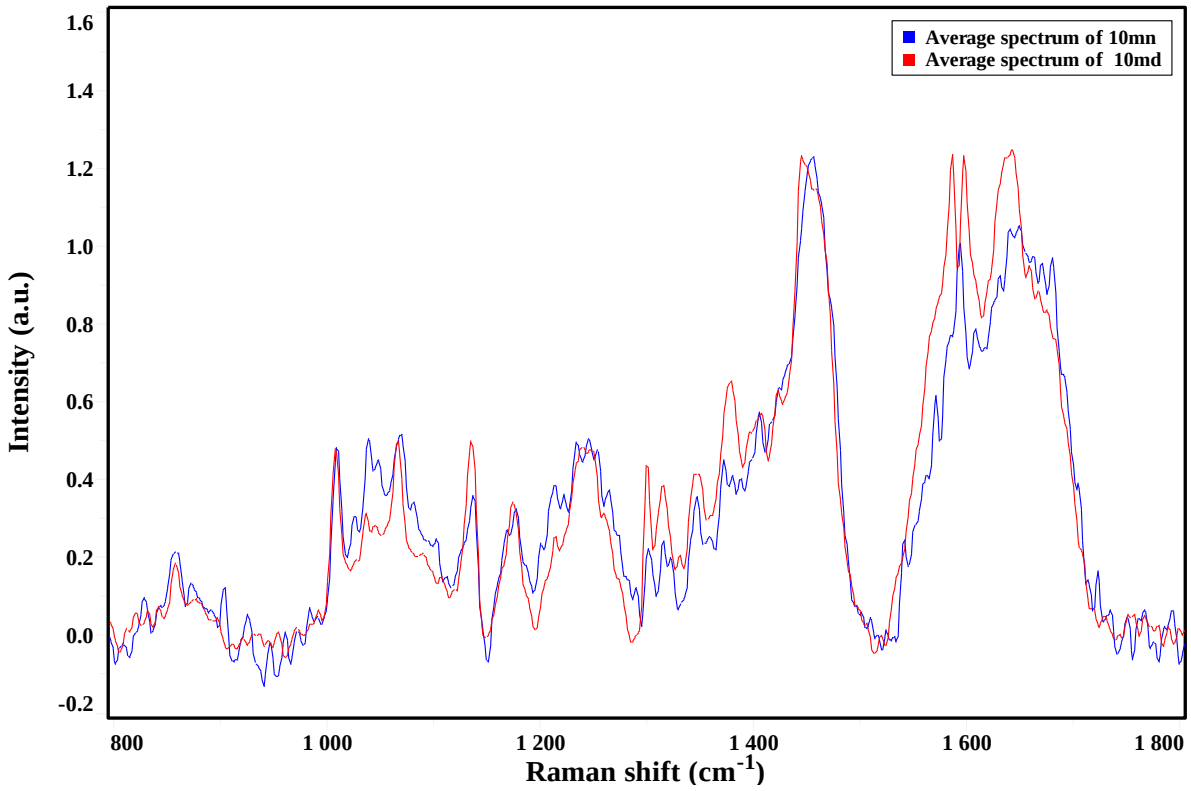
1 600
1 800
Raman shift (cm-1)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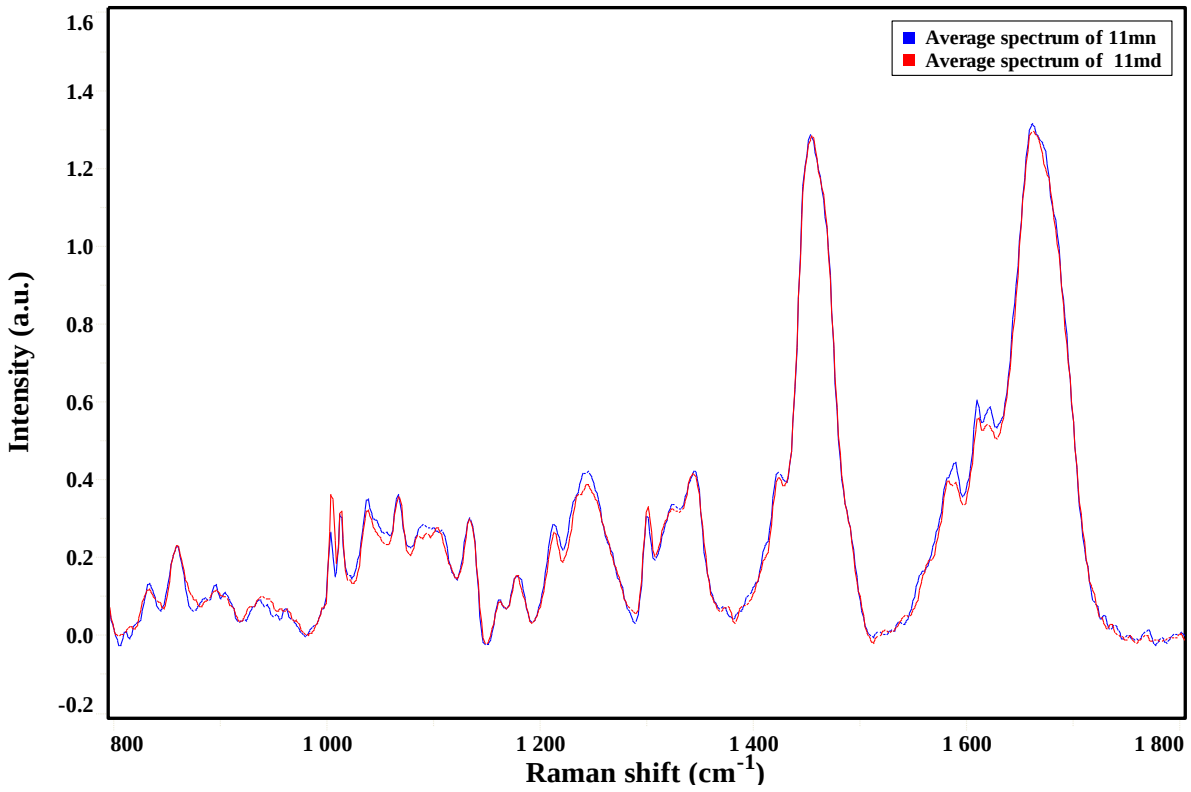
1 800
Raman shift (cm-1)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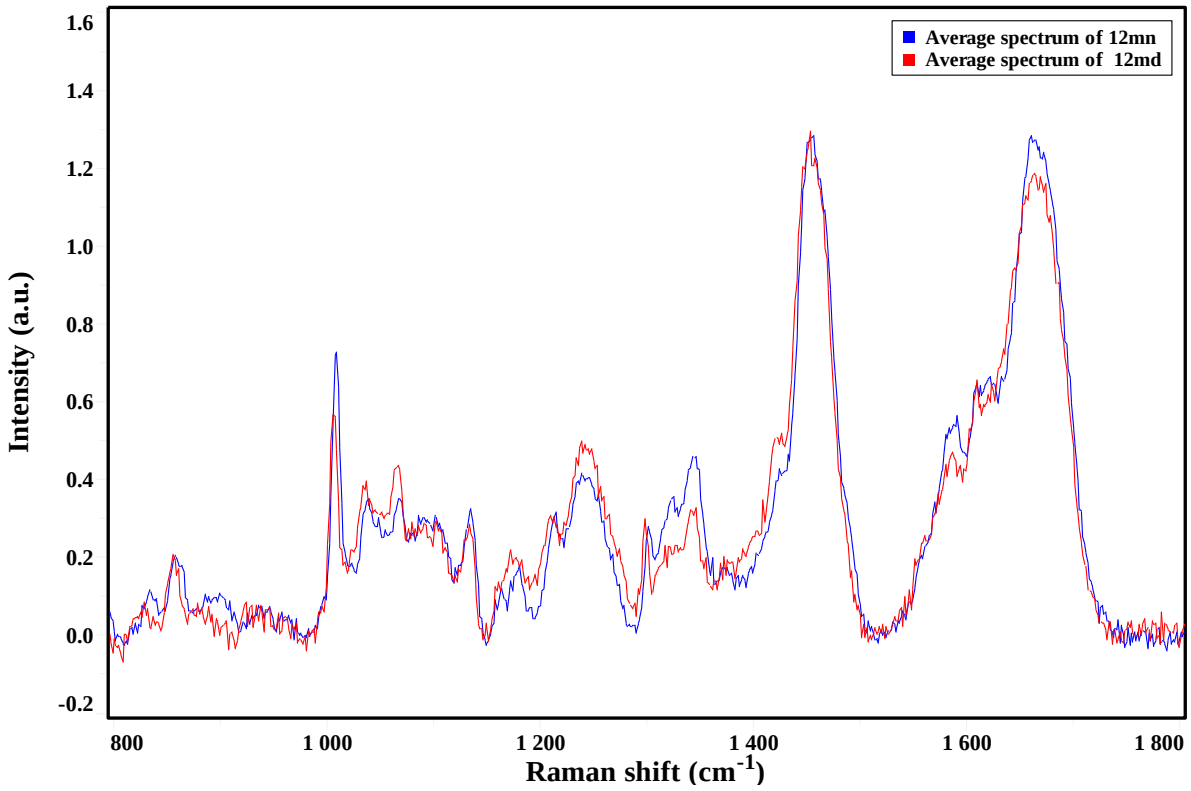
1 200
1 400
1 600
1 800
Raman shift (cm-1)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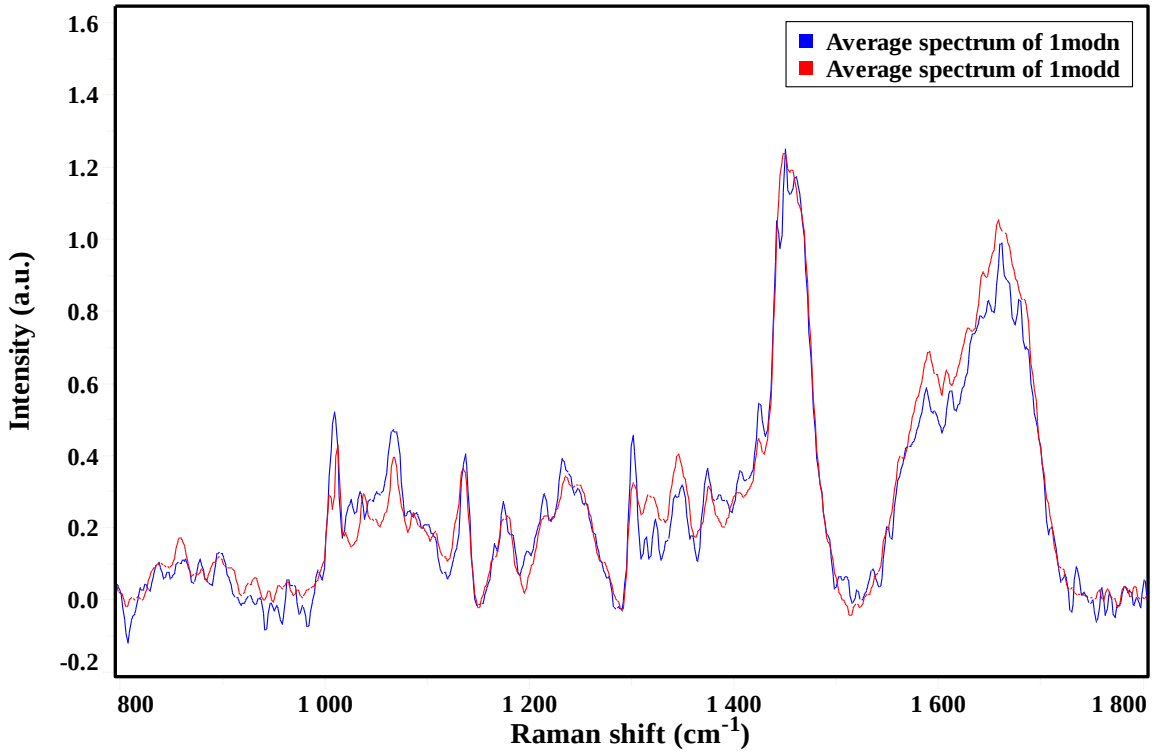
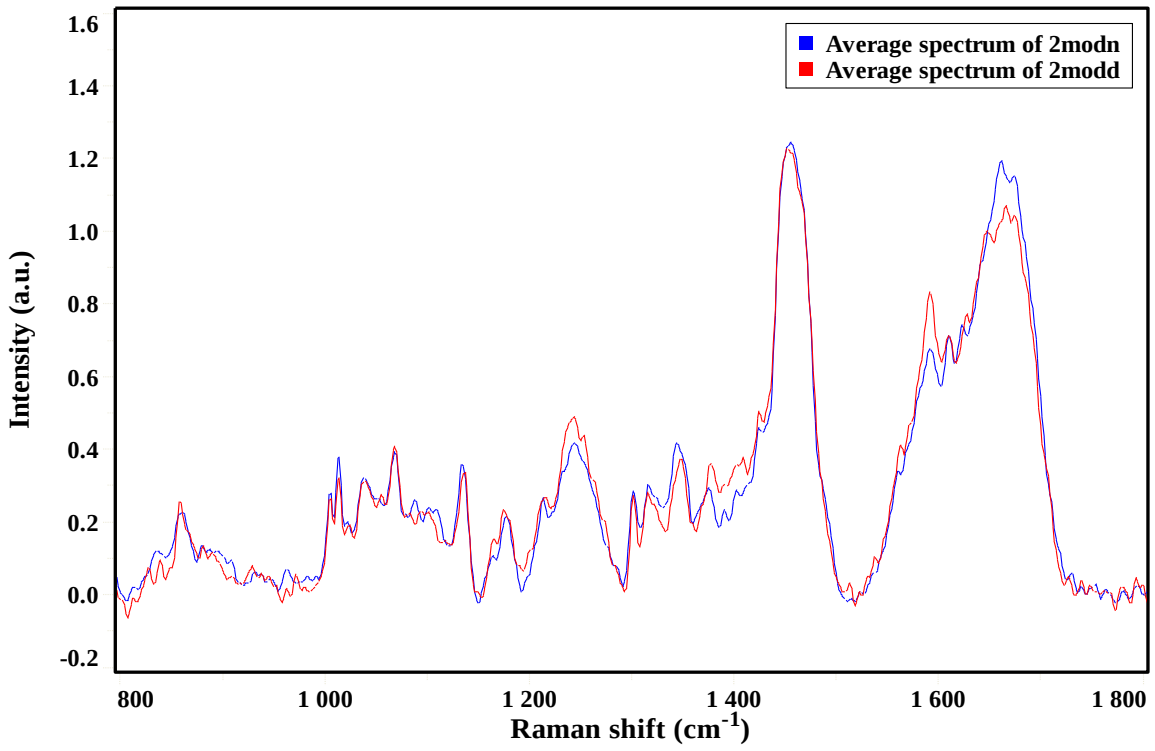
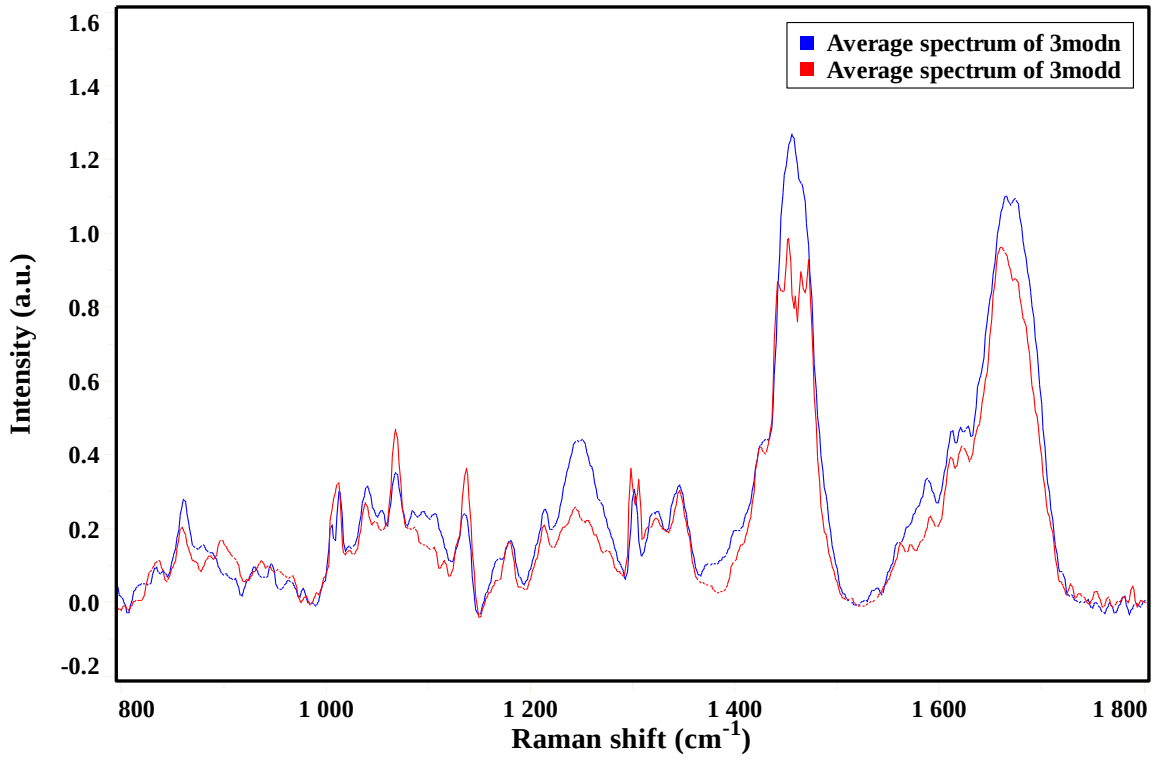
-0.2
800
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)

2.2. Appendix (2-B): Pair-wise average spectra of moderate dysplasia tissue group.

(modn= morphologically normal, modd= moderate dysplastic tissue)



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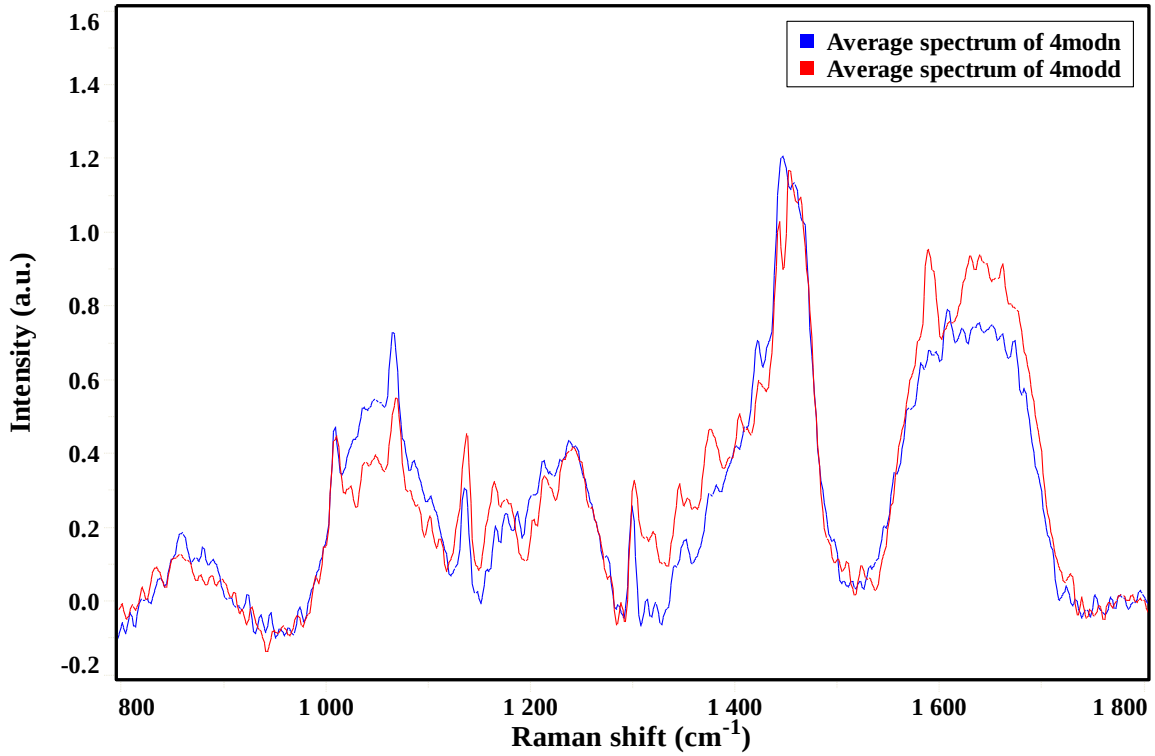
Raman shift (cm-1)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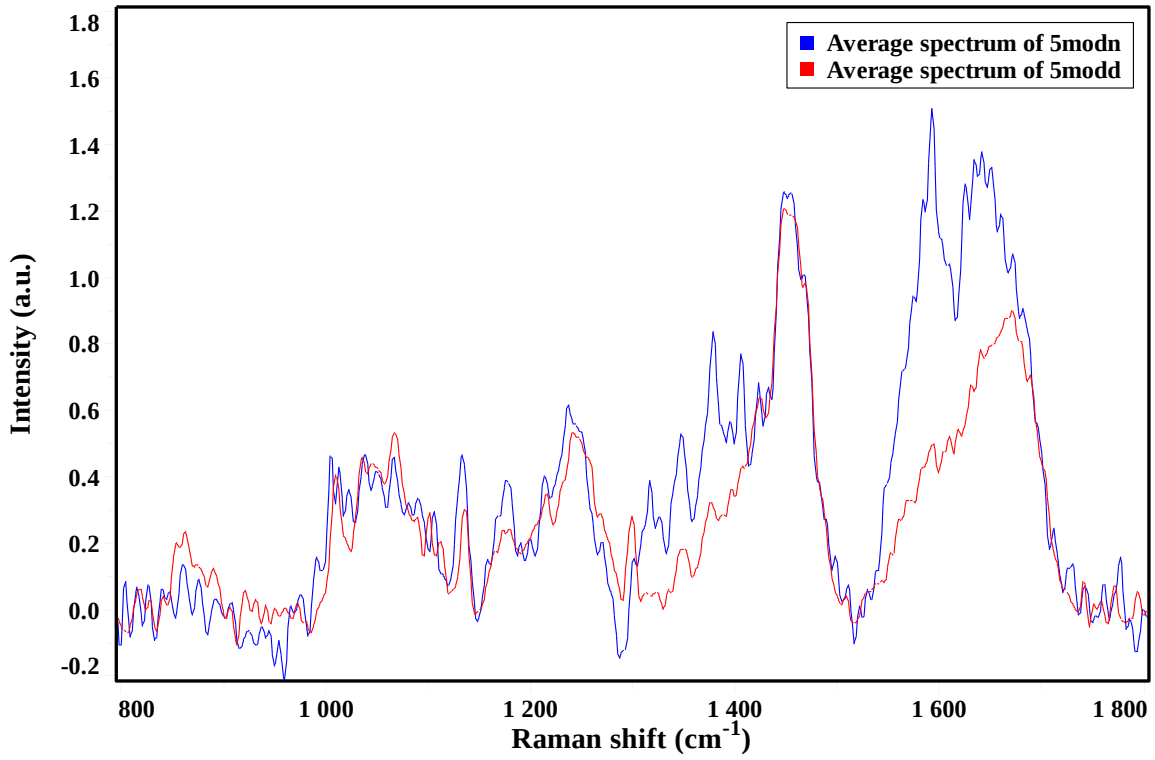
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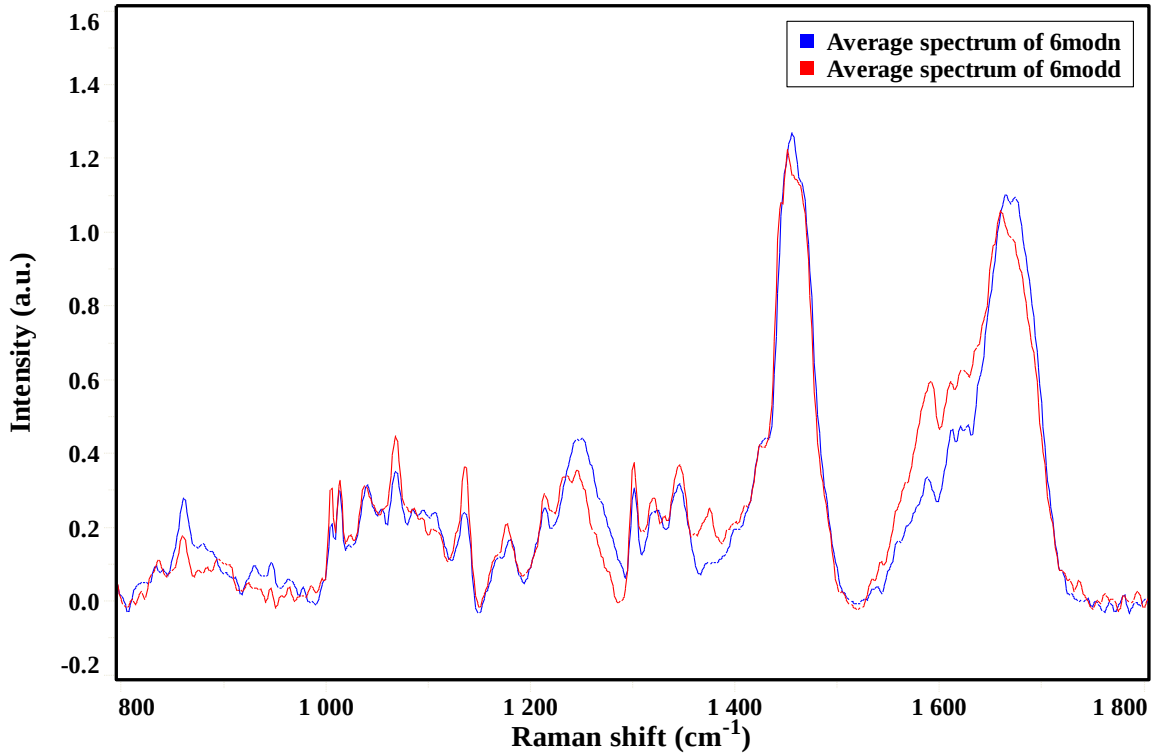
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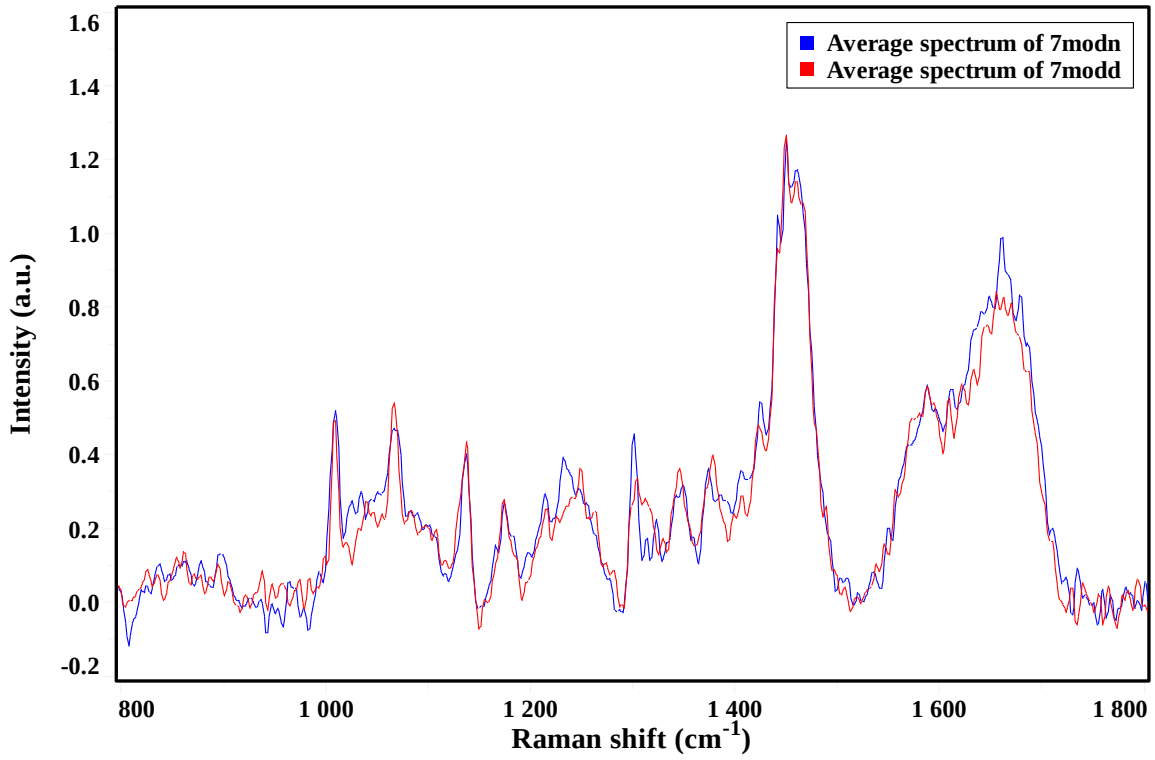
Raman shift (cm-1)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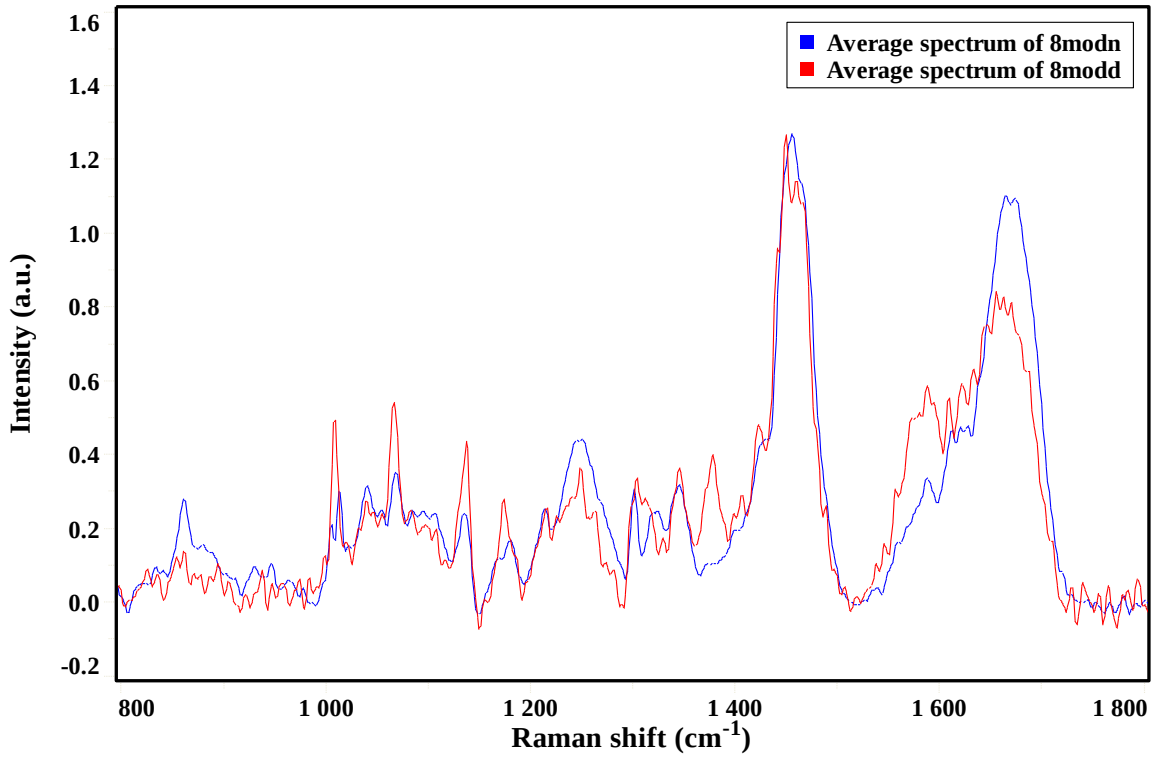
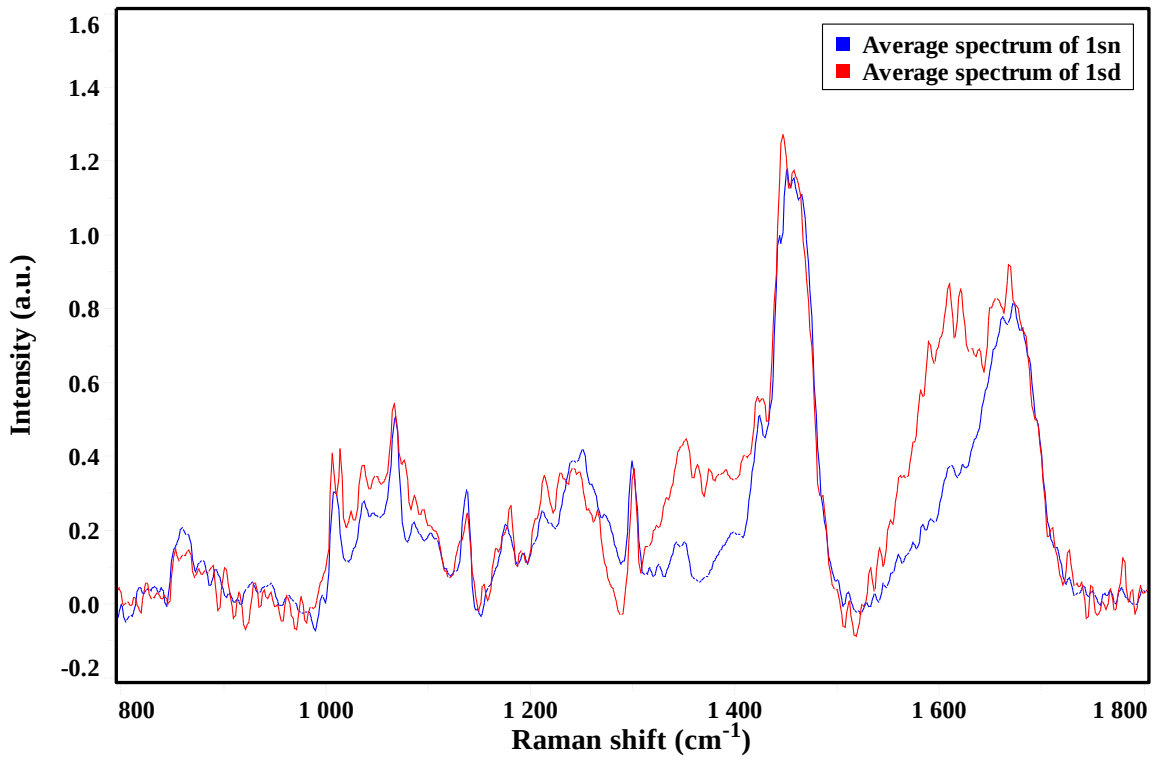
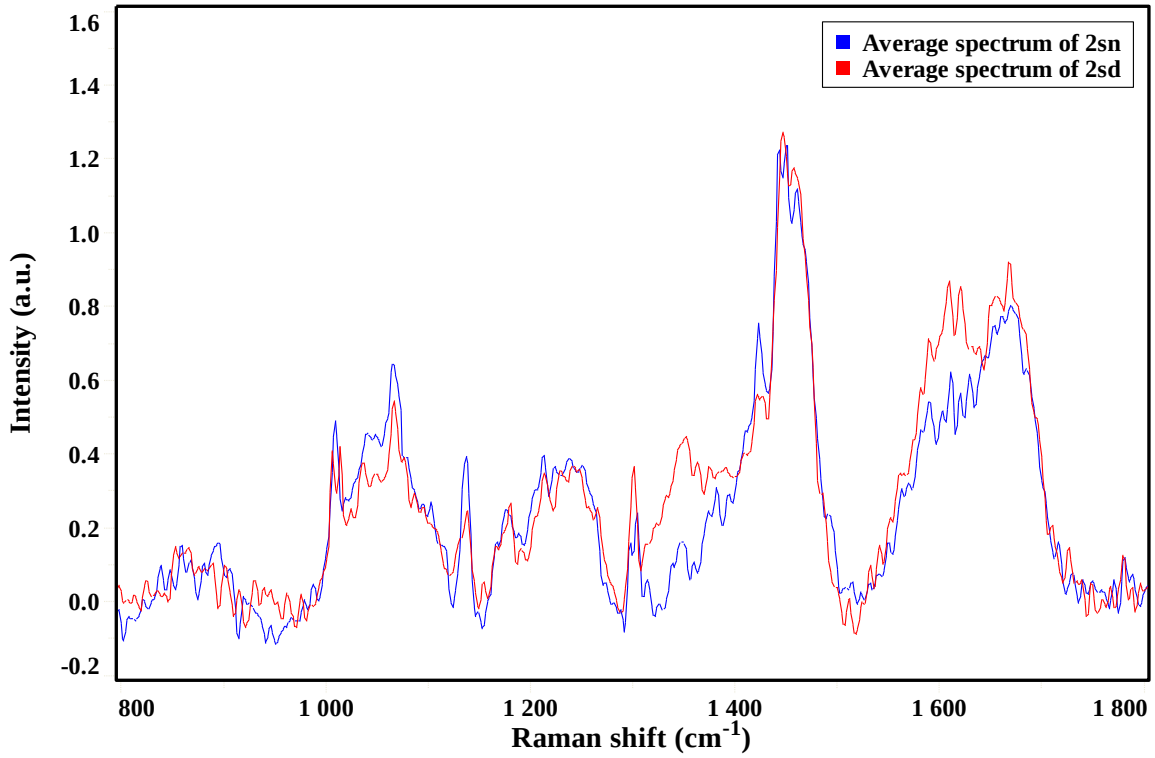
800
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)

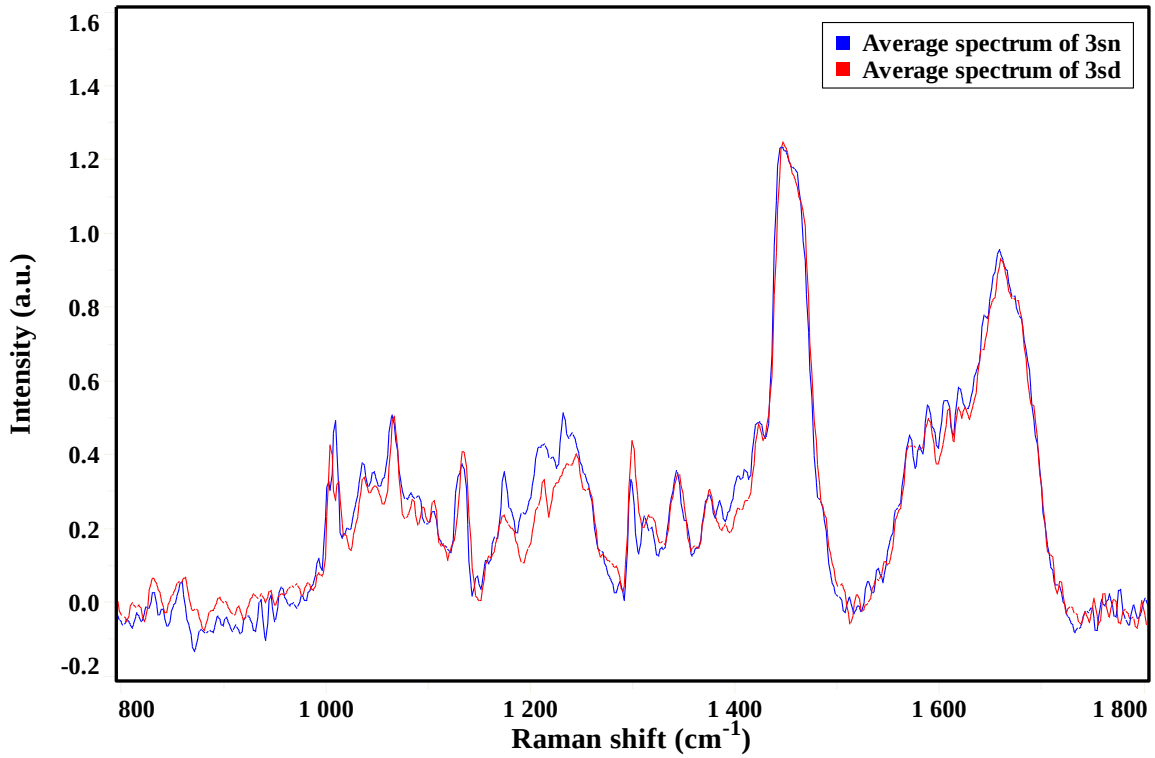

Average spectrum of 8modn
Average spectrum of 8modd
Intensity (a.u.)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
800
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)

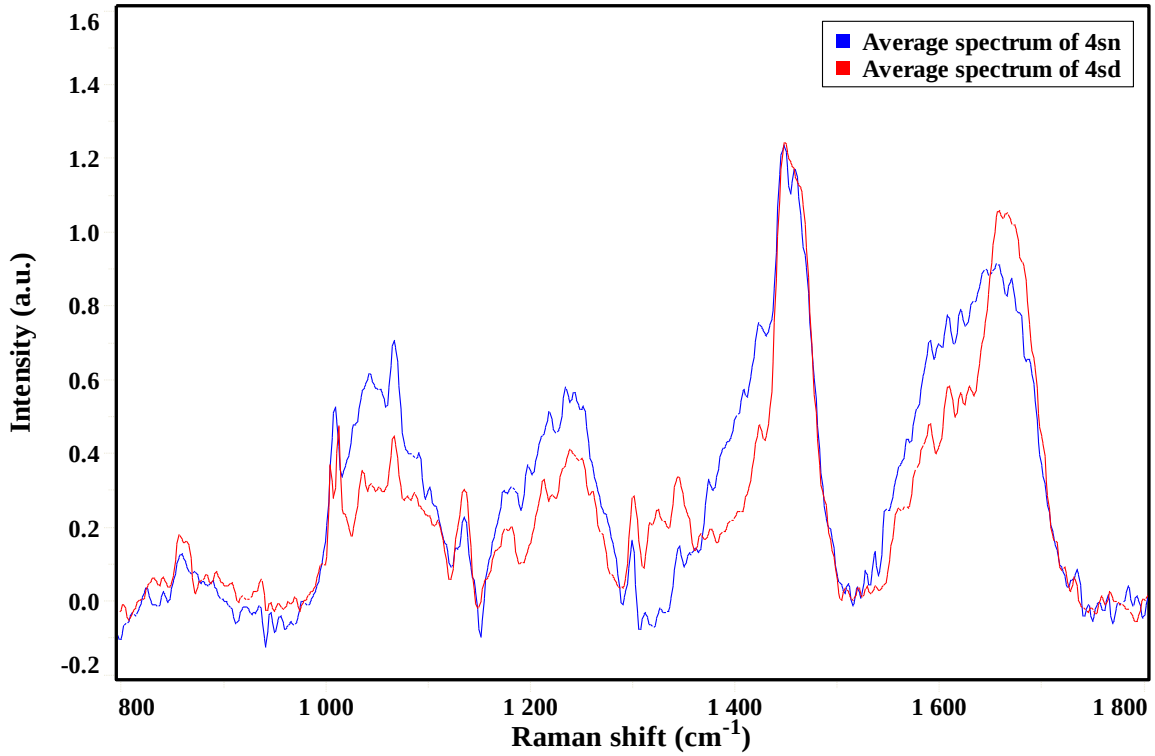
2.3. Appendix (2-C): Pair-wise average spectra of severe dysplasia tissue group.

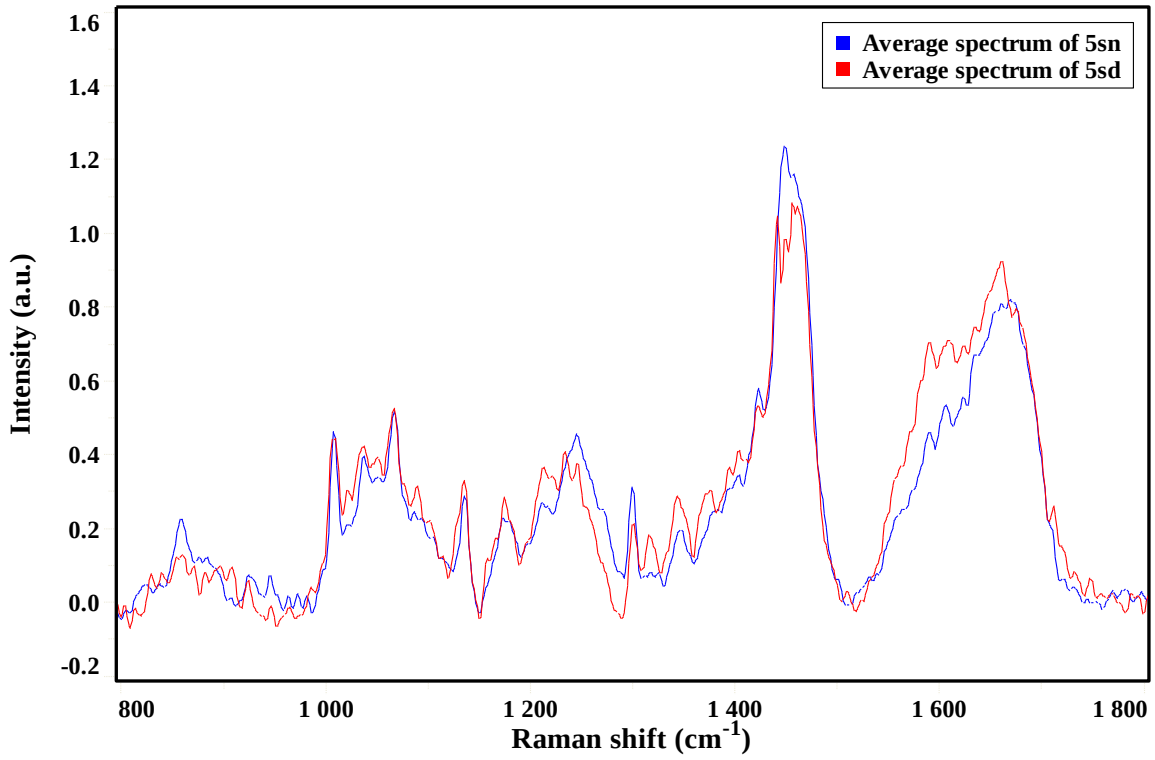
(sn= morphologically normal, sd= severe dysplastic tissue)

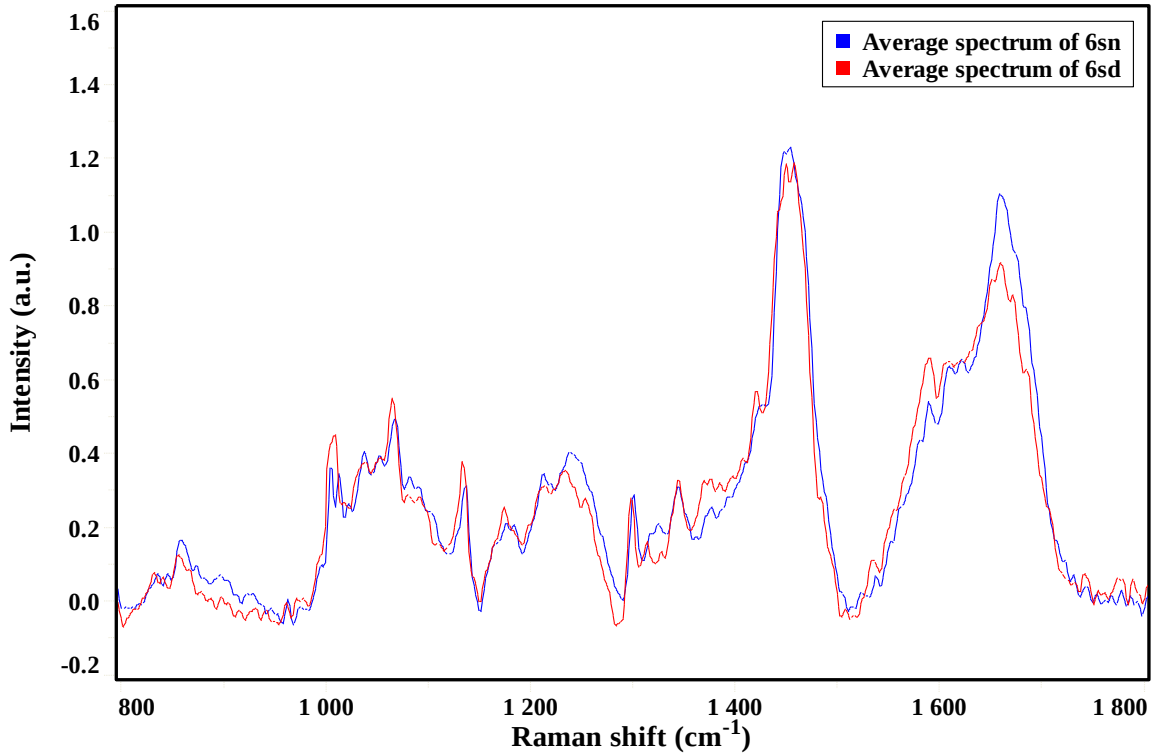





Average spectrum of 3sn
Average spectrum of 3sd
Intensity (a.u.)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
800
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)

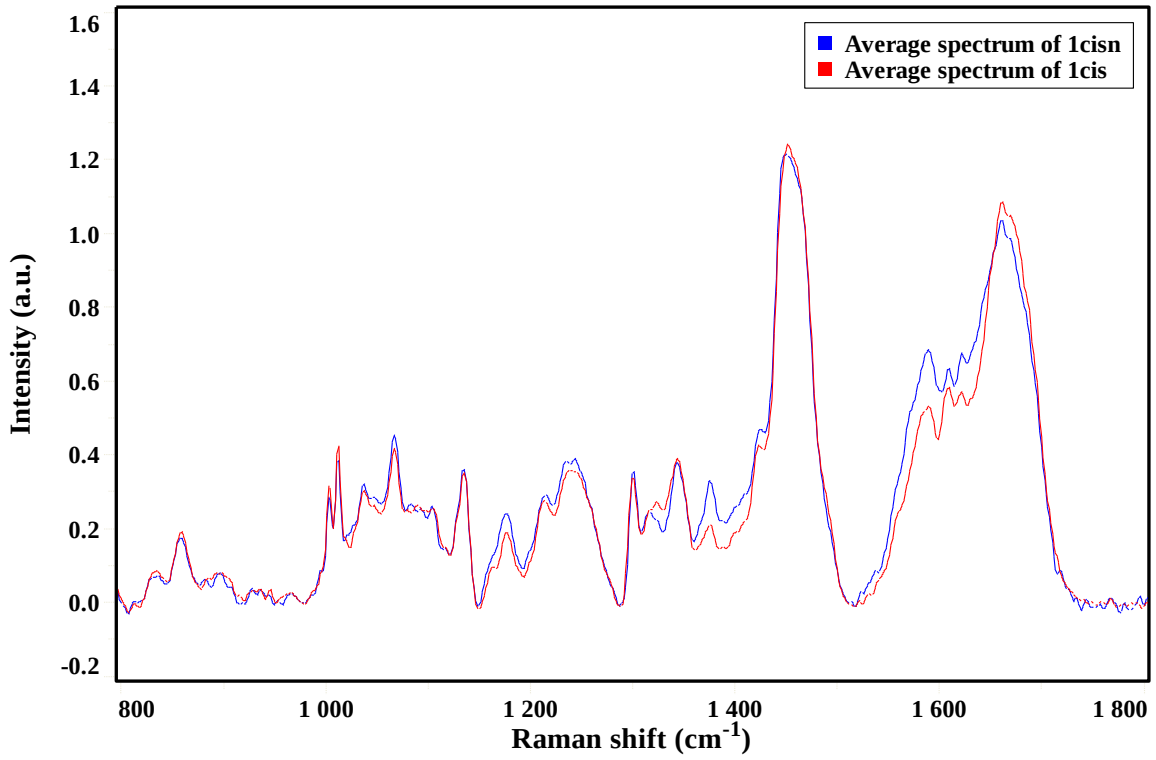

Average spectrum of 4sn
Average spectrum of 4sd
Intensity (a.u.)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
800
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)

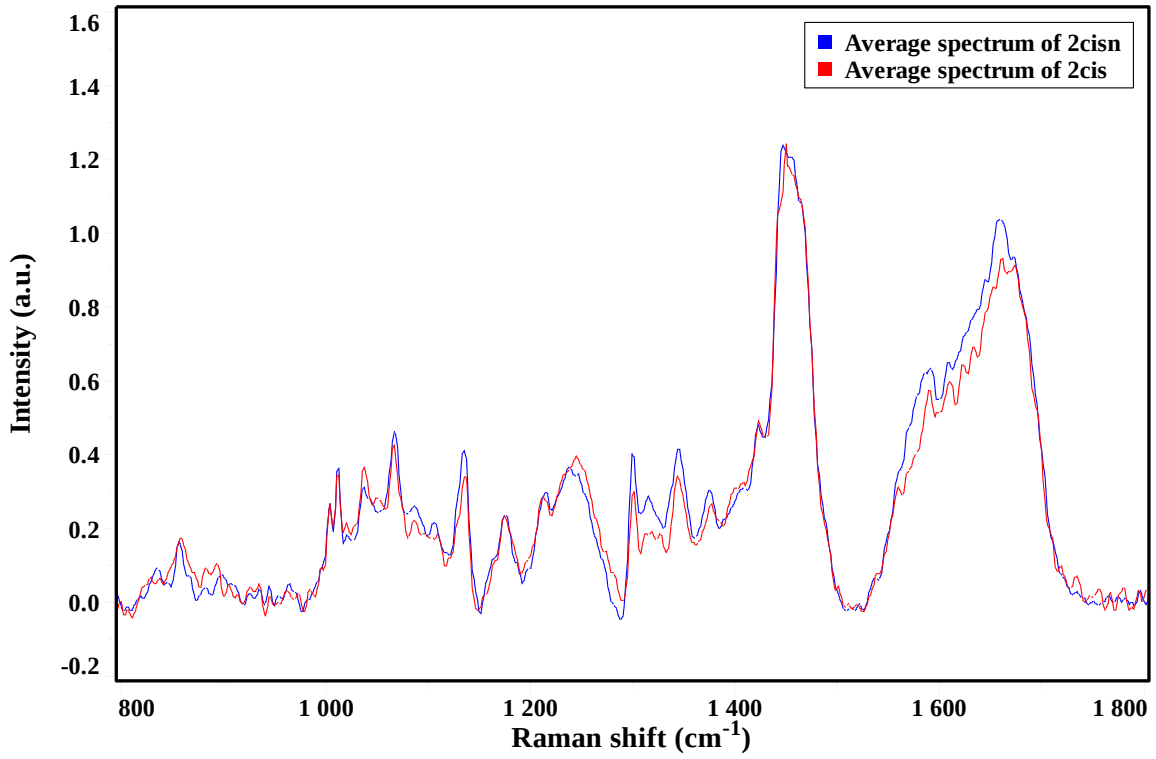

Average spectrum of 5sn
Average spectrum of 5sd
Intensity (a.u.)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
800
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)

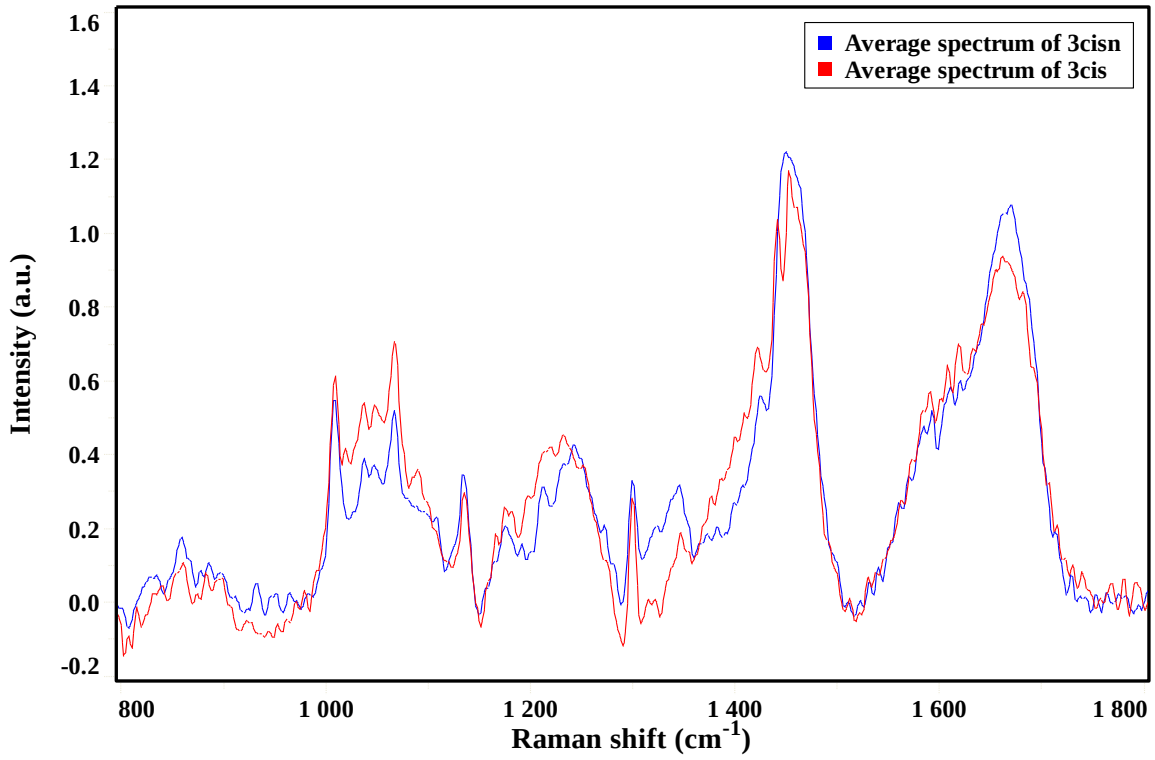

Average spectrum of 6sn
Average spectrum of 6sd
Intensity (a.u.)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
800
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)

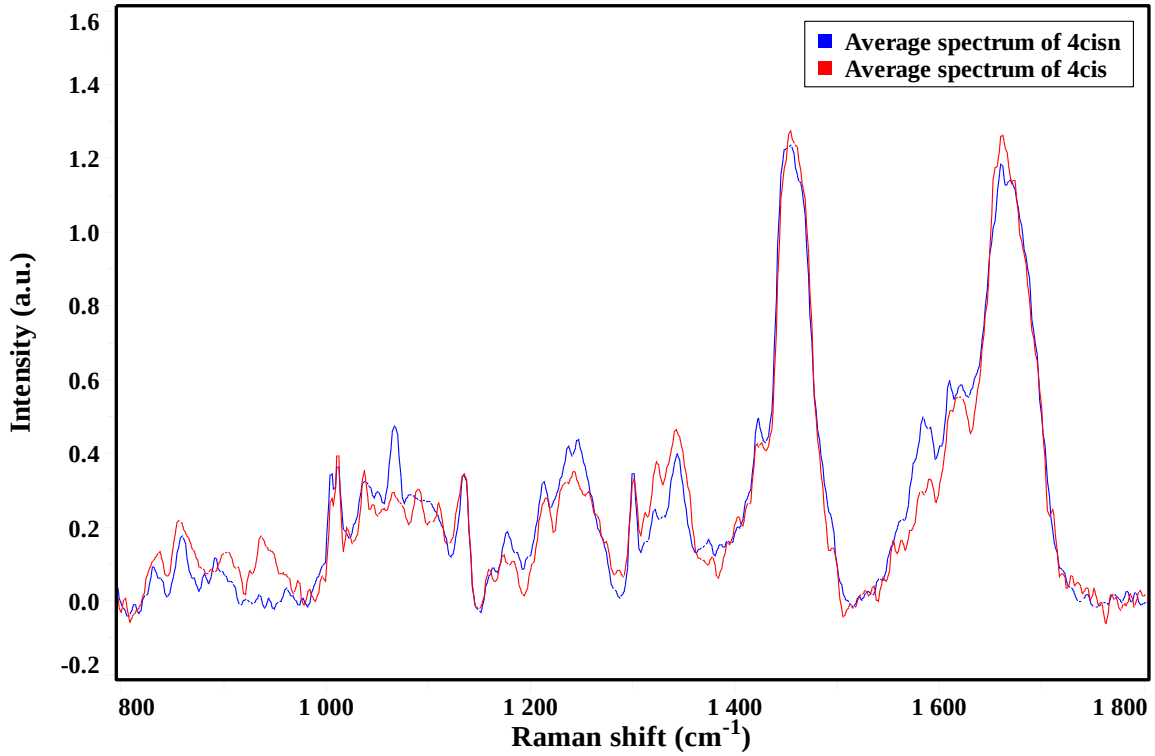
2.4. Appendix (2-D): Pair-wise average spectra of CIS tissue group.

(cisn= morphologically normal tissue, cis= carcinoma *in situ*)



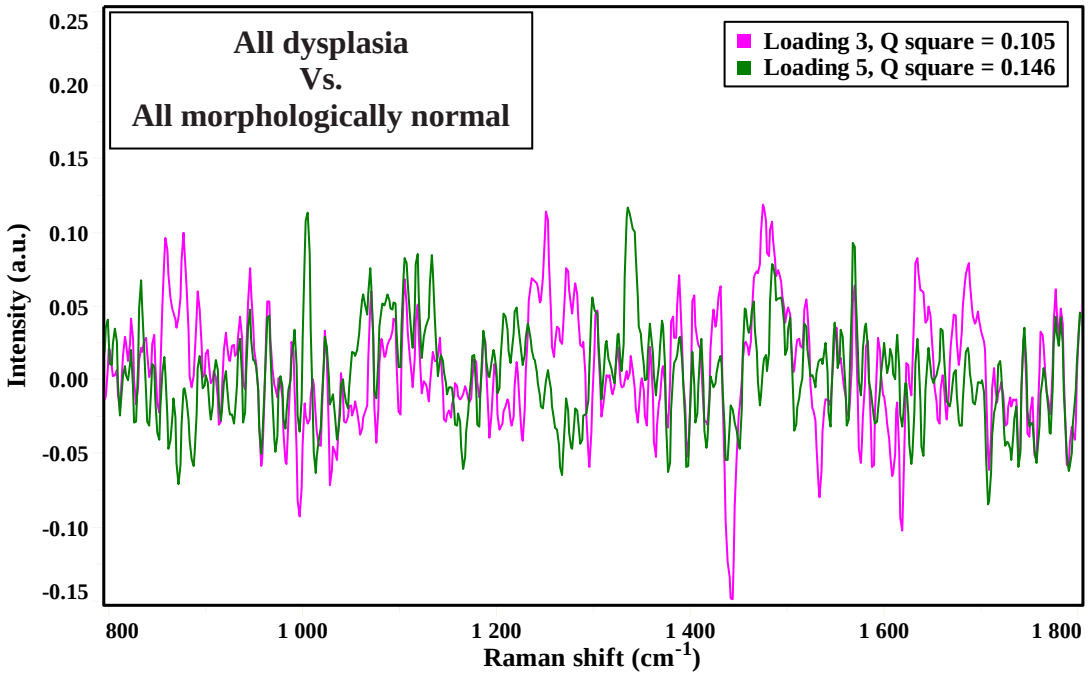



Average spectrum of 3cisn
Average spectrum of 3cis
Intensity (a.u.)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
800
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)

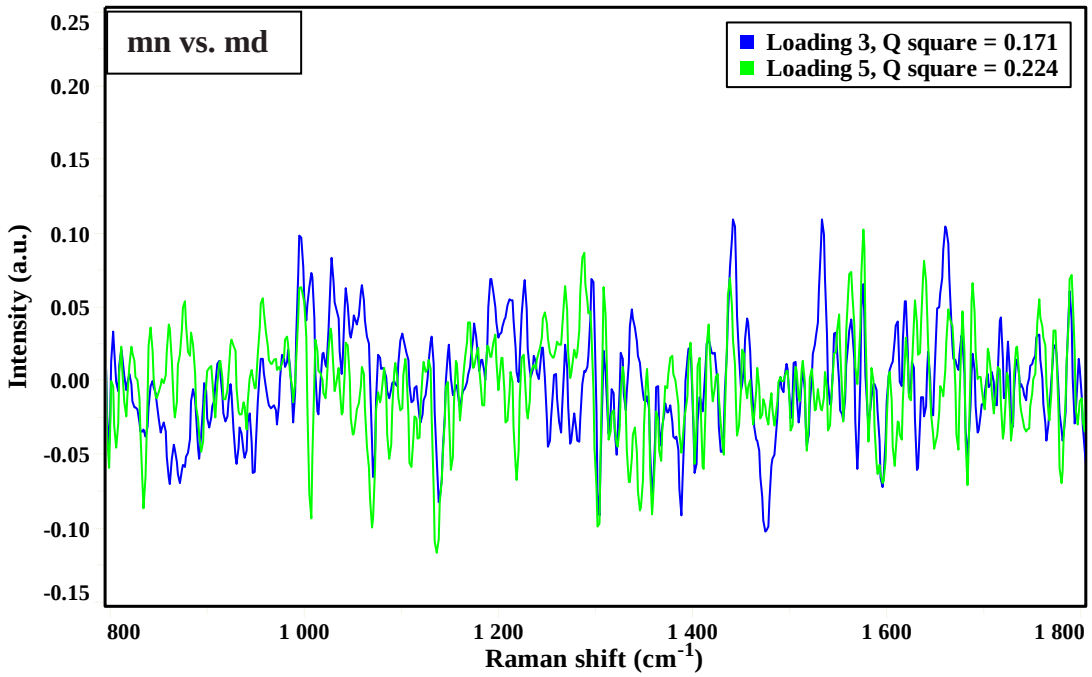

Average spectrum of 4cisn
Average spectrum of 4cis
Intensity (a.u.)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
800
1 000
1 200
1 400
1 600
1 800
Raman shift (cm-1)

2.5. Appendix (2-E): Partial Least Square Component Loadings.

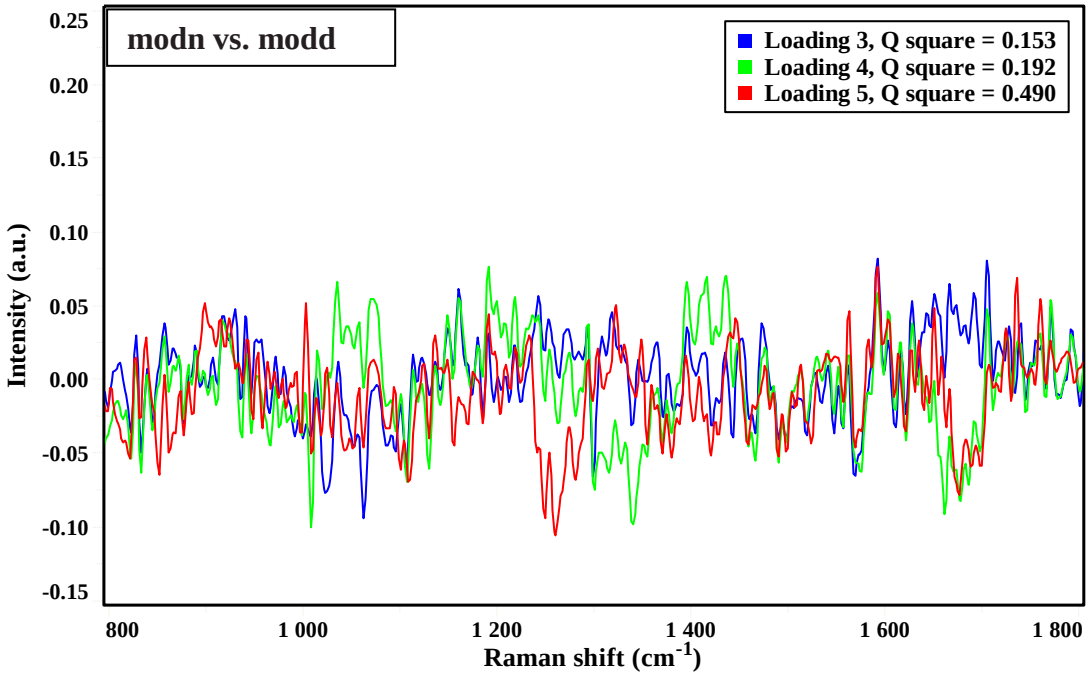
The significant partial least square component loadings plots for the dysplasia against morphologically normal tissue spectra for WHO grading systems and binary grading system with their Q^2 values.



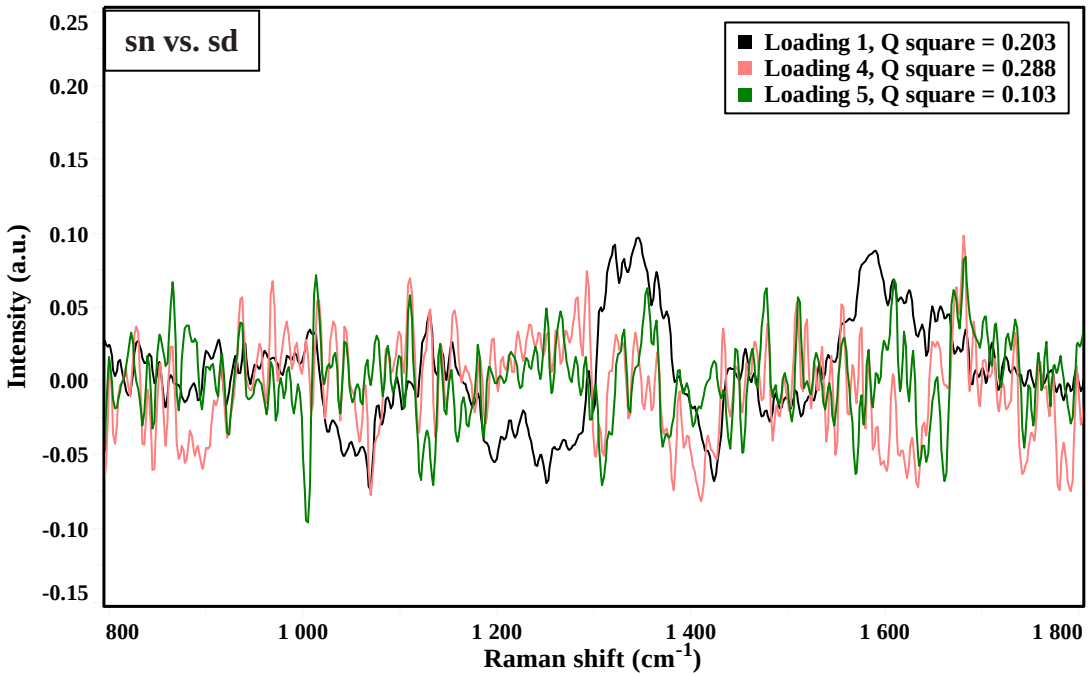
Loadings used for group classification between all morphologically normal against all dysplastic tissue samples.



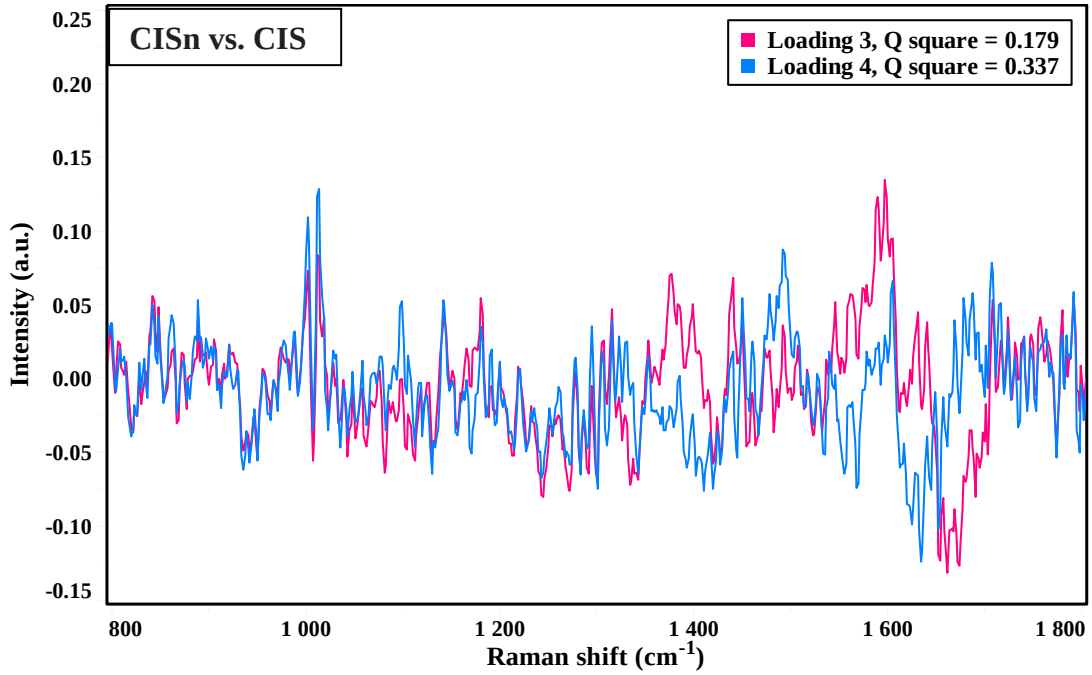
Loadings used for group classification between mild dysplasia and morphologically normal tissue samples.



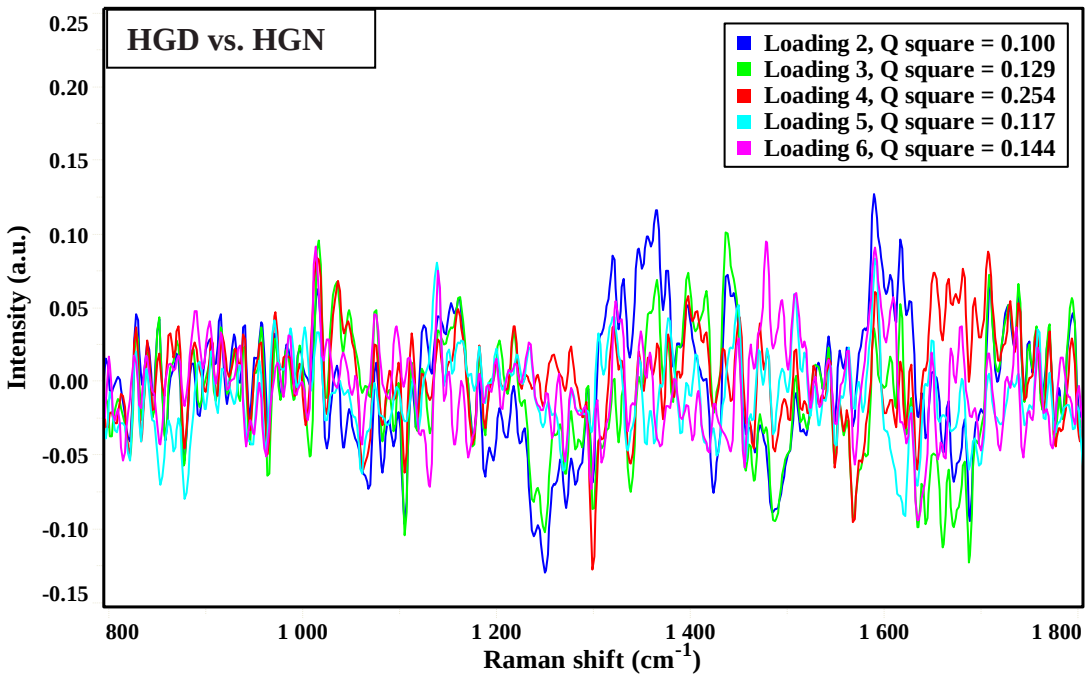
Loadings used for group classification between moderate dysplasia and morphologically normal tissue samples.



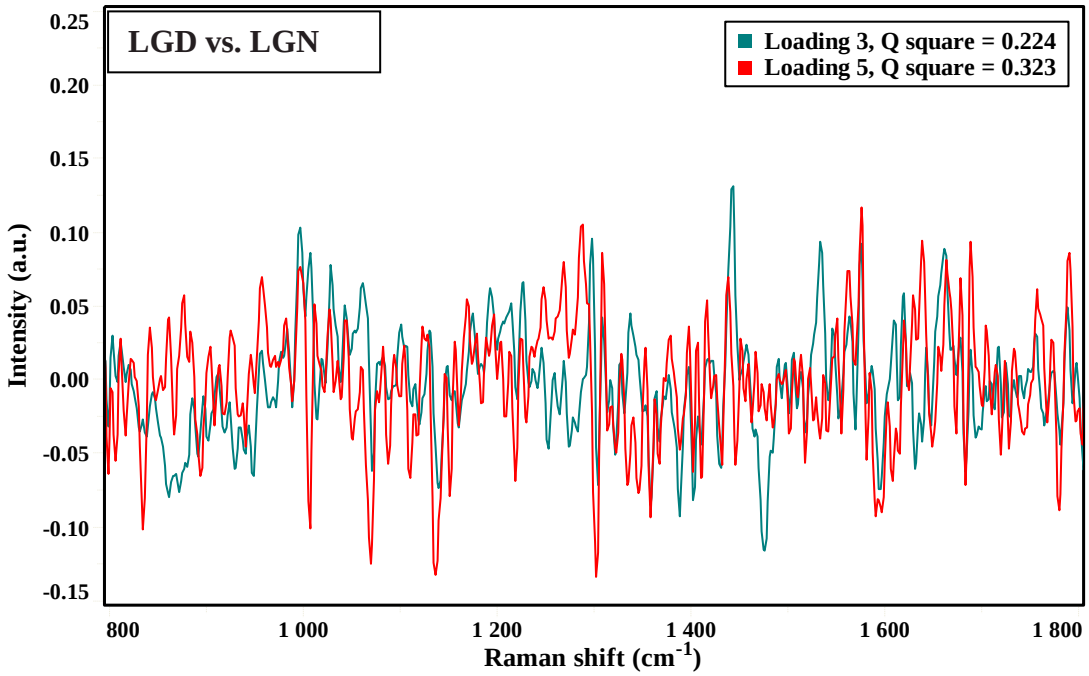
Loadings used for group classification between severe dysplasia and morphologically normal tissue samples.



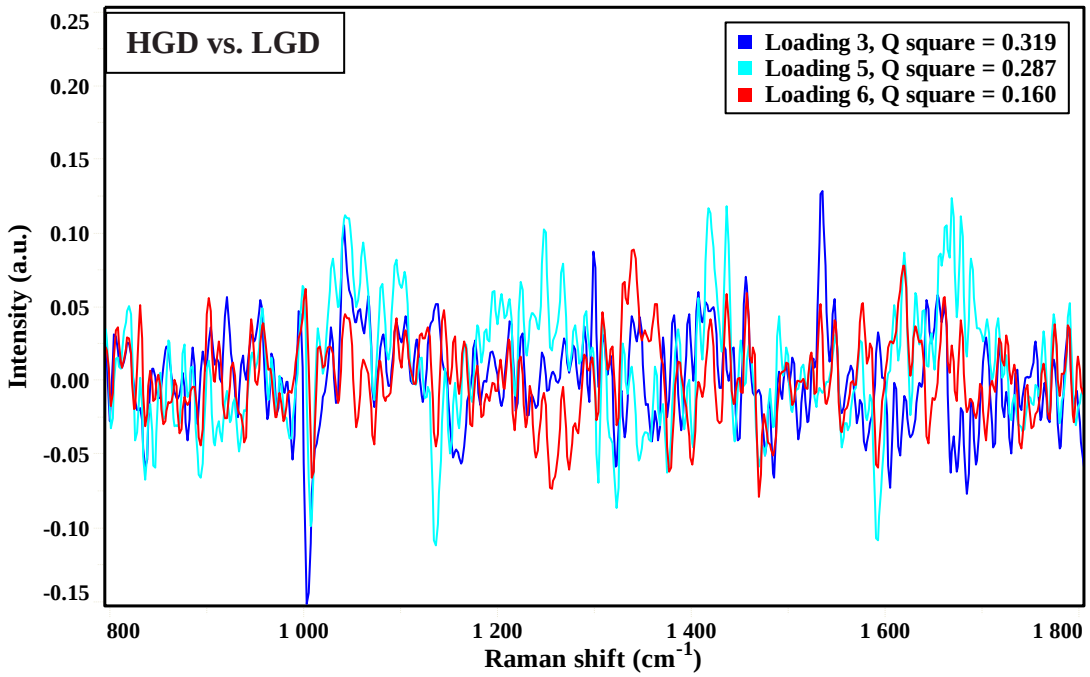
Loadings used for group classification between CIS and morphologically normal tissue samples.



Loadings used for group classification between high grad dysplasia and morphologically normal tissue samples.



Loadings used for group classification between low grad dysplasia and morphologically normal tissue samples.



Loadings used for group classification between high grade and low grade dysplasia tissue samples.