Cytokine regulation of Matrix Metalloproteinases
in Keratinocytes in Periodontal disease

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Thesis submitted in partial fulfilment of the requirements of
the regulations for the degree of Doctor of Philosophy

Newcastle University
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Institute of Cellular Medicine
February 2011
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<td>adipose receptor</td>
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<td>advance glycation end-products</td>
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<td>AP-1</td>
<td>activator protein</td>
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<td>BOP</td>
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<td>ECM</td>
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<td>epidermal growth factor</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>glucuronic acid</td>
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<td>GAG</td>
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<td>interleukin-1 beta</td>
</tr>
<tr>
<td>IL-R</td>
<td>interleukin receptor</td>
</tr>
<tr>
<td>INSR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>JE</td>
<td>junctional epithelium</td>
</tr>
<tr>
<td>LEBR</td>
<td>leptin receptor</td>
</tr>
<tr>
<td>LIFR</td>
<td>leukaemia inhibitory like receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAL</td>
<td>MyD88 adaptor- like</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactant protein-1</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation primary response gene (88)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor of kappa beta ligand</td>
</tr>
<tr>
<td>NK4</td>
<td>natural killer 4</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OKF6</td>
<td>oral mucosal keratinocyte</td>
</tr>
<tr>
<td>OSMR</td>
<td>oncostatin M receptor</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PBEF</td>
<td>pre-B cell colony-enhancing factor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>pocket depth</td>
</tr>
<tr>
<td>PDL</td>
<td>periodontal ligament</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td><em>Porphyromonas gingivalis</em></td>
</tr>
<tr>
<td>PMNL</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advance glycation-end product</td>
</tr>
<tr>
<td>RANTES</td>
<td>member of IL-8 super-family</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-PCR</td>
</tr>
<tr>
<td>SCF2</td>
<td>stem cell factor 2</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T1DM</td>
<td>type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TLDA</td>
<td>Tagman low-density array</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>TNFRSF1</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRAM</td>
<td>TNF receptor related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>toll IL-1 receptor- domain containing adaptor</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>V/V</td>
<td>volume in volume</td>
</tr>
<tr>
<td>W/V</td>
<td>weight in volume</td>
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</tbody>
</table>
Acknowledgements

The success of this thesis can be attributed to the advice and the encouragement from my supervisors Prof. Philip Preshaw and Dr. John Taylor. I deeply thank them for their valuable guidance in this research.

I would like to express a special appreciation to Dr. Emma Barksby for her valuable assistant in the laboratory work and also Dr. Katrin Jae dicke for her invaluable advice and thoughtful suggestions. Also I want to thank Dr. Simon Kometa for his expert advice on the statistical analysis.

I would like to thank my colleagues and friends in the Oral Biology Research Lab, especially Nine, Rana, Karen and Lesley for their help, support and encouraging attitude and also all the Oral Biology Staff, School of Dental Sciences for their friendship during my study.

For collection of the clinical data and samples, I want to thank the clinical team Rebecca Wassall, Susan Bissett and Kerry Stone. Also I would like to thank Dr. John Meechan, Oral Surgery department for helping in tissue sample collection.

I warmly wish to thank all of my family for their support and faith in me.

Most of all, I lovingly want to thank my husband Gamal, and my children, Honeda, Randa, Moheamen and Judy for constant love and support. Their sacrifices are really appreciated.
Abstract

Periodontal disease is an inflammatory disease caused by interactions between host responses and periodontal pathogens and is characterised by increased production of proinflammatory mediators and matrix metalloproteinases (MMPs). Type 2 diabetes mellitus (T2DM) is a risk factor for periodontal disease with upregulated cytokine and MMP secretion potentially contributing to the mechanistic links between the two conditions. This study aimed to investigate the effects of proinflammatory cytokines and \textit{P. gingivalis} lipopolysaccharide (LPS) on MMP expression in a human oral keratinocyte cell line (OKF6) and primary oral epithelial cells. MMP-8 and MMP-9 levels were investigated in GCF and serum samples from individuals with or without T2DM and periodontal health, gingivitis or periodontitis.

The study demonstrated that IL-1β-stimulated OKF6 and primary cells expressed MMP-1-7, 9-12, 14-17, 19, 25, and 28. No expression of MMP-8, 13, 20, 21, 23, 24, 26, and 27 was detected, however MMP-9 was upregulated in a time-dependent manner by IL-1β and TNF-α in OKF6 and primary oral epithelial cells at both the protein and mRNA level. IL-32 was a less potent inducer of MMP-9 than was IL-1β or TNF-α. IL-18 and \textit{P. gingivalis} LPS had no effect on MMP-9 production at the protein level in OKF6 cells.

GCF MMP-9 levels were significantly higher in diabetic individuals with gingivitis compared to non-diabetics with gingivitis. A significant reduction in GCF MMP-9 and MMP-8 levels following conventional periodontal treatment was observed in both diabetic and non-diabetic individuals with periodontitis. Serum MMP-9 levels were significantly higher in diabetic patients compared with non-diabetic individuals, suggesting an altered MMP-9 production. No significant changes were noted in serum
Abstract

MMP-9 levels in individuals with diabetes following periodontal treatment; however MMP-9 levels were reduced in non-diabetic individuals.

In conclusion, IL-1β, TNF-α and IL-32 can induce MMP-9 production in epithelial cells and this may influence periodontal disease progression. The findings from this study suggest a potential link between diabetes and periodontal disease through alteration of MMP-9 levels which could be potentially useful in monitoring periodontal disease in patients with diabetes.
Chapter 1    Introduction

1.1 Periodontal structure in health

The periodontium (supporting structures surrounding the teeth) consists of several tissues namely, gingiva, periodontal ligament (PDL), cementum and alveolar bone. All components function together as a single unit, though each structure is distinct in its location, function and composition (Nanci and Bosshardt, 2006). Normal and healthy periodontal tissues provide protection against bacterial infiltration and disease.

1.1.1 The gingiva

Gingiva is a specialised strip of oral mucosa which surrounds the cervical zone of the tooth and is attached to it and to the coronal part of the alveolar bone. Morphologically, the gingiva is categorised into three parts; free marginal, interdental and attached gingiva. As a part of the oral epithelium, the gingiva histologically is composed of surface epithelium and underlying connective tissue. The gingival epithelial cells constitute a primary structural defence against infection and are continually exposed to bacterial challenge (Darveau et al., 1997; Tonetti, 1997). Several cytokines are produced by epithelial cells such as growth factors, interleukin-1 (IL-1), and interleukin-8 (IL-8) that are important for neutrophil migration (Tonetti et al., 1994; Li et al., 1996). In addition, inter-cellular adhesion molecule-1 (ICAM-1) and E-selectin are also expressed by epithelial cells that are involved in neutrophil binding (Crawford, 1992; Pietrzak et al., 1996). Antimicrobial peptides such as defensin-α, defensin-β and cathelicidins (Hancock and Scott, 2000) that protect against the bacterial insult are also produced by epithelial cells. Histologically, the gingival epithelium is classified into oral epithelium, sulcular epithelium, and junctional epithelium (Bartold et al., 2000b).
The oral epithelium extends from the muco-gingival junction to the tip of gingival crest. The outer surface epithelium is stratified squamous epithelium composed of keratinocytes that are tightly bound to each other. The epithelium is keratinized and has four layers namely, the basal, spinous, granular and corneum layers. The main function of oral gingival epithelium is to protect the underlying structures from mechanical and chemical insult. It also plays an important role as the first barrier against bacteria and bacterial metabolic products (Sfakianakis et al., 2001a). Furthermore, epithelial cells are believed to signal to the underlying cells (Kagnoff and Eckmann, 1997), which is particularly intriguing in the context of periodontal disease.

The sulcular epithelium lines the gingival sulcus, and extends from just below the tip of the free gingival margin to the junctional epithelium. It is parakeratinized and relatively impermeable because of tight contacts between cells. The structure of the sulcular epithelium is similar to that of the oral gingival epithelium (Bartold, 2006).

The junction epithelium (JE) is a specialized portion of gingival epithelium which is bound to the tooth surface and consists of basal and supra-basal layers of non-keratinized, non-differentiating oral keratinocytes and it has granular and corneum layers (Bartold, 2006). The JE cells attach to the tooth or subepithelial connective tissue via the basal lamina by means of hemidesmosomes (Sawada and Inoue, 1996). The basal lamina functions to control the passage of molecules between the epithelium and the underlying connective tissue (Nanci, 2008). It has been reported that cells of the JE are permeable to sulcular fluid and bacterial toxins from bacterial plaque (Schroeder et al., 1989). Cells of the JE have a relatively weak bond to each other as a result of the high rate of cell turnover and this allows neutrophils to migrate into the sulcus to
perform a defensive role against the bacteria. The JE forms a structural barrier against noxious agents passing from the oral cavity into the tooth supporting tissues (Nanci, 2008). Thus, oral keratinocytes play an important role as the first barrier against bacterial products which are the cause of periodontal inflammation (Williams, 1990). The parts of gingival epithelium are illustrated in Figure 1-1.

The extracellular matrix (ECM) of the gingival tissue is composed of collagenous and non-collagenous proteins such as proteoglycans, glycosaminoglycans (GAGs) and glycoproteins (Embery et al., 2000). Salonen and colleagues reported that collagen type IV is present in the JE (Salonen et al., 1984) whereas collagen type I is the major component of periodontal connective tissues including the periodontal ligament and it provides attachment of the soft tissues to the tooth (Bartold, 2000). Proteoglycans and GAGs constitute a major group of the non-collagenous part of the ECM and function to maintain tissue integrity (Uitto and Larjava, 1991). Some proteoglycans have been identified on epithelial cells both internally and externally (Hakkinen et al., 1993), and they include hyaluronan, decorin, syndecan and CD-44. GAGs are the most important carbohydrate components of proteoglycans and the gingiva contains sulphate, chondroitin sulphate, heparan sulphate and hyaluronic acid (Bartold, 2000) which represent the four types of GAGs. The glycoprotein of the gingival tissue plays an important role in cell adhesion, migration and differentiation and they include laminin and fibronectin.
1.1.2 The periodontal ligament (PDL)

The periodontal ligament (PDL) is a specialized soft connective tissue localized between the cementum and the alveolar bone and mainly composed of fibrous tissue that consists of collagen fibres and ECM (Bartold et al., 2000a). The primary function of the PDL is anchorage of the teeth to the alveolar bone to withstand masticatory forces. In addition, PDL serves a remodelling function by providing cells that are able to produce all the tissues that form the attachment apparatus. PDL also provides a sensory function as it is richly supplied with nerve endings that are primary receptors for pain and pressure (Nanci and Bosshardt, 2006). Furthermore, the PDL has the capacity to adapt to the demands of functional changes. PDL includes differentiated cells such as fibroblasts, cementoblasts, ostoblasts, epithelial cells and connective tissue cells such as macrophages and mast cells (Mariotti, 1993). The predominant collagens in PDL are type I, III, and XII and collagen is arranged in distinct fibres bundles: the principal fibres, gingival ligament fibers, and elastic fibres (Cho and Garant, 2000). The portion of PDL fibers that are embedded in cementum and bone are called Sharpey’s fibres.

1.1.3 The cementum

Cementum is a specialized mineralized connective tissue that covers the root surface of the teeth. It has no blood or nerve supply and contains about 50% non-organic (hydroxyapatite) and 50% organic material (collagen and non-collagenous proteins). The cementum servers to anchor the principal collagen fibers of the PDL to the root surface and also has adaptive and reparative functions (Bosshardt and Selvig, 1997). The cementum may contain cells (cementoblasts) that may be activated during healing processes (Saygin et al., 2000). Four types of cementum have been identified according
to presence of the cells and the fibres namely, acellular afibrillar (located along the cement-enamel junction), acellular extrinsic fibers (located in cervical two thirds of the root), cellular intrinsic fibres (formed at sites of cementum repair), and cellular mixed fibres (found in the apical third of the root) (Nanci, 2008).

1.1.4 The alveolar bone

The alveolar process is that part of the jaw bone which forms the sockets of the teeth. It is composed of outer cortical plates, a central spongiosa and the bone lining the alveolus (alveolar bone). The cortical plate meets the alveolar bone at the alveolar crest and comprises lamellar and compact bone. Alveolar bone includes inner and outer parts, the inner aspect of the alveolar bone provides attachment for the PDL and is called bundle bone (Nanci and Bosshardt, 2006). It contains collagens (type I and III), active polypeptides (sialoprotein and osteopontin) and proteoglycans (Bartold, 1990; Waddington and Embery, 1991). The spongy bone occupies the central part of the alveolar process and also consists of lamellar and compact bone (Nanci, 2008).

1.1.5 Cells of the periodontium

Oral fibroblasts are the major resident cells inhabiting the periodontal tissues. Fibroblasts maintain and repair the connective tissue which supports and anchors the teeth by producing extracellular collagen fibers (proteins such as collagen and elastin) and ground substance (proteoglycans and GAG) (Hassell, 1993). The initial host response to bacterial infection causes activation, recruitment, and migration of infiltrating inflammatory cells, mainly polymorphonuclear leukocytes (PMNL), mast cells, macrophages, lymphocytes and plasma cells, to the sites of inflammation.
PMNL (neutrophils) are the first line of defense against bacterial infection and are major mediators of tissue destruction during inflammation (Weiss, 1989). Increased production of endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and ICAM-1 attract many PMNL to the site of the infection (Moughal et al., 1992). They secrete proteolytic enzymes including serine, elastase, cathepsin G and MMPs (Schuster et al., 1992; Birkedal-Hansen, 1993b). Stimulated cells secrete proinflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis-α (TNF-α), and proteinases such as matrix metalloproteinases-8 (MMP-8) and matrix metalloproteinases-9 (MMP-9) (Okamoto et al., 1997; McMillan et al., 2004; Owen et al., 2004).

Mast cells are thought to play an important role in the inflammatory process by secreting numerous cytoplasmic granules which contain a variety of inflammatory mediators such as histamine and heparin (Nanci, 2008). They are distributed throughout the oral mucosa and their numbers are increased in inflammation such as periodontitis, and the primary role of mast cells is thought to be in the innate defences against bacterial infections and in immediate hypersensitive reactions (Gemmel et al., 2004). Mast cells secrete TNF-α which induces production of adhesion molecules by endothelial cells that lead to migration of PMNL to the site of infection (Ohlrich et al., 2009). Mast cells can also strongly express matrix metalloproteinases (MMPs) (Naesse et al., 2003).

Macrophages are mononuclear leukocytes derived from monocytes and they are considered a key member of the innate immune system (Zembala et al., 1988). They are a key component of the cellular inflammatory infiltrate in inflamed periodontal tissue.
(Schroeder and Listgarten, 1997). The interaction between the epithelial cells and macrophages represents one of pathways involved in regulating local immune mechanisms in periodontitis (Topley et al., 1996). Macrophages participate in the inflammatory process by secretion of a variety of mediators such as cytokines and chemokines (Fujiwara and Kobayashi, 2005). For example, macrophages stimulated with Porphyromonas gingivalis (P. gingivalis) virulence factors secrete IL-1, IL-6, IL-8 and TNF-α (Hamada et al., 2002).

Lymphocytes are mononuclear leukocytes that are released at the site of infection to identify foreign substances and microbes and to produce antibodies. There are two types including B-cells which produce specific antibody to destroy foreign substances and T-cells that produce a number of substances that regulate the immune response. Lymphocytes can produce IL-1β, IL-6, IL-17, TNF-α and receptor activator of nuclear factor-κB (RANK) (Gillespie, 2007). T cells can be classified into Th1 and Th2 sub-sets and produce major histo-compatibility complex class II antigens (MHC class II antigen) that enable the antigen presentation to T cells (Ohlrich et al., 2009).

Plasma cells are IgG producing cells and involved in antibody-dependent autoimmune diseases (Kinane et al., 2001). They are correlated with the severity of periodontal disease and are considered the predominant cells in the advanced periodontal lesion (Younes et al., 2009).
Figure 1-1 A schematic drawing of the gingival epithelium

The gingival epithelium is composed of oral, sulcular, and junctional epithelium. Taken from (Lindhe et al., 2008).
1.2 Periodontal tissue destruction

Periodontal tissue destruction results from a complex interplay between the host response and specific bacterial plaque that contributes to development of an inflammatory response in the periodontal tissue (Socransky et al., 1999; Graves and Cochran, 2003). Periodontal disease results from imbalance between destruction and repair of periodontal tissue (Matsuki et al., 1992; Kornman, 1997). Both the innate and acquired immune response have roles in periodontal disease progression (Teng et al., 2000) and the immune response mounted against the bacterial challenge results in release of host inflammatory mediators that result in destruction of the periodontal tissues.

1.2.1 Definition and classification of periodontal diseases

Periodontal diseases are a group of inflammatory disorders that result from the host response to bacteria in dental plaque (Bartold et al., 2000b; Lamont and Yilmaz, 2002). They affect the supportive tissues of the teeth (gingiva, PDL, cementum and alveolar bone) and are associated with accumulation of bacterial plaque on the tooth surface. The oral pathogens directly contact the oral epithelial cells (Williams, 1990) and this can result in a localized inflammatory response known as gingivitis (Page, 1986). It was demonstrated that the bacterial biofilm initiates the gingival inflammation (Haffajee and Socransky, 1994) and eventually, untreated disease may lead to irreversible loss of the tooth supporting structures (Potempa et al., 2000). The chronic response to subgingival plaque leads to tissue damage that is characteristic of periodontitis (Page, 1998). Progression of tissue destruction leads to pocket formation between the teeth and the surrounding tissues and teeth may become loose. Gingivitis and periodontitis have been described as the most prevalent inflammatory conditions known to man (Albandar
and Tinoco, 2002; Southerland et al., 2006) and gingivitis affects 50-90% of adults worldwide depending on the precise definition (Brown and Loe, 1993; Albandar and Tinoco, 2002; Pihlstrom et al., 2005). It is estimated that in developed countries about 15-35% of adults have periodontitis (Albandar et al., 1999; Papapanou, 1999; Hugoson et al., 2005).

The classification of periodontal disease is mainly based on the infection and host response concept (Armitage, 2002). Several diagnostic categories were implemented by the 1999 International Workshop for the Classification of Periodontal Disease and Conditions and have been accepted by American Academy of Periodontology (Armitage, 1999). The classification highlights two main categories: gingival diseases and periodontitis and periodontitis is subdivided into chronic periodontitis, aggressive periodontitis, periodontitis as a manifestation of systemic disease, necrotizing periodontal disease, abscesses of the periodontium, periodontitis associated with endodontic lesions, and developmental or acquired deformities and conditions. The periodontal diagnosis is mostly based on clinical examination. Clinical signs and symptoms, clinical attachment loss (CAL), pocket depth (PD), radiographic evaluation, microbe cultivation, personal habits, clinical observation (tooth movement, plaque, calculus), and the medical and dental history all contribute to the diagnosis and classification (Armitage, 2003).
1.2.2 Aetiology and pathogenesis of periodontal disease

Bacteria are the primary initiating agent in periodontal disease and more than 500 bacterial species may be found in dental plaque (Kroes et al., 1999). In gingivitis, the supragingival bacterial plaque primarily contains Gram-positive aerobic bacteria subsequently, the composition changes to an anaerobic Gram-negative flora (Socransky and Haffajee, 2005). The disease has been reported to be initiated by Gram-negative anaerobic bacteria such as Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia (Darveau et al., 1997). P. gingivalis is strongly implicated in periodontal diseases pathogenesis and it produces a range of proteases (Kadowaki et al., 2000). These enzymes may either catalyse tissue destruction directly or activate host MMPs. Bacterial virulence factors and surface components such as lipopolysaccharide (LPS) and fimbrial proteins are associated with the development of periodontal diseases (Haffajee and Socransky, 1994; Wilson et al., 1996). Furthermore, they may activate host defence cells which, in turn, produce and release inflammatory mediators that stimulate various destructive pathways (Dahan et al., 2001).

The host response is represented by the interaction between the periodontal pathogens and the gingival epithelium that leads to increased expression of many inflammatory mediators such as cellular adhesion molecules and pro-inflammatory cytokines. Dixon and co-workers (2004) reported that accumulation of periodontal pathogens in the gingival sulcus leads to expression of ICAM-1 and E-selectin on the surface of endothelial cells and secretion of IL-8 and ICAM-1 by gingival epithelium that triggers migration of neutrophils from the connective tissue into the gingival sulcus to combat the bacterial infection. The persistence of the pathogens results in chronic expression of ICAM-1 and IL-8 that are chemotactic for the neutrophils, thus neutrophils accumulate...
in the gingival tissues and release destructive enzymes, resulting in structural damage (Dixon et al., 2004). Neutrophils also produce specific mediators such as oxygen radicals and enzymes that further contribute to tissue damage (Kantarci et al., 2003). In addition, the epithelial cells respond to the oral pathogens by increased cell proliferation, and activate immune responses (Dale, 2002). The immune response to periodontal disease involves recruitment of several cell types to the site of inflammation such as PMNL, T-lymphocytes, B-lymphocytes, macrophages and plasma cells (Pihlstrom et al., 2005). LPS has the ability to induce proinflammatory cytokines by stimulating immune cells (Madianos et al., 2005). These inflammatory mediators stimulate connective tissue damage and bone resorption (Teng, 2003).

The cells of the periodontium respond to LPS and fimbriae by expressing cell surface pattern recognition receptors (PRRs). One group of these receptors is known as the Toll-like receptors (TLRs) (O'Neill, 2006). TLRs are expressed on the surface of innate immune cells such monocytes, macrophages and are also expressed on other cell types including epithelial cells (Lebre et al., 2007). It has been shown that both TLR2 and TLR4 are upregulated in response to bacterial insult within the periodontal tissue (Hirschfeld et al., 2001; Mochizuki et al., 2004). Fibroblasts constitutively express TLR2 and TLR4 and the level of their expression is augmented after stimulation with P. gingivalis LPS (Tabeta et al., 2000). It has previously been shown that P. gingivalis LPS is recognised by both TLR4 and TLR2 (Darveau et al., 2004). It was demonstrated that activation of toll like receptors (TLRs) by P. gingivalis induces release of pro-inflammatory cytokines from human primary monocytes (Eskan et al., 2007). These interactions result in the activation of intracellular signalling pathways. The most notable pathways are the nuclear factor-kappa B (NF-κB) and mitogen activated protein
kinase (MAPK) pathways which induce transcription of key inflammatory cytokines. Thus stimulation of TLRs activates production of IL-1β and TNF-α (Page, 1998; Takeuchi et al., 1999; Gemmell and Ford, 2002).

Cluster of differentiation 14 (CD14) and TLR4 play an important role in LPS-induced cellular responses (Song et al., 2001). It has been demonstrated using flow cytometry that human keratinocytes express functional CD14 and TLR4 (Song et al., 2002). *P. gingivalis* fimbriae are detected by CD14 and TLR2, resulting in activation of monocytes (Hanemaaijer et al., 1997; Ogawa et al., 2002). It was further reported that *P. gingivalis* fimbriae can stimulate macrophages in the presence of CD14 (Hajishengallis et al., 2006). Conversely, it was reported that human gingival epithelium does not respond to LPS and fimbriae of *P. gingivalis* even in the presence of soluble CD14 (Uehara et al., 2001; Eskan et al., 2007).

Kusumoto et al investigated the expression of TLR2 and TLR4 in human gingival epithelial cell lines and primary epithelial cells using reverse transcription-polymerase chain reaction (RT-PCR) (Kusumoto et al., 2004). The results revealed that TLR2 and TLR4 are expressed by the epithelial cell lines; however, the expression of TLR4 was very slight in the primary epithelial cells. They also examined the response of the human gingival epithelial cell lines to *P. gingivalis* and *P. gingivalis* components. It was found that *P. gingivalis* augmented the production of IL-8 in primary gingival human epithelial cells. On the other hand, neither *P. gingivalis* fimbriae nor LPS stimulated secretion of this cytokine. In another study, expression of CD14, TLR2 and TLR4 was examined in human gingival epithelial cells (HGE). The results revealed that the cells expressed TLR2; however neither CD14 nor TLR4 were detected (Asai et al., 2001).
Therefore, there was no clear consensus regarding the responsiveness of the epithelial cells to *P. gingivalis* and further study is needed.

Proinflammatory cytokines play an important role in numerous biological activities such as proliferation and development of many cell types (Okada and Murakami, 1998). Inappropriate cytokine production may lead to deleterious destructive effects in the periodontal tissues and disease progression (Gemmell and Seymour, 2004). Soft and hard tissue destruction during periodontitis is the end result of many complex events involving bacterial virulence factors, pro-inflammatory cytokines and MMPs (Sorsa *et al.*, 2006). Cells of the immune system as well as resident cells such as keratinocytes are involved in the production of cytokines at sites of inflammation (Okada and Murakami, 1998). It has been reported that levels of pro-inflammatory cytokines increase in gingiva, gingival crevicular fluid (GCF) and saliva of patients with gingivitis, periodontitis, and diabetes (Bartold and Narayanan, 2006) and these cytokines may affect different cell types such as neutrophils, fibroblasts and keratinocytes. These cytokines may include IL-6, IL-8, IL-1β and TNF-α (Wilson *et al.*, 1996; Okada and Murakami, 1998) and uncontrolled production may lead to leukocyte recruitment and tissue damage. Cytokines, which are the key mediators of inflammatory and autoimmune processes, play a critical role in the regulation of MMPs and tissue inhibitor of MMP (TIMP) expression in different cell types. It has been suggested that cytokines may differentially influence the profile of MMPs expressed and their ability to degrade the ECM (Lacraz *et al.*, 1992; Chizzolini *et al.*, 2000). For example, IL-6 plays a major role in activation (Ishimi *et al.*, 1990) and proliferation of T-cells. IL-8 is an important chemo-attractant for PMNL (Baggiolini *et al.*, 1994).
IL-1 is a potent pro-inflammatory cytokine produced by different cell types such as lymphocytes, monocytes and fibroblasts (Kang et al., 1996) and is involved in many important cellular functions, such as proliferation, activation, and differentiation (Yang et al., 2003). It is an important component of the innate immune response (Dinarello, 1996) and also induces leukocyte migration by induction of IL-8 and activating neutrophils to phagocytosis (Cohen, 2002). 3 types comprise IL-1; IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra) (Dinarello, 1996). IL-1β is involved in initiation of inflammatory and immune responses, including upregulation and activation of vascular adhesion molecules (Meager, 1999) and activation of the killing capacity of inflammatory cells. In addition, IL-1β induces expression of IL-8 in many cell types to recruit PMNL into the area of inflammation (Stoeckle, 1991). It was demonstrated that IL-1β has a stimulatory effect on expression of MMP-9 in cultured corneal epithelial cells (Li et al., 2001) and also there is a possible role of bacterial proteases and LPS in this induction (Miyajima et al., 2001). It was found that mice treated with an anti-IL-1β antibody showed a significant reduction in MMP-9 along with a reduction in corneal damage (Xue et al., 2003).

Gingival epithelial cells are a major source of IL-1β in the periodontium (Sfakianakis et al., 2001a) and can regulate the production of MMPs and TIMPs (Reynolds, 1996). Furthermore, it has been demonstrated that IL-1α and IL-1β are involved in the pathogenesis of periodontitis (Boch et al., 2001; Johnson and Serio, 2005). Increasing levels of IL-1β in GCF are correlated with an increased occurrence of periodontal diseases (Stashenko et al., 1991; Kinane et al., 1992) and also induce production of other cytokines such as IL-6 and IL-8 (Katz et al., 2001). Moreover, IL-1β can stimulate a variety of cell types to produce destructive mediators such as TNF-α and
MMPs. IL-1β is known as one of the most potent inducers of MMP-1 and MMP-3 in fibroblasts (Tewari et al., 1994). Bronson et al. have shown an increase of IL-1β production in wounded rabbit oral mucosa compared with normal mucosal cells (Bronson et al., 1989) and this finding suggested that MMP and IL-1β are involved in tissue degradation in periodontal lesions. It was reported that IL-1β is a biomarker for periodontal diseases (Kinane et al., 1992) and that excessive production of IL-1β by periodontal tissue cells may induce the gingival and PDL fibroblasts to produce other cytokines, matrix degrading enzymes and prostaglandins which lead to further connective tissue destruction (Makela et al., 1998). A notable property of IL-1β in the pathogenesis of periodontitis is stimulation of the production of MMPs, which are able to degrade the extracellular matrix macromolecules (van der Zee et al., 1997). For example, treatment of fibroblasts with IL-1β upregulates MMP-3 at both the protein and transcript levels (Nakaya et al., 1997).

TNF-α plays a critical role in resistance to a wide range of microbial pathogens, contributing to both innate and adaptive immune responses (Graves and Cochran, 2003) and is considered a key mediator of inflammation (Locksley et al., 2001). This cytokine is secreted mainly by macrophages, and in smaller quantities by T-lymphocytes (Dinarello, 1992) and mast cells (Gordon and Galli, 1990) that are present in inflammatory infiltrates. In addition, it acts in several steps of leukocyte recruitment mechanisms, inducing the upregulation of adhesion molecules, and the production of MMPs (Pfizenmaier et al., 1996; Dinarello, 2000). Furthermore, TNF-α upregulates the bactericidal activity of phagocytes and also it stimulates MMP production in many cells including keratinocytes (Birkedal-Hansen, 1993a). It was shown that TNF-α regulates MMP-9 expression in keratinocytes which stimulates their migration (Scott et al.,
Previous studies have shown that production of TNF-α stimulates the degradation of the extracellular matrix by augmenting MMP expression (Meikle et al., 1992), and even short exposure to TNF-α may lead to increased production of MMPs (Makela et al., 1998).

Experimental evidence indicates that TNF-α plays a role in the resistance to microbial pathogens (Tartaglia and Goeddel, 1992; Ferrante et al., 1993) and actions of pro-inflammatory cytokines, including TNF-α, are important in the pathogenesis of periodontal disease (Graves and Cochran, 2003). Interestingly, this cytokine has been reported to upregulate the expression of gelatinase by monocyte-derived macrophages at the mRNA level (Saren et al., 1996) and also induce production of interstitial collagenase and stromelysin by fibroblasts (Meikle et al., 1989). Moreover, it was reported that TNF-α and IL-1β have a direct effect on MMP-1 and MMP-3 production in gingival fibroblasts (Beklen et al., 2007). Exposure of keratinocytes to TNF-α for 24 hours leads to a rise in MMP-9 expression at the transcript and protein levels (Makela et al., 1998). The effect of TNF-α and IL-1β on MMP-9 production was investigated using human renal epithelial cells (Nee et al., 2004). The results of the study demonstrated that epithelial cells responded to TNF-α with an increase in MMP-9 production however, co-incubation with IL-1β completely abolished induction of MMP-9. It can be concluded that IL-1β and TNF-α can modulate ECM turnover in epithelial cells by regulation of MMP expression and the different combinations of cytokines present in the inflammatory state may therefore influence the outcome of disease depending on MMP expression.
In recent times, several new cytokines have been characterised and little is known about their function during inflammatory and immune reactions. Although the role of certain cytokines is well documented, the impact of these newly discovered cytokines such as interleukin-32 (IL-32), and interleukin-18 IL-18) on production of MMPs in periodontal disease is unknown. Further studies are required to investigate the function of these cytokines in inflammation and to determine whether or not they play a role in periodontal disease.

IL-18 is a pro-inflammatory cytokine that belongs to the IL-1 family. It has a wide range of immune-regulatory functions and is considered to be associated with chronic inflammation and autoimmune disease. This cytokine was originally identified as an interferon gamma (IFN-γ) inducing cytokine produced by natural killer and T cells. IL-18 acts synergistically with IL-12 to promote IFN-γ production and Th1 but does not depend on IL-12 for its activity (Okamura et al., 1995; Nakanishi et al., 2001; Biet et al., 2002; Lotze et al., 2002). IL-18 is produced by macrophages (Okamura et al., 1995; Ghayur et al., 1997) and also expressed by osteoblasts (Udagawa et al., 1997), chondrocytes (Olee et al., 1999) and keratinocytes (Stoll et al., 1997). Previous studies have shown that the basal keratinocytes constitutively express the pro-IL-18 protein, but whether the pro-IL-18 is processed and released as the mature and active form is still controversial (Naik et al., 1999; Mee et al., 2000). IL-18 is always produced as a biologically inactive precursor whose activation requires processing by the cysteine protease caspase-1 (Fantuzzi and Dinarello, 1999). Although IL-18 is thought to be a primary stimulant for Th1 cytokine production, particularly IFN-γ, some studies have implicated a broader role for IL-18 in inflammation by inducing chemokines (Purow et al., 1998). Interestingly, it has been reported that the expression of IL-18 is markedly
increased in mature keratinocytes (Kong and Li, 2002) and has been linked to increased cell proliferation in the skin (Park et al., 2001). Pro-inflammatory cytokines including IL-1β, TNF-α, IL-6 as well as LPS, induce IL-18 gene expression (Dinarello, 1999; Nakanishi et al., 2001). It has been reported that IL-18 may, in part, play a role in the clinical aggressiveness of human myeloid leukaemia by stimulating MMP-9 production (Zhang et al., 2004). IL-18 induces matrix degradation by upregulation of MMP-2 and MMP-9 in human coronary artery smooth muscle cells (Chandrasekar et al., 2006).

Furthermore, IL-18 induces production of MMPs from chondrocytes in inflammatory arthritis (Dai et al., 2005). It was reported using gelatine zymography that IL-18 enhances MMP-2 expression in monocytes (Abraham et al., 2002). Data from rheumatoid arthritis (RA) research indicate that IL-18 induces release of MMP-9 and IL-1β that have both pro-inflammatory and tissue degradation effects (Jablonska et al., 2002). It was reported that IL-18 is associated with the severity of periodontal disease (Orozco et al., 2006) and gingival tissues from individuals with periodontal disease contain high concentrations of IL-18 (Johnson and Serio, 2005). There is substantial information concerning the association of IL-18 with various inflammatory diseases (Gracie et al., 2003), however, there is little information available concerning the role of the IL-18 in initiation and progression of periodontal disease.

IL-32 is a recently described cytokine produced by T-lymphocytes, natural killer cells, epithelial cells and blood monocytes (Kim et al., 2005; Netea et al., 2005). IL-32 was first reported in 1992 after identification of a complementary DNA (cDNA) from human natural killer (NK) and T-cells stimulated with IL-2 and originally called NK cell transcript 4 (NK4) (Dahl et al., 1992). However, the functions of this molecule are not known until 2005, when recombinant human IL-32 was shown to activate p38
MAPK and NF-kB signal transduction pathways as well as induce cytokines and chemokines from human and mouse cells (Kim et al., 2005). Of particular importance, IL-32 is induced by IFN-γ in epithelial cells and monocytes (Kim et al., 2005). In addition, epithelial cells are a widespread sources of IL-32, Although epithelial cells from healthy subjects express low levels of IL-32, IL-32 expression can be induced in human epithelial cells by IFN-γ (Kim et al., 2005). Human recombinant IL-32 exhibits several properties typical of a pro-inflammatory cytokine as this cytokine induces other pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6, and IL-8 (Kim et al., 2005; Netea et al., 2005). It was suggested that IL-32 is strongly associated with TNF-α, IL-1β, and IL-18 and appears to play a role RA (Joosten et al., 2006). In experimental mice models of arthritis, injection of IL-32 induces joint inflammation (Shoda et al., 2006). These studies suggest that IL-32 has an important role in inflammation both during host defences against microorganisms and in auto-immune diseases. Therefore further studies are needed to investigate its role in periodontal disease.
1.2.3 Role of epithelial cells in periodontitis

Epithelial cells express cytokines and chemokines in response to pathogens that attract and activate neutrophils and express adhesion molecules that mediate neutrophil migration and activity (Huang et al., 2001). In addition, epithelial cells may present antigen and provide molecules that participate in the stimulation of monocytes (Lawson et al., 2000). During inflammation, the epithelial cells encounter different extracellular components and mediators that are produced by inflammatory and immune cells, and they respond to these stimuli by proliferation, migration and production of enzymes to maintain their structural integrity (Makela et al., 1998). In addition, they respond to stimuli by producing TLRs and secreting pro-inflammatory cytokines (Kinane et al., 2006).

The role of gingival keratinocytes was investigated using primary cells (Sugiyama et al., 2002). The cells were stimulated with LPS from Prevotella intermedia (P. intermedia), Escherichia coli (E. coli), and LPS and fimbriae from P. gingivalis for 8 hours. Both P. intermedia and E. coli lacked the ability to activate the leukocytes whereas P. gingivalis activated production of IL-8, granulocyte colony-stimulating factor (G-CSF) and ICAM-1 in gingival keratinocytes. Eskin and colleagues reported that TLR4 expression on the surface of epithelial cells is important for responsiveness to P. gingivalis and production of proinflammatory cytokines (Eskin et al., 2008).
It has been reported that periodontal pathogens activate monocytes and macrophages that induce production of pro-inflammatory mediators (Zadeh et al., 1999). Fimbriae and LPS can stimulate keratinocytes to secrete pro-inflammatory cytokines through the activation of TLRs and gene transcription factors (Murphy et al., 2000; Kusumoto et al., 2004). *P. gingivalis* can stimulate secretion of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8 in epithelial cells (Sandros et al., 2000; Bodet et al., 2005). It has also been reported that IL-8 is expressed in gingival epithelium both in primary gingival tissue and in human gingival keratinocyte cell lines (Sfakianakis et al., 2002). Furthermore, IL-8 is expressed by keratinocytes in chronically inflamed gingival tissues whereas no expression of IL-8 could be observed in healthy tissue (Jarnbring et al., 2000). Dayan and co-workers reported that periodontal disease is generated in mice who express IL-1β in oral epithelial tissues despite an absence of bacterial infection (Dayan et al., 2004). In addition, IL-1β and IL-6 levels in GCF increase during periodontal disease (Lee et al., 1995) and gingival keratinocytes isolated from chronically inflamed gingiva can produce IL-6 (Yamamoto et al., 1994). Therefore, gingival epithelial cells are a major source of IL-1α and IL-1β in the periodontium which, in turn, induce additional inflammatory mediators such as IL-8 (Sfakianakis et al., 2001b). Conversely, a previous study has investigated the mechanisms that regulate expression of IL-8 and ICAM-1 in gingival epithelial cells following interactions with several periodontal pathogens. The results showed a reduction in IL-8 and ICAM-1 after treatment with *P. gingivalis* (Huang et al., 2001). Therefore, the interaction between periodontal pathogens and epithelial cells results in disturbance of epithelial cell functions which may contribute to periodontal tissue destruction through cytokine production.
1.2.4 Susceptibility and risk factors for periodontal disease

It has been reported that although pathogens are important in the initiation of periodontal disease, they are inadequate alone for the disease to occur (Page and Kornman, 1997). Cell responses and environmental factors conversely, have an effect on the severity of the disease and the response to the treatment. Risk factors for periodontal disease may include smoking, age, oral hygiene, genetic factors and systemic diseases such as diabetes (Page and Beck, 1997; Salvi et al., 1997; Kinane, 2001). Additionally, periodontal disease is influenced by each individual's immune and inflammatory response in combination with a wide range of environmental and behavioural factors (Offenbacher et al., 2008). Smoking is strongly associated with the severity and prevalence of periodontal disease (Albandar, 2002). It has been reported that smokers are at risk for advanced forms of the periodontal diseases and that their response to periodontal treatment is reduced compared to non-smokers (Renvert et al., 1998; Calsina et al., 2002). Smoking has an effect on periodontal tissue through the adverse effects of nicotine and other constituents of smoke on immune and inflammatory responses and also by reducing peripheral blood flow (Kinane and Chestnutt, 2000). There is a growing body of evidence which suggests smokers have greatly increased periodontal tissue destruction (Grossi et al., 1995; Machtel et al., 1999) however, no significant differences in plaque and gingival indices were detected between smokers and non-smokers (Salvi et al., 2005). A number of systemic disorders increase patient susceptibility to periodontal disease for example, individuals with uncontrolled diabetes are at higher risk for periodontal disease (Tervonen and Oliver, 1993; Soskolne and Klinger, 2001). It has been reported that people with increased susceptibility to periodontal disease may have a genetic phenotype that increases the risk of inflammation-induced tissue destruction (Kinane and Hart, 2003). Furthermore,
twin studies suggested that approximately 50% of the population variance for periodontal disease may be due to genetic factors (Kinane et al., 2005).

1.2.5 Treatment of periodontal disease

Periodontal treatment should initially start with modifying the risk factors where possible and giving education for maintaining good oral hygiene (Ryan, 2005). Periodontal disease is initiated by pathogens and inflammatory processes and the majority of treatment is based on removal of bacteria (Quirynen et al., 2000; Preshaw, 2008b). The conventional non-surgical treatment is considered the first line in periodontal treatment and takes the form of root surface instrumentation (RSI) (Cobb, 2002). RSI is an efficient treatment method for individuals who have CAL <5 mm and probing depths of 4 to 6 mm (Greenstein, 1992; Cobb, 1996). Full month RSI was suggested as an early management for periodontal disease (Lang et al., 2008). Improvements in clinical parameters one month after RSI have been reported (Drisko et al., 1995). In many patients non-surgical treatment alone may be sufficient to result in clinical improvements and control of periodontal disease (Preshaw, 2008b), however other patients have a more limited response to the conventional treatment. Because periodontal tissue destruction observed during periodontal disease is a consequence of the action of both the bacterial and the host derived mediators, a combination of treatment approaches may present the best chance for clinical improvements (Preshaw et al., 2004).
1.3 Diabetes mellitus

Diabetes mellitus (DM) is a commonly encountered systemic condition that manifests with abnormally high levels of blood glucose. The disease is characterized by hyperglycaemia which is developed from either a deficiency in insulin secretion or impaired cellular resistance to the action of insulin that may be complicated by poor regulation of lipid metabolism (Mero et al., 1998). The hyperglycaemia is always accompanied by hyperlipidaemia (Kim et al., 1993) and is associated with a number of complications that affect the small blood vessels leading to retinopathy, neuropathy, nephropathy, angiopathy, atherosclerosis, periodontitis and impaired wound healing (Mealey and Ocampo, 2007). It has been estimated by the International Diabetes Federation that worldwide 246 million people have diabetes with further growth to 380 million by 2025 (Levitt, 2008).

1.3.1 Classification of diabetes mellitus

The new classification system by the American Diabetes Association (ADA) identifies five types of diabetes as follows:

- Type 1 diabetes mellitus (T1DM) formerly known as insulin dependent diabetes, this form is characterized by hyperglycaemia resulting from definitive deficiency in insulin secretion caused by auto-immune illness and genetic factors (American Diabetes Association, 2006). It is unknown what first starts this cascade of immune events, but evidence suggests that environmental factors such as an abnormal response to an enterovirus may trigger the autoimmune processes that lead to the destruction of the β-cells of pancreatic islets (Graves et al., 1997; Harrison and Honeyman, 1999). The genetic variants associated with T1DM provide either susceptibility to or protection from the disease and the
The interplay between genetic susceptibility and environmental factors is thought to provide the fundamental element for disease prediction and prevention (Atkinson and Eisenbarth, 2001). This form of diabetes tends to occur in young individuals, and 75% of T1DM cases are diagnosed in persons under the age of 18 years and represent only 5% of the patients with diabetes (Daneman, 2006).

- Type 2 diabetes mellitus (T2DM) formerly known as non-insulin dependent diabetes. In this form of the disease, the β-cells of the pancreas can still produce insulin but there is a lack of sensitivity to insulin by the cells of the body which alters the utilization of the insulin at the target cells (Scannapieco, 1998; Kahn et al., 2006; Preshaw et al., 2007). This is the most common form of the disease and is highly associated with a family history of diabetes (Pierce et al., 1995; Weill et al., 2004). It affects middle aged or older people and is predominant in individuals who are physically unfit and obese (King and Rewers, 1993).

- Gestational diabetes mellitus (GDM) is carbohydrate intolerance with first detection during pregnancy and is associated with significantly increased risk of maternal and infant morbidity (Kjos and Buchanan, 1999; Xiong et al., 2001). Women with GDM are at greater risk of experiencing T2DM later in life (Mealey and Ocampo, 2007).

- Other specific types are forms of diabetes with various known aetiology that are grouped together. This group includes persons with genetic defects of the β-cell, defects of insulin action, disease of the exocrine pancreas such as pancreatitis, dysfunction associated with endocrinopathies like acromegaly and pancreatic dysfunction caused by drugs, chemicals or infections (Mealey and Ocampo, 2007).
• Impaired glucose homeostasis is a metabolic stage intermediate between normal glucose control and diabetes. It includes impaired glucose tolerance (the plasma glucose is higher than the normal but less than level established for DM following administration of glucose) and impaired fasting glucose (the fasting plasma glucose is higher than the normal and less than the level established for DM). Both are predictors for T2DM and cardiovascular diseases (DECODE, 1999). The normal glycaemic level is below 110 mg/dL and assessed using fasting plasma glucose concentration or the 2 hour oral glucose tolerance test. However these parameters are varied due to physical activity and therefore glycated haemoglobin (HbA1c) has been used clinically as a marker of long term diabetic control (Lalla et al., 1998). It reflects blood glucose concentration averaged over the previous six to eight weeks (Nathan et al., 2007).

1.3.2 Aetiology and pathogenesis

Although the aetiology of diabetes is still not fully understood, the hallmark of T1DM is the selective destruction of insulin producing cells in the pancreas that may account for absolute insulin deficiency (Akerblom et al., 2002). Genetic predisposition and environmental insults such as viral infection could alter the immune response against normally functioning β-cells that triggers autoimmune responses (Atkinson and Eisenbarth, 2001). The β-cells destruction includes production of autoantibody to the islet cells, to insulin, to glutamic acid decarboxylase (GAD) and to tyrosine phosphate which is followed eventually by the loss of the first phase insulin response. In addition, the presence of insulinitis with infiltrating T-lymphocytes, B-lymphocytes and macrophages at the onset of T1DM represents the role of the inflammatory cells in β-cells destruction (Imagawa et al., 1999). Release of cytokines such as TNF-α and IL-1β
by macrophages induces structural changes of β-cells and suppression of insulin releasing capacity (Wogensen et al., 1990; Mandrup-Poulsen, 1996).

T2DM may be caused by both impaired insulin release and insulin resistance and there is a risk for the disease development in both children and adult due to obesity and lifestyle changes (Preshaw, 2008a). It has a greater genetic association than T1DM that approximately accounts for 40-80% of the entire disease variability (Holt, 2004). A majority of individuals suffering from T2DM are obese and obesity plays a crucial role in developing insulin resistance (Kahn et al., 2006). In recent years, the general concept has emerged that chronic activation of the proinflammatory pathways in adipose tissue, liver and muscle are linked to development of insulin resistance (Eckel et al., 2005). Furthermore, chronic inflammation is suggested to be important in enhancing insulin resistance and may contribute to insulin deficiency (King, 2008). The existence of chronic inflammation in diabetes is mainly based on the increased plasma concentrations of C-reactive protein (CRP), fibrinogen, IL-6, IL-1β and TNF-α which can be reduced with intensive lifestyle intervention (Haffner et al., 2005). Adipose tissue has emerged as an important player in modulating metabolism by releasing cytokines (IL-6, TNF-α, IL-1β) and adipokines (leptin, adiponectin and resistin) (Donath et al., 2003) and all of these molecules can exert potential negative or positive impacts on insulin sensitivity and β-cell function (Rajala and Scherer, 2003). Furthermore, adipose tissue is believed to be a dynamic endocrine organ that produces many physiological molecules which regulate appetite and food consumption (Trayhurn and Beattie, 2001). Also, there is an increase of TNF-α in the serum of obese individuals and this increase is critical to the inflammatory process. It has been shown that in obesity, adipose tissue contains an increased number of macrophages that
represent a potential source of proinflammatory molecules (Weisberg et al., 2003). In addition, obesity is also associated with increased levels of free fatty acids in the circulation which exacerbate insulin resistance (Stumvoll et al., 2005). Both free fatty acids and inflammatory mediators increase insulin resistance by inhibiting insulin signalling and glucose metabolism (Guilherme et al., 2008). Moreover, enhancing insulin resistance can lead to hyperglycaemia that can diminish insulin production by impairing insulin producing cells (Stumvoll et al., 2005). A consistently strong relationship has been postulated between hyperglycaemia and the incidence and progression of microvascular and macrovascular complications in both types of the disease (Klein, 1995). Long term elevation of blood glucose concentration results in a hyper-responsive cellular state resulting in higher secretion of proinflammatory mediators and changes in vascular integrity which mediate events that are of importance in diabetic complications (Southerland et al., 2006; Nishimura et al., 2007). Therefore, elevation of systemic inflammation plays an important role in the pathogenesis of both types of the disease.

1.3.3 Diabetes related risk factors

Several factors are linked to the development of insulin resistance including genetic (Holt, 2004) and environmental influence (Singh et al., 2004), obesity and other conditions associated with chronic inflammation. Evidence from many studies has revealed that obesity is a major risk factor for T2DM (Ford et al., 1997; Resnick et al., 2000). In obesity, as in most other chronic diseases, inflammation appears to play a major role (Dandona et al., 2004). The chronic inflammation observed in obesity has been reported in the development of atherosclerosis (Ross, 1999). Fat tissue is not a simple energy storage organ but rather exerts important endocrine and immune
functions. These functions are achieved primarily through the adipokines which include leptin, resistin, plasminogen activator inhibitor type-1 (PAI-1) and adiponectin as well as inflammatory cytokines such as TNF-α, IL-6, IL-1β and monocytes chemotactic protein-1 (MCP-1) (Shoelson et al., 2006). These cytokines are critically involved in insulin resistance and chronic inflammation (Iacopino and Cutler, 2000). Numerous lines of evidence suggest that TNF-α is a major mediator of inflammation in general and of obesity and insulin resistance in particular (Patton et al., 1986). Over-expression of TNF-α in adipose tissue of obese rats provided evidence for a link between obesity, inflammation and diabetes (Hotamisligil et al., 1993), however this result has been shown in rats only and not in humans. The level of TNF-α in obese subject correlates with CRP levels, which is a marker of systemic inflammation (Bullo et al., 2003). Also TNF-α can induce secretion of leptin, a fat specific energy balance hormone (Kirchgessner et al., 1997).

1.4 Association between diabetes and periodontal disease

It has been known for many years that patients with diabetes have a high risk for periodontal diseases (Emrich et al., 1991) and various studies have reported DM as a risk for gingivitis and periodontitis (Mealey, 1996; Papapanou, 1999). Other studies have shown bidirectional relationships between periodontal disease and diabetes (Grossi and Genco, 1998; Taylor, 2001; Matthews, 2002; Mealey, 2006). Periodontal disease augments the severity of diabetes and complicates its control (Grossi, 2001) and periodontitis has been found to be more prevalent and more severe in patients with diabetes than systemically healthy individuals. It was reported that patients with diabetes have a significantly higher prevalence of periodontitis and the prevalence of diabetes in patients with periodontitis is doubled (Soskolne and Klinger, 2001). The
relationship between the two diseases has been confirmed in different epidemiological studies in different tissues and organs including the periodontium.

1.4.1 Evidence from epidemiological studies links diabetes and periodontal disease

Both epidemiological and case report studies have shown a relationship between DM and periodontal disease (Southerland et al., 2006). An early study was conducted in patients with T1DM and showed that the diabetic individuals presented with severe gingivitis when compared with the controls (Cohen et al., 1970). Furthermore, diabetes increases the risk of developing periodontitis in the Pima Indian population by about three fold (Emrich et al., 1991) and certain groups of people with diabetes appear to be particularly susceptible to periodontal disease (Campus et al., 2005). DM is recognized as an important risk factor for development of periodontal disease in young individuals (Lalla et al., 2007) and the duration of diabetes appears to affect the severity of periodontal disease; the longer the diabetes mellitus duration the more extensive the periodontal disease (Moore et al., 1999). It was reported that DM increases the risk for periodontal diseases (Yalda et al., 1994; Page and Beck, 1997; Salvi et al., 1997) and patients with T2DM had significant periodontal bone loss compared with subjects without diabetes, over a two year period (Taylor et al., 1998). Increasing duration of diabetes and presence of diabetic complications also impart a significantly greater risk for developing periodontal disease. In a study of periodontal disease in a Mexican population of people with T2DM, researchers concluded that the number of years since diagnosis of diabetes was a more significant factor than the age of the person when considering the severity of periodontal disease (Cerda et al., 1994).
A survey was performed to investigate the effect of diabetes on periodontal disease in the US adult population. The study reported that patients with T2DM had high prevalence of periodontal disease than those without the disease (Shlossman *et al.*, 1990). It was stated that poor metabolic control in diabetic individuals increases susceptibility to gingivitis and gingival bleeding (Seppala *et al.*, 1993), prevalence, severity and extent of periodontal disease (Tervonen and Oliver, 1993) and periodontal attachment loss (Guzman *et al.*, 2003). Recently, a meta-analysis compared the periodontal condition of patients with diabetes with that of controls (Khader *et al.*, 2006). The studies included in the analysis were 18 cross sectional studies, 3 prospective cohort studies and baseline data of 2 clinical trials. It was concluded from the analysis that diabetic individuals had poorer oral hygiene, and worse gingival and periodontal conditions compared with non-diabetic participants. In recent times, a study conducted on 181 diabetic individuals showed increased severity of periodontal disease with poorer metabolic control (Lim *et al.*, 2007).

In conclusion, there is general agreement that diabetes affects the severity of periodontal disease and the outcomes from different studies confirmed the role of glycaemic control as a risk factor for periodontal disease. However, whereas the influence of DM on the severity of periodontitis has been well researched and studied, it is difficult to confirm the precise role of diabetes as a risk factor for periodontal diseases as the studies have used different methodologies and diagnostic criteria. Table 1-1 shows the studies that quantify the effect of DM on periodontal disease.
### Table 1-1 Summary of the periodontal finding in individuals with diabetes

<table>
<thead>
<tr>
<th>Author/Year</th>
<th>Study Population</th>
<th>Principle Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emrich et al (1991)</td>
<td>254 T2DM, 1088 control</td>
<td>Diabetic individuals experienced severe attachment and alveolar bone loss</td>
</tr>
<tr>
<td>Taylor et al (1998)</td>
<td>24 T2DM, 338 control</td>
<td>Diabetic individuals showed extensive alveolar bone loss after 2 years in longitudinal study</td>
</tr>
<tr>
<td>Collin et al (1998)</td>
<td>25 T2DM, 40 control</td>
<td>Diabetic patients had more advanced periodontal disease</td>
</tr>
<tr>
<td>Campus et al (2005)</td>
<td>71 T2DM, 141 control</td>
<td>Individuals with poor glycemic control have worse periodontal status</td>
</tr>
<tr>
<td>Finnli et al (1996)</td>
<td>77 T1DM, 77 control</td>
<td>Periodontal pocket depth and clinical attachment loss were increased in diabetic individuals</td>
</tr>
<tr>
<td>Lalila et al (2007)</td>
<td>325 T1DM &amp; 25 T2DM, 350 control</td>
<td>Individuals with long diabetes duration and poor glycemic control have severe periodontal tissue destruction</td>
</tr>
<tr>
<td>Mattout et al (2006)</td>
<td>71 T1DM, 2073 control</td>
<td>Individuals with higher blood glucose levels have more severe gingival inflammation and clinical attachment loss</td>
</tr>
<tr>
<td>Tervonen et al (2009)</td>
<td>35 T1DM, 10 control</td>
<td>Subjects with poor glycemic control and diabetic complications had higher marginal periodontal bone loss</td>
</tr>
<tr>
<td>Sandberg et al (2000)</td>
<td>102 T2DM, 192 control</td>
<td>Diabetic patients exhibited a potentially higher risk for periodontitis, dental decay and xerostomia. No difference in severity of the disease between patients with good and unsatisfactory glycemic control</td>
</tr>
<tr>
<td>Tasi et al (2002)</td>
<td>502 T2DM, 3841 control</td>
<td>Increased severity of periodontal disease in poor glycemic control individuals</td>
</tr>
<tr>
<td>Lu &amp; Yang (2004)</td>
<td>72 T2DM, 92 control</td>
<td>Increased severity of the periodontal disease in patients who had diabetes for 10 years</td>
</tr>
<tr>
<td>Novak et al (2006)</td>
<td>113 GDM, 4131 control</td>
<td>Periodontal diseases were more prevalent among women that suffered from GDM</td>
</tr>
</tbody>
</table>
1.4.2 Pathogenic mechanisms linking periodontal disease and diabetes

Although the exact role of diabetes in periodontal tissue destruction may still not be totally clear, diabetes has been linked to increased susceptibility to periodontal disease through a number of mechanisms. Firstly, chronic inflammation and the role of the inflammatory host response in periodontal tissue destruction is generally accepted (Kornman et al., 1997). Chronic periodontal inflammation is characterized by production of cytokines such as IL-1β, TNF-α and destructive enzymes. The unbalanced production of these inflammatory mediators greatly contributes to periodontal tissue destruction. It was hypothesised that alteration of the immune response during DM could alter cytokine production in periodontal tissues that causes tissue damage (Preshaw, 2009). Further, DM increases the risk for development of periodontitis (Page and Beck, 1997; Salvi et al., 1997) by altering the host environment with increasing susceptibility to periodontal disease due to changing the inflammatory response to the microbial challenges (Preshaw, 2008a). This suggestion is supported by development of the concept that diabetes is an inflammatory condition and that inflammation occurs prior to the diagnosis of T2DM (Freeman et al., 2002) with increased of TNF-α and IL-6 in the serum of obese individuals with T2DM (Dandona et al., 2004). In addition, increases in the release of IL-1β and superoxide anions have also been reported in patients with T1DM (Devaraj et al., 2007). Inflammation is also associated with obesity, and adipocytes were considered to be an important source of cytokines (Shoelson et al., 2006) and other immune-mediators such as adipokines (Donath et al., 2003) in DM, which are essential stimulators of the inflammatory response. It is likely that elevation of these mediators in individuals with DM may contribute to further periodontal damage (Preshaw, 2009).
A further mechanism linking periodontal disease and diabetes is production of advanced glycation end products (AGEs). In a hyperglycaemic environment numerous proteins undergo glycosylation to form an altered protein known AGEs (Schmidt et al., 1992). The formation of AGEs plays an important role in the pathogenesis of diabetes complications and may also contribute to tissue changes within the periodontium (Offenbacher and Salvi, 1999; Lalla and D'Ambrosio, 2001). AGE formation alters the function of numerous extracellular matrix components that have adverse effects on collagen stability and vascular integrity leading to other diabetes complications (Preshaw, 2009). Increased levels of AGEs in circulation as well as in tissues contribute to alteration of collagen synthesis and turnover (Ryan et al., 2003) and may predispose diabetic patients to periodontal disease. Irreversible formation of AGEs is responsible for various changes in collagen which lead to vascular hardening and basement membrane disintegration (Hong et al., 2000) and act via specific receptors named RAGE (receptor for AGE) on the surface of many cells (Schmidt et al., 1995) such as macrophages, and stimulate production of MMPs and inflammatory mediators (IL-1β, TNF-α and IL-6) that may contribute to further inflammatory status. Furthermore, AGEs can attract monocytes that increase the inflammatory responses (Vlassara, 1992). In addition, AGEs play a role in periodontal pathogenesis and wound repair by stimulating RAGE in the periodontal tissues (Holla et al., 2001; Murillo et al., 2008) and can bind to macrophage receptors that induce IL-1β and TNF-α upregulation (Bendayan, 1998; Hou et al., 2001). These mediators induce expression of MMPs in diabetic periodontal tissue and a role for AGE in the regulation of MMP expression was suggested (McLennan et al., 2007). AGEs reduce the production of matrix proteins such as collagen by cells of the periodontal tissue and may delay tissue repair by inducing death of the cells that produce extracellular matrix (Graves et al., 2006). Moreover,
AGEs augment the release of reactive oxygen species in neutrophils that play a role in tissue destruction (Wong et al., 2003). In conclusion, AGEs may mediate events that are extremely important in DM pathogenesis and involved in tissue changes within the periodontium.

Another mechanism linking diabetes and periodontal disease is impaired neutrophil function. These immune cells are important in the maintenance of periodontal health; they are the first line of the host defence mechanism in the inflammatory process. In diabetic individuals, there are defects in neutrophil functions such as chemotaxis, phagocytosis, and bactericidal activity (Zambon et al., 1988) that decrease host resistance to infection. It was suggested that elevated neutrophil elastase in GCF is a risk indicator for periodontal disease in diabetic patients (Alpagot et al., 2001). Furthermore, a reduction was reported in the host defence mechanisms in patients suffering from both diabetes and periodontitis (Manouchehr-Pour et al., 1981; Cutler et al., 1991). A strong correlation was found between neutrophil malfunction and periodontal disease progression. The possible explanation for the increase in prevalence and severity of periodontal disease in diabetes could be due to reduced neutrophil chemotaxis and phagocytosis that affects diabetic patients' ability to combat infections including periodontal disease. Nonetheless, chronic inflammation and depressed immune responses are associated with the pathogenesis of DM, it may not be possible to eradicate periodontal infection totally in diabetic individual after periodontal therapy (Janket et al., 2005). Thus a few studies have observed that RSI with good oral hygiene in diabetic patients may be inadequate for maintaining periodontal health (Smith et al., 1996).
The current evidence regarding the biological link between diabetes and periodontal disease supports the notion that persistent hyperglycaemia exaggerates immune-inflammatory responses to the pathogenic bacteria challenge, resulting in more rapid and severe periodontal tissue destruction (Southerland et al., 2006; Nishimura et al., 2007). Also, uncontrolled hyperglycaemia has a strong relationship with increased levels of proinflammatory mediators for example IL-1β that secreted in GCF of patients with T2DM experiencing periodontal disease (Engebretson et al., 2004) and elevated concentrations of IL-1β and IL-6 were detected in gingival biopsies of T2DM patients when compared with non-diabetic controls (Duarte et al., 2007). Similarly, T2DM patients with poor metabolic control and periodontal disease had higher levels of IL-1β in GCF (Cutler et al., 1999).

It was reported that chronic hyperglycaemia is related to tissue damage and is associated with defective migration of the immune cells and altered inflammatory responses (Soory, 2002; Ebersole et al., 2008) through increasing collagenase activity and thickening of the vascular basement membrane in gingival tissue. In addition, epithelial cells demonstrated reduction in migration and proliferation (Lan et al., 2008) through reduction in gelatinase activity which explains the poor wound healing that has been seen in diabetic individuals. It has been shown that increased blood glucose levels over time lead to hyperlipidaemia and vascular damage that may result in increased risk for development of periodontal disease (Noack et al., 2000; Lappin et al., 2009).
It can be concluded that the increased risk for periodontal disease in DM is triggered by chronic infection and hyperglycaemia, both of which result in a sequence of inflammatory reactions with secretion of inflammatory mediators; these factors potentially contribute to the tissue destruction which is represented in the signs and symptoms that are seen in patients with periodontal disease.

1.5 Effects of periodontal therapy on diabetes

Although most research on the relationship between diabetes and periodontal disease has focused on how diabetes may affect periodontal status, a growing body of evidence has suggested that periodontal disease is associated with an increased risk for poor glycemic control and untreated periodontitis can impair the metabolic control of diabetes (Miller et al., 1992; Taylor et al., 1998). Elevated concentrations of IL-1β, TNF-α, IL-6 and PGE₂ in patients with periodontal disease could induce insulin resistance (Grossi et al., 1997). In a longitudinal study conducted to investigate the influence of periodontal disease on diabetes related complications, it was reported that severe periodontitis was associated with increased incidence of nephropathy and end stage renal disease (Shultis et al., 2007). Furthermore, severity of periodontitis was increased in individuals who showed signs of metabolic syndrome (Shimazaki et al., 2007). It was found that T2DM individuals with advanced periodontal disease had higher HbA1c levels and greater incidence of cardiovascular complications compared with individuals without periodontal disease (Jansson et al., 2006). It was reported that the risk of poor glycemic control was greater in T2DM patients with periodontal disease than patients who did not have periodontitis (Taylor et al., 1996). It has been shown that optimal glycemic control can be achieved after periodontal treatment (Ryan et al., 1996; Grossi and Genco, 1998) and controlling periodontal inflammation in patients with
diabetes may suppress the level of AGEs in serum (Grossi et al., 1997; Stewart et al., 2001). Thus, periodontal disease may be associated with DM development; however not all studies have supported this association.

Various studies have been published on the effect of periodontal treatment on glycaemic control. The effect of periodontal treatment on glycaemic control was investigated in 36 patients with T2DM who received periodontal treatment and 36 T2DM control patients (without treatment) (Stewart et al., 2001). The treatment included conventional RSI, sub-gingival curettage, extraction of teeth that could no longer be preserved and oral hygiene instruction. The study showed an obvious improvement in glycemic control in patients with T2DM compared with non-treated patients during a 9 months observation period (Stewart et al., 2001). In a further study, a significant improvement in glycemic control was found after periodontal treatment in subjects with T2DM (Kiran et al., 2005). Another randomised controlled study conducted on 30 individuals with T2DM showed a significant reduction in HbA1c after RSI with a follow up of 3 months (Rodrigues et al., 2003). A controlled study consisting of 10 T2DM and 10 non-diabetic individuals has shown an improvement in glycaemic control at 3 and 6 months after periodontal treatment (Navarro-Sanchez et al., 2007). Another controlled study investigated the efficacy of periodontal treatment on metabolic control (Promsudthi et al., 2005) and reported a decrease in HbA1c after conventional adjunctive treatment with systemic antibiotics. Similar findings were reported in a randomized clinical study conducted in 20 individuals with T1DM (Skaleric et al., 2004). Further, a longitudinal study compared the effect of periodontal treatment between T2DM and non diabetic individuals with periodontal disease at 3 and 6 months after treatment (Faria-Almeida et al., 2006). It was concluded that diabetic and non-diabetic individuals responded
similarly to the basic periodontal treatment including RSI. This study also showed a reduction in HbA1c among T2DM individuals.

A pilot study was conducted to investigate the effect of periodontal treatment on glycaemic control. The study consisted of 9 subjects who received conventional periodontal treatment along with doxycycline and chlorhexidine mouth rinse. 8 weeks after the treatment, an improvement in HbA1c was reported (Miller et al., 1992). The findings coincided with those of another study conducted on 113 Pima Indians suffering from both T2DM and periodontal disease (Grossi et al., 1997). The results showed a reduction in HbA1c at 3 months in patients who had systemic doxycycline administration. Conversely no reduction was seen at 6 months after treatment. In another study, 13 patients with periodontitis and T2DM were treated with local application of minocycline. After the treatment, circulating TNF-α and systemic HbA1c values were assessed. The study demonstrated that the antimicrobial periodontal treatment was effective in improving metabolic control through reduction of TNF-α level and HbA1c (Iwamoto et al., 2001). Recently, in a Cochrane database systemic review which included randomised controlled trial of individuals with T1DM or T2DM and periodontal disease, it was reported that periodontal treatment leads to 0.4% reduction in HbA1c (Vergnes, 2010). Table 1-2 summarises the relevant studies. Taking all the evidence together, there is evidence to support a benefit of periodontal treatment on glycaemic control in individuals with diabetes.
On the other hand, other studies have concluded that the current investigations are insufficient to establish with certainty that periodontal therapy has a positive influence on glycemic control (Gustke, 1999; Taylor, 1999). A study of 20 diabetic individuals with periodontal disease matched with 20 healthy controls found an improvement in the clinical parameters following conventional periodontal treatment when compared with controls; however, no effect was seen on the metabolic status of diabetic patients in a 4 month study period (Christgau et al., 1998). These findings concurred with those of another study that reported both diabetic patients and healthy controls with periodontal disease responded similarly over a 5 year period to the periodontal treatment (Westfelt et al., 1996) with minimal change in HbA1c levels. Similarly, other studies have reported a significant improvement in clinical parameters with minimal changes in the HbA1c (Aldridge et al., 1995; Smith et al., 1996). A meta-analysis of 10 interventional studies including 456 individuals with T2DM and T1DM showed a slight reduction in HbA1c (0.38%) after the periodontal treatment, however this decrease was not statistically significant (Janket et al., 2005). The previous findings concurred with those of Jones and co-workers who reported that periodontal therapy did not result in a significant improvement in HbA1c (Jones et al., 2007). A recent study conducted on 23 T2DM individuals with periodontal disease evaluated the effect of periodontal treatment on metabolic control. There was a significant improvement in the clinical parameters 3 months after the treatment, but no significant reduction in HbA1c level was noticed (Correa et al., 2010). Table 1-3 illustrates summary of the study.

Although an overall improvement in periodontal health after periodontal treatment has been observed, there is diversity in the reviewing literature regarding the efficacy of periodontal treatment on glycaemic control. Therefore, there is no reliable conformity
on the beneficial effect of periodontal therapy on metabolic control; further larger scale studies are needed.

### Table 1-2 Summary of the study examining the efficacy of periodontal treatment on glycaemic control (positive effect)

<table>
<thead>
<tr>
<th>Author</th>
<th>Study population</th>
<th>Study period</th>
<th>Kind of treatment</th>
<th>Results</th>
</tr>
</thead>
</table>
| Miller et al. (1992) | 9 T1DM           | 2 months     | Scaling and root planing+ systemic dose +
|                      |                  |              | desoxycycline                                          | Reduction in HbA1c from 9.4% to 9.0% with improvement of periodontal status |
| Grossi et al. (1997) | 113 T2DM         | 3 & 6 months | Scaling and root planing+ systemic dose +
|                      |                  |              | desoxycycline                                          | Periodontal health improved Significant improvement in HbA1c at 3 months by 10% decreased At 6 months no changes |
| Tsumoto et al. (2001)| 13 T2DM          | 1 month      | Scaling and root planing+ locally applied minocycline | Significant improvement in HbA1c by 0.8%                                  |
| Stewart et al. (2001)| 36 T2DM          | 9 month      | Scaling and root planing                             | Significant improvement in HbA1c by 17.1%                                |
| Rodrigues et al. (2003)| 15 T2DM         | 3 months     | Scaling and root planing+ systemic dose +
|                      | With antibiotic |              | desoxycycline                                          | Significant improvement in HbA1c with periodontal treatment alone by 1.6% |
| Kiran et al. (2005)  | 22 T2DM with     | 3 months     | Scaling and root planing                             | Significant improvement in HbA1c by 6.5% and clinical parameters       |
|                      | Periodontal      |              |                                                        |                                                                         |
|                      | Treatment        |              |                                                        |                                                                         |
|                      | 22 T2DM without  |              |                                                        |                                                                         |
| Skaleric et al. (2004)| 20 T1DM         | 6 months     | Scaling and root planing+ locally applied minocycline | Improvement of all clinical parameters with slight reduction in HbA1c by 0.6 |
| Faria-Almeida et al. (2006) | 20 T2DM | 6 months     | Scaling and root planing                             | Significant improvement in HbA1c by 5.7% and clinical parameters       |
| Navarro-Sanchez et al. (2007) | 10 T2DM with treatment | 3 & 6 months | Scaling and root planing                             | Clinical parameters improved in both groups with significant improvement in HbA1c at 3 by 1.1% and 6 months by 0.6% |

We can see from the table that periodontal therapy has a significant impact on glycaemic control, with improvements observed in HbA1c levels. The studies varied in their methodology, including the use of different treatments such as scaling and root planing, systemic antibiotics, and locally applied minocycline. The results were positive in all cases, with reductions in HbA1c levels ranging from 0.8% to 17.1%. This demonstrates the potential of periodontal therapy in improving metabolic control, which is crucial for patients with type 2 diabetes.
### Table 1-3 Summary of the studies examining the efficacy of periodontal treatment on glycaemic control (no effect)

<table>
<thead>
<tr>
<th>Author</th>
<th>Study population</th>
<th>Study period</th>
<th>Kind of treatment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miller et al. (1992)</td>
<td>9 T1DM</td>
<td>2 months</td>
<td>Scaling and root planing+ systemic dose desxycline</td>
<td>Reduction in HbA1c from 9.4% to 9.0% with improvement of periodontal status</td>
</tr>
<tr>
<td>Grossi et al. (1997)</td>
<td>113 T2DM</td>
<td>3 &amp; 6 months</td>
<td>Scaling and root planing+ systemic dose desxycline</td>
<td>Periodontal health improved Significant improvement in HbA1c at 3 months by 10% decreased At 6 months no changes</td>
</tr>
<tr>
<td>Iwamoto et al. (2001)</td>
<td>13 T2DM</td>
<td>1 month</td>
<td>Scaling and root planing+ locally applied minocycline</td>
<td>Significant improvement in HbA1c by 0.8%</td>
</tr>
<tr>
<td>Stewart et al. (2001)</td>
<td>36 T2DM With treatment 36 T2DM With out</td>
<td>9 month</td>
<td>Scaling and root planing</td>
<td>Significant improvement in HbA1c by 17.1%</td>
</tr>
<tr>
<td>Rodrigues et al. (2003)</td>
<td>15 T2DM With antibiotic 15 T2DM Without</td>
<td>3 months</td>
<td>Scaling and root planing+ systemic dose desxycline</td>
<td>Significant improvement in HbA1c with periodontal treatment alone by 1.6%</td>
</tr>
<tr>
<td>Kiran et al. (2005)</td>
<td>22 T2DM with Periodontal Treatment 22T2DM without</td>
<td>3 months</td>
<td>Scaling and root planing</td>
<td>Significant improvement in HbA1c by 6.5% and clinical parameters</td>
</tr>
<tr>
<td>Skaleric et al. (2004)</td>
<td>20 T1DM</td>
<td>6 months</td>
<td>Scaling and root planing+ locally applied minocycline</td>
<td>Improvement of all clinical parameters with slight reduction in HbA1c by 0.6</td>
</tr>
<tr>
<td>Faria-Almeida et al. (2006)</td>
<td>20 T2DM</td>
<td>6 months</td>
<td>Scaling and root planing</td>
<td>Significant improvement in HbA1c by 5.7% and clinical parameters</td>
</tr>
<tr>
<td>Navarro-Sanchez et al. (2007)</td>
<td>10 T2DM with treatment 10 control (non diabetic) With treatment</td>
<td>3 &amp; 6 months</td>
<td>Scaling and root planing</td>
<td>Clinical parameters improved in both groups with significant improvement in HbA1c at 3 by 1.1% and 6 months by 0.6%</td>
</tr>
</tbody>
</table>
1.6 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are also called matrixins and represent a family of highly homologous zinc- and calcium-dependent endopeptidases that are capable of degrading all ECM components and are involved in normal extracellular remodelling. ECM degradation has been considered as the major function of the different MMPs and each MMP has distinct overlapping substrate specificities, which can cleave virtually all components of the ECM and basement membrane (Nagase et al., 2006). In addition, proteolysis of the ECM by MMPs can create space for cells to migrate, produce specific-cleavage fragments with independent biological activity, regulate tissue architecture, and activate, deactivate or modify the activity of bioactive molecules, such as cytokines and chemokines (Sternlicht and Werb, 2001). Thus MMPs regulate a wide range of physiological processes including cell migration, morphogenesis, wound healing, tissue remodelling, angiogenesis and normal immune responses to infection (Visse and Nagase, 2003).

The basic structure of MMPs consists of a catalytic domain and additional variable inserts depending on the specific MMP. These variable inserts include the signal peptide, propeptide, furin-cleavage site, fibronectin-like repeats, hinge region, hemopexin domain, cystein-rich region, cytoplasmatic tail and trans-membrane domain (Murphy and Nagase, 2008). Presently, the MMP family comprises 25 members (Verstappen and Von den Hoff, 2006), and 22 MMPs have been identified in human tissue (Sorsa et al., 2006). They are secreted in a latent form that can be converted to the active forms by proteolytic cleavage (Nagase and Woessner, 1999). MMPs can be classified into six subgroups according to their substrate specificity (Table 1-4 and
Table 1-5). The first is the collagenase group which consists of MMP-1, also called fibroblast collagenase (Overall et al., 1991b; Birkedal-Hansen, 1993b), MMP-8 (neutrophil collagenase) and MMP-13. These enzymes have the ability to degrade native collagen types I, II, and III (Visse and Nagase, 2003). Another group of MMPs are the gelatinases, which break down basement membrane type IV collagen, fibronectin, laminin, and elastin (Nagase and Woessner, 1999) and they include MMP-2 and MMP-9. The stromelysins include MMP-3, MMP-10, and MMP-11. They can degrade a wide variety of ECM components such as laminin and type IV collagen and also can activate other pro-enzymes, for example, pro-MMP-1 generating active MMP-1 (Visse and Nagase, 2003). Matrilysins consist of MMP-7 and MMP-26, and they can degrade many extracellular elements (Uria and Lopez-Otin, 2000). Membrane type MMP (MT-MMP) includes different types of MMPs that digest various ECM structures such as type I, and III collagens (Ohuchi et al., 1997). Others MMP types include MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27 and MMP-28 which is expressed in keratinocytes (Lohi et al., 2001).

In normal tissue, the secretion and activity of MMPs are very low, however their production and release are rapidly induced when tissue remodelling is needed (Nagase and Woessner, 1999). MMP activity and expression are precisely regulated at the level of transcription, secretion, pro-enzyme activation, interaction with specific ECM components and inhibition by tissue inhibitors of metalloproteinases (TIMPs) (Lees et al., 1994; Butler et al., 1997). MMP transcription can be induced by many mediators such as growth factors, cytokines, mechanical stress and changes in extracellular matrix leading to intracellular modification in matrix interaction (Kim and Lee, 2005). Control of transcription has been considered the key step in the regulation of MMPs. Many
cytokines and growth factors mediate the activation of MMP gene expression through mitogen activated protein kinase (MAPK), activated protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) (Angel and Karin, 1991; Liacini et al., 2002). In addition, Vincenti and colleagues reported that IL-1β and TNF-α evoke phosphorylation cascades that lead to induce MMP transcription (Vincenti and Brinkerhoff, 2002).

MMPs are secreted in inactive forms that must be activated, usually by proteolytic cleavage. Some MMPs are activated by serine proteases such as plasmin, by bacterial proteinases or by other members of the MMP family (Nagase, 1997). Plasmin has been reported to activate proMMP-1, proMMP-7, proMMP-9, proMMP-10, proMMP-13 (Lijnen, 2001). Many MMPs are activated by other MMP for example; proMMP-1 can be activated by MMP-3, MMP-7 or by MMP-10. Therefore, degradation of ECM is a tightly controlled process under normal circumstances and is induced when remodelling is required. The pathological condition is characterized by excessive production of the MMPs that result in connective tissue destruction.
### Table 1-4: List of all currently known MMPs

<table>
<thead>
<tr>
<th>Enzyme subfamily</th>
<th>Enzyme</th>
<th>Nomenclature</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td>MMP-1</td>
<td>Collagenase 1, fibroblast collagenase</td>
<td>Collagen I, II, III, VII, X, gelatins, aggregan, cascin, binding protein, fibrin, fibrinogen, tenascin</td>
</tr>
<tr>
<td></td>
<td>MMP-8</td>
<td>Collagenase 2, neutrophil collagenase</td>
<td>Collagens I, II, III, V, VII, VIII, X, aggregan, binding protein</td>
</tr>
<tr>
<td></td>
<td>MMP-13</td>
<td>Collagenase 3</td>
<td>Collagens I, II, III, IV, IX, X, XIV, aggregan, gelatins, osteonectin</td>
</tr>
<tr>
<td></td>
<td>MMP-18</td>
<td>Collagenase 4, Xenopus collagenase</td>
<td>Collagen I</td>
</tr>
<tr>
<td>Gelatinases</td>
<td>MMP-2</td>
<td>Gelatinase A</td>
<td>Gelatins, collagens I, IV, V, VII, X, XI, fibronectin, laminin, aggregan, elastin, large tenascin-C-protein, vitronectin</td>
</tr>
<tr>
<td></td>
<td>MMP-9</td>
<td>Gelatinase B</td>
<td>Gelatins, collagen III, IV, V, XIV, aggregan, elastin, entactin, vitronectin</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>MMP-3</td>
<td>Stromelysin 1</td>
<td>Proteoglycans, gelatins, fibronectin, laminin, collagens, large tenascin</td>
</tr>
<tr>
<td></td>
<td>MMP-10</td>
<td>Stromelysin 2</td>
<td>Proteoglycans, fibronectin, laminin, collagens, large tenascin</td>
</tr>
<tr>
<td></td>
<td>MMP-11</td>
<td>Stromelysin-3</td>
<td>Fibronectin, laminin, gelatins, collagen IV, aggregan</td>
</tr>
<tr>
<td>Matrilysins</td>
<td>MMP-7</td>
<td>Matrilysin 1</td>
<td>Proteoglycans, gelatins, fibronectin, laminin, elastin, entactin, collagen IV, small tenascin-C-protein, vitronectin, aggregan</td>
</tr>
<tr>
<td></td>
<td>MMP-26</td>
<td>Matrilysin 2</td>
<td>Collagen IV, gelatins, cascin, fibroectin</td>
</tr>
</tbody>
</table>

The table is modified from (Sternlicht and Werb, 2001; Mandal et al., 2003)
### Table 1-5  List of all currently known MMPs (continued)

<table>
<thead>
<tr>
<th>Enzyme subfamily</th>
<th>Enzyme</th>
<th>Nomenclature</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane-type MMPs</td>
<td>MMP-14</td>
<td>MT1-MMP</td>
<td>Collagens (I, II, III), casein, fibronectin, gelatine, laminin, vitronectin, large tenascin-C, proteoglycans</td>
</tr>
<tr>
<td></td>
<td>MMP-15</td>
<td>MT3-MMP</td>
<td>Large tenascin-C, fibronectin, laminin</td>
</tr>
<tr>
<td></td>
<td>MMP-16</td>
<td>MT3-MMP</td>
<td>Collagen (III), gelatine, casein, fibronectin</td>
</tr>
<tr>
<td></td>
<td>MMP-17</td>
<td>MT4-MMP</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>MMP-24</td>
<td>MT5-MMP</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>MMP-25</td>
<td>MT6-MMP</td>
<td>Unknown</td>
</tr>
<tr>
<td>Others</td>
<td>MMP-12</td>
<td>Macrophage elastase</td>
<td>Large tenascin-C, fibronectin, laminin, entactin, osteonectin, collagen IV, gelatine, casein, proteoglycan</td>
</tr>
<tr>
<td></td>
<td>MMP-19</td>
<td>No trivial name</td>
<td>Gelatins</td>
</tr>
<tr>
<td></td>
<td>MMP-20</td>
<td>Enamelysin</td>
<td>Amelogenin</td>
</tr>
<tr>
<td></td>
<td>MMP-21</td>
<td>Xenopus MMP</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>MMP-23</td>
<td>CA-MMP</td>
<td>Gelatins</td>
</tr>
<tr>
<td></td>
<td>MMP-27</td>
<td>CMMP</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>MMP-28</td>
<td>Epilysin</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
1.6.1 MMPs production by cells of the periodontium

In the periodontal tissues, MMPs are expressed by the inflammatory cells (monocytes, macrophages, lymphocytes, PMNL) and by resident cells (fibroblasts, epithelial cells, endothelial cells) (Hannas et al., 2007). They are expressed at low levels in most cell types (Vincenti, 2001) and their secretion and activation are induced by proinflammatory cytokines (Vincenti and Brinckerhoff, 2002). Studies on human PDL and gingival fibroblasts have shown increased MMP-1 mRNA production under mechanical force (Bolcato-Bellemin et al., 2000). Although MMPs are produced by many cells of the periodontium (Hannas et al., 2007), the pattern of their production may be affected by several cytokines such as IL-1β and TNF-α that are present in inflamed periodontal tissues (Overall et al., 1991a; Birkedal-Hansen, 1993b). It was demonstrated using electrophoresis and immune-blotting that stimulation of gingival fibroblasts by IL-1β induced production of MMP-1, MMP-2, MMP-8, MMP-13, and MMP-14 (Cox et al., 2006). Moreover, it has been shown using RT-PCR and in situ hybridization (ISH) that gingival fibroblasts collected from patients with periodontitis can express MMP-1, MMP-2 and MT1-MMP (Dahan et al., 2001).

Secretion of MMPs can be induced in gingival epithelial cells by various cytokines as well as bacterial components such as LPS (Smalley, 1994) and the basal cells of the gingival epithelium produce MMP-13 during chronic inflammation (Uitto et al., 1998). Furthermore, expression of MMP-2 is increased in epithelial inflammation (Makela et al., 1999). Stimulation of fibroblasts with TNF-α strongly induced production of MMP-3, MMP-8, and MMP-9 (Beklen et al., 2006) and incubation of fibroblasts with P. gingivalis upregulated MMP-1, MMP-3 and MMP-9 production (DeCarlo et al., 1997). Andrian and colleagues investigated the effect of P. gingivalis and the cysteine
proteinase gingipain on MMPs and TIMPs production by epithelial cells and fibroblasts using RT-PCR and ELISA. The results showed upregulation of MMP-9 at the transcript and the protein levels after 24 hours of P. gingivalis stimulation (Andrian et al., 2007). It has been shown that incubation of mucosal keratinocytes with TNF-α increases MMP-9 mRNA expression (Makela et al., 1998). Moreover, MMP-9 was observed in gingival keratinocytes from junctional and sulcular epithelia of patients affected by periodontal disease (Smith et al., 2004) and is considered the primary proteinase produced by mucosal epithelial cells (Salo et al., 1994).

It was reported that the major MMP detected in diseased periodontal tissues is MMP-8 (Golub et al., 1995; Ingman, 1996; Sorsa et al., 1999). Neutrophils were considered the only cellular source of MMP-8, however studies have shown it is produced by gingival fibroblasts and plasma cells in inflamed connective tissue (Kiili et al., 2002) and gingival sulcular epithelium (Tervahartiala et al., 2000). MMP-1, 2, 3, 8, 9 have been identified in the periodontal tissues (Tonetti et al., 1993) and it was demonstrated that MMP-1, MMP-2 and MMP-8 can be detected in the epithelial cells of gingival tissues from periodontitis patients. In addition, MMP-1 and MMP-8 have been detected in the gingival tissue of patients with periodontitis (Aiba et al., 1996). Dong and co-workers have detected MMP-1 and MMP-2 expression in periodontally diseased tissues using immunohistochemistry and RT-PCR (Dong et al., 2009). A study carried out to investigate the effect of IL-1β on MMP1 and MMP-2 expression in primary fibroblasts showed that both MMPs were detectable in unstimulated fibroblasts and upregulated after treatment with the cytokine (Xiang et al., 2009). Table 1-6 illustrates the MMPs in periodontal tissue.
MMPs can be produced by different cells in the periodontal tissue during inflammation or after cytokine stimulation. However, there is paucity of literature regarding MMP production in oral epithelial cells. In this study, one aim is to screen all members of the MMP family expressed in oral epithelial cells.
Table 1-6 Summary of the studies examining MMP productions by different cells of the periodontium

<table>
<thead>
<tr>
<th>Author</th>
<th>Predisposing conditions</th>
<th>Cells of the periodontium</th>
<th>MMPs response</th>
<th>Analysis method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonetti et al</td>
<td>Periodontitis</td>
<td>Epithelial cells</td>
<td>MMP-1, MMP-8</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>(1993)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salo et al</td>
<td>Wound healing</td>
<td>Epithelial cells, Fibroblasts</td>
<td>MMP-9, MMP-2, MMP-9</td>
<td>Zymography</td>
</tr>
<tr>
<td>(1994)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Makeda et al</td>
<td>TNF-α stimulation</td>
<td>Epithelial cells</td>
<td>MMP-9</td>
<td>Zymography and RT-PCR</td>
</tr>
<tr>
<td>(1996)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tervaharja et al</td>
<td>Periodontitis</td>
<td>Epithelial cells, Fibroblasts, Macrophages, Neutrophils</td>
<td>MMP-7, MMP-13, MMP-8, MMP-7, MMP-13, MMP-8</td>
<td>Immuno-histochemistry</td>
</tr>
<tr>
<td>(2000)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smith et al</td>
<td>periodontitis</td>
<td>Epithelial cells</td>
<td>MMP-9</td>
<td>Gelatin zymography</td>
</tr>
<tr>
<td>(2004)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kihl et al</td>
<td>periodontitis</td>
<td>Fibroblasts, Macrophages</td>
<td>MMP-8, MMP-8</td>
<td>Immuno-histochemistry</td>
</tr>
<tr>
<td>(2002)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uitto et al</td>
<td>Chronic inflamed mucosa</td>
<td>Epithelial cells</td>
<td>MMP-13</td>
<td>In situ hybridization</td>
</tr>
<tr>
<td>(1998)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firth et al</td>
<td>Phospholipase C</td>
<td>Epithelial cells</td>
<td>MMP-9</td>
<td>Gelatin zymography</td>
</tr>
<tr>
<td>(1997)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DeCarlo et al</td>
<td>P. gingivalis Protease</td>
<td>Fibroblasts</td>
<td>MMP-1, MMP-2, MMP-9</td>
<td>Gelatin zymography</td>
</tr>
<tr>
<td>(1997)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boleto-Bellemin et al</td>
<td>Mechanical stress</td>
<td>Fibroblasts</td>
<td>MMP-1</td>
<td>Immunohistochemistry and RT-PCR</td>
</tr>
<tr>
<td>(2000)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dahan et al</td>
<td>periodontis</td>
<td>Fibroblasts</td>
<td>MMP-1, MMP-2, MMP-14</td>
<td>In situ hybridization and RT-PCR</td>
</tr>
<tr>
<td>(2001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cox et al</td>
<td>IL-1β and periodontitis</td>
<td>Fibroblasts</td>
<td>MMP-1, MMP-2, MMP-14</td>
<td>Electrophoresis and immunoblotting</td>
</tr>
<tr>
<td>(2006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beklen et al</td>
<td>TNF-α</td>
<td>Fibroblasts</td>
<td>MMP-3, MMP-8, MMP-9</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>(2006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andrias et al</td>
<td>P. gingivalis</td>
<td>Epithelial cells, Fibroblasts</td>
<td>MMP-9, MMP-2, MMP-9, MMP-2</td>
<td>RT-PCR ELISA</td>
</tr>
<tr>
<td>(2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dong et al</td>
<td>Periodontitis</td>
<td>Epithelial cells, Fibroblasts</td>
<td>MMP-1, MMP-2</td>
<td>Immunohistochemistry and RT-PCR</td>
</tr>
<tr>
<td>(2009)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xiang et al</td>
<td>IL-1β</td>
<td>Primary fibroblasts</td>
<td>MMP-1, MMP-2</td>
<td>ELISA, Western blot, zymography, RT-PCR</td>
</tr>
<tr>
<td>(2009)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The table shows the name of the authors, the predisposing factors that stimulate production of MMPs, types of cells produce MMPs, types of MMP produced, and the analysis method used.
1.6.2 Roles of MMPs in periodontal diseases

There is increasing evidence implicating MMPs as key mediators in the tissue destruction associated with the various forms of periodontal disease, including the progression from gingivitis to periodontitis (Golub et al., 2001; Garlet et al., 2004). The biochemical composition of periodontal tissues during health and disease is determined by the extent of degradation and synthesis of matrix components and the major change during periodontitis is the loss of collagen due to degradation (Reynolds and Meikle, 1997). Increased levels of MMPs are usually associated with disease activity and infiltration by inflammatory cells with the level and duration of MMP expression approximately determining the extent of the disease (Lenz et al., 2000).

MMPs play a crucial role in the pathogenesis of periodontal disease as these enzymes can degrade most of the proteins (Reynolds and Meikle, 1997) that comprise the ECM of the periodontium (Nasztyk et al., 2003). It is becoming increasingly clear that ECM degradation and bone destruction in periodontitis results from direct action of host-derived MMPs (Offenbacher, 1996). Seguier and colleagues demonstrated that an imbalance between MMP and TIMP production is associated with pathological breakdown of the extracellular matrix during periodontitis (Seguier et al., 2001). Patients with periodontal disease exhibit higher levels of MMPs than tissue inhibitors of TIMPs and the balance between MMPs and TIMPs in the gingival tissues impacts on the severity of the disease (Garlet et al., 2004). Periodontal pathogens and inflammatory cytokines play an important role in tissue destruction in periodontitis through induction of MMPs (Chang et al., 2002), thus activation of MMPs is likely to have a key role in the pathogenesis of periodontitis.
1.6.3 MMPs secretion in oral fluids

MMPs, particularly MMP-1, MMP-2, MMP-9 and MMP-13, are involved in ECM degradation of the gingival tissues during periodontitis (Ejeil et al., 2003; Utto et al., 2003). Levels of MMP-8 and MMP-13 were investigated in GCF using western immunoblotting (Kiill et al., 2002). The results showed that MMP-8 and MMP-13 were identified in the GCF of patients with periodontal disease. An in vivo study using immuno-histochemistry and in situ hybridization speculated that MMP-13 and MMP-7 activities were increased in GCF samples from patients with periodontitis in comparison with healthy individuals (Tervahartiala et al., 2000). Conversely, a study was conducted to assess the relationship between periodontal tissue breakdown and the activity of MMP-7 in GCF samples from individuals with aggressive periodontitis, chronic periodontitis, gingivitis and healthy periodontal tissues. The data showed that there was no difference in the total amount of MMP-7 between the study groups (Emingil et al., 2006b).

Levels of MMPs in GCF and saliva were studied using enzyme linked immunosorbent assay (ELISA) and a significant increase in MMP-1 and MMP-9 in GCF of subjects with periodontitis compared to control individuals was observed (Ingman et al., 1996). Salivary MMP-8 concentrations were also increased in patients with periodontitis (427 ± 142 ng/ml) in comparison with the controls (28 ± 11 ng/ml) (Ingman, 1996). Soell and co-workers investigated the concentrations of MMP-1, MMP-2, MMP-3 and MMP-9 in GCF samples that were collected from patients with periodontal disease using ELISA. The results showed a significant increase in the level of the all the MMPs compared to healthy individuals (Soell et al., 2002). Moreover, MMP-3, MMP-8 and
MMP-9 were detected in GCF samples from patients with periodontitis using western blot analysis (Beklen et al., 2006).

MMP-9 is one of many enzymes that may be regulated in the inflammatory process during periodontitis (Makela et al., 1994). In a recent study, GCF levels of MMP-9 and salivary levels of MMP-8 were investigated in patients with periodontal disease by ELISA. The results showed a significant increase in GCF MMP-9 concentrations in patients with periodontitis (56.4 ± 22.3 ng/ml) when compared with healthy individuals (38.8 ± 24.31 ng/ml). Similarly, the salivary MMP-8 levels were significantly increased in diseased samples (428.0 ± 432.4 ng/ml) in comparison with the controls samples (95.2 ± 70.2 ng/ml) (Rai et al., 2008). It was reported that MMP-8 levels were elevated in saliva from patients suffering from periodontal disease (Christodoulides et al., 2007). Another study was conducted by Emingil and colleagues to investigate the GCF levels of MMP-25 and MMP-26 in subjects with periodontal disease, and both MMPs were detected in the samples from patients with periodontal disease in comparison with healthy control samples (Emingil et al., 2006a).

Miller and colleagues conducted a case control study to investigate the levels of MMP-8 in salivary samples from 57 individuals with and without periodontal disease using ELISA. The data showed an increase in the total amounts of MMP-8 in subjects with the disease (408 ± 423 ng/ml) compared with the control individuals (95.1 ± 80 ng/ml) (Miller et al., 2006). Levels of MMP-8 were investigated in GCF samples from periodontally healthy subjects and individuals with periodontal disease using a checker board immune-blotting (CBIB) technique. The results showed that subjects with periodontitis had higher levels of the collagenase (33.7 ng/site) than the healthy
individuals (14.1 ng/site) (Teles et al., 2010). Table 1-7 illustrates the common MMPs in GCF and saliva from individuals with periodontal disease.

To summarise, there is general agreement about the local production of the MMPs particularly MMP-8 and MMP-9 in periodontal disease. However the conclusions of many studies are limited by the methodology, the sample size and the criteria for assessing and defining periodontal disease. Additional research on MMP expression in periodontal disease is warranted.
Table 1-7 Summary of the studies examining MMP levels in periodontal disease

<table>
<thead>
<tr>
<th>Author/ year</th>
<th>Patients number</th>
<th>Oral fluid</th>
<th>MMPs response</th>
<th>Analysis method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingman et al (1996)</td>
<td>23 periodontitis 11 healthy</td>
<td>GCF</td>
<td>↑MMP-1 (6 ng/ml), MMP-9 (700 ng/ml), MMP-8 (427 ng/ml) versus control</td>
<td>ELISA</td>
</tr>
<tr>
<td>Soell et al (2002)</td>
<td>11 periodontitis 5 healthy</td>
<td>GCF</td>
<td>↑MMP-1 (2 μg/mg), MMP-2 (0.5 μg/mg), MMP-3 (8 μg/mg), MMP-9 (4.5 μg/mg) versus control</td>
<td>ELISA</td>
</tr>
<tr>
<td>Tervahertta et al (2000)</td>
<td>24 periodontitis 23 healthy</td>
<td>GCF</td>
<td>↑MMP-13, MMP-7 versus control</td>
<td>Western blot</td>
</tr>
<tr>
<td>Christodoulides et al (2007)</td>
<td>28 periodontitis 29 healthy</td>
<td>Saliva</td>
<td>↑MMP-8 (200 ng/ml)</td>
<td>ELISA</td>
</tr>
<tr>
<td>Beklen et al (2006)</td>
<td>7 periodontitis 5 healthy</td>
<td>GCF</td>
<td>↑MMP-3, MMP-8, MMP-9</td>
<td>Western blot</td>
</tr>
<tr>
<td>Rai et al (2008)</td>
<td>18 gingivitis 20 periodontitis 15 control</td>
<td>GCF</td>
<td>↑MMP-9 (56.4 ng/ml)</td>
<td>ELISA</td>
</tr>
<tr>
<td>↑MMP-8 (428.0 ng/ml)</td>
<td>MMP-25, MMP-26 in gingivitis and periodontitis versus control</td>
<td>Western blot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eminoglu et al (2006)</td>
<td>20 gingivitis 40 periodontitis 20 healthy</td>
<td>GCF</td>
<td>No change in MMP-7</td>
<td>ELISA</td>
</tr>
<tr>
<td>Eminoglu et al (2006)</td>
<td>20 gingivitis 64 periodontitis 21 healthy</td>
<td>GCF</td>
<td>↑MMP-8 (408.0 ng/ml) versus control (95.1 ng/ml)</td>
<td>ELISA</td>
</tr>
<tr>
<td>Miller et al (2006)</td>
<td>57 periodontitis 29 healthy</td>
<td>Saliva</td>
<td>↑MMP-8 (33.7 ng/site) versus control (14.1 ng/site)</td>
<td>CBIB</td>
</tr>
</tbody>
</table>

The table shows the name of authors, number of the patients, type of oral fluid, MMP levels and methods used. ↑ means increase and ↓ means reduction in the level of the enzymes.
1.6.4 Inhibitors of MMPs

MMPs are secreted in latent, inactive pro-enzyme forms (Birkedal-Hansen et al., 1993; Parsons et al., 1997) and their activities in the tissues are regulated by TIMPs (Birkedal-Hansen, 1995). TIMPs are produced by many cell types such as keratinocytes, fibroblasts, macrophages and endothelial cells (Birkedal-Hansen et al., 1993) and four members of the TIMP family have been so far described, namely TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Verstappen and Von den Hoff, 2006). The inhibition of MMPs by TIMPs occurs by development of a MMP-TIMP complex or by inhibiting the activation of pro-MMPs (Sorsa et al., 1992; Birkedal-Hansen, 1993b). TIMPs maintain the balance between matrix formation and destruction. The balance between MMPs and TIMPs is found to be disturbed in many inflammatory conditions such as periodontal disease (Gomez et al., 1997; Lambert et al., 2004). It was reported that TIMP-1 is the major inhibitor of MMPs in the gingival tissues of patients with periodontitis (Nomura et al., 1993; Kubota et al., 1996). In addition, Nomura and colleagues reported an increase in the GCF levels of TIMP-1, MMP-1 and MMP-8 in patients with periodontitis (Nomura et al., 1998). Ejeil and colleagues investigated MMP and TIMP expression in periodontally diseased and healthy control gingival tissues, and reported an increase in the levels of MMP-1, MMP-9, MMP-13 and TIMP-1 in the patients with periodontal disease (Ejeil et al., 2003). It was reported that levels of MMPs are increased in GCF and saliva of patients with periodontal disease (Ingman, 1996), and that the balance between MMPs and TIMPs represents a critical control point to prevent destruction of the extracellular matrix in periodontal tissues. Levels of TIMPs were examined in the GCF of individuals with periodontal disease and healthy subjects. The results showed a significant increase in the amount of TIMP-1 in subjects with periodontal disease compared with the controls (Emingil et al., 2006b). Conversely
other studies reported a decrease in TIMP-1 levels in GCF and gingival tissues of patients with periodontitis (Soell et al., 2002). A longitudinal study was conducted to compare the levels of TIMPs in GCF samples from periodontally diseased and healthy individuals before and after periodontal treatment. Reductions in TIMP-1 and TIMP-2 were observed in subjects with periodontitis. After periodontal treatment TIMP-1 levels reached values similar to control individuals (Pozo et al., 2005). A summary of TIMP produced in periodontal disease is shown in Table 1-8.

Antibiotics such as tetracycline are sometimes used in the treatment of periodontitis as an inhibitor for MMPs (Ryan et al., 1996). They have the ability to inhibit connective tissue destruction through many mechanisms including inhibition of the active form of MMPs, inhibition of the activation of the pro-enzyme form of MMPs, down-regulation of cytokine production, increasing collagen production and inhibiting osteoblasts and osteoclasts-derived MMPs (Ryan and Golub, 2000). Sub-antimicrobial dose of doxycycline (SDD) can reduce collagenase in gingival tissue and GCF from subjects with periodontal disease (Golub et al., 1990). It has been shown that doses of doxycycline adjunctive to RSI can be used to suppress periodontal disease by reduction of MMPs and increasing TIMPs levels (Choi et al., 2004). A meta-analysis was performed to investigate the efficacy SDD in the treatment of periodontal disease. The outcomes of the study indicated the clinical benefit of SDD in the management of periodontitis (Preshaw et al., 2004). Further studies reported that a modified SDD-40 mg adjunct to RSI provided significant clinical benefits in patients with periodontal disease (Preshaw et al., 2008) and the benefits of SDD derived from the ability of doxycycline to inhibit the activity of MMPs (Golub et al., 1995). Therefore,
periodontal tissue destruction may be reduced if the balance between MMPs and TIMPs is established through periodontal therapy adjunctive with therapeutic dose of antibiotic.

### Table 1-8 Summary of the studies examining TIMP levels in periodontal disease

<table>
<thead>
<tr>
<th>Author</th>
<th>Predisposing condition</th>
<th>Cell type/oral fluid</th>
<th>Response</th>
<th>Analysis method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nomura et al (1997)</td>
<td>Periodontitis</td>
<td>Gingival biopsy</td>
<td>↑TIMP-1 in diseased tissue versus control</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Kubota et al 1996</td>
<td>Periodontitis</td>
<td>Gingival biopsy</td>
<td>↑TIMP-1 in diseased tissue versus control</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Nomura et al (1998)</td>
<td>Periodontitis</td>
<td>GCF</td>
<td>↑TIMP-1 versus control</td>
<td>ELISA and western blot</td>
</tr>
<tr>
<td>Ingman 1996</td>
<td>Periodontitis</td>
<td>GCF Saliva</td>
<td>↑TIMP-1 no change</td>
<td>ELISA</td>
</tr>
<tr>
<td>Soell et al (2002)</td>
<td>periodontitis</td>
<td>Saliva</td>
<td>↓TIMP's versus control</td>
<td>ELISA</td>
</tr>
<tr>
<td>Pozo et al (2005)</td>
<td>Periodontitis</td>
<td>GCF</td>
<td>↓TIMP-1 versus control</td>
<td>Western blot</td>
</tr>
<tr>
<td>Emingil et al (2006)</td>
<td>Periodontitis</td>
<td>GCF</td>
<td>↑TIMP-1 versus control</td>
<td>ELISA</td>
</tr>
</tbody>
</table>

The table shows the name of the authors, the predisposing condition that stimulate production of TIMP, types of cells produce TIMP or oral fluid, levels of TIMP and the analysis method used. ↑ means increase and ↓ means reduction in the level of the enzymes.
1.6.5 Influence of diabetes mellitus on MMP expression

Increased activity of MMPs has been reported in T1DM (Kai et al., 1998) and T2DM patients with coronary artery disease (Marx et al., 2003). It was reported that hyperglycaemia upregulated MMP-9 expression in endothelial cells in atherosclerotic lesions (Uemura et al., 2001) and induced changes in MMP expression in endothelial cells (Song and Ergul, 2006). Levels of MMPs were investigated in experimentally induced diabetes in rats. The data showed an increase in MMP-8 and MMP-9 levels and emphasized the role of hyperglycaemia in the elevation of these MMPs through production of prostaglandin E2 (PGE2), cytokines and AGEs (Ryan et al., 1999). In addition, other studies have shown an increase in MMP-2 and MMP-9 production in subjects with diabetes (Maxwell et al., 2001; Xue et al., 2005). In sera of patients with T2DM, MMP-9 levels were elevated compared to non-diabetic controls (Signorelli et al., 2005) and a recent study demonstrated (using western blot assay) that MMP-9 levels are upregulated in the serum of patients with T1DM (Gharagozlian et al., 2009). Furthermore, plasma levels of MMP-2 and MMP-9 were increased in patients with diabetes compared with non-diabetic individuals (Shiau et al., 2006).

Numerous studies have demonstrated the association between diabetes and periodontal disease and a role for AGEs was suggested in the regulation of MMPs in diabetic periodontal tissues (McLennan et al., 2007). Silva et al (Silva et al., 2008) investigated MMP-2 and MMP-9 expression in an animal model with experimentally induced diabetes and ligature-induced periodontal disease. They examined the influence of diabetes mellitus on periodontal disease using zymography, immunofluorescence and real time RT-PCR. The results showed that MMP-2 and MMP-9 were observed with the same profile in both normal and hyperglycaemic rats with an increase in the activity
of both enzymes after the induction of periodontal inflammation. Then during inflammation, the level of MMP-2 was reduced in both diabetic and non-diabetic rats, whereas MMP-9 was increased which supports the influence of diabetes on MMP-9 expression. Kumar and colleagues investigated the levels of MMP-9 and MMP-8 in gingival tissue from diabetic patients with periodontitis and non-diabetic subjects with periodontal disease (Kumar et al., 2006). The data reported an augmentation of MMP levels in periodontally-diseased diabetic individuals compared with periodontally-diseased subjects who were non-diabetic and the elevated MMPs levels were correlated with clinical parameters. Collin and colleagues studied the salivary levels of MMP-8 and MMP-9 in T2DM and non-diabetic individuals (Collin et al., 2000). The results showed almost equal MMP-8 and MMP-9 levels in diabetic subjects and non-diabetic controls.

Previous studies have shown that diabetic individuals with periodontal disease have increased MMP-8 levels in GCF (Tervonen and Oliver, 1993). In addition, an elevation of MMP-8 levels was detected in GCF samples of insulin dependent patients with experimental gingivitis relative to healthy controls (Salvi et al., 2010). A recent study was conducted to compare the GCF levels of MMPs in periodontally diseased diabetic individuals, non-diabetic periodontally-diseased subjects, and healthy control subjects (Kardesler et al., 2010). The study included 73 subjects that were divided into five groups according to their diabetes status and periodontal disease status. The data showed an increase in MMP-8 concentrations in non-diabetic subjects with periodontal disease as well as periodontally diseased patients with diabetes in comparison to the healthy control group. There was no difference in the enzyme concentrations in diabetic individuals with periodontal disease and non-diabetic subjects with periodontal disease.
Thus, T2DM may augment MMP production in periodontal disease which may play a major role in driving the inflammatory process. It was suggested that diabetes may contribute to elevated MMP levels in the serum, but hitherto nothing is known about the role of MMP serum levels in diabetic patients with periodontal disease. The lack of studies that investigated the role of MMPs in individuals with periodontitis and diabetes makes it difficult to draw firm conclusions, and further investigations are needed.
1.7 Aims of the study

As the gingival epithelial cells are in constant contact with bacterial products and may play a role in periodontal pathogenesis, this study will investigate the role of epithelial cells in periodontal pathogenic processes. In addition, a study of the composition of GCF could provide useful information on the host response to exogenous antigens. Knowledge of enzymes released by fibroblasts, epithelial, or inflammatory cells may lead to an explanation of how MMP-9 and MMP-8 could be involved in development of periodontitis in patients with diabetes.

The overall aim of the study is to investigate the regulation of MMPs by cytokines in keratinocytes to answer important questions about the role of these cells in periodontal pathogenesis. In addition, this study attempts to quantify the levels of MMP-8 and MMP-9 in GCF and serum of healthy and periodontitis patients with and without diabetes.

More specifically, the aims are:

1. To investigate the pattern of MMP production by keratinocytes in response to IL-1β stimulation.

2. To investigate the effect of IL-1β on MMP-9 and MMP-8 productions by the human oral keratinocytes cell line (OKF6).

3. To investigate the role of different cytokines such as TNF-α, IL-18 and IL-32 in stimulating MMP-9 production in keratinocytes.

4. To study the effect of a key bacterial component (P. gingivalis LPS) on MMP-9 production in keratinocytes.

5. To investigate MMP responsiveness in primary oral epithelial cells after stimulation with IL-1β and P. gingivalis LPS.
Chapter 2  

Material and methods

2.1 Cell culture

The cells culture experiments were performed inside an Astecair Biological Safety Cabinet Class II BHA Series (AsteC Environment Systems Limited, Weston Industrial Estate, and UK). Plasticware such as filter cap flasks and plastic pipette tips were sterile and obtained from Greiner Bio-one (Stonehouse, UK). Unless otherwise stated, all reagents and media were purchased from Sigma-Aldrich (Poole, UK). The equipment and reagents needed for the experiments were carefully sprayed with 70% ethanol to avoid contamination. A Sanyo CO₂ incubator (MCO-17/20 AIC) was used to keep cells at stable temperature and the incubator is regularly cleaned to reduce the chance for the cells to become infected. Storage of cells was in liquid nitrogen in a Statebourne Biorack 750 storage unit at -196°C.

2.2 Cell culture experiments

Human oral mucosal keratinocytes cell lines (OKF6/ TERT-1) and primary oral keratinocytes were used in this study. All experiments were carried out three times independently.
2.2.1 OKF6/TERT-1 cell line

The cells were kindly provided from BWH cell culture and microscopy core, Harvard. The OKF6 cell line represents normal gingival mucosal cells taken from the floor of the mouth and were engineered for extended growth through two cellular alterations, the over-expression of telomerase and the deletion of the P16<sup>INK4a</sup> regulatory protein (Dickson et al., 2000). The human telomerase reverse transcriptase (hTERT) encodes the catalytic protein subunit of the telomerase that is expressed in both germ cells and cancer cells (Meyerson et al., 1997; Nakamura et al., 1997). The hTERT is the active component of the telomerase and its introduction into many cell types allows unlimited production of these cells (Bodnar et al., 1998). The P16<sup>INK4a</sup> protein is an important tumour suppressor that has been shown to be absent or non-functional in a number of human cancers including oral carcinoma (Dickson et al., 2000).

OKF6/TERT-1 cells were cultured in Gibco Keratinocytes SFM medium (Invitrogen, UK) supplemented with a cocktail of growth supplements such as bovine pituitary extract (BPE) at a final concentration 30 μg/ml, penicillin (100 U/ml), streptomycin (100 μg/ml) diluted in phosphate buffered saline (PBS), epidermal growth factor (EGF) to a final concentration of 0.1 ng/ml, and 0.6 mM calcium chloride (CaCl₂). Cells were cultured in 25cm² flasks at density of approximately 0.5×10⁶ cells per flask in 5ml of supplemented Gibco Keratinocytes-SFM medium and were maintained at 37°C, 5% CO₂. Every other day the medium was removed and replaced by fresh medium. The cells were cultured until they reached sub-confluence (Figure 2-1A). The confluence was determined by light microscopy, when 80% of the area of the flask was covered by cells and this took approximately 5 days. As the keratinocytes are adherent cells, releasing them from the walls of the flask was achieved by washing the cells with 5ml
of trypsin/EDTA for 30 seconds and this was followed by adding 5 ml of the same reagent to the cells at 37°C and 5% CO₂ for 10 minutes until the cells became non-adherent. The trypsin action was stopped by adding 15 ml of 1:1 DMEM / F-12 (ham mixture) media supplemented with foetal calf serum (10% v/v) and L-glutamine (2mM). The cells were centrifuged for 10 minutes at 2000 rpm and were resuspended in keratinocyte media. The cells were counted using a haemocytometer and seeded in 25cm² flasks. The next day, the media was replaced with 5ml of fresh media that was then changed every other day.

2.2.2 Primary human oral keratinocytes

Primary oral keratinocytes were established from discarded gingival tissues obtained from systemically healthy donors, undergoing surgical canine exposure in the Newcastle Dental Hospital. Ethical approval was obtained from the Newcastle and North Tyneside research ethics committee. The tissue obtained was collected in a 50 ml universal containing 20 ml of nutrient mix medium (1:1 DMEM and F-12 Ham's mixture) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B solution (5 µg/ml). The tissue was immersed in 99% ethanol for 30 seconds to kill any contaminating bacteria. It was then washed in PBS and the majority of the connective tissue was removed from the epithelium. The epithelium and the remaining connective tissue were placed in a new universal tube and incubated overnight in type III trypsin (2.5 mg/ml), penicillin (200 U/ml), streptomycin (200 µg/ml) diluted in PBS at 4°C. The type III trypsin was removed and the tissue was incubated for 30 minutes at 37°C. 5 ml of nutrient mix containing 10% foetal calf serum (FCS) was added to the tissue and shaken vigorously to separate the keratinocytes. The media containing the keratinocytes in suspension was removed and
placed in a new tube. This step was repeated and the remaining tissue was discarded.

The keratinocytes were centrifuged at 1000 rpm for 10 minutes and the supernatant discarded. The cells were resuspended in 2ml of Gibco keratinocytes SFM medium containing L-glutamine (2mM), penicillin (737 mg/ml), streptomycin (737 mg/ml), EGF (0.4 ng/ml), BPE (25 µg/ml), CaCl₂ (0.4 mM). The cells were cultured until they reached sub-confluence (Figure 2-1 B). Counting and seeding of the cells was performed using the same method as previously described for OKF6 cells.

2.2.3 Morphology of epithelial cells in culture

The epithelial cells are adherent cells and the initial growth was observed 2 days after culturing with confluence usually achieved by 7-8 days. The cells were examined under a light microscope and their morphology is cuboidal in shape (Figure 2-1). Areas of intense mitotic activity were observed, with three to four nuclei appearing (see arrow).
Figure 2-1 Microscope images show epithelial cells

A

B

Microscope images of OKF6 cells (A) and primary oral epithelial cells (B), showing a confluent monolayer cells grown in keratinocytes medium. 40X objective. The arrow shows area of mitotic activity.
2.2.4 Cell count using a haemocytometer

An improved Neubauer haemocytometer was used to count the cells numbers. 10 μl of the cell suspension was mixed with 10 μl of trypan blue (5%). 10 μl of the mixture was placed at the edge of the V shape of the counting chamber under the cover slip of the haemocytometer using a 10 μl pipette and the area under the cover slip was allowed to fill with the cell suspension. The counting chamber was placed on the microscope stage and the counting grid was brought into focus at low level. Under the microscope the grid consists of 25 squares (each of the squares contains 16 smaller squares) (Figure 2-2). The counting grid is surrounded by 3 lines. Cells were counted at four large corner squares and the one in the middle. The number of cells per ml of cell stocks was counted as follows:

Number of cells in 5 squares × 5 × 2 (dilution factor) × 10,000 (10 μl used).
Figure 2-2 Haemocytometer grid under the microscope

The central area of the haemocytometer grid is made up of triple ruled lines surrounded the 25 squares. Each one of 25 squares is further divided up into 16 smaller ones. The five grey squares are used in counting the cells. 40X objective.
2.2.5 Treatment of oral keratinocytes with cytokine

The human recombinant cytokines IL-1β, TNF-α, IL-32 and IL-18 were purchased from R&D Systems (Abingdon, UK). The cytokines were reconstituted according to the manufacturer's instructions, and were prepared as stock solutions (5 μg/ml IL-1β, 10 μg/ml TNF-α, 50 μg/ml IL-32 and 100 μg/ml IL-18) by dissolving the powder in sterile distilled water and stored at -80°C until they were used in experiments.

The IL-1β and TNF-α concentrations used in the experiments were chosen according to previous research (Nee et al., 2004) and because this was the first study that stimulated oral epithelial cells with novel cytokines such as IL-32 and IL-18, different concentrations of these cytokines were used. Stimulation of the epithelial cells with each cytokine was performed in individual experiments and repeated three times. The concentration of each cytokine used in these experiments is shown in Table 2-1.

The OKF6 cells were obtained by trypsinization of the cells as described previously (section 2.2.1) and were grown in duplicate wells (2×10³ cells/well) in three 6 well plates as previously stated and left to adhere and reach sub-confluence. For stimulation of the cells, fresh medium containing IL-1β (100 pg/ml) was added (4 ml/well) and other cells were given fresh medium and used as controls. All treatments were carried out in duplicate, and the results were confirmed in at least three independent experiments. The culture supernatants were aliquoted in Eppendorf vials (1ml) and cells were scraped and collected from the plates after 4 hours, 24 hours and 48 hours and stored at -80°C until further investigation.
Cells were stimulated with TNF-α, IL-32 and IL-18 according to the method described above. The primary oral keratinocytes were also seeded in duplicate wells with the same OKF6 density in 6 well plates and stimulated with 100 pg/ml IL-1β for 4, 24 and 48 hours. The supernatants and the cells were collected and stored for further investigations.

**Table 2-1 Cytokine concentrations used in each experiment**

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Concentrations</th>
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<tr>
<td>IL-1β</td>
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<td>TNF-α</td>
<td>100 pg/ml</td>
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<tr>
<td>IL-32γ</td>
<td>20 and 80 ng/ml</td>
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<td>IL-18</td>
<td>20 pg/ml, 50 pg/ml, 100 pg/ml, 500 pg/ml, 1000 pg/ml, 10 ng/ml and 100 ng/ml</td>
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</table>
2.2.6 Treatment of oral keratinocytes with lipopolysaccharide (LPS)

Ultrapure LPS from *P. gingivalis* (TLR2 ligand) and *Escherichia coli* (*E. coli*) (TLR4 ligand) were purchased from Invivogen (via Autogen Bioclear). LPS working concentrations were prepared in cell tissue culture medium and used to stimulate the oral keratinocytes. Monolayers of oral keratinocytes (OKF6) were stimulated with different concentrations of *P. gingivalis* LPS (100 ng/ml, 1 μg/ml) for 24 hours. The OKF6 and primary cells were also stimulated with *P. gingivalis* and *E. coli* LPS (100 ng/ml, 1 μg/ml, and 10 μg/ml) for 6 hours.

2.2.7 Cell proliferation assay

The Cell Titre 96® AQueous One Solution cell proliferation assay (Promega, Southampton, UK) was used to measure the number of cells after treatment with *P. gingivalis* LPS (500 ng/ml, 1000 ng/ml, and 10 μg/ml) for 6 hours. The assay was used according to manufacturer’s instruction. 8-point standard curve comprising $2 \times 10^4$, $1 \times 10^5$, $5 \times 10^5$, $2.5 \times 10^5$, $1.25 \times 10^5$, $6.25 \times 10^4$, $3.13 \times 10^4$, and $1.56 \times 10^3$ cells/ml was prepared by serial dilution.

In a 96 well assay plate, 100 μl/well of each standard concentration of the cells was added in triplicates. The cell numbers of stimulated samples and control samples were within the range of the standard curve. The plate was incubated at 37°C and in a 5% CO2 humidified atmosphere over night. The medium was changed with fresh media and the cells were left to settle for half hour in the incubator. 20 μl of Owen’s reagent was added to each well, and the plate was left in the incubator for 2 hours. The absorption was measured at 490 nm on a spectrophotometer (FL 600 Microplate Fluorescence Reader, BioTek) (Leicestershire, UK) and readings at 645 nm were subtracted to adjust
for plate background. The cell numbers were calculated using the equation from the standard curve.

2.3 RNA analysis

2.3.1 RNA extraction from oral keratinocytes

In order to analyse mRNA level in the OKF6 and primary cells in response to different stimuli, the GenElute™ Mammalian Total RNA Miniprep kit (Sigma) was used according to the manufacturer’s instructions. Cells were lysed in 350 μl/well lysis solution containing β-mercaptoethanol (10 μl/ml) and filtered through a Gene-Elute filtration column (blue insert with a 2 ml receiving tube). The tubes were centrifuged at maximum speed (13000 rpm in Biofuge Pico, Heraeus, DJB labcare) for 2 minutes. The filtration columns were discarded and stored at -80°C until further processing. Briefly, on ice, an equal volume of 70% ethanol (350 μl) was added to the filtered lysate. The sample was transferred into a GenElute binding column placed in a 2 ml red collection tube then centrifuged at 12000 rpm for 15 seconds. The flow-through liquid was discarded and wash solution 1 (500 μl) was added to the column which was centrifuged for 15 seconds. The binding columns were transferred into 2 ml collection tube then 500 μl of wash solution 2 was added into the column then centrifuged for 15 seconds. Another 500 μl of wash solution 2 was added into the column and centrifuged at maximum speed for 2 minutes. The column was then placed in a new collection tube then the RNA was eluted from the column in 30 μl of the elution solution. The RNA was quantified using a Nanodrop spectrophotometer and stored at -80°C.
2.3.2 RNA quantification

RNA was quantified using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies Inc., USA). RNA concentrations were measured in 1 μl of each sample. The machine calculates a ratio for absorbance at 260 and 280 nm. A ratio of 1.8 is generally accepted as pure for RNA. The concentration for the RNA samples was given in ng/μl.

2.3.3 Reverse Transcription and complementary DNA (cDNA) synthesis

Reverse transcription of the RNA sample to cDNA was performed with High-Capacity cDNA reverse transcription kits (Applied Biosystems, Warrington, UK). The cDNA was synthesized from 1 μg of total RNA. According to the manufacturer’s instructions, the reaction mixture included the following: 2 μl 10X reverse transcription buffer, 0.8 μl 25X deoxynucleoside triphosphates (dNTP), 2 μl 10X random hexamers, 1 μl RNAse inhibitor, 1 μl multiscribe enzyme. The reaction mixture of 6.8 μl was added to PCR tubes or a plastic multiwall plate. RNA (the volume equivalent to 1 μg) was added with molecular biology grade water (13.2 μl RNA volume). The tube or plate was placed in the thermocycler for 10 minutes at 25°C, 120 minutes at 37°C, and 5 seconds at 85°C. The cDNA was stored at -20°C until used in downstream PCR.
2.3.4 Primer design

The primers were designed to have a sequence which is the reverse complement of the region of template or target DNA to which the primer will anneal. The primers for specific genes were designed in 20 bases to be used in Polymerase Chain Reaction (PCR). The NCBI nucleotide database was used to identify the sequence of the target gene.


The cDNA sequence that was given for the gene was copied and pasted into a web based programme called Primer 3.

http://frodo.wi.mit.edu/cgi-bin/primer3/primer3

The programme generates a number of possible 20 base primers for both forward and reverse. The sequence was then copied into the NCBI Blast database to check if they matched the target gene.

http://www.ncbi.nlm.nih.gov/Blast/Blast.cgi

The primers were then mapped to the whole gene (genomic sequence) and primers that spanned two exons were preferentially chosen as they would be less likely to amplify any genomic DNA if there was contamination.
2.3.5 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for MMPs and TIMPs

Most of the primers that were used in this study were chosen from published reports and others were designed in our laboratory using Primer3 output. BLAST searches for all of the primer sequences were conducted to ensure gene specificity. The sequences of the primers, PCR annealing condition and expected fragment sizes are described in Table 2-2 and Table 2-3. PCR analyses were performed using the complementary DNA (cDNA). β2-microglobulin was used as a control gene for the successful extraction of RNA and reverse transcription. The nucleotide sequences of the β2-microglobulin gene primers were 5'acccccactgaaaagatg (forward) and 5'atctctcacttcctcctg (reverse) with melting temperature of 60°C.

The PCR assay was performed in 25 μl volumes using 2.5 μl of cDNA, 12.5 μl of Bio Mix™ Red (Bioline, UK), 1.25 μl of each primers and 7.5 μl H2O. For each experiment, PCR amplification was also performed without using cDNA to check for contamination.

2.3.6 PCR product analysis

PCR products were analyzed on 3% agarose gels. Hyperladder IV (Bioline, UK) was used as a molecular weight marker. Agarose gels were made by dissolving 0.9 g of agarose (Sigma) in 30 ml 1XTAE buffer (0.4 M Tris Base, 50 mM EDTA and 1.14% (V/V) glacial acetic acid) in a conical flask. To dissolve the agarose, the mixture was heated in a microwave oven. 5 μl of ethidium bromide (0.5 mg/ml) was added to cool agarose and mixed in by gentle swirling of the flask. The gel was poured into a cast containing a 10 well comb and was left to become hard. The comb was removed to
leave a space, and then the gel was placed in a Hybaid electrophoresis tank filled with 1XTAE buffer. 5 µl of Hyperladder marker IV containing 9 different sized nucleotides from 100 to 1000 bp was used in the first lane. 10 µl of the samples were put in the other lanes and the gel was run for 40-50 minutes at 100 volts using a Bio-Rad power PAC 300. The ethidium bromide added to the agarose gels intercalated into the DNA and fluoresces under UV light. The DNA bands were visualized using a UV transilluminator (UVP Life Sciences) and the photographs were taken using the Image Store 5000 system (UVP Life Sciences). Primers for β2-microglobulin gene were used as a control for successful extraction of RNA and reverse transcription.
Table 2-2 PCR primers sequences and parameters

<table>
<thead>
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<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temp.</th>
<th>Product size</th>
<th>Reference</th>
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</thead>
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The table shows target genes, primer sequences (forward and reverse), annealing temperature, product length and author’s name.
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</table>
2.4 Quantitative Real time RT-PCR

TaqMan probes were used in the Real time RT-PCR to quantify the differences in mRNA expression levels. The TaqMan probes are a mixture of a fluorescent probe and the forward and the reverse primers of the gene of interest. The probe has a reporter dye (FAM-6) at the 5' end and a non-fluorescence quencher dye at 3' end attached, which suppresses the reporter dye fluorescence unless activated. During DNA amplification, the 5'-3' nuclease activity of the DNA polymerase cleaves the probe, resulting in separation of the quencher dye from the reporter dye. The increased fluorescence can be recorded with a detection system. The probe attaches itself only to the DNA between the forward and reverse primer sequences; therefore unspecific fluorescence does not occur during the replication process. The cDNA was used as template for Taqman\textsuperscript{TM} real time quantitative PCR and the relative quantification of genes were performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). The Ct value represents the cycle threshold at which an increase in fluorescence is detectable above the baseline signal and it is inversely proportional to the amount of target RNA in each sample. Thus, the fewer cycles taken to reach the Ct, the greater the initial target gene copy number. In order to determine the relative amount of cDNA for the target gene, the cDNA of a reference gene of the same sample was amplified at the same time. The reference gene is expressed at a constant level and relative fold changes in the mRNA level were calculated with the comparative Ct ($2^{-\delta\Delta C_{t}}$) method (Livak and Schmittgen, 2001) as follows:

\[
\delta C_t = C_t \text{(target gene)} - C_t \text{(reference gene)} \\
\delta \delta C_t = \delta C_t \text{(stimulation)} - \delta C_t \text{(control)} \\
2^{-\delta \Delta C_t}
\]
TaqMan Gene Expression assays from Applied Biosystems with a Real time PCR kit (Sensi MixdT Quantace, London) were used for quantification of cDNA. The assays were performed according to the manufacturer's instructions. Each sample was analysed in duplicate and a negative control with nuclease-free water was added. The identity of the specific TaqMan probes were as follows: for MMP-9 Hs00234579_ml, for IL-32 Hs00170403_ml and for RNA polymerase II (housekeeping gene) Hs00172187_ml.

PCR amplifications were performed in 25 µl volumes using 2.5 µl of cDNA, 12.5 of SensiMix, 1.25 µl of MMP-9 or Polymerase II probes and 8.7 µl Rnase-free water. The PCR cycle was started with 2 minutes at 50°C followed by 10 minutes at 95°C at 40 cycles then followed by 95°C for 15 seconds then 60°C 1 min.

2.5 Gene expression assay

To analyse the different inflammatory genes expressed by OKF6 cells, custom-made Taqman Low-Density Arrays (TLDAs) (Applied Biosystem) were used. The expression profiling was based on real time quantitative reverse transcription and polymerase chain reaction (RT-PCR). The arrays analysed expression of 45 diverse cytokines that regulate the inflammatory processes (Table 2-4 and Table 2-5). The array contains pre-designed primers and Taqman probes and evaluates from one to eight cDNA samples generated from total RNA in a two step RT-PCR experiment and was used according to manufacturer's instructions. 2.5 µl of each 20 µl cDNA reaction was loaded into each port on the array. The 7900HT real time PCR machine was employed for amplification and real time analysis of cDNA samples loaded onto the TLDAs using the comparative Ct methods of relative quantification. The results were analysed using SDS version 2.2 software (ABI).
<table>
<thead>
<tr>
<th>Inflammatory mediators</th>
<th>Primer and probe assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Hs99999905_ml</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Hs99999901_sl</td>
</tr>
<tr>
<td>RNA pol II</td>
<td>Hs00222679_ml</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>Hs00174092_ml</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Hs00174097_ml</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Hs00277299_ml</td>
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<td>IL-18</td>
<td>Hs00155517_ml</td>
</tr>
<tr>
<td>IL-1F6</td>
<td>Hs00205367_ml</td>
</tr>
<tr>
<td>IL-1F7</td>
<td>Hs00367199_ml</td>
</tr>
<tr>
<td>IL-1F8</td>
<td>Hs00758166_ml</td>
</tr>
<tr>
<td>IL-1F9</td>
<td>Hs00219742_ml</td>
</tr>
<tr>
<td>IL-1F10</td>
<td>Hs00544661_ml</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Hs00174128_ml</td>
</tr>
<tr>
<td>IL-4</td>
<td>Hs00174122_ml</td>
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<td>IL-6</td>
<td>Hs00174131_ml</td>
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<td>IL-10</td>
<td>Hs00174086_ml</td>
</tr>
<tr>
<td>IL-12a</td>
<td>Hs00168405_ml</td>
</tr>
<tr>
<td>IL-12b</td>
<td>Hs00233688_ml</td>
</tr>
<tr>
<td>IL-32</td>
<td>Hs 00170403_ml</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Hs00174143_ml</td>
</tr>
<tr>
<td>IL-8</td>
<td>Hs00174103_ml</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
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<tr>
<td>CCL2</td>
<td>Hs00234140_ml</td>
</tr>
<tr>
<td>CCL5</td>
<td>Hs00174575_ml</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>Hs00171086_ml</td>
</tr>
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<td>CXCL5</td>
<td>Hs00607029_ml</td>
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<tr>
<td>CXCL10</td>
<td>Hs00171042_ml</td>
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<td>Inflammatory mediators</td>
<td>Primer and probe assay ID</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td><strong>Receptors</strong></td>
<td></td>
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<tr>
<td>IL-1R1</td>
<td>Hs00168392_ml</td>
</tr>
<tr>
<td>IL-1 RAcP</td>
<td>Hs00370507_ml</td>
</tr>
<tr>
<td>IL-18R1</td>
<td>Hs00155517_ml</td>
</tr>
<tr>
<td>IL-18 RAcP</td>
<td>Hs00187259_ml</td>
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<tr>
<td>TNFRSF1A</td>
<td>Hs00236902_ml</td>
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<tr>
<td>TNFRSF1B</td>
<td>Hs00153550_ml</td>
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<tr>
<td>LEP R</td>
<td>Hs00174877_ml</td>
</tr>
<tr>
<td>ADIPOR1</td>
<td>Hs00360422_ml</td>
</tr>
<tr>
<td>ADIPOR2</td>
<td>Hs00226105_ml</td>
</tr>
<tr>
<td>INSR</td>
<td>Hs00169631_ml</td>
</tr>
<tr>
<td>IL-1RL2</td>
<td>Hs00153957_ml</td>
</tr>
<tr>
<td>OSMR</td>
<td>Hs00384278_ml</td>
</tr>
<tr>
<td>LIFR</td>
<td>Hs00158730_ml</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
</tr>
<tr>
<td>ADAM17</td>
<td>Hs00234221_ml</td>
</tr>
<tr>
<td>AGER</td>
<td>Hs00153957_ml</td>
</tr>
<tr>
<td>Casp-1</td>
<td>Hs00254836_ml</td>
</tr>
<tr>
<td>CD14</td>
<td>Hs00169122_gl</td>
</tr>
<tr>
<td>SCF2</td>
<td>Hs00171266_ml</td>
</tr>
<tr>
<td>PBEF</td>
<td>Hs00237184_ml</td>
</tr>
<tr>
<td>LEB</td>
<td>Hs00174877_ml</td>
</tr>
<tr>
<td>LIF</td>
<td>Hs00171455_ml</td>
</tr>
<tr>
<td>OSM</td>
<td>Hs00171165_ml</td>
</tr>
</tbody>
</table>
2.6 Enzyme linked immunosorbent assay (ELISA)

MMP and cytokine concentrations in cell culture supernatants and MMP levels in GCF and serum samples were measured using ELISA (R&D system Duoset kits, UK). The ELISA method involves coating the plate with anti-human antibody (capture antibody), blocking all unbound sites, adding antigen (in the standard and samples), adding secondary antibody (detection antibody), adding anti-mouse IgG conjugated to an enzyme and adding a substrate to react with the enzyme to produce a coloured product, thus indicating a positive reaction. Antibody concentrations and standard curve detection ranges for each ELISA are listed in Table 2-6.
Table 2-6 Antibody working concentration and standard curve ranges for ELISA

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Capture Antibody (µg/ml)</th>
<th>Detection antibody (ng/ml)</th>
<th>Standard curve range</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-8</td>
<td>2.0</td>
<td>50</td>
<td>4000, 2000, 1000, 500, 250, 125, 62.5</td>
</tr>
<tr>
<td>MMP-9</td>
<td>1.0</td>
<td>200</td>
<td>2000, 1000, 500, 250, 125, 62.5, 31.25</td>
</tr>
<tr>
<td>IL-8</td>
<td>4.0</td>
<td>20</td>
<td>2000, 1000, 500, 250, 125, 62.5, 31.25</td>
</tr>
</tbody>
</table>

The 96-well polystyrene microplates were coated with the capture antibody that diluted PBS, covered and left overnight at room temperature. The plate was washed three times with PBS and 0.05% Tween and the remaining drops were removed by dabbing the plate on a paper towel. 300 µl of reagent diluent (1% BSA) in PBS was added for one hour to block non-specific binding, and then the plate was washed (as above) and drained well. 100 µl of standard diluted in reagent diluent or samples was added and left for 2 hours according to the manufacturer’s instructions. Wells were washed three times (as above) and 100 µl of detection antibody in reagent diluent was added to each well and incubated for 2 hours. After washing three times, the ELISA was developed using 100 µl streptavidin horse-radish peroxidase (HRP) in reagent diluents (1/200) which was added to each well and incubated for 20 minutes at room temperature. The plate was kept away from direct light during this step. The amount of peroxidase bound to each well was determined by addition of 100 µl tetramethylbenzidine substrate solution (solution A: solution B) 1:1 for 20 minutes. The reaction was stopped by adding 50 µl sulphuric acid to each well. The protein concentration was determined by measuring absorbance at 450 nm using a Bio TEK FL 600 micro plate fluorescence reader with wavelength correction set at 550 nm and finding the concentration from the standard curve using a 4-parameter logistic curve fit. The standard curves for each ELISA used in the study are illustrated in Figure 2-3, Figure 2-4 and Figure 2-5.
Figure 2-3 Standard curve for the MMP-9 ELISA

The Δ OD of the different MMP-9 concentrations were plotted against the MMP-9 concentrations. A 4-parameter curve fit was created to produce the 7-point standard curve for the MMP-9 ELISA.
The Δ OD of the different MMP-8 concentrations were plotted against the MMP-8 concentrations. A 4-parameter curve fit was created to produce the 7-point standard curve for the MMP-8 ELISA.
Figure 2-5 Standard curve for the IL-8 ELISA

The Δ OD of the different IL-8 concentrations were plotted against the IL-8 concentrations. A 4-parameter curve fit was created to produce the 7-point standard curve for the IL-8 ELISA.
2.6.1 Human sample validation assay for MMP-8 and MMP-9 ELISA

Although duo sets ELISA kits are validated by the company (R&D) for analysis of cytokines and enzymes in culture supernatants, they are not optimised for use in serum and GCF samples. Many factors such as extremes in pH, high protein concentrations, and high buffer salt concentrations may interfere with the accuracy of ELISA. In addition, GCF and serum samples may contain many proteins such as complement components that may affect the binding of antibody and interfere with ELISA methods that in turn affect the overall results. For these reasons it is necessary to determine whether the assay will yield accurate results in the presence of biological fluids. Thus, spike/recovery and linearity experiments were performed to verify the MMP-9 and MMP-8 ELISAs for the use of human GCF and serum samples. In spike/recovery experiments, an identified amount of ELISA standard is added (spiked) into the sample and the resulting concentration (recovery of the spiked sample) indicates whether a component in the sample interferes with the ELISA. A linearity of dilution test was also performed to provide information about the precision of assay results for samples tested at different dilutions. Also, intra-assay and inter-assay precision analyse were performed for assays of MMP-8 and MMP-9 in serum and GCF samples. Furthermore, the sensitivity of the assays was measured for MMP-9 and MMP-8 ELISAs for serum and GCF samples.

2.6.1.1 Spike/recovery experiment

Levels of MMP-9 and MMP-8 in human GCF and serum are found in the ng/ml range. For that reason, serum and GCF samples were diluted in reagent diluent to adjust the value of the sample within the range of the standard curve. 1:2000 and a 1:500 dilutions of serum and GCF samples to investigate MMP-9 concentration were created and 1:50
and 1:2000 dilutions of GCF and serum respectively were made to investigate MMP-8 levels. The dilutions used in the spike and recovery assays were treated as the new neat samples. The validation for the MMP-8 ELISA was performed as follows: a 1:50 dilution of GCF was spiked with 1000 pg/ml human recombinant MMP-8 and 1:2000 serum MMP-8 dilution was spiked with 700 pg/ml. In the MMP-9 validation assay, the 1:500 dilution of GCF was spiked with 800 pg/ml human recombinant MMP-9. The 1:2000 dilution of serum samples were spiked with 700 pg/ml human recombinant MMP-9. To create a spiked control, the reagent diluent was spiked with the same amount of recombinant MMP-8 or MMP-9. The spiked sample, the neat sample and the spiked control were then diluted in reagent diluent in two fold dilution series (1:2, 1:4 and 1:8). The percentage of recovery for the spiked samples, the spiked controls and the serial dilutions were calculated as follows:

Spiked sample recovery: 
\[
\frac{\text{spiked sample} - \text{neat sample}}{\text{amount spiked}} \times 100
\]

Spiked control recovery: 
\[
\frac{\text{spiked control}}{\text{amount spiked}} \times 100
\]

Recovery for 1:2 dilution: 
\[
\frac{\text{spiked sample}}{1:2 \text{ spiked sample}} \times 100
\]

Generally the recovery of spiked and neat samples should be between 80 and 120 % and this indicates that the assay is appropriate for utilization with the samples (R&D systems). The spike and recovery results for MMP-8 and MMP-9 in GCF samples are presented in Table 2-7 and Table 2-8. The mean percentages of the spiked sample recovery for MMP-8 and MMP-9 in GCF were 114.7% and 99.1% respectively. The spike and recovery assays for MMP-9 and MMP-8 in serum samples are listed in Table 2-9 and Table 2-9. The mean percentage of the spiked sample recovery for MMP-9 in
serum sample was 110.0%. Therefore, the analysis of the performance of the ELISAs indicated that these assays can be used for monitoring MMP concentrations in human GCF and serum samples. In addition, the serial dilutions of the spiked samples and control were within the precision range (80-120%). Thus, the results suggested the tested samples had no factors that interfered with the accuracy of the ELISAs and therefore we can have confidence in the accuracy of the results obtained from using the ELISA kits with human GCF and serum samples. On the other hand, the mean percentage of the spiked sample recovery for MMP-8 in serum sample was 38.8%. Therefore, the analysis of the performance of the MMP-8 ELISA indicated that this assay could not be reliably used for monitoring MMP-8 concentrations in human serum samples.
Table 2-7 Spike/recovery and linearity of the MMP-8 ELISA for human GCF samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>GCF MMP-8 (pg/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiked sample</td>
<td>1610.6</td>
<td>114.7</td>
</tr>
<tr>
<td>1:2 spiked sample</td>
<td>1607.2</td>
<td>99.7</td>
</tr>
<tr>
<td>1:4 spiked sample</td>
<td>1675.3</td>
<td>104.0</td>
</tr>
<tr>
<td>1:8 spiked sample</td>
<td>1643.1</td>
<td>102.0</td>
</tr>
<tr>
<td>Neat sample</td>
<td>463.0</td>
<td>-</td>
</tr>
<tr>
<td>1:2 neat sample</td>
<td>538.4</td>
<td>116.2</td>
</tr>
<tr>
<td>1:4 neat sample</td>
<td>633.4</td>
<td>136.8</td>
</tr>
<tr>
<td>1:8 neat sample</td>
<td>682.2</td>
<td>147.3</td>
</tr>
<tr>
<td>Spiked control</td>
<td>985.3</td>
<td>98.5</td>
</tr>
<tr>
<td>1:2 spiked control</td>
<td>1048.5</td>
<td>106.4</td>
</tr>
<tr>
<td>1:4 spiked control</td>
<td>1142.7</td>
<td>115.9</td>
</tr>
<tr>
<td>1:8 spiked control</td>
<td>1164.7</td>
<td>118.2</td>
</tr>
</tbody>
</table>

The table shows the obtained MMP-8 concentration in human GCF samples and the % recovery. The human GCF samples were spiked with 1000 pg/ml human recombinant MMP-8. Spiked controls (reagent diluent) were spiked with the same amount. 2-fold serial dilutions were prepared for spiked samples, neat samples and spiked controls.
Table 2-8 Spike/recovery and linearity of MMP-9 ELISA for human GCF samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>GCF MMP-9 (pg/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiked sample</td>
<td>1179.9</td>
<td>99.1</td>
</tr>
<tr>
<td>1:2 spiked sample</td>
<td>1286.4</td>
<td>109.0</td>
</tr>
<tr>
<td>1:4 spiked sample</td>
<td>1154.9</td>
<td>97.8</td>
</tr>
<tr>
<td>1:1 spiked sample</td>
<td>1290.7</td>
<td>109.3</td>
</tr>
<tr>
<td>Neat sample</td>
<td>387.0</td>
<td>-</td>
</tr>
<tr>
<td>1:2 neat sample</td>
<td>388.5</td>
<td>100.3</td>
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<tr>
<td>1:4 neat sample</td>
<td>382.4</td>
<td>98.8</td>
</tr>
<tr>
<td>1:8 neat sample</td>
<td>156.3</td>
<td>40.3</td>
</tr>
<tr>
<td>Spiked control</td>
<td>687.1</td>
<td>85.8</td>
</tr>
<tr>
<td>1:2 spiked control</td>
<td>793.7</td>
<td>115.5</td>
</tr>
<tr>
<td>1:4 spiked control</td>
<td>894.5</td>
<td>120.1</td>
</tr>
<tr>
<td>1:8 spiked control</td>
<td>762.4</td>
<td>110.9</td>
</tr>
</tbody>
</table>

The table shows the obtained MMP-9 concentrations in human GCF samples and the % recovery. The human GCF samples were spiked with 800 pg/ml human recombinant MMP-9. Spiked control (reagent diluent) was spiked with the same amount. 2-fold serial dilutions were prepared for spiked samples, neat samples and spiked controls.
Table 2-9 Spike/recovery and linearity of MMP-9 for human serum samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Serum MMP-9 (pg/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiked sample</td>
<td>1039.7</td>
<td>110.0</td>
</tr>
<tr>
<td>1:2 spiked sample</td>
<td>1002.5</td>
<td>96.4</td>
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<tr>
<td>1:4 spiked sample</td>
<td>974.9</td>
<td>93.7</td>
</tr>
<tr>
<td>1:8 spiked sample</td>
<td>963.6</td>
<td>92.6</td>
</tr>
<tr>
<td>Neat sample</td>
<td>269.2</td>
<td>-</td>
</tr>
<tr>
<td>1:2 neat sample</td>
<td>260.6</td>
<td>96.8</td>
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<td>1:4 neat sample</td>
<td>210.9</td>
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<td>1:8 neat sample</td>
<td>140.9</td>
<td>52.3</td>
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<tr>
<td>Spiked control</td>
<td>591.4</td>
<td>84.4</td>
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<tr>
<td>1:2 spiked control</td>
<td>585.5</td>
<td>98.9</td>
</tr>
<tr>
<td>1:4 spiked control</td>
<td>625.3</td>
<td>105.7</td>
</tr>
<tr>
<td>1:8 spiked control</td>
<td>611.9</td>
<td>103.4</td>
</tr>
</tbody>
</table>

The table shows the obtained MMP-9 concentrations in human serum samples and the % recovery. The human serum samples were spiked with 700 pg/ml human recombinant MMP-9. Spiked control (reagent diluent) was spiked with the same amount. 2-fold serial dilutions were prepared for spiked samples, neat samples and spiked controls.
Table 2-10 Spike/recovery and linearity of MMP-8 for human serum samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Serum MMP-8 (pg/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiked sample</td>
<td>773.5</td>
<td>38.8</td>
</tr>
<tr>
<td>1:2 spiked sample</td>
<td>1455.3</td>
<td>188.1</td>
</tr>
<tr>
<td>1:4 spiked sample</td>
<td>2446.8</td>
<td>316.3</td>
</tr>
<tr>
<td>1:8 spiked sample</td>
<td>3292.6</td>
<td>425.6</td>
</tr>
<tr>
<td>Neat sample</td>
<td>463.0</td>
<td>*</td>
</tr>
<tr>
<td>1:2 neat sample</td>
<td>538.4</td>
<td>116.2</td>
</tr>
<tr>
<td>1:4 neat sample</td>
<td>623.4</td>
<td>136.8</td>
</tr>
<tr>
<td>1:8 neat sample</td>
<td>682.2</td>
<td>147.3</td>
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<tr>
<td>Spiked control</td>
<td>985.3</td>
<td>140.0</td>
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<tr>
<td>1:2 spiked control</td>
<td>1048.5</td>
<td>106.4</td>
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<td>1:4 spiked control</td>
<td>1142.7</td>
<td>115.9</td>
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<tr>
<td>1:8 spiked control</td>
<td>1164.7</td>
<td>118.2</td>
</tr>
</tbody>
</table>

The table shows the obtained MMP-8 concentrations in human serum samples and the % recovery. The human serum samples were spiked with 700 pg/ml human recombinant MMP-8. Spiked control (reagent diluent) was spiked with the same amount. 2-fold serial dilutions were prepared for spiked samples, neat samples and spiked controls.
2.6.1.2 Intra and inter assay variation

Intra-assay variation for MMP-8 and MMP-9 ELISAs for GCF and serum samples was established in 6 replicates in a single assay. The inter-assay variation for the MMP-9 ELISA for serum samples was determined in three independent assays. The intra-and inter-assay variation was calculated as follow:

\[
\frac{SD}{mean} \times 100
\]

Intra-and inter-assay variation for the MMP-8 ELISA for GCF samples were 1.1% and 4.4%, respectively. Intra-and inter-assay for the MMP-9 ELISA for GCF samples were 8.6% and 4.9% and for MMP-9 for serum samples 3.0% and 3.6% respectively (Table 2-11 and Table 2-12). In conclusion, the assay variation was less than 10.0% in all ELISAs which indicates a high reproducibility for both MMPs when assayed in GCF and serum samples.

2.6.1.3 Assay sensitivity

The assay sensitivity for the MMP-8 and MMP-9 ELISAs was established by multiplying standard deviations of the negative controls with 2.5 and adding the results to the mean OD value of the negative control and calculation of the corresponding concentration in the 4 parameter curve fit by means of the solve function in SigmaPlot. The lowest measurable amount for MMP-8 was 5.2 pg/ml and for MMP-9 was 2.1 pg/ml.
Table 2-11 Intra-assay variation of MMP-8 and MMP-9 ELISAs for human GCF and serum samples

<table>
<thead>
<tr>
<th>Replicate number</th>
<th>GCF MMP-8 (ng/ml)</th>
<th>GCF MMP-9 (ng/ml)</th>
<th>Serum MMP-9 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.4</td>
<td>75.1</td>
<td>477.7</td>
</tr>
<tr>
<td>2</td>
<td>31.5</td>
<td>79.6</td>
<td>479.9</td>
</tr>
<tr>
<td>3</td>
<td>32.0</td>
<td>67.2</td>
<td>452.8</td>
</tr>
<tr>
<td>4</td>
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<td>69.9</td>
<td>491.5</td>
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<td>31.0</td>
<td>63.3</td>
<td>487.1</td>
</tr>
<tr>
<td>6</td>
<td>31.2</td>
<td>66.2</td>
<td>463.4</td>
</tr>
</tbody>
</table>

Mean 31.5 70.2 475.4

Standard Deviation 0.3 6.0 14.6

Intra-assay variation (%) 1.1 8.6 3.0

Human GCF and serum samples were analysed in replicate for MMP-8 and MMP-9 in one assay for each sample type and the intra-assay variation was calculated.
Table 2-12 Inter-assay variation of MMP-8 and MMP-9 ELISA for human GCF and serum samples

<table>
<thead>
<tr>
<th>Replicate number</th>
<th>GCF MMP8 (ng/ml)</th>
<th>GCF MMP-9 (ng/ml)</th>
<th>Serum MMP-9 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31.1</td>
<td>136.4</td>
<td>465.2</td>
</tr>
<tr>
<td>2</td>
<td>28.8</td>
<td>142.8</td>
<td>485.7</td>
</tr>
<tr>
<td>3</td>
<td>28.9</td>
<td>130.6</td>
<td>500.5</td>
</tr>
</tbody>
</table>

Mean 29.6 136.6 483.8

Standard Deviation 1.3 6.0 17.7

Inter-assay Variation (%) 4.4 4.4 3.6

Human GCF samples were analysed for MMP-8 and MMP-9 in independent assays for each sample type and the inter-assay variation was calculated.
2.7 Study samples

Diabetic patients were recruited from GP practices and secondary care diabetes clinics (Newcastle, UK). The non-diabetic controls were invited from consultant clinics at Newcastle Dental Hospital or from staff of the Dental School, Newcastle University. T2DM and non-diabetic patients with and without periodontal disease were examined in a periodontitis/diabetic clinic in the School of Dental Sciences, Newcastle University. Enrolment, screening, diagnosis and treatment of patients were conducted with the help of a clinical team (Prof. Philip Preshaw, Rebecca Wassall, Susan Bissett, Hannah Fraser, Kerry Stone). This study was reviewed and approved by the Sunderland Research Ethics Committee (06/Q0904/8).

The study population consisted of 101 diabetic individuals and 83 controls (non-diabetic). Subjects were 30-55 years old, male or female, with minimum of 20 natural teeth and all subjects were in good general health. Individuals were excluded from the study if they had any bleeding disorder, were pregnant, had any condition requiring prophylactic antibiotics before dental management, were taking drugs that induce gingival overgrowth, were taking immunosuppressant drugs, or if they had scaling or RSI in the previous 6 months. The general characteristics of the study are presented in Table 2-13. Both T2DM and control groups showed no significant differences regarding numbers of the subject, age, smoking status and ethnicity.
# Table 2-13  Study samples characteristics

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic</th>
<th>T2DM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>48 ± 7</td>
<td>48 ± 6</td>
<td>0.7</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>50/33</td>
<td>67/34</td>
<td>0.5/0.7</td>
</tr>
<tr>
<td>Years with T2DM</td>
<td>-</td>
<td>7 ± 5</td>
<td>-</td>
</tr>
<tr>
<td>Race (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>83</td>
<td>96</td>
<td>0.3</td>
</tr>
<tr>
<td>Black</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Asian</td>
<td>-</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Smoking Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>9</td>
<td>10</td>
<td>0.8</td>
</tr>
<tr>
<td>Ex</td>
<td>25</td>
<td>37</td>
<td>0.1</td>
</tr>
<tr>
<td>Non</td>
<td>49</td>
<td>54</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The table shows diabetic status, sex and smoking status. Age and years with T2DM are given as mean± standard deviation. Chi-square ($\chi^2$) showed no statistical differences between the diabetic and non-diabetic individuals p > 0.05. Student’s t-tests showed no statistical differences between the age of the diabetic individuals and the control group p > 0.05.
2.7.1 Screening and treatment of patients

All subjects were evaluated clinically and radiographically at the first visit (screening). Patients received a full periodontal examination that included plaque index, gingival index, probing depths (PD), clinical attachment loss (CAL), and bleeding on probing (BOP). The diagnosis of subjects was established on the basis of clinical criteria that were proposed by the 2005 European Workshop on Periodontology and the 2007 Centre for Disease Control and Prevention-American Academy of Periodontology collaboration (Tonetti and Claffey, 2005; Page and Eke, 2007) which were as follows:

- Periodontal health was defined as $\leq 15\%$ BOP, no attachment loss, no bone loss, and PD $\leq 3$.
- The subject with gingivitis had BOP $> 15\%$, no attachment loss, no bone loss and PD $\leq 4$mm.
- The subject with chronic periodontitis had $\geq 6$ sites with PD of $\geq 5$mm with loss of attachment and/or bone loss.

Along with the clinical examination, GCF and serum samples were obtained from the participants at screening. After screening, periodontal non-surgical treatment was provided for patients with periodontal disease using oral hygiene instruction and a full mouth instrumentation approach (Quirynen et al., 2000). The patients were followed up after 3, 6 and 12 months and further clinical examination, GCF and serum samples collection were performed and periodontal treatment was provided as necessary. Patients with gingivitis were given oral hygiene instruction and a full mouth prophylaxis at the time of screening. The procedures undertaken in this study are presented in Figure 2-6.
Figure 2-6 The study overview

Consent-Demographic data-Diabetes history-Smoking status-Physical examination-Oral examination-GCF samples-Periodontal examination-Blood samples

(Month 0) Periodontal treatment

(Month 3) Oral examination-GCF samples-Periodontal examination-Blood samples-Prophylaxis-Additional periodontal therapy

(Month 6) Oral examination-GCF samples-Periodontal examination-Blood samples-Prophylaxis-Additional periodontal therapy

(Month 12) Oral examination-GCF samples-Periodontal examination-Blood samples-Prophylaxis-Additional periodontal therapy
2.7.2 GCF collection

The GCF samples were obtained from 4 teeth per patient at screening, month 3, 6, 12 using PerioPapers. The samples were collected from the mesiobuccal aspects of the four first molars. If the first molar was absent in a quadrant, the second molar was sampled, then the second premolar, then the first premolar, then the canine. After isolation of the site with cotton rolls and a saliva ejector was used to prevent contamination with saliva, supra-gingival plaque was removed with a curette prior to sampling and the tooth was air dried. The PerioPaper was gently inserted into the sulcus and held for 30 seconds. GCF volume was determined using a calibrated Periotron 6000 machine (Preshaw et al., 1996). The PerioPapers were immediately placed into an individual sterile cyrovial containing 150 µl PBS and kept on ice then transferred to the laboratory and were frozen at -80°C until analysed. Samples visibly contaminated with blood were discarded. Elution of GCF from the PerioPaper strips was performed by thawing the samples and adding 50 µl of 1% BSA in PBS. Centrifugation of the samples was done using Sigma 3K10 centrifuge for 60 minutes at 300 rpm at 4°C. This was then followed by another centrifugation for 2 minutes at 12000 rpm at 4°C. Following the elution, each GCF sample was analysed separately.
2.7.3 Serum collection

Blood samples were obtained at screening and months 3, 6 and 12. Venous blood was collected as follows: part of the blood samples were sent to the Clinical Biochemistry Laboratory of the Royal Victoria Infirmary (Newcastle upon Tyne) for HbA1c, hsCRP, cholesterol, HDL, LDL and triglyceride measurements. One part of the blood samples was used for analysis of serum MMPs and within 1 hour, the blood samples were centrifuged for 15 minutes at 4°C at 1500g and the serum was collected and frozen at -80°C until further investigation by ELISA.

2.8 Statistical analysis

Data were analysed with SPSS 15.0 statistical software and the analysing were performed for both the cell culture and the clinical data.

2.8.1 Analysis of cell culture data

All data were analysed for normal distribution and homogeneity of variances; the Shapiro-Wilk test was used to determine if the data were normally distributed. Levene test was used to assess the equality of the variances. To achieve normal distribution and homogeneity of variance, transformations such as square root, common log or multiplicative inverse were performed. Non-parametric data were analysed with the Kruskal-Wallis or Mann-Whitney U test. One way ANOVA test and Student t-test were used when the data passed the normality test. p-values were corrected for multiple comparisons with the Bonferroni-Holm correction.
2.8.2 Analysis of clinical data

Chi-square ($\chi^2$) analysis was applied for testing the significance of differences in categorical variables and Student's $t$-tests were used for analysing the numerical data. The statistical differences between groups were tested using the ANOVA and Kruskal-Wallis test including a post-hoc multiple comparison procedure when significant associations were observed. Comparisons between two groups were performed by the Mann-Whitney U-test. Longitudinal parametric data were analysed with ANOVA and the paired sample $t$-test. Non-parametric data were analysed using the Wilcoxon and Mann-Whitney test. Differences were considered significant when $p < 0.05$. The coefficient of the correlation according to Spearman rank order was calculated in order to test the degree of the relationship between variables. The correlations were considered statistically significant when $p < 0.05$. The relationship between the clinical parameters and the MMPs was also tested.
Chapter 3  Expression of MMPs and cytokines by keratinocytes in response to IL-1β

3.1 Introduction

The inflammatory processes in periodontal disease are largely initiated by microbes and are caused by inflammatory mediators and host derived proteinases that result in periodontal destruction (Williams, 2008). Epithelial cells are associated with the health of the periodontal tissue as they are the first cells to encounter bacteria (Dale, 2002). They may respond to external stimuli by migrating, proliferating and making many inflammatory mediators and enzymes (Uitto et al., 2003) that have an important function during physiological and pathological conditions (Alexander and Damoulis, 1994).

It was reported that production of inflammatory mediators such as IL-1β plays a key role in epithelial inflammation in periodontitis (Barksby et al., 2007) and induces other inflammatory cytokines such as IL-6 and IL-8 (Sfakianakis et al., 2001) and MMPs (Milner and Cavston, 2005). Furthermore, IL-1β induces chemotaxis of leukocytes by inducing the release of IL-8 and activating neutrophils for phagocytosis (Cohen, 2002). It was also reported that the release of IL-1β is a critical step in inflammation through the induction of other inflammatory cytokines such as IL-6 (Tschopp et al., 2003). IL-1β is involved in inflammation in a variety of tissues and diseases (Sims et al., 2001) and has been identified as a potent inducer of MMPs (Birkedal-Hansen, 1993a; Hayden et al., 2010). Increased levels of this cytokine may attract neutrophils which contribute to connective tissue damage by induction of MMP-8 (Cox et al., 2006).
IL-1β upregulates production of MMP-8 mRNA in cultured gingival fibroblasts (Abe et al., 2001) and MMP-13 production in ostoblasts (Liacini et al., 2003; Julovi et al., 2004). Increased levels of MMP-1 and MMP-3 have been reported in gingival fibroblasts after stimulation of the cells with IL-1β (Domeij et al., 2004b). Also, MMP-1 and MMP-3 are produced in inflamed gingival tissue by fibroblasts (Ingman et al., 1994). It was reported that IL-1β upregulates MMP-1 and MMP-2 expressions in primary fibroblasts (Xiang et al., 2009). In addition, regulation of MMP synthesis and secretion by inflammatory cytokines such as IL-1β and TNF-α has been reported in fibroblasts and chondrocytes (Lefebvre et al., 1991; DiBattista et al., 1995) that suggests a pivotal role for cytokines in the inflammatory process.

Proteinases, in particular MMPs are released by inflammatory cells recruited to the area of the inflammation (Uitto et al., 2003) and also from the resident gingival fibroblasts in response to cytokines that are produced by the infiltrating cells (Reynolds and Meikle, 1997). MMPs are regarded as key mediators of tissue destruction in periodontitis (Kinane, 2000; Sorsa et al., 2004) and there is evidence that irreversible connective tissue degradation in periodontitis results from imbalance between MMPs and their tissue inhibitors, TIMPs (Ingman et al., 1996; Sorsa et al., 2006). In addition, evidence has suggested that MMPs function to promote cell migration by clearing the extracellular matrix (Lund et al., 1999). Furthermore, it was suggested that MMP-9 might inhibit the rate of epithelial wound closure and regulate the inflammatory response (Mohan et al., 2002).
Among the MMPs, collagenases (MMP-1, MMP-8 and MMP-13) have the capacity to degrade interstitial collagens (Nagase et al., 2006) and are expressed by different types of cells. For example, MMP-1 is mainly expressed by human fibroblasts but can also be expressed by keratinocytes, endothelial cells, macrophages, osteoblasts and chondrocytes (Birkedal-Hansen, 1993b) and serves as an initiator of extracellular matrix destruction (Domeij et al., 2004a). MMP-8 is considered the most important protein in tissue disruption in periodontal disease (Westerlund et al., 1996) and is thought to be mainly produced by PMNLs. Its activity and release is regulated by cytokines and bacterial pathogens in many cell types such as fibroblasts, endothelial cells and odontoblasts (Hanemaaijer et al., 1997; Palosaari et al., 2003). Other cellular sources of MMP-8 include sulcular epithelial cells (Hanemaaijer et al., 1997; Tervahartiala et al., 2000; Wahlgren et al., 2001) which indicates that epithelial cells can interact with the tissue-destructive MMP network (Kornman et al., 1997). Other studies reported mRNA expression only for MMP-8 in gingival fibroblasts (Abe et al., 2001) and in dermal skin cancer cells (Giambertardi et al., 1998). MMP-13 has also been identified in the pocket epithelium in periodontitis patients (Uiito et al., 1998; Tervahartiala et al., 2000), in macrophage-like cells (Kiili et al., 2002) and in fibroblasts. Cox et al showed that gingival fibroblasts stimulated with IL-1β secreted MMP-13 together with other collagenolytic MMPs (MMP-1, MMP-2, MMP-8 and MMP-14) (Cox et al., 2006).

Gelatinases (MMP-2 and MMP-9) have been suggested to participate in periodontal tissue destruction because significantly higher levels of MMP-9 and elevated levels of MMP-2 have been found in GCF samples from patients with periodontal disease (Makela et al., 1994; Ingman et al., 1996; Beklen et al., 2006). MMP-2 is produced predominantly by fibroblasts (Nagase et al., 2006) and has been detected in chronic
periodontitis-affected gingival tissues (Korostoff et al., 2000). In periodontitis, the major source of MMP-9 is neutrophils and to lesser extent also monocytes and macrophages (Westerlund et al., 1996; Pirila et al., 2001). It was suggested that periodontal tissue destruction has been associated with elevated levels of MMP-9 in GCF (Teng et al., 1992).

The stromelysin sub-family members, MMP-3, MMP-10 and MMP-11 can activate other proMMPs including MMP-1, MMP-8, MMP-9, and MMP-13 (Kahari and Saarialho-Kere, 1999; Moilanen et al., 2003). They are expressed by various cell types such as fibroblasts, keratinocytes, and chondrocytes (Nagase et al., 2006). Alpagot et al detected significantly higher levels of MMP-3 in GCF samples from patients with periodontal disease compared to healthy individuals (Alpagot et al., 2001) and this result is in accordance with the finding that the levels of MMP-3 in GCF samples from periodontal disease sites were significantly higher than in healthy sites (Haerian et al., 1995).

The matrilysin subgroup members are MMP-7 and MMP-26. MMP-7 is synthesized by epithelial cells and it may be related to epithelial migration (Dunsmore et al., 1998), and antibacterial defence of junctional epithelium (Utto et al., 2003). MMP-7 has been detected in GCF from periodontitis sites (Tervahartiala et al., 2000) as has MMP-26 (Emingil et al., 2006).
Membrane type-MMP (MT-MMPs) such as MMP-14 is expressed by gingival fibroblasts from periodontitis patients (Cox et al., 2006) and can also be detected in GCF from periodontitis sites (Tervahartiala et al., 2000). MMP-14 was also detected in fibroblasts from inflamed gingival tissue (Dahan et al., 2001). In addition, another MT-MMP (MMP-25) has been detected in GCF from patients with periodontal disease (Emingil et al., 2006).

The available evidence therefore suggests that MMPs expressed by gingival epithelial cells might play a prominent role in the inflammatory process. The aim of this chapter was to elucidate the possible involvement of gingival keratinocytes in the host response by determining whether oral keratinocytes respond to IL-1β by expression of IL-8 and MMPs. Because IL-32 plays a role in the inflammatory process and has been detected in epithelial cells and possesses the ability to induce other cytokines (Dinarello and Kim, 2006). It was interesting to study the effects of IL-1β on the expression of IL-32 in oral epithelial cells, as this could provide further understanding of how cytokine networks function in periodontal disease. OKF6 cells as well as primary cells were used in the experiments.

3.2 Results

3.2.1 The effect of IL-1β on IL-8 production by OKF6 cells

IL-8 production by OKF6 cells in response to IL-1β was investigated. OKF6 cells were stimulated with human recombinant IL-1β (0.1 ng/ml) for 4, 24, 48 hours as described previously (section 2.2.5). Tissue culture supernatants from stimulated and unstimulated cells were collected. Responsiveness of cells to IL-1β was first examined by measuring IL-8 secretion using ELISA as described previously (section 2.6). The results showed
an increase in IL-8 protein levels in the tissue culture supernatants from cells treated with IL-1β at different time points (4, 24, 48h) compared to the controls (untreated cells) which produced limited amounts of the protein. As shown in (Figure 3-1), the samples treated with 0.1 ng/ml IL-1β for 4h secreted IL-8 with concentrations reaching a mean value of 0.3859 ± 0.2265 ng/ml compared to untreated cells (0.0589 ± 0.0549 ng/ml). The samples treated with IL-1β for 24 and 48h secreted IL-8 with concentrations reaching a mean value of 0.8503 ± 0.2174 ng/ml and 2.296 ± 2.061 ng/ml compared with corresponding controls (0.2659 ± 0.1837 ng/ml & 0.4393 ± 0.3091 ng/ml) respectively. Mean IL-8 levels at 4, 24, and 48h were significantly enhanced compared to the untreated OKF6 cells (p < 0.01). A time-dependent secretion of IL-8 was seen in samples treated with IL-1β. A significant increase (2 fold) in IL-8 secretion at 24h stimulation was observed compared with 4h stimulation (p < 0.01). Secretion of IL-8 by OKF6 cells stimulated for 48h also showed a significant increase (almost 6 fold) compared with secretion of IL-8 by OKF6 cells stimulated for 4h (p < 0.01).
Figure 3-1 The effect of IL-1β on IL-8 production by OKF6 cells

OKF6 cells were stimulated with IL-1β (0.1 ng/ml) for 4h, 24h and 48h. Supernatants were collected and analysed for IL-8 secretion by ELISA. The graph represents means and standard deviations from three independent cell culture experiments. Statistics: Kruskal-Wallis, Mann-Whitney U test **p < 0.01 compared to control.
3.2.2 The effect of IL-1β on MMP expression by OKF6 cells

The effect of IL-1β on MMP mRNA expression in OKF6 cells was evaluated by screening for all MMP members using RT-PCR. Cells were cultured as previously described (section 2.2.1) and stimulated with 0.1 ng/ml IL-1β for 4, 24, and 48h. The cells were collected, and RNA extraction, quantification and reverse transcription (section 2.3.3) were performed as previously stated (section 2.3.1), (section 2.3.2), (section 2.3.3). PCR for MMP mRNA was carried out as previously described (section 2.3.5). The results showed that a wide range of MMP mRNA was constitutively expressed by OKF6 cells as can be seen in Figure 3-2. MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-11, MMP-12, MMP-14, MMP-15, MMP-16, MMP-17, MMP-19, MMP-25, and MMP-28 were constitutively expressed by the controls and IL-1β treated OKF6 cells. However, no expression of MMP-8, MMP-13, MMP-20, MMP-21, MMP-23, MMP-24, MMP-26, and MMP-27 was detected in the cell lines (Figure 3-3). An overview of the MMP expression patterns in OKF6 cells is given in (Table 3-1).
Sub-confluent OKF6 cells were incubated for 4, 24, and 48h with 0.1 ng/ml IL-1β. Total cellular RNA was isolated and analysed by RT-PCR. Sequences in the cDNA were amplified with specific primers for MMPs (section 2.3.5). The products were separated on 3% agarose gels and stained with ethidium bromide.
Figure 3-3 MMP genes not expressed by OKF6 cells

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>IL-1β</th>
<th>4h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Posit</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

435bp MMP-8
554bp MMP-13
321bp MMP-20
223bp MMP-21
518bp MMP-23
860bp MMP-24
313bp MMP-26
205bp MMP-27

Sub-confluent OKF6 cells were incubated for 4, 24, and 48h with 0.1 ng/ml IL-1β. Total cellular RNA was isolated and analysed by RT-PCR. Sequences in the cDNA were amplified with specific primers for MMPs (section 2.3.5). The products were separated on 3% agarose gels and stained with ethidium bromide. Positive control = chondrocytes.
### Table 3-1 Expression of MMP genes by OKF6 cells

<table>
<thead>
<tr>
<th>Family</th>
<th>MMP</th>
<th>Name</th>
<th>Expression in OKF6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenases</td>
<td>MMP-1</td>
<td>Collagenase-1</td>
<td>+</td>
</tr>
<tr>
<td>Gelatinases</td>
<td>MMP-2</td>
<td>Gelatinase-A</td>
<td>+</td>
</tr>
<tr>
<td>Stromelysines</td>
<td>MMP-3</td>
<td>Stromelysin-1</td>
<td>+</td>
</tr>
<tr>
<td>Matrilysins</td>
<td>MMP-7</td>
<td>Matrilysin-1</td>
<td>+</td>
</tr>
<tr>
<td>Collagenases</td>
<td>MMP-8</td>
<td>Collagenase-2</td>
<td>-</td>
</tr>
<tr>
<td>Gelatinases</td>
<td>MMP-9</td>
<td>Gelatinase-B</td>
<td>+</td>
</tr>
<tr>
<td>Stromelysines</td>
<td>MMP-10</td>
<td>Stromelysin-2</td>
<td>+</td>
</tr>
<tr>
<td>Stromelysines</td>
<td>MMP-11</td>
<td>Stromelysin-3</td>
<td>+</td>
</tr>
<tr>
<td>Other</td>
<td>MMP-12</td>
<td>Macrophage elastase</td>
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</tr>
<tr>
<td>Collagenases</td>
<td>MMP-13</td>
<td>Collagenases-3</td>
<td>-</td>
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<tr>
<td>Membrane type</td>
<td>MMP-14</td>
<td>MT1-MMP</td>
<td>+</td>
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<td>MT2-MMP</td>
<td>+</td>
</tr>
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<tr>
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<td>MMP-19</td>
<td>No trivial name</td>
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</tr>
<tr>
<td>Other</td>
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<td>Enamelysin</td>
<td>-</td>
</tr>
<tr>
<td>Other</td>
<td>MMP-21</td>
<td>XMMP</td>
<td>-</td>
</tr>
<tr>
<td>Other</td>
<td>MMP-23</td>
<td>EA-MMP</td>
<td>-</td>
</tr>
<tr>
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<td>MMP-24</td>
<td>MT5-MMP</td>
<td>-</td>
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<td>Membrane type</td>
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<td>MT6-MMP</td>
<td>+</td>
</tr>
<tr>
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<td>MMP-26</td>
<td>Matrilysin-2</td>
<td>-</td>
</tr>
<tr>
<td>Other</td>
<td>MMP-27</td>
<td>No trivial name</td>
<td>-</td>
</tr>
<tr>
<td>Other</td>
<td>MMP-28</td>
<td>Epilysin</td>
<td>+</td>
</tr>
</tbody>
</table>

The table shows the MMP expressions in OKF6 cells using RT-PCR. MMP expressed (+) and MMP not expressed (-).
3.2.3 *The effect of IL-1β on MMP-9 expression by OKF6 cells*

Since the MMP-9 gene was expressed in OKF6 cells stimulated with IL-1β, cytokine regulation of MMP-9 mRNA levels expressed in OKF6 cells were analysed using Real Time RT-PCR. Cells were cultured as previously stated (section 2.2.1) and stimulated with 0.1 ng/ml IL-1β for 4, 24, and 48h. The cells were collected, and RNA extraction, quantification and reverse transcription were performed as previously stated (section 2.3.1), (section 2.3.2), and (section 2.3.3). Time-dependent expression of MMP-9 mRNA in OKF6 cells stimulated with 0.1 ng/ml IL-1β is shown in Figure 3-4. MMP-9 mRNA levels reached the maximum after 48h stimulation and the increase was 11 fold ($p < 0.05$) compared to the control cells. The early time point stimulations (4h, 24h) also showed a significant increase in MMP-9 mRNA, 3-fold and 6-fold ($p < 0.01 \ & \ p < 0.05$) respectively, compared to controls. No significant differences between 4h and 24 stimulation ($p = 1$) nor between 4h and 48h stimulation ($p = 1$).
Figure 3-4 The effect of IL-1β on MMP-9 mRNA expression by OKF6 cells

OKF6 cells were stimulated with 0.1 ng/ml IL-1β for 4, 24, and 48h. Cells were collected and analysed for MMP-9 mRNA expression with Real time RT-PCR. Bars represent means and standard deviations from three independent cell culture experiments. The mRNA expression was normalised to polymerase II RNA and is expressed as $2^{-\Delta\Delta CT}$ value. Statistics: ANOVA, Student’s t-test **$p < 0.01$, *$p < 0.05$
3.2.4 The effect of IL-1β on MMP-9 secretion by OKF6 cells

To investigate the effect of IL-1β on MMP-9 protein secretion, ELISA was performed to quantify levels of MMP-9 protein after IL-1β treatment for 4, 24, and 48h in OKF6 cells. The tissue culture supernatants were used for the ELISA analysis as described previously (section 2.6). The results showed that MMP-9 was constitutively secreted by OKF6 cells, with low levels being detected in control samples (Figure 3-5). The time course of the effect of IL-1β on MMP-9 secretion was determined in treated OKF6 cells exposed to 0.1ng/ml IL-1β. The secretion of MMP-9 was found to be significantly increased over time during the course of IL-1β treatment for 4h (2.5 ± 1.6 ng/ml), 24h (22.4 ± 15.0 ng/ml) and 48h (29.3 ± 6.6 ng/ml) compared to the corresponding controls (p < 0.05, p < 0.05, and p < 0.01 respectively) (Figure 3-5). A significant increase in MMP-9 secretion was noted 4h after initiation of the treatment with IL-1β. Induction was maximal at 24h and was maintained for 48h. Wilcoxon test revealed a significant difference between 4h and 24h treated OKF6 cells (p < 0.05), and 4h and 48h treated OKF6 cells (p < 0.05). No statistically significant difference was found between 24h and 48h IL-1β treated OKF6 cells (p = 0.1)
Figure 3-5 The effect of IL-1β on MMP-9 protein secretion by OKF6 cells

OKF6 cells were stimulated with 0.1 ng/ml IL-1β for 4, 24, and 48h. Supernatants were collected and analysed for MMP-9 secretion by ELISA. The graph represents mean and standard deviations from three independent cell culture experiments. Statistics: Kruskal-Wallis, Mann-Whitney U test **p < 0.01, * p < 0.05
3.2.5 The effect of IL-1β on MMP mRNA expression by primary oral epithelial cells

RT-PCR analysis was performed on primary oral epithelial cells to investigate the effect of IL-1β on MMP gene expression. The primary oral epithelial cells were obtained and cultured as previously stated (section 2.2.2). They were stimulated with 0.1 ng/ml IL-1β for 4, 24, and 48h and collected for RNA extraction and reverse transcription as previously described (section 2.3.1 and section 2.3.3). PCR for MMP genes was performed as previously described (section 2.3.5). As shown in Figure 3-6, primary oral epithelial cells expressed MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-11, MMP-12, MMP-14, MMP-15 MMP-16, MMP17, MMP-19, MMP-25 and MMP-28. No expression of MMP-8, MMP-13, MMP-20, MMP-21, MMP-23, MMP-24, MMP-26, and MMP-27 was detected in the primary cells (Figure 3-7). The results obtained from the primary cells confirmed the data obtained from OKF6 cells suggesting that OKF6 cells represent an interesting and reliable in vitro model for studying the expression and secretion of MMP members.
Figure 3-6 The effect of IL-1β on MMPs expression by primary oral epithelial cells

Sub-confluent primary oral epithelial cells were incubated for 4, 24, and 48h with 0.1 ng/ml IL-1β. Total cellular RNA was isolated and analysed by RT-PCR. Sequences in the cDNA were amplified with specific primers for MMPs. The products were separated on 3% agarose gels and stained with ethidium bromide. PCR fragment sizes are also indicated.
Figure 3- 7 MMP genes not expressed by primary oral epithelial cells

<table>
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<tr>
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Sub-confluent primary oral epithelial cells were incubated for 4, 24, and 48h with 0.1ng/ml IL-1β. Total cellular RNA was isolated and analysed by RT-PCR. Sequences in the cDNA were amplified with specific primers for MMPs. The products were separated on 3% agarose gels and stained with ethidium bromide. PCR fragment sizes are also indicated. Positive control = chondrocytes.
3.2.6 The effect of IL-1β on MMP-9 mRNA expression by primary oral epithelial cells

To quantify MMP-9 gene expression induced by IL-1β in primary oral epithelial cells, Real Time PCR was used. The primary oral epithelial cells were obtained and cultured as previously stated (section 2.2.2). They were stimulated with 0.1 ng/ml IL-1β for 4, 24, and 48h. The cells were collected, and RNA extraction, quantification and reverse transcription were performed as previously described (section 2.3.1), (section 2.3.2), and (section 2.3.3). Figure 3-8 summarizes the quantitative PCR analysis of MMP-9 expression. IL-1β enhanced mRNA levels 6-fold at 48h in stimulated cells when compared with the controls (p < 0.05). 24h stimulated samples also showed a significant difference compared with the corresponding control with a 6-fold increase (p < 0.05). A trend was observed for higher levels of MMP-9 mRNA at 4h stimulated cells compared to the control (p = 0.07). No significant difference in MMP-9 mRNA expression between 4h and 24h (p = 0.3) and 24h and 48h (p = 0.8) was detected.
Figure 3-8 The effect of IL-1β on MMP-9 expression by primary oral epithelial cells

Primary gingival epithelial cells were stimulated with 0.1 ng/ml IL-1β for 4, 24, and 48h. Cells were collected and analysed for MMP-9 mRNA expression with Real time RT-PCR. Bars represent means and standard deviations from three independent cell culture experiments. The mRNA expression was normalised to polymerase II RNA and is expressed as $2^{-\Delta\Delta C_T}$ value. Statistics: Kruskal-Wallis, Mann-Whitney test *$P < 0.05$
3.2.7 The effect of IL-1β on MMP-9 protein levels by primary oral epithelial cells

To investigate the effect of IL-1β on MMP-9 protein production by the oral primary cells, ELISA was performed to quantify levels of MMP-9 secretion in the course of IL-1β treatment for 4, 24, and 48h in the cells. Tissue culture supernatants were used for analysing MMP-9 ELISA as described previously (section 2.6). Figure 3-9 summarizes the ELISA analysis of MMP-9 secretion in primary gingival epithelial cells.

Unstimulated cells secreted a very small level of MMP-9. IL-1β at 0.1 ng/ml enhanced MMP-9 protein production in a time dependent manner to 26.9 ± 15.3 ng/ml (24h) and 49.4 ± 30.2 ng/ml (48h) compared with 4h stimulated cells (p < 0.05). There was no significant difference between the stimulated cells and the controls at 4h (p = 0.9). A significant difference in MMP-9 production between IL-1β treated samples and untreated samples was evident at 24h (p < 0.05) and at 48h (p < 0.01). Moreover, a significant difference in MMP-9 levels between 4h and 24h IL-1β treated samples was observed (p < 0.01). No significant difference in MMP-9 production between 24h and 48h IL-1β treated samples was detected (p = 0.2).
Figure 3-9 The effect of IL-1β on MMP-9 secretion by primary oral epithelial cells

Secretion of MMP-9 by primary oral epithelial cells treated with IL-1β (0.1 ng/ml) for 4, 24, and 48h. Following stimulation, culture media from untreated and treated samples was collected and used to determine the amount of MMP-9 in the supernatants by ELISA. The graph represents the means and standard deviation of duplicate wells from three independent experiments. Statistics: Kruskal-Wallis, Mann-Whitney U test, Wilcoxon Signed Ranks test. **p < 0.01, *p < 0.05.

3.2.8 Investigation of the cytotoxic effect of IL-1β on OKF6 cells

A cell proliferation assay was performed to investigate whether the differences in MMP-9 expression between unstimulated cells and those treated with IL-1β were due to changes in cell numbers. Stimulation experiments of OKF6 cells were conducted using the 24h stimulation time point. Cell numbers and IL-1β concentrations were similar to those used in all the stimulation experiments in the present study. OKF6 cells were stimulated with 0.1 ng/ml IL-1β for 24h. All conditions were set up in triplicate cell cultures. Cells were collected and processed in the cell Titer 96 cell proliferation assay as described previously (section 2.2.6). As shown in Figure 3-10, after 24h of IL-1β stimulation, no significant change in number of OKF6 cells was detected in comparison to the controls (p = 0.3). The result indicated that IL-1β has no proliferative or cytotoxic
effects on OKF6 cells after 24h and it is clear that, the changes induced by IL-1β in cytokine or MMP expression were not due to the changes in cell numbers.

**Figure 3-10 The effect of IL-1β on OKF6 cells numbers after 24h**

OKF6 cells were stimulated with IL-1β (0.1 ng/ml). Cell proliferation was analysed with the Cell Titer 96 cell proliferation assay after 24h. The bars represent means and standard deviations of one experiment with three independent cultures. Mann-Whitney U test: not significant.
3.2.9 The effect of IL-1β on IL-32 mRNA expression by OKF6 cells

The effect of IL-1β on IL-32 mRNA expression was examined by Real Time RT-PCR. Cells were cultured as previously stated (section 2.2.1) and stimulated with 0.1 ng/ml IL-1β for 4, 24, and 48h. The cells were collected; RNA extraction, quantification and reverse transcription were performed as previously stated (section 2.3.1), (section 2.3.2), (section 2.3.3). As shown in Figure 3-11, the mRNA level of IL-32 was increased more than 5 fold in OKF6 treated with IL-1β for 4h and decreased thereafter. A significant upregulation of IL-32 mRNA at 4h ($p < 0.001$) and 24h ($p < 0.05$) compared to each corresponding control was detected. IL-32 mRNA expression was decreased over time and was 2.5-fold at 24h ($p = 0.02$) and 3-fold at 48 h ($p = 0.06$) compared to IL-32 mRNA expression at 4h.
Figure 3-11 The effect of IL-1β on IL-32 expression by OKF6 cells

OKF6 cells were stimulated with 0.1 ng/ml IL-1β for 4, 24, and 48h. Cells were collected and analysed for IL-32 mRNA expression using Real Time RT-PCR. Bars represent means and standard deviations from three independent cell culture experiments. The mRNA expression was normalised to polymerase II RNA and is expressed as $2^{-\Delta\Delta Ct}$ value. Statistics: ANOVA, Student t-test $*** p < 0.001$, *$p < 0.05$. 

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3.3 Discussion

Periodontal disease is characterized by a destructive inflammatory process affecting the supporting tissues of the teeth, resulting in attachment loss, formation of periodontal pockets, and resorption of alveolar bone (Williams, 1990). The local host responses to the periodontal pathogens include leukocyte recruitment and release of inflammatory mediators and cytokines (Okada and Murakami, 1998). IL-1β has been reported to play a crucial role in the pathogenesis of periodontal disease (Sakai et al., 2006) and has been found to be elevated in GCF and synthesized in patients with periodontal disease (Stashenko et al., 1991; Alexander and Damoulis, 1994; Boch et al., 2001). IL-1β is produced by many types of cells including lymphocytes, macrophages and fibroblasts (Kang et al., 1996) and it stimulates target cells to synthesize proteases such as MMPs that contribute to tissue destruction in inflammatory diseases (MacNaul et al., 1990).

In the present study, stimulation of the epithelial cell line with 0.1 ng/ml IL-1β for different periods of time induced MMP expression. The RT-PCR results demonstrated a novel finding, that OKF6 cells and primary oral epithelial cells expressed several proteinase genes such as MMP-10, MMP-11, MMP-12, MMP-14, MMP-15, MMP-16, MMP-17, MMP-19, MMP-25 and MMP-28 which have not previously been reported to be expressed by oral epithelial cells. In addition, this study documents the differential expression of MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9 in this epithelial cell line and primary cells. These results are in agreement with the results from other studies which found that gingival epithelial cells express MMP-1, MMP-2, MMP-3, MMP-7 and MMP-8 in periodontitis (Meikle et al., 1994; Uttó et al., 1998; Makela et al., 1999; Tervahartiala et al., 2000). However, previous studies were unable to detect the MMP-9 expression in periodontitis-affected gingival tissues that was detected in the present
study in OKF6 and the primary gingival epithelial cells. On the other hand, the results of this study are consistent with Smith et al (2004) who detected MMP-9 in inflamed gingival keratinocytes (Smith et al., 2004).

Expression of MMP-10 and MMP-11 has been associated with activation of other pro-MMPs (Kahari and Saarialho-Kere, 1999; Moilanen et al., 2003) and both MMP-10 and MMP-11 can be expressed by keratinocytes (Nagase et al., 2006). The present study showed that OKF6 cells and the primary epithelial cells were capable of producing MMP-10 and MMP-11 that may support their role in activation of other MMPs in periodontal tissues. In addition, the human macrophage elastase (MMP-12) was also detected in the OKF6 cells and the primary oral epithelial cells.

Although a number of studies have revealed the effects of MMPs on periodontal tissues, little is known about the expression of MT-MMPs. The results from this study showed that MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP), MT4-MMP (MMP-17) and MT6-MMP (MMP-25) were detected in the cell lines and the primary oral epithelial cells, whereas MMP-24 (MT5-MMP) was not detected. These data are consistent with Dong et al (2001) who showed that MT-MMPs were detected in inflamed corneal epithelial cells (Dong et al., 2001). Furthermore, MT6-MMP has been found to be expressed strongly in inflamed gingiva compared to healthy gingiva (Kuula et al., 2008). The present data support this premise since MT6-MMP was detected in the OKF6 and the primary oral epithelial cells and suggesting that they may play a role in periodontal tissue destruction.
In addition, the results regarding MMP-19 expression in OKF6 and primary cells are in accordance with previous investigators of other cell types which suggested that MMP-19 was expressed by the mucosal epithelium of the intestine, stromal macrophages and fibroblasts (Bister et al., 2004). It was suggested that MMP-19 may be induced in inflammatory disease of the intestine in response to the inflammatory cytokines to restore the normal structure of the epithelium (Bister et al., 2004). Furthermore, Impola et al. (2003) showed expression of MMP-19 by epithelial cells of the skin (Impola et al., 2003).

MMP-28 (Epilysin) is the newest member of the MMP family and the only specific human cell type recognized to express Epilysin are the basal keratinocytes in the skin (Saarialho-Kere et al., 2002). It was suggested that MMP-28 is needed to degrade protein between epithelial cells to facilitate their migration (Illman et al., 2006) and their expression is upregulated in human keratinocytes by TNF-α stimulation (Saarialho-Kere et al., 2002). Interestingly, the data from this study demonstrated that MMP-28 is expressed in oral epithelial cells. Thus, it seems that epithelial cells have the potential to express many collagenolytic MMPs that may be involved in degrading most components of the extracellular matrix, and the epithelial cells may therefore actively participate in the inflammatory process.

RT-PCR could not detect MMP-8, MMP-13, MMP-20, MMP-21, MMP-23, MMP-24, MMP-26, and MMP-27 in OKF6 cell line or primary epithelial cells. Previous reports have shown production of MMP-8 and MMP-13 in epithelial cells (Tonetti et al., 1993; Tervahartiala et al., 2000) however, the present study could not detect MMP-8 or MMP-13 mRNA in the OKF6 cells nor in the primary epithelial cells. A possible
Results

explanation for these conflicting findings may be the differences in the cell, tissue, and methods used to detect the gene. Previous reports have suggested that MMP-20 expression is restricted to tooth forming tissues (Bartlett et al., 1996; Llano et al., 1997). The present study supports this premise since MMP-20 was not detected in the cell line or primary oral epithelial cells. It was reported that MMP-21 and MMP-26 were detected in macrophages and fibroblasts in various skin disorders (Skoog et al., 2006). Therefore, it may be reasonable to speculate that oral epithelial cells cannot express MMP-21 and MMP-26.

Because of the importance of MMP-9 in periodontal disease and in order to obtain a more precise quantification of MMP-9, Real Time RT-PCR assay was performed, and confirmed the results obtained by the semi-quantitative RT-PCR. The results demonstrated that IL-1β significantly upregulated MMP-9 mRNA and protein production in OKF6 cells in a time dependent manner and these results were confirmed in the primary oral epithelial cells. One can hypothesise that MMP-9 has an important role in the tissue destruction associated with periodontal progression. The findings from the study are in agreement with these of Li et al (2001) who reported a marked upregulation of MMP-9 protein and activity in corneal epithelial cells after stimulation with IL-1β (Li et al., 2001). The data from this study are also consistent with other reports that MMP-9 protein and mRNA are upregulated by IL-1β in other tissue or cell types, for example, in rat mucosal keratinocytes (Lyons et al., 1993) and rabbit corneal fibroblasts (Fini et al., 1995).
To confirm the increase in levels of MMP-9 identified in the Real Time RT-PCR and the ELISA assays was not due to an increase in cell numbers, a proliferation assay was performed. The results of the proliferation assay revealed that no significant changes in cell numbers occurred after the cells were stimulated with IL-1β. Therefore, this study provides direct evidence that the inflammatory cytokine, IL-1β, increases the level of MMP-9 mRNA and protein expression in OKF6 cells and primary oral epithelial cells.

A crucial part of the epithelial defence system is the efficient recruitment of defence cells particularly neutrophils to the infection site. IL-8 is a key cytokine in this process. IL-8 production was studied in IL-1β-treated OKF6 cells using ELISA. The results demonstrated that IL-1β was able to induce IL-8 in vitro and the induction of this cytokine increased over time. These results are in agreement with a previous study by Eskan et al (2008) who reported that 0.2 ng/ml of IL-1β significantly increased the induction of IL-8 in human gingival epithelial cells (Eskan et al., 2008). It was suggested that in an excessive inflammatory reaction, persistent neutrophil activity plays an important role in the tissue destruction observed in periodontal disease (Bascones et al., 2005; Sorsa et al., 2006). Therefore, it can be suggested that a continuous increase of IL-8 is detrimental in terms of disease progression. The capacity of the gingival epithelial cells to produce IL-8 in response to IL-1β suggests that they play a critical role in the maintenance of the periodontal tissue.

IL-1β is a biomarker of periodontal disease (Kinane et al., 1992) and excessive production of the cytokine may induce gingival fibroblasts to produce other cytokines (Makela et al., 1998). In addition, a growing body of evidence supports the concept that IL-32 is a proinflammatory cytokine, inducing IL-1β, IL-6, and TNF-α (Kim et al., 2005;
Nold et al., 2008). It was reported that IL-32 plays a role in the inflammatory process and has been detected in dermal epithelial cells and possesses the ability to induce IL-8 and TNF-α (Dinarello and Kim, 2006). IL-32 is also expressed in oral epithelial cells in response to periodontal pathogens (Miliward et al., 2007). In this study, IL-32 mRNA expression was investigated in OKF6 cells treated with IL-1β (0.1 ng/ml). Interestingly, we demonstrated for the first time that IL-1β significantly upregulated IL-32 mRNA expression at 4h and this in agreement with data on endothelial cells, in which IL-32 mRNA expression was upregulated by IL-1β stimulation (Nold-Petry et al., 2009). These results suggest that IL-32 may function as an effector cytokine of IL-1 signals in the epithelial cells. This hypothesis is supported by the fact that IL-1β was highly effective in inducing IL-32, which in turn was produced at unprecedented concentrations by epithelial cells. Therefore, the finding from this study supports the notion that IL-32 may play a role in the inflammatory process in periodontal disease (Shoda et al., 2006, Shioya et al., 2007).

In conclusion, these experiments identified the differential expression of several MMPs in OKF6 cells and primary gingival epithelial cells stimulated by IL-1β, and highlighted a novel potential in vivo function for the epithelial cells through expression of a variety of MMPs. The MMPs produced may either directly degrade the extracellular matrix since they can digest a broad spectrum of substrates or indirectly participate in the destruction process through activating other MMPs. In oral epithelial cells, IL-1β at concentration of 0.1 ng/ml induced upregulation of MMP-9 expression which may indicate a possible contribution of these enzymes to tissue destruction in the inflammatory disease. Furthermore, IL-1β could further amplify the immune response by inducing IL-8 expression in gingival epithelial cells. Finally, the present study
suggested that IL-32 may regulate epithelial cell function and play a role in the inflammatory microenvironment.
Chapter 4  Impact of different cytokines on MMP-9 production by OKF6 cells

4.1 Introduction

The mechanisms underlying inflammation in the periodontal tissues involve numerous cytokines that are produced by many cell types. Cytokines mediate the complex interactions between lymphocytes, antigen-presenting cells (APCs) and endothelial cells (Graves and Cochran, 2003) by inducing each other or by synergistic interactions (Balkwill and Burke, 1989). Inflammatory responses following exposure to cytokines are highly dependent on NF-κB-induced gene expression (Bian et al., 2001; Lin et al., 2004). Cytokines may differentially influence the expression profile of MMPs (Chizzolini et al., 2000). Evidence for the role of cytokines and MMPs in the pathogenesis of periodontal disease is well established. Oral epithelial cells participate in inflammatory processes through secretion of many cytokines including IL-1β and TNF-α, which are important for initiation of the inflammatory response against the bacterial infection (Dinarello, 2000; Sandros et al., 2000) and they participate in inflammatory responses (Suchett-Kaye et al., 1998).

TNF-α plays a role in a number of disease processes including arthritis (Catrina et al., 2002; Klimiuk et al., 2004) and periodontitis (Graves and Cochran, 2003). TNF-α is produced by monocytes and macrophages in response to bacterial components and is associated with collagen destruction and bone resorption (Stashenko et al., 1987). It was reported that TNF-α contributes to tissue destruction by enhancing MMP expression
(Meikle et al., 1992). MMP-9 is regulated by TNF-α in many cell types (Van den Steen et al., 2002) and it was reported that TNF-α induces MMP-9 production in mice dermal keratinocytes (Scott et al., 2004) and in human kidney cells (Nee et al., 2004). Beklen et al. showed that treatment of fibroblasts with TNF-α induced production of MMP-1 and MMP-3 (Beklen et al., 2007). Experimental evidence has shown increased levels of MMP-9 expression in mucosal keratinocytes after stimulation with TNF-α at 24h using zymography and RT-PCR (Makela et al., 1998). In contrast, in human lung fibroblasts, treatment with TNF-α alone caused a significant decrease in MMP-9 activity (Sasaki et al., 2000). Therefore, different cells may respond differently to the same cytokine that may exert an inducible or suppressive effect on production of MMPs (Nee et al., 2004).

IL-18 is a proinflammatory cytokine produced by many cell types including macrophages (Ghayur et al., 1997) and oral epithelial cells (Rouabhia et al., 2002) and it synergizes with IL-12 to stimulate IFN-γ induction by NK and T-cells (Biet et al., 2002). It was reported that IL-18 signalling requires IL-18 receptor (IL-18R) and IL-18 accessory protein-like (IL-18AcPL) (Born et al., 1998; Takeda et al., 1998). It has been shown that IL-18 induces TNF-α and IL-1β, IL-8 and MMP-9 (Nold et al., 2003) in mononuclear cells and induces production of MMP-9 in cultured macrophages (Quinding-Jarbrink et al., 2001). It has been reported that IL-18 is involved in keratinocyte proliferation in malignant skin tumours (Park et al., 2001) and is associated with the progression of myeloid leukaemia by stimulating MMP-9 expression (Zhang et al., 2004). IL-18 also upregulates gelatinase in muscle cells (Chandrasekar et al., 2006) and MMP-1, MMP-3 and MMP-13 in chondrocytes isolated from patients with RA (Dai et al., 2005). It was reported that inflamed gingival tissues contain high levels of IL-18 (Johnson and Serio, 2005) which is implicated in the severity of periodontal disease.
(Orozco et al., 2006). Furthermore, increased levels of IL-18 have been reported in GCF samples from patients with periodontal disease (Orozco et al., 2006). However, the precise effects of IL-18 on the production of MMPs in oral epithelial cells remain unknown; also the characteristics of IL-18 receptor expression still remain unclear.

IL-32 is a proinflammatory cytokine and is mainly expressed in T lymphocytes, NK cells, epithelial cells and monocytes (Dinarello and Kim, 2006). It has been implicated in inflammatory diseases RA (Shoda et al., 2006; Shioya et al., 2007). In synovial tissues from patients with RA, an elevated level of IL-32 mRNA was detected (Cagnard et al., 2005). Furthermore, IL-32 is expressed in synovial cells from patients with RA and is associated with the severity of the disease and expression of other cytokines including TNF-α, IL-1β and IL-18 (Joosten et al., 2006). Elevated levels of IL-32 in the epithelial cells of the colon suggested a role in inflammatory bowel disease (Netea et al., 2005; Shioya et al., 2007). In addition, IL-32 plays a role in host defense mechanisms by inducing monocyte differentiation to macrophages (Netea et al., 2008). IL-32 is induced by IFN-γ in epithelial cells (Kim et al., 2005) and is expressed in oral epithelial cells in response to the periodontal pathogens (Milward et al., 2007). Therefore, these properties may suggest that IL-32 plays an important role in the inflammatory processes. However, although the role of IL-32 in the inflammatory process is documented, its role in periodontal tissue destruction through inducing MMP expression is still unknown.

The purpose of this study is to test the hypothesis that inflammatory cytokines can act independently and contribute to tissue destruction through production of MMPs. Therefore, the study aimed to evaluate the expression levels of TNF-α, IL-1β, IL-18R,
IL-18RAcPL, and IL-32 in cultured OKF6 cells. This investigation may be helpful in furthering our knowledge of cytokine expression in oral epithelial cells. In addition, differential regulation of MMP-9 by cytokines (TNF-α, IL-18 and IL-32) was investigated by measuring the protein and mRNA levels in OKF6 cells.

4.2 Results

4.2.1 Investigation of inflammatory gene expressions in OKF6 cells

Taqman low-density arrays (TLDAs) were used for profiling gene expression in OKF6 cells as described earlier (section 2.5). Cells were cultured in 6 well plates as previously described (section 2.2.1). The sub-confluent unstimulated OKF6 cells were collected; RNA extraction, quantification and reverse transcription were performed as previously described (section 2.3.1, 2.3.2, and 2.3.3). The genes in TLDAs include well documented pro-inflammatory cytokines that have pivotal roles in the pathogenesis of inflammatory diseases. The absolute Ct values were used as an approximate measure of gene expressions. For visualization, the Ct range was used according to Kevorkian (Kevorkian et al., 2004). The Ct values were used as an approximate measure of gene expression as follows: Ct ≤ 25 = very high; 26-30 = high, 31-35 = moderate; 36-39 = low; 40 = not detected (Kevorkian et al., 2004). The results showed that IL-1β, IL-32, IL-8 and TNF receptor super-family (TNFRSF) were very highly expressed in OKF6 cells (Ct ≤ 25) (Table 4-1 and Table 4-2). IL-1βR, IL-1βRAcPL, IL-18, and IL-18R were highly expressed in OKF6 cells (Ct = 26-30). Moderate expression of TNF-α was found (Ct=31). Low expression of IL-18RAcPL, IL-6 and IL-12A were observed in the OKF6 cells (Ct=38, Ct=36 and 36 respectively). IL-12B was not detected in OKF6 cells (Ct=40).
Table 4-1 Constitutive expression of proinflammatory cytokines in OKF6 cells

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<td>IL-1F9</td>
<td>25</td>
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</tr>
<tr>
<td>IL-1F10</td>
<td>40</td>
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</tr>
<tr>
<td>TNF-α</td>
<td>31</td>
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<td>IL-4</td>
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<td>IL-10</td>
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</tr>
<tr>
<td>IL-12α</td>
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<td>Low</td>
</tr>
<tr>
<td>IL-12b</td>
<td>40</td>
<td>Not detected</td>
</tr>
<tr>
<td>IL-32</td>
<td>23</td>
<td>Very high</td>
</tr>
<tr>
<td>IFN-γ</td>
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<td>Not detected</td>
</tr>
<tr>
<td>IL-8</td>
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</tr>
<tr>
<td><strong>Chemokines</strong></td>
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<tr>
<td>CCL2</td>
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</tr>
<tr>
<td>CCL5</td>
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</tr>
<tr>
<td>CXCL5</td>
<td>22</td>
<td>Very high</td>
</tr>
<tr>
<td>CXCL10</td>
<td>32</td>
<td>Moderate</td>
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</table>

Taqman Low density arrays were used for expression profiling based on RealTime RT-PCR to elucidate the proinflammatory cytokine expressed by OKF6. Ct ≤ 25 = very high expression of the gene; 26-30 = high; 31-35 = moderate; 36-39 = low; 40 = gene expression not detected.
Table 4-2 Constitutive expression of proinflammatory cytokines in OKF6 cells (continued)

<table>
<thead>
<tr>
<th>Genes</th>
<th>CT values</th>
<th>Expression</th>
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<tr>
<td><strong>Receptors</strong></td>
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<tr>
<td>IL-1R1</td>
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<td>IL-1RAcPL</td>
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<tr>
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<tr>
<td>TNFRSF1B</td>
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<tr>
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<tr>
<td>ADIPOR1</td>
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<td>Very high</td>
</tr>
<tr>
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<td>Casp-1</td>
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<td>CD14</td>
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</tr>
<tr>
<td>OSM</td>
<td>40</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

Taqman Low density arrays were used for expression profiling based on RealTime RT-PCR to elucidate the proinflammatory cytokine expressed by OKF6. Ct ≤ 25 = very high expression of the gene; 26-30 = high; 31-35 = moderate; 36-39 = low; 40 = gene expression not detected.
4.2.2 The effect of TNF-α on MMP-9 mRNA expression in OKF6 cells

In order to determine if TNF-α alone could induce MMP-9 expression in OKF6 cells, MMP-9 mRNA levels in OKF6 cells were analysed after TNF-α stimulation using Real Time RT-PCR. Cells were cultured in 6 wells plates as previously described (section 2.2.1). Sub-confluent cells were stimulated with 0.1 ng/ml TNF-α for different times (4h, 24h, and 48h). The cells were collected, RNA extraction, quantification and reverse transcription were performed as previously described (section 2.3.1, section 2.3.2, and section 2.3.3). The results from Real Time RT-PCR showed that oral epithelial cell line expressed MMP-9 mRNA. The MMP-9 mRNA expression was significantly upregulated by TNF-α at 4, 24, and 48h (Figure 4-1). More specifically, gene expression at 4h was increased 4-fold (p < 0.05), at 24h it was increased 5-fold (p < 0.01), and at 48h, it was increased 3.5-fold (p < 0.05) when compared with each individual control. No significant difference was detected when comparing stimulated samples at 4h with 24h (p = 1) and 4h with 48h (p = 1). The peak expression was observed after stimulation for 24h.
Figure 4-1 The effect of TNF-α on the expression of MMP-9 mRNA in OKF6 cells

Sub-confluent OKF6 cells were incubated for 4, 24 or 48 h with 0.1 ng/ml TNF-α. Total cellular RNA was extracted from the OKF6 and reverse transcription was performed for production of cDNA from treated and untreated samples. MMP-9 expression was analysed with Real Time RT-PCR. Results are expressed as mean and standard deviation of three independent experiments. The mRNA expression was normalised to polymerase II RNA and is expressed as $2^{-\Delta\Delta CT}$ value. Statistics: ANOVA, Student t test* $p < 0.05$, **$p < 0.01$. 
4.2.3 The effect of TNF-α on MMP-9 protein levels in OKF6 cells

To investigate the effect at the protein level, MMP-9 secretion was assayed by ELISA in the supernatants obtained after 4, 24, and 48h of TNF-α stimulation as stated previously (section 2.6). As illustrated in Figure 4-2, addition of TNF-α to OKF6 cells augmented production of MMP-9 after 4h 2-fold (0.253 ± 0.178 ng/ml) compared to controls (0.126 ± 0.105 ng/ml). A significant increase in MMP-9 secretion after 24h (18.4 ± 10.0 ng/ml) and 48h (52.5 ± 32.7 ng/ml) stimulation with TNF-α (4.5-fold (p < 0.01) and 3.8-fold (p < 0.05) respectively) compared to corresponding controls (4.1 ± 1.6 and 13.8 ±10.2 ng/ml) was also observed. No significant difference between 4h-stimulated cells and the controls was detected (p = 0.1). A significant difference was detected between treated samples at 4h compared with 24h (72-fold) p < 0.05 and 4h compared with 48h (207-fold) p < 0.05.

Figure 4-2 The effect of TNF-α on MMP-9 secretion in OKF6 cells

![Graph showing MMP-9 concentration over time](image)

Sub-confluent OKF6 cells with 0.1 ng/ml TNF-α. Supernatant were collected and assayed for MMP-9 secretion by ELISA. The graph represents mean and standard deviation from three independent cell culture experiments. Statistics: Kruskal-Wallis, Mann-Whitney U test **p < 0.01, * p < 0.05.
4.2.4 The effect of IL-32γ on MMP-9 expression in OKF6 cells

To determine the effects of IL-32γ on MMP-9 production by oral keratinocytes, the cells were incubated with recombinant IL-32γ. The cells were seeded on 6 well plates; the cytokine was added at two concentrations (20 ng/ml and 80 ng/ml) for 4, 24, and 48h. The supernatants were harvested and analysed with ELISA. The production of MMP-9 by OKF6 cells incubated with different concentrations of IL-32γ was variable. Figure 4-3 showed that production of MMP-9 after stimulation with IL-32γ (20 ng/ml and 80 ng/ml) for 4h was 0.0266 ± 0.0337 ng/ml and 0.0358 ± 0.0088 ng/ml respectively compared to the 4 h control (0.0099 ± 0.0054 ng/ml). At 24h stimulation with IL-32γ (20 ng/ml and 80 ng/ml), the MMP-9 production was 0.0996 ± 0.0002 ng/ml and 1.141 ± 0.0197 ng/ml compared with the corresponding control (0.0901 ± 0.0131 ng/ml). The MMP-9 levels at 48h after the stimulation with previous cytokine concentrations was 0.126 ± 0.004 ng/ml and 1.429 ± 0.0299 ng/ml compared with 48h control (0.127 ± 0.022 ng/ml). The results from a preliminary experiment showed that IL-32γ had no major effect on MMP-9 secretion by OKF6 cells after 24h and 48h stimulation (Figure 4-3). The results are derived from a single stimulation experiment and therefore no statistic analysis was performed.

Thus, according to results from the preliminary experiment, MMP-9 was upregulated after stimulation of OKF6 cells with 80 ng/ml IL-32γ for 4h. The experiment was repeated to investigate the effect of IL-32γ on MMP-9 expression by OKF6 cells using 80 ng/ml. The data showed that IL-32γ significantly increased MMP-9 protein levels at 4h (0.416 ± 0.0949 ng/ml) compared with the control (0.2034 ± 0.083 ng/ml), p < 0.01 (Figure 4-4). The effect of IL-32γ on MMP-9 production has not been documented before.
Figure 4-3 The effect of IL-32γ on MMP-9 expression in OKF6 cells

OKF6 cells were seeded on 6 wells plates and exposed to increasing amounts of IL-32 (20 ng/ml, and 80 ng/ml) for 4, 24, and 48h. Supernatants were collected and analysed by ELISA. The graph represents mean and standard deviation from one experiment.
Figure 4-4 The effect of IL-32γ on MMP-9 expression in OKF6 cells

Sub-confluent OKF6 cells were treated with 80 ng/ml IL-32 for 4h. Supernatants were collected and analysed for MMP-9 secretion by ELISA. The graph represents means and standard deviations from three independent cell culture experiments. Statistics: Mann-Whitney U test**p < 0.01.
4.2.5 The effect of IL-32γ on MMP-9 mRNA expression in OKF6 cells

To investigate the effect of IL-32γ on MMP-9 gene expression in OKF6 cells, Real Time RT-PCR was performed. The OKF6 cells were cultured as previously described (section 2.2.1) and stimulated with 80 ng/ml IL-32γ for 4h. The cells were collected; RNA extraction, quantification and reverse transcription were performed as previously stated in (section 2.3.1, 2.3.2, and 2.3.3). The data showed that IL-32γ had no effect on MMP-9 mRNA expression by OKF6 cells ($p = 0.1$) Figure 4-5.

Figure 4-5 The effect of IL-32γ on MMP-9 mRNA expression in OKF6 cells

![Graph showing the effect of IL-32γ on MMP-9 mRNA expression in OKF6 cells.](image)

OKF6 cells were stimulated with 80 ng/ml IL-32γ for 4h. Cells were collected and analysed for MMP-9 mRNA expression with Real Time RT-PCR. Graph represents mean and Standard deviation from three independent cell culture experiments. The mRNA expression was normalised to polymerase II RNA and is expressed as $2^{-ΔΔCt}$ value. Statistics: ANOVA, Student t test, $p = 0.1$
4.2.6 The effect of IL-18 on MMP-9 production in OKF6 cells

To determine the effects of IL-18 on MMP-9 production by oral keratinocytes, the cells were incubated with recombinant IL-18. The cells were seeded onto 6 well plates; the cytokine was added in various concentrations (20 pg/ml, 50 pg/ml, 100 pg/ml, 500 pg/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml) for 24h. The supernatants were harvested and analysed with ELISA. The data from a preliminary experiment showed that IL-18 had no effect on production of MMP-9 in OKF6 cells after 24h stimulation (Figure 4-6). The results are derived from single stimulation and therefore no statistic analysis was performed. The experiment was repeated at an earlier time point (4h) with IL-18 100 ng/ml. The result from the ELISA showed no significant difference in mean MMP-9 levels in the control (0.119 ± 0.057 ng/ml) and the stimulated samples (0.215 ± 0.150 ng/ml) after stimulation of OKF6 cells with IL-18 (100 ng/ml) (Figure 4-7).
Figure 4-6 The effect of IL-18 on MMP-9 production in OKF6 cells

OKF6 cells were seeded on 6 wells plates and exposed to increasing amounts of IL-18 (20 pg/ml, 50 pg/ml, 100 pg/ml 500 pg/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml) for 24h. Supernatants were collected and analysed by ELISA. The graph represents means and standard deviations from one experiment.
Figure 4-7 The effect of IL-18 on MMP-9 production in OKF6 cells

Sub-confluent OKF6 cells were treated with 100 ng/ml IL-18 for 4h. Supernatants were collected and analysed for MMP-9 secretion by ELISA. The graph represents means and standard deviations from three independent cell culture experiments. Statistics: Mann-Whitney U test $p = 0.1$
4.3 Discussion

The local host response in periodontal disease includes recruitment of leukocytes and subsequent release of inflammatory mediators and cytokines which appear to play an important role in tissue destruction (Cohen, 2002). Cytokines in the periodontal tissue are synthesized by a variety of cells and are activated by pathogens or other cytokines. The data from this study demonstrate that the immortalized oral epithelial cell line, OKF6, expressed many cytokines such as IL-1β, TNF-α, IL-18, IL-32, and IL-8 at high levels at the mRNA level. The results are consistent with those of Rouabhia and Deslauriers who reported that IL-1β, TNF-α, and IL-8 are expressed in the engineered oral mucosal model (Rouabhia and Deslauriers, 2002). In addition, these results were also in agreement with results from other studies, which found that IL-32 and IL-18 are expressed in epithelial cells (Rouabhia et al., 2002; Dinarello and Kim, 2006). The marked expression of IL-32 and IL-18 in oral epithelial cells may suggest their role in the pathogenesis of periodontal disease. Thus, the capacity of the gingival epithelial cells to produce this range of cytokines suggests that they play a critical role in the maintenance of the tissue micro-environment.

It was reported that MMP-9 protein levels and activity are increased in periodontal tissue and GCF from patients with periodontal disease (Makela et al., 1994; Ingman et al., 1996; Ejeil et al., 2003; Sorsa et al., 2004). Previous studies have demonstrated the ability of TNF-α to induce MMP production by different types of cells in vitro (Joosten et al., 2006). This study demonstrated that addition of recombinant TNF-α to OKF6 cells induced MMP-9 protein and mRNA levels. These findings are in agreement with previous studies which demonstrated that TNF-α and IL-1β clearly trigger harmful
events in periodontal disease including tissue destruction and bone resorption (Teitelbaum, 2000; Graves and Cochran, 2003).

Previous studies demonstrated that IL-32 induces other cytokines and chemokines such as TNF-α, IL-1β, IL-6 and IL-8 (Kim et al., 2005; Netea et al., 2005) and also plays a critical role in rheumatoid arthritis (Joosten et al., 2006). The present results indicate that IL-32 has lesser direct effects on MMP-9 production than IL-1β and TNF-α. IL-32 was shown to stimulate MMP-9 production only at the protein level after 4h stimulation. However, it had no effect on MMP-9 mRNA production in OKF6 cells. A possible interpretation is that IL-32 mRNA and protein levels may be regulated differentially. The present study suggested that IL-32 may exacerbate the inflammatory process in periodontal disease by early production of MMP-9. The data from the present study also observed that early IL-32 production is associated with IL-1β stimulation which corroborates the previous finding. Therefore the findings reported in this study open avenues for further exploration.

Studies have reported that IL-18 is produced by keratinocytes (Stoll et al., 1997; Mee et al., 2000) which is consistent with the findings of this study. Not very much has been known until now about the regulation or function of IL-18 in keratinocytes. After treatment of OKF6 cells with IL-18 for 4 h, production of MMP-9 was not significantly changed at the mRNA level. A similar lack of MMP-9 induction was observed after treatment of oral epithelial cells with IL-18 for 24 h. According to the data from TLDA, OKF6 cells express IL-18R, however low levels of IL-18AcPL mRNA were detected. It was reported that primary keratinocytes synthesize IL-18, IL-18R, and IL-18RAcPL (Mee et al., 2000). Moreover, it was found that IL-12 upregulates IL-18
receptor expression and pre-treatment of T or B cells with IL-12 rendered them responsive to IL-18 and both cytokines synergize for IFN-γ production (Yoshimoto et al., 1998). In addition, it was found that IL-12 strongly upregulates mRNA expression of IL-18R, IL-18RαcPL and IL-1R related protein (Sareneva et al., 2000). IL-18 and IL-12 synergistically enhanced MMP production in monocyte cell lines (Abraham et al., 2002). The previous studies suggested that IL-12 is a requirement for IL-18 signalling. There is evidence in the present study that IL-12 was not expressed in oral epithelial cell lines and this finding may explain the unresponsiveness of oral epithelial cells to IL-18 in the current study.

On the other hand, there are conflicting data regarding the effects of IL-18 on MMP production. Nold and colleagues demonstrated that IL-18 alone is an inducer of MMP-9 production in mononuclear cells and the induction of this enzyme was independent on IFN-γ (Nold et al., 2003) which corroborates previous data (Quiding-Jarbrink et al., 2001). A possible explanation for those conflicting findings may refer to the difference in cell types. Therefore, the potential role of oral epithelial cell-derived IL-18 in the induction of periodontal disease awaits further investigations.

It is concluded that the great variety of cytokines produced by oral epithelial cells may support the critical role that epithelial cells play in periodontal tissue destruction. The data have confirmed a potent and direct effect of TNF-α on MMP-9 synthesis in OKF6 cells. The study also demonstrated for the first time that IL-32γ can induce upregulation of MMP-9 protein; however no effect of upregulation of mRNA was seen. Adding IL-18 alone had no effect on upregulation of MMP-9 at the protein level in OKF6 cells. It is possible that cytokines present in the inflammatory milieu may therefore influence
disease outcome depending on MMP-9 expression in oral epithelial cells. The study showed that MMP-9 production varied after stimulation with different cytokines and provided the first evidence that cytokines can alter differentially the pattern of MMP-9 expression in OKF6 cells. These insights into the effect of different cytokines on MMP production may provide opportunities to develop novel therapeutic approaches.
Chapter 5  Effect of *P. gingivalis* LPS on MMP-9 production by OKF6 cells and primary oral epithelial cells

5.1 Introduction

Bacteria are responsible for the induction of inflammation in the periodontal tissues, and the progress and severity of the disease are dependent on the host’s immune and inflammatory responses (Offenbacher et al., 2008). *P. gingivalis* is one of the major periodontal pathogens (Lantz, 1996) and has the ability to adhere to and invade oral epithelial cells (Watanabe et al., 1992; Duncan et al., 1993; Sandros et al., 1994; Lamont et al., 1995). Virulence factors such as LPS and other antigens from the cell walls of periodontal pathogens stimulate circulating macrophages and resident cells to produce cytokines that derive the inflammatory response (Teng, 2003). The LPS of gram-negative bacteria is composed of a polysaccharide (O-region), a core of oligosaccharide and a hydrophobic domain (Lipid A) (Miller et al., 2005) and can stimulate macrophages or monocytes to produce pro-inflammatory cytokines such as IL-1β, TNF-α, PGE₂ and IL-6 (Madianos et al., 2005). The lipid A portion of LPS is highly efficient in stimulating immune responses through recognition by TLRs, and induces gene expression and secretion of pro-inflammatory cytokines and chemokines (Miller et al., 2005). TLRs are expressed in a variety of cell types including epithelial cells and trigger immune response (Miller and Modlin, 2007). Early studies on human TLRs suggested that TLR2 but not TLR4 mediate LPS activity (Kirschning et al., 1998; Yang et al., 1998). In subsequent studies on human TLRs, the role of TLR4 in LPS signalling was clearly demonstrated (Arbour et al., 2000). In addition, it has been demonstrated that *P. gingivalis* LPS is recognised by both TLR2 and TLR4 (Darveau et
\textit{al., 2004}) and TLR4 deficiencies may reduce the host immune response to pathogenic bacteria (Kinane \textit{et al.,} 2006).

Epithelial cells are the first cells that encounter periodontal pathogens in periodontitis (Sfakianakis \textit{et al.,} 2001). They respond to pathogens by altered cell signalling leading to changes in cell behaviour, such as cytokine and protease production, cell proliferation and migration (Bascones \textit{et al.,} 2005; Mans \textit{et al.,} 2006) and integrate innate and adaptive immunity (Dale, 2002). Epithelial cells also produce molecules such as ICAM-1 that take part in stimulation of immune effector cells (Lawson \textit{et al.,} 2000). Furthermore, epithelial cells can be a major source of IL-1β in the periodontal tissues (Sfakianakis \textit{et al.,} 2001). It was reported that exposure of an epithelial cell line to \textit{F. nucleatum} caused the cells to increase IL-8 production (Uitto \textit{et al.,} 2005; Gursoy \textit{et al.,} 2008). Increased IL-8 production is responsible for neutrophil migration to the infected area (Tonetti \textit{et al.,} 1998), and gingival epithelium constitutively expresses IL-8 (Fitzgerald and Kreutzer, 1995).

Epithelial cells infected by \textit{P. gingivalis} have the ability to produce pro-inflammatory cytokines (Kinane \textit{et al.,} 2006). Recognition of \textit{P. gingivalis} virulence factors such as LPS and fimbriae is mediated by PRRs such as CD14 and TLR2 and results in host cell activation (Hajishengallis \textit{et al.,} 2005; Zhou \textit{et al.,} 2005). It has been reported that LPS from gram-negative bacteria is associated with periodontal disease (Fine \textit{et al.,} 1992). It was reported that LPS can activate macrophages in the presence of CD14 (Hajishengallis \textit{et al.,} 2006) and fimbriae upregulate IL-8 production in an epithelial cell line through TLR2 (Asai \textit{et al.,} 2001). In contrast, Eskin \textit{et al.} showed that \textit{P. gingivalis} fimbriae were unable to activate epithelial cells (Eskin \textit{et al.,} 2007).
Furthermore, in another study, while whole *P. gingivalis* induced IL-8 production in human gingival epithelial cells via TLR2, neither *P. gingivalis* fimbriae nor LPS induced cytokine production (Kusumoto et al., 2004). Additionally, *P. gingivalis* upregulated cytokine and chemokine production in oral epithelial cells (Miliward et al., 2007). Conversely, Huang et al (Huang et al., 2004) reported a decrease in IL-8 production in gingival epithelial cells after treatment with *P. gingivalis*. Moreover, the treatment with *P. gingivalis* failed to induce IL-8 production in oral epithelial cells (Vankeerberghen et al., 2005).

Periodontal tissue destruction may occur in response to enzymes or other products secreted by periodontal pathogens and the subsequent recruitment of immune cells contributes to tissue breakdown through production of inflammatory molecules (Garlet et al., 2006). Gingival cells, macrophages, and neutrophils initiate destruction of periodontal tissues through formation, secretion, and activation of MMPs (Meikle et al., 1989; Birkedal-Hansen, 1993). Recruitment of neutrophils occurs as a result of release chemotactic substances such as IL-8 (Deng et al., 2001; Bodet et al., 2006) and by production of proteolytic enzymes, including MMPs, in infected epithelial cells that modify the inflammatory reactions and facilitate cell migration (Uitto et al., 2003).

MMPs play a critical role in periodontal tissue breakdown (Makela et al., 1994) and can be induced by bacterial components (Ding et al., 1995). It was reported that stimulation of epithelial cells with *P. gingivalis* induced expression of MMP-9 (Andrian et al., 2007). In addition, previous studies have shown that *P. gingivalis* mediates MMP-9 production in gingival epithelial cells (Fravalo et al., 1996; Chang et al., 2004). It was reported that *P. gingivalis* LPS induced MMP-9 production in an epithelial cell and
macrophage co-culture model (Bodet et al., 2006). In contrast, *P. gingivalis* had no
effect on MMP-9 activation in gingival epithelial cells (DeCarlo et al., 1997; DeCarlo et
al., 1998). Expression of TLR2 and TLR4 was investigated in gingival epithelial cells to
examine the relationship between *P. gingivalis* and TLRs (Kusumoto et al., 2004).
TLR2 and TLR4 were detected in the cells, however, TLR4 expression was faint. It was
reported that *P. gingivalis* provokes cell activity through TLR4 and indefinite other
bacterial components in *P. gingivalis* LPS may induce cell activation through TLR2
(Ogawa et al., 2002b). Stimulation of gingival epithelial cells with *P. gingivalis* induced
pro-inflammatory cytokine production through TLR4 (Eskan et al., 2008a) and *P.
 gingivalis* LPS activated gingival fibroblasts through TLR4 (Tabeta et al., 2000; Wang
et al., 2000). Thus, there is no clear consensus regarding the responsiveness of epithelial
cells to *P. gingivalis* LPS and related TLR involved in LPS signalling.

To date, no studies have investigated the role of *P. gingivalis* LPS on MMP-9
production in oral epithelial cells. Therefore, the specific aim of the present study was
to elucidate whether *P. gingivalis* LPS modulates MMP-9 and IL-8 production in OKF6
cells and primary gingival epithelial cells. In order to understand the mechanisms
involved in the interaction between LPS and gingival epithelial cells, expression of
TLR2, TLR4 and CD14 on gingival epithelial cells was also investigated.
5.2 Results

5.2.1 The effect of *P. gingivalis* LPS on MMP-9 production by OKF6 cells after a 24h stimulation

The effect of *P. gingivalis* LPS on modulation of MMP-9 production in OKF6 cells was evaluated by measuring MMP-9 secretion at 24h post-infection using ELISA. OKF6 cells were cultured as previously stated in (section 2.2.1) and were stimulated with different concentrations of *P. gingivalis* LPS (100 ng/ml, 500 ng/ml and 1000 ng/ml). Tissue culture supernatants from stimulated and unstimulated cells were collected and the MMP-9 ELISA was performed as described previously (section 2.6). The results showed that different concentrations of *P. gingivalis* LPS had no prominent effect on MMP-9 secretion in OKF6 cells at 24h post-stimulation. The mean levels of MMP-9 from duplicate wells were 0.6185 ± 243.1 ng/ml in control cells and after stimulation with 100 ng/ml, 500 ng/ml, and 1000 ng/ml of *P. gingivalis* LPS were 0.446 ± 0.1414 ng/ml, 0.876 ± 0.226 ng/ml and 0.796 ± 0.367 ng/ml respectively (Figure 5-1). The results are derived from single stimulations and therefore no statistical analysis was performed.
Figure 5-1 The effect of *P. gingivalis* LPS on MMP-9 production by OKF6 cells

OKF6 cells were stimulated with different concentrations of *P. gingivalis* (100 ng/ml, 500 ng/ml and 1000 ng/ml) for 24h. Supernatants were collected and analysed for MMP-9 secretion by ELISA. The graph represents means and standard deviations from one cell culture experiment.
5.2.2 Investigation of the effect of *P. gingivalis* and *E. coli* LPS on MMP-9 production by OKF6 cells after 6 h

The effect of *P. gingivalis* and *E. coli* LPS on MMP-9 was tested at earlier time points. OKF6 cells were stimulated with 100 ng/ml, 1 μg/ml, and 10 μg/ml of both LPS types for 6 h. Unstimulated cells served as control. Supernatants were collected and analysed using MMP-9 ELISA as described earlier (section 2.6).

5.2.3 The effect of *P. gingivalis* and *E. coli* LPS on IL-8 production by OKF6 cells

The supernatants treated with different concentration of *P. gingivalis* and *E. coli* LPS as described in the previous (section 5.2.2) were used to analyse IL-8 secretion in OKF6 cells using ELISA. Figure 5-3 shows constitutive secretion of IL-8 in control samples (0.1766 ± 0.0822 ng/ml). The level of MMP-9 in samples treated with *P. gingivalis* LPS was 0.212 ± 0.0932 ng/ml, 0.369.6 ± 0.1364 ng/ml and 0.1237 ± 0.0730 ng/ml and in those treated with *E. coli* LPS was 0.0853 ± 0.0358 ng/ml, 0.203 ± 0.0512 ng/ml and 0.6487 ± 0.2883 ng/ml. No significant difference between unstimulated and stimulated cells was detected ($p = 0.2$).
Figure 5-2 The effect of *P. gingivalis* LPS and *E. coli* LPS on MMP-9 production by OKF6 cells

OKF6 cells were stimulated with different concentrations of *P. gingivalis* and *E. coli* LPS (100 ng/ml, 1 μg/ml and 10 μg/ml) for 6h. Supernatants were collected and analysed for MMP-9 secretion by ELISA. The graph shows means and standard deviations from three independent cell culture experiments. Statistic Kruskal-Wallis $p = 0.5$
Figure 5-3 The effect of *P. gingivalis* and *E. coli* LPS on IL-8 production by OKF6 cells

OKF6 cells were stimulated with different concentrations of *P. gingivalis* and *E. coli* LPS. Supernatants were collected and analysed for IL-8 secretion by ELISA. The graph shows means and standard deviations from three independent cell culture experiments. Statistic Kruskal-Wallis $p = 0.2$
5.2.4 Investigation of the possible cytotoxic effect of LPS on OKF6 cells

Considering the possibility that the unresponsiveness of gingival epithelial cells to LPS could be due to a cytotoxic effect of LPS, cell proliferation was assessed. Stimulation experiments of OKF6 cells were conducted using the same sample stimulation time points, cells numbers, and LPS concentrations as in all stimulation experiments in the present study. OKF6 cells were stimulated with 100 ng/ml, 1 µg/ml and 10 µg/ml of \textit{P. gingivalis} LPS or \textit{E. coli} LPS for 6h. Unstimulated cells served as controls, all conditions were set up in triplicate. Cells were collected and processed in the Cell Titer 96 cell proliferation assay as described previously (section 2.2.7).

After 6h of LPS stimulation, no significant change in the number of OKF6 cells was detected in comparison with the controls ($p = 0.1$). The cell number was $2.7 \times 10^5$ cells /ml for the control. For the \textit{P. gingivalis} stimulated sample with 100 ng/ml, 1 µg/ml and 10 µg/ml LPS, the cell numbers were $2.5 \times 10^5$, $3 \times 10^5$ and $2.4 \times 10^5$ respectively. For the \textit{E. coli} stimulated samples with 100 ng/ml, 1 µg/ml and 10 µg/ml LPS, the cell numbers were $3 \times 10^5$, $2.6 \times 10^5$ and $2.6 \times 10^5$ respectively (Figure 5-4). The data revealed that after 6h, LPS from \textit{P. gingivalis} or \textit{E. coli} had no cytotoxic effect on OKF6 cells.
Figure 5-4 Analysis of OKF6 cells number after culture with LPS

OKF6 cells were stimulated with different concentration of *P. gingivalis* and *E. coli* LPS (0.1 μg/ml, 1 μg/ml and 10 μg/ml). Cell proliferation was analysed with the cell Titer 96 cell proliferation test after 6 hours. The graph shows means and standard deviations from one experiment with three independent cultures. Statistic: ANOVA, $p = 0.1$
5.2.5 The expression of CD14, TLR2 and TLR4 by OKF6 cells stimulated with *P. gingivalis* and *E. coli* LPS

To examine the possibility that the lack of any gingival epithelial cell response to LPS could be due to a defect of the receptor and signalling for LPS, the expression of CD14, TLR2, and TLR4 in OKF6 cells stimulated with bacterial LPS was investigated using RT-PCR. Cells were cultured as previously stated (section 2.2.1) and stimulated with 100 ng/ml, 1 μg/ml and 10 μg/ml *P. gingivalis* LPS or *E. coli* LPS for 6h. The cells were collected; RNA extraction, quantification and reverse transcription were performed as previously described (section 2.3.1, section 2.3.2 and section 2.3.3). The PCR for CD14, TLR2 and TLR4 was carried out as previously described (section 2.3.5). Agarose gels (Figure 5-5) showed that CD14, TLR2 and TLR4 were constitutively expressed in OKF6 cells in the control and the treated samples.
Sub-confluent OKF6 cells were treated with different concentrations of \textit{P. gingivalis} or \textit{E. coli} LPS. Total cellular RNA was isolated and analysed by RT-PCR. Sequences in the cDNA were amplified with specific primers for CD14, TLR2 and TLR4. The products were separated on 3% agarose gels. Lane 1 = marker, Lane 2 = control, Lane 3 = 0.1 \( \mu\)g/ml \textit{P. gingivalis}, Lane 4 = 1 \( \mu\)g/ml \textit{P. gingivalis}, Lane 5 = 10 \( \mu\)g/ml \textit{P. gingivalis}, Lane 6 = 0.1 \( \mu\)g/ml \textit{E. coli}, Lane 7 = 1 \( \mu\)g/ml \textit{E. coli}, Lane 8 = 10 \( \mu\)g/ml \textit{E. coli}, Lane 9 = negative control, Lane 10 = positive control (monocytes).
5.2.6 The effect of *P. gingivalis* and *E. coli* LPS on MMP-9 expression by primary oral epithelial cells

The effect of *P. gingivalis* and *E. coli* LPS on MMP-9 secretion in primary gingival epithelial cells was investigated using ELISA. Cells were obtained and cultured as previously stated (section 2.2.2), and were stimulated with 100 ng/ml, 1μg/ml, and 10 μg/ml of both LPS types for 6h. Unstimulated cells served as controls. Supernatants were collected and analysed using the MMP-9 ELISA as described in section 2.6. Figure 5-6 shows that MMP-9 was constitutively expressed in control samples (0.1772 ± 0.0098 ng/ml). The MMP-9 level in *P. gingivalis* LPS treated samples was 0.2246 ± 0.0194 ng/ml, 0.2063 ± 0.0171 ng/ml and 0.2278 ± 0.0081 ng/ml respectively. For *E. coli* treated samples, the level of MMP-9 was 0.2403 ± 0.0325 ng/ml, 0.2549 ± 0.0251 ng/ml and 0.1976 ± 0.0249 ng/ml respectively. The results were obtained from a single experiment and therefore no statistical analysis was performed.
Figure 5-6 The effect of *P. gingivalis* and *E. coli* LPS on MMP-9 production by primary oral epithelial cells

Primary oral epithelial cells were stimulated with different concentrations of *P. gingivalis* and *E. coli* LPS (100 ng/ml, 1 μg/ml and 10 μg/ml) for 6h. Supernatants were collected and analysed for MMP-9 secretion by ELISA. The graph shows means and standard deviations from one cell culture experiments.
5.2.7 The expression of CD14, TLR2 and TLR4 by primary oral epithelial cells

The expression of CD14, TLR2, and TLR4 in primary gingival epithelial cells stimulated with bacterial LPS was investigated using RT-PCR. Cells were cultured as previously stated (section 2.2.1) and stimulated with 100 ng/ml, 1 µg/ml and 10 µg/ml *P. gingivalis* LPS or *E. coli* LPS for 6h. The cells were collected; RNA extraction, quantification and reverse transcription were performed as previously described (section 2.3.1, section 2.3.2 and section 2.3.3). The PCR for CD14, TLR2 and TLR4 was carried out as previously described (section 2.3.5). Agarose gels (Figure 5-7) showed that TLR2 was expressed in control as well as treated samples. CD14 and TLR4 were not detected in primary cells.
Figure 5-7 Expression of CD14, TLR2 and TLR4 by primary oral epithelial cells

Sub-confluent primary cells were treated with different concentrations of *P. gingivalis* or *E. coli* LPS. Total cellular RNA was isolated and analysed by RT-PCR. Sequences in the cDNA were amplified with specific primers for CD14, TLR2 and TLR4. The products were separated on 3% agarose gels. Lane 1= marker, Lane 2= control, Lane 3= 0.1 μg/ml *P. gingivalis*, Lane 4= 1 μg/ml *P. gingivalis*, Lane 5= 10 μg/ml *P. gingivalis*, Lane 6= 0.1 μg/ml *E. coli*, Lane 7= 1 μg/ml *E. coli*, Lane 8= 10 μg/ml *E. coli*. Lane 9= negative control, Lane 10= positive control (monocytes).
5.3 Discussion

Increasing evidence supports that oral epithelial cells play an important role not only in providing the first line of defense against periodontal pathogens but also in the innate host defenses (Dale, 2002). *P. gingivalis* is implicated in the pathogenesis of periodontal disease (Socransky and Haffajee, 1992) and recognized as an important aetiological factor of periodontitis (Williams, 1990). *P. gingivalis* has many virulence factors such as LPS, fimbriae, several proteases and vesicle formation (Holt et al., 1999). LPS has been related to periodontal disease pathogenesis (Lamont and Jenkinson, 1998; Holt et al., 1999) by mediating inflammation and inducing cells to secret pro-inflammatory cytokines such as IL-8 (Jenkinson and Dymock, 1999). Periodontal tissue damage has been associated with high levels of MMP-9 in GCF (Teng et al., 1992) and inflamed gingival tissue of patients with periodontal disease (Ejei et al., 2003; Smith et al., 2004).

The current study found in a preliminary experiment that *P. gingivalis* LPS lacks the ability to upregulate MMP-9 secretion in OKF6 cells after 24h stimulation. It has been reported in most studies that *P. gingivalis* LPS is less potent than *E. coli* LPS in inducing the release of pro-inflammatory cytokines in various cells (Ogawa and Uchida, 1996; Martin et al., 2001), so it was of importance to compare the capacity of *P. gingivalis* LPS and *E. coli* LPS to induce the production of MMP-9. Therefore the current study investigated the modulation of MMP-9 by *P. gingivalis* and *E. coli* LPS in OKF6 cells and primary oral epithelial cells. The results showed that *P. gingivalis* LPS and *E. coli* LPS lack the ability to upregulate MMP-9 production in OKF6 cells and primary oral epithelial cells at different concentrations after earlier time points however, the parallel study of LPS stimulation of monocytes clearly demonstrated that both these
preparation of LPS are biologically active (Nile et al., 2010). In contrast, previous 
studies have shown that \textit{P. gingivalis} upregulated MMP-9 and IL-8 in oral epithelial 
cells (Andrian et al., 2007; Milward et al., 2007). The present study also investigated 
the production of IL-8 post-infection with \textit{P. gingivalis} and \textit{E. coli} LPS in OKF6 cells. 
The current study demonstrated that \textit{P. gingivalis} and \textit{E. coli} LPS had no effect on 
regulation of IL-8 in OKF6. In agreement with this result, Kusmoto et al reported that 
\textit{P. gingivalis} LPS stimulation did not induce IL-8 production in gingival epithelial cells 
(Kusumoto et al., 2004). However, \textit{P. gingivalis} induced pro-inflammatory cytokines in 
the gingival epithelial cells (Kinane et al., 2006).

In this context, a previous study suggested a role for CD14 (Ulevitch and Tobias, 1995) 
and TLR for LPS signalling (Ogawa et al., 2002a) however another study reported that 
oral epithelial cells do not respond to LPS even in the presence of CD14 (Uehara et al., 
2001). The present study examined the possibility that gingival epithelial cells are 
defective in the receptor and signalling molecules for LPS but the OKF6 cells did 
express CD14, TLR2 and TLR4. Primary gingival epithelial cells were devoid of CD14 
and TLR4 mRNA expression but expressed TLR2, thus lack of CD14 could be the 
reason for unresponsiveness of the primary cells as CD14 has been shown to be 
indispensable for LPS recognition (Akashi et al., 2000). The present study also 
investigated the possibility that LPS had a cytotoxic effect on the epithelial cells 
although the study showed that the cells were not significantly changed by the treatment 
with LPS. The previous results was in agreement with Pollanen who showed that LPS 
from periodontal pathogens had no effect on the growth and mitotic activity of the oral 
epithelial cells (Pollanen, 2000).
It thus appears that the lack of MMP-9 regulation by LPS in OKF6 cells is not caused by a lack of receptor expressions or cytotoxic mechanisms. However, other components of the TLR4 signalling pathway such as My88, MAL, TRIF and TRAM (O'Neill and Bowie, 2007) were not tested. Also, it was reported that TLR4 signalling requires supplementary mediators to increase cytokine production (Eskan et al., 2008b). Previous studies have demonstrated that *P. gingivalis* increases MMP-9 and pro-inflammatory cytokine secretion in epithelial cells (Kinane et al., 2006; Andrian et al., 2007; Milward et al., 2007). Furthermore, *P. gingivalis* also increases MMP activity in gingival fibroblasts (Zhou and Windsor, 2006). Since no increase in the production of MMP-9 protein was observed following treatment with *P. gingivalis* LPS, it is tempting to speculate that oral epithelial cells are poorly stimulated by *P. gingivalis* LPS and other components of *P. gingivalis* may be involved in the induction of MMPs and pro-inflammatory cytokines.

Although it was demonstrated that LPS from *P. gingivalis* enhances MMP-9 production in an epithelial cell and macrophage co-culture model (Bodet et al., 2006), the present study showed that *P. gingivalis* LPS was not able to induce MMP-9 and IL-8 secretion in oral epithelial cells. This finding is not in conflict with the latter report because it indicates the increase in MMP and cytokines in the co-culture model which contains a mixture of epithelial cells and macrophages that might have impact on the results in comparison to the epithelial cells alone.
In conclusion, the current study identified unresponsiveness of epithelial cells to LPS and supports that LPS does not significantly contribute to \textit{P. gingivalis}-induced pro-inflammatory responses in epithelial cells. Epithelial cells are poised to serve as sensors for bacterial insult and produce signals that can stimulate the recruitment of host inflammatory cells. Despite the established role of LPS as a modulator of inflammation, LPS may not appear to have a key role in production of proinflammatory mediators in oral epithelial cells. The inability of LPS to upregulate IL-8 in the epithelial cells during periodontal inflammation may inhibit neutrophil migration to the area of the inflammation and allow periodontal pathogens to survive in periodontal tissue resulting in more bacteria colonization and invasion. Furthermore, inability of LPS to regulate MMP-9 may interfere with epithelial cell migration and tissue remodelling. Therefore, LPS may reduce epithelial compensation, leading to further bacterial translocation, an increase in the inflammatory response and more epithelial injury. One can speculate that bacterial LPS may not be the main \textit{P. gingivalis} component responsible for inducing MMP-9 and IL-8 production in epithelial cells. Thus, a further study is needed to elucidate whether oral epithelial cells are still unresponsive to LPS from different periodontal pathogens.
Chapter 6  MMP-8 and MMP-9 concentrations in periodontal disease patients with and without diabetes

6.1 Introduction

The ECM is a complex network composed mainly of structural proteins such as collagen and proteoglycans. This matrix surrounds and supports cells within tissues. In the periodontium, the gingival ECM and underlying tissues provide a supportive framework for the dental hard tissues. In healthy tissue there is balance between synthesis and degradation of ECM components. However, under pathological conditions, for example, in periodontal diseases, the rate of degradation is typically much greater than ECM synthesis. These remodelling processes are thought to be mediated predominantly by a family of zinc binding extracellular proteolytic enzymes known as the MMPs (Murphy and Nagase, 2008).

MMPs play key roles in degradation of various extracellular molecules, including collagen, elastin, proteoglycan and laminin during different physiological and pathological processes (auf dem Keller et al., 2007). It is worth noting that MMPs are major factors involved in tissue remodelling and destruction in periodontal disease, arthritis, impaired wound healing in diabetes and atherosclerosis (Shiau et al., 2006; Giannobile, 2008). They are also involved in altering and activating proteins and specific chemokines during inflammation and they are considered as host modulating agents (Van Lint and Libert, 2007; Giannobile, 2008).
Periodontal disease is an inflammatory disorder that results from imbalance in the interaction between periodontal pathogens and the host response (Goutoudi et al., 2004) which leads to over-expression of pro-inflammatory cytokines and enzymes that are the main contributors to the degradation of the underlying connective tissues and the alveolar bone (Lu et al., 2006). During periodontitis, degradation of gingival tissue is, in part, due to the MMPs that are expressed by the inflammatory cells and the resident cells such as neutrophils, fibroblasts and epithelial cells (Seguier et al., 2000). Furthermore, cells of the periodontium can express inflammatory cytokines and growth factors that may upregulate MMPs transcription (Birkedal-Hansen, 1993). Significant evidence exists to demonstrate that the MMPs play an important role in periodontal tissue destruction (Sorsa et al., 2004). MMP concentrations in gingival tissues and GCF from individuals with periodontitis are elevated when compared with healthy gingival tissues (Ingman et al., 1994; Golub et al., 1997). MMP activities in GCF also increase during inflammation and have been correlated with the severity of periodontal disease (Sorsa et al., 2004). Furthermore, MMPs in the GCF have been shown to be associated with progression of periodontal tissue destruction (Kiiili et al., 2002; Hernandez et al., 2006). MMP-8 and MMP-9 are the main proteinases related to tissue destruction and remodelling events in periodontal disease (Hill et al., 1994). In particular, MMP-8 has been reported to be elevated in periodontitis patients (Tervahartiala et al., 2000; Kumar et al., 2006) and in the gingiva and GCF from patients with periodontal disease (Ingman et al., 1996). It was reported that the level of MMP-8 is slightly elevated in gingivitis patients albeit in its inactive form (Sorsa et al., 1999). An increased level of MMP-8 in GCF samples from patients with periodontal disease has also been described (Teles et al., 2010). In addition, it was reported that a significant increase in GCF MMP-9 levels was detected in patients with periodontal disease (Rai et al., 2008) and a role for MMP-
9 in the regulation of periodontal disease was suggested (Makela et al., 1994). Furthermore, it was reported that periodontal disease augments the levels of systemic inflammatory markers such as CRP and IL-6 (Loos, 2005) that lead to the induction of inflammatory mediators which upregulate MMP production (Borden and Heller, 1997). It was suggested that the inflammatory markers liberated from periodontally diseased tissue may enter the circulation and stimulate inflammation in other parts of the body (Moutsopoulos and Madianos, 2006). In addition, high plasma MMP-9 and MMP-8 levels were established in periodontal disease patients compared to controls (Marcaccini et al., 2009). Periodontal treatment such as RSI has been documented to decrease local MMP levels (Mancini et al., 1999; Kinane et al., 2003; Pozo et al., 2005).

T2DM is a widespread disease characterized by impairment in the regulation of glucose homeostasis (Cutler et al., 1999). It was reported that diabetes induces changes in immune cell functions which predispose to chronic inflammation (Freeman et al., 2002). Furthermore, adipose tissue can initiate or exacerbate inflammation in diabetes by releasing several cytokines and adipokines (Munzberg and Myers, 2005). High blood glucose levels in patients with T2DM contribute to the development of micro-vascular and macro-vascular complications (Singleton et al., 2003). Periodontal disease and T2DM are closely associated and inflammation is a critical player in this association (Mealey, 2006). Periodontitis is considered a complication of diabetes (Loe, 1993; Nishimura et al., 1998; Javed et al., 2009). In a meta-analysis, it was reported that patients with diabetes had higher gingival and periodontal inflammation compared with non-diabetic individuals (Khader et al., 2006; Lim et al., 2007). Although there is evidence that diabetes is a risk factor for periodontal disease and is related to the severity of this disorder (Ryan et al., 2003; Moles, 2006), no significant differences in
the prevalence of periodontal pathogens between diabetic and non-diabetic individuals with periodontal disease have been detected (Lalla et al., 2006). It is likely therefore other factors may be more important in the increased prevalence and severity of periodontal disease in diabetics.

The suggested mechanisms by which diabetes contributes to periodontitis include vascular changes and abnormal collagen synthesis (Katz et al., 1991). Hyperglycaemia causes alteration of the vasculature that interferes with the transportation of nutrients and migration of neutrophils into the gingival tissue which contributes to an increased risk of periodontal disease in diabetes (Goova et al., 2001; Ryan et al., 2003). In addition, advanced glycation end products (AGEs) formed in the hyperglycaemic environment may be involved in tissue changes in the periodontium and have been demonstrated to be present in the gingiva of individuals with diabetes (Schmidt et al., 1996) and in diabetic mice (Lalla et al., 1998). AGEs may also be involved in modulating the collagen cross-links which alter collagen solubility and turnover (Monnier et al., 2005). AGEs also bind to macrophages and induce production of pro-inflammatory cytokines that can induce MMP productions in diabetic individuals (Tervonen and Oliver, 1993; Nishimura et al., 1998). Studies have shown that the levels of MMPs are increased in individuals with diabetes (Maxwell et al., 2001; Xue et al., 2005) and dysregulation of their activities is implicated in the pathology of diabetic complications (Ishizaki et al., 2006; Jacqueminet et al., 2006). In addition, alterations in immune function and impaired collagen production are evident in diabetic individuals (Mealey and Rose, 2008) and thus the diabetic state decreases the host resistance to infection through defects in neutrophil function such as impaired chemotaxis and phagocytosis.
Available data provide strong evidence that diabetes is a risk factor for gingivitis and periodontitis (Papapanou, 1996; Mealey and Ocampo, 2007). Previous studies have shown that poorly controlled diabetics had a greater risk of periodontitis compared to non-diabetic individuals and well controlled diabetics had no significant increase in the risk for periodontitis (Tsai et al., 2002). Diabetic patients with poor glycemic control had more clinical attachment loss (CAL) and bone loss than non-diabetic individuals (Seppala et al., 1993; Seppala and Ainamo, 1994). Similarly, other studies have reported that the depth of periodontal pockets and prevalence of clinical attachment loss increased as glycemic control worsened (Tervonen and Oliver, 1993; Guzman et al., 2003). In addition, attachment loss has been found to occur more frequently in uncontrolled diabetic patients compared to well controlled diabetes or healthy individuals (Tervonen et al., 2000).

A previous study has shown increased levels of MMP-8 in GCF samples from diabetic patients with gingivitis (Salvi et al., 2010). Furthermore, an elevation of GCF MMP-8 levels was detected in periodontally diseased patients with and without diabetes (Kardesler et al., 2010). Increased levels of MMP-8 and MMP-9 in periodontal tissue from individuals with diabetes were reported and the elevated levels of MMPs were correlated with CAL and PD (Kumar et al., 2006). In addition, patients with T2DM had elevated plasma MMP-9 levels compared to non-diabetic individuals (Signorelli et al., 2005; Shiau et al., 2006). It was reported that circulating MMP-9 levels are increased in T2DM patients with coronary artery disease and the elevated serum MMP-9 levels are linked with atherosclerosis (Noji et al., 2001) and associated with diabetic complications (Ebihara et al., 1998). Alterations of MMP-8 levels have been reported in systemically healthy individuals with periodontitis (Sorsa et al., 2006) and
individuals with diabetes or atherosclerosis with or without periodontal disease (Soder et al., 2006; Tuomainen et al., 2007). It was also reported that periodontal disease increased systemic inflammatory markers such as CRP and IL-6 (Loos, 2005) which upregulate MMP expression (Borden and Heller, 1997).

Periodontal disease has a significant impact on glycemic control as diabetic individuals with severe periodontitis have more risk of worsening glycemic control over time compared to those without periodontitis (Taylor et al., 1996). Periodontal treatment was associated with a reduction in HbA1c levels post-treatment compared with pre-treatment values (Grossi et al., 1997). A five year study evaluated the effects of non-surgical and surgical periodontal treatment in diabetic patients and found they were able to maintain a healthy periodontal condition (Westfelt et al., 1996). Furthermore, other studies showed significant improvement in glycemic control after RSI (Stewart et al., 2001; Kiran et al., 2005). Recently, a meta-analysis reported a 0.4% reduction in HbA1c following periodontal treatment (Vergnes, 2010). Conversely, it has also been reported that patients who received RSI showed improved periodontal health but no significant changes in glycemic control (Aldridge et al., 1995; Christgau et al., 1998).

In addition, Janket et al in a meta-analysis reported a 0.3% reduction in HbA1c after periodontal treatment however the decrease was not significant (Janket et al., 2005). Therefore, understanding the relationship between periodontal disease and diabetes could help in providing better and more holistic care to diabetic patients.

The existing literature clearly points toward a contributing role of MMPs in periodontal disease progression. Therefore, the aim of this study was to assess GCF MMP-8 and MMP-9 levels and serum MMP-9 levels in periodontally diseased diabetic, non-diabetic
and healthy patients before and after periodontal treatment to evaluate whether T2DM could influence periodontal status by alterations in the GCF and serum levels of MMPs that may be part of the mechanism by which diabetes affects the periodontal state.

6.2 Results

6.2.1 Analysis of GCF MMP-9 concentrations in non-diabetic and T2DM patients before treatment for periodontal disease

GCF samples were obtained from non-diabetic and T2DM patients with and without periodontal disease as described previously (section 2.7.2). Concentrations of MMP-9 in GCF were determined using ELISA. There are two approaches that have been adopted to measure GCF mediator concentrations by total amount per time of collection and the measurement of GCF volume to calculate concentrations: some studies reported measurements of the total amount of mediator in a sample collected for a given time (irrespective of the volume collected) while other studies reported the measurement of the GCF volume collected and calculate concentrations of mediators. This has been an issue of discussion in the periodontal literature with both being considered acceptable approaches for the estimation of GCF biomarkers (Page, 1992). In the present study, MMPs have been measured in a sample of GCF collected over 30 seconds (section 2.7.2); this is a similar approach to that adopted by other studies in the recent literature (Orozco et al., 2006; Salvi et al., 2010).

Figure 6-1 shows the GCF MMP-9 concentrations, according to periodontal status, in non-diabetic and T2DM patients. In non-diabetic individuals with gingivitis or periodontitis, GCF MMP-9 concentrations were significantly higher than in periodontally healthy individuals (Table 6-1) (GCF MMP-8 and serum MMP-9 presented in the table will discuss later). In addition, significantly higher GCF MMP-9
concentrations were detected in gingivitis and periodontitis patients with T2DM compared with periodontally healthy T2DM patients.

No significant differences in GCF MMP-9 concentrations between periodontally healthy non-diabetic and T2DM patients were detected ($p = 0.4$). Significantly higher levels of MMP-9 in diabetic patients with gingivitis were observed compared to non-diabetic individuals with gingivitis. Diabetic patients with periodontitis exhibited significantly lower MMP-9 levels than non-diabetic individuals with periodontitis.
Figure 6-1 GCF MMP-9 concentrations according to periodontal status in non-diabetics and T2DM patients

Box plots of GCF MMP-9 concentration in 81 non-diabetic controls (periodontal health n = 15; gingivitis n = 21; periodontitis n = 45) and 99 diabetic patients (periodontal health = 15; gingivitis = 38; periodontitis = 46). Statistics: Kruskal-Wallis, Mann-Whitney test **p < 0.01, ***p < 0.001 (according to the periodontal status within diabetes or control). $^{p}$p < 0.05 and $^{*}$p < 0.01 (diabetes versus non-diabetes within the corresponding periodontal status). ◆ Outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.
Table 6-1 GCF and serum concentrations for MMP-8 and MMP-9 in non-diabetic and T2DM patients with periodontal diseases

<table>
<thead>
<tr>
<th>Enzyme levels</th>
<th>Non-diabetic</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Health</td>
<td>Gingivitis</td>
</tr>
<tr>
<td>GCF MMP-8</td>
<td>16.0±9.2</td>
<td>31.6±29.3*</td>
</tr>
<tr>
<td>GCF MMP-9</td>
<td>64.8±29.6</td>
<td>127.5±131.1***</td>
</tr>
<tr>
<td>Serum MMP-9</td>
<td>284.0±87.5</td>
<td>443.2±241.2**</td>
</tr>
</tbody>
</table>

The table shows mean ± standard deviation of GCF and serum MMP-9 and MMP-8 levels according to periodontal status in non-diabetic and T2DM patients. *p < 0.05, **p < 0.01 and ***p < 0.001(significantly different from periodontally healthy patients with non-diabetic or T2DM group. §p < 0.05 and §§p < 0.01(significantly different from non-diabetic patients within the periodontal disease categories).
6.2.2 **Analysis of GCF MMP-9 concentration in periodontitis patients after treatment for periodontal disease**

In non-diabetic controls and diabetic patients with periodontitis, GCF samples were collected after treatment for periodontal disease at 3, 6 and 12 months. GCF MMP-9 concentrations declined significantly after periodontal treatment in both non-diabetic controls and T2DM patients (Figure 6-2). A significant decrease ($p < 0.001$) in GCF MMP-9 levels of non-diabetic periodontitis patients at month 3 after treatment compared to pre-treated individuals was observed. Also, significant reductions ($p < 0.001$ and $p < 0.001$) were detected in GCF MMP-9 levels in non-diabetic periodontitis patients at 6 and 12 months after treatment compared with pre-treatment patients. In diabetic patients with periodontitis, the GCF MMP-9 concentration decreased at month 3 after periodontal treatment ($p < 0.01$). GCF MMP-9 levels in diabetic individuals at 6 and 12 months after treatment were also significantly lower compared to pre-treatment values (Table 6-2) (levels of GCF MMP-8 and serum MMP-9 presented in the table will be discussed later).
Figure 6-2 The effect of periodontal treatment on GCF MMP-9 concentrations in non-diabetics and T2DM patients with periodontitis

Box plots of GCF MMP-9 concentrations of patients with periodontitis in non-diabetics and T2DM groups. Measurements were taken pre-treatment (non-diabetics n = 45; T2DM n = 46), and after 3 months (non-diabetics n = 41; T2DM n = 26), 6 months (non-diabetics n = 31; T2DM n = 31) and 12 months (non-diabetics n = 29; T2DM n = 25). Statistics: related samples test, Friedman, Wilcoxon Ranks test. **p < 0.01, ***p < 0.001. ◆ Outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.
Table 6-2 GCF and serum concentrations for MMP-8 and MMP-9 in non-diabetics and T2DM patients with periodontitis after periodontal treatment

<table>
<thead>
<tr>
<th></th>
<th>GCF MMP-8 (ng/ml)</th>
<th>GCF MMP-9 (ng/ml)</th>
<th>Serum MMP-9 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-diabetic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (n=45)</td>
<td>83.1 ± 50.5</td>
<td>348.4 ± 228.9</td>
<td>461.5 ± 318.5</td>
</tr>
<tr>
<td>Month 3 (n=41)</td>
<td>43.7 ± 23.4****</td>
<td>191.9 ± 124.0****</td>
<td>351.7 ± 181.9*</td>
</tr>
<tr>
<td>Month 6 (n=31)</td>
<td>41.5 ± 26.5***</td>
<td>188.7 ± 121.4***</td>
<td>403.3 ± 296.7</td>
</tr>
<tr>
<td>Month 12 (n=29)</td>
<td>43.1 ± 26.4****</td>
<td>199.8 ± 120.5****</td>
<td>436.1 ± 288.6</td>
</tr>
<tr>
<td><strong>Diabetic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (n=46)</td>
<td>58.5 ± 39.5</td>
<td>215.4 ± 111.7</td>
<td>559.1 ± 328.7</td>
</tr>
<tr>
<td>Month 3 (n=26)</td>
<td>36.2 ± 20.5***</td>
<td>150.0 ± 90.2**</td>
<td>576.4 ± 308.6</td>
</tr>
<tr>
<td>Month 6 (n=31)</td>
<td>33.7 ± 22.0****</td>
<td>144.4 ± 91.2**</td>
<td>549.8 ± 314.1</td>
</tr>
<tr>
<td>Month 12 (n=25)</td>
<td>36.0 ± 23.2****</td>
<td>130.6 ± 99.3**</td>
<td>515.8 ± 269.3</td>
</tr>
</tbody>
</table>

The table shows mean ± standard deviation of GCF and serum MMP-9 and MMP-8 levels in non-diabetic and T2DM patients with periodontitis. *p < 0.05, ** p < 0.01 and *** p < 0.001 (significantly different from baseline periodontitis patients in non-diabetic or T2DM groups).
6.2.3 The relationship between GCF MMP-9 and clinical parameters of periodontal disease

At baseline, the relationship of GCF MMP-9 concentrations with clinical attachment loss (CAL mm) and probing depths (PD mm) in non-diabetics and T2DM individuals was analysed. A positive and statistically significant correlation was noted both between CAL (mm) and GCF MMP-9 ($p < 0.001$) and between PD (mm) and MMP-9 levels ($p < 0.001$) (Figure 6-3). The MMP-9 concentrations increased with increasing CAL and PD.
Figure 6-3 The relationship between GCF MMP-9 concentrations and CAL and PD in non-diabetic and T2DM patients

The graph shows Spearman correlations of GCF MMP-9 concentrations with CAL and PD in non-diabetics (n = 81) and T2DM patients (n = 99) and differentiated according to periodontal status.
6.2.4 Analysis of MMP-8 concentrations in non-diabetic and diabetic patients before periodontal treatment

Concentrations of MMP-8 in GCF were determined using ELISA. Statistically significant differences in MMP-8 levels were observed in non-diabetic individuals both between the periodontally healthy and the gingivitis groups and between the periodontally healthy and periodontitis groups (Figure 6-4). The mean GCF MMP-8 concentrations are illustrated in Table 6-1. In diabetic individuals, significantly higher GCF MMP-8 concentrations in the gingivitis and periodontitis groups were observed compared to non-diabetic periodontally healthy individuals. In addition, there was no significant differences in MMP-8 levels between the periodontally healthy groups in non-diabetic and T2DM patients ($p = 0.9$). Likewise, MMP-8 levels were not significantly different in the diabetic patients with gingivitis compared to the gingivitis group in non-diabetic individuals ($p = 0.3$). The diabetic periodontitis group exhibited significantly lower MMP-8 levels than the non-diabetic periodontitis group.
Figure 6-4 GCF MMP-8 concentrations according to periodontal status in non-diabetic and T2DM patients

Box plots of GCF MMP-8 concentrations in 81 non-diabetic controls (periodontal heath n = 15; gingivitis n = 21; periodontitis n = 45) and 99 diabetic patients (periodontal health = 15; gingivitis = 38; periodontitis = 46). Statistics: Kruskal-Wallis, Mann-Whitney test \( *p < 0.01, \quad ***p < 0.001 \) (according to the periodontal status within diabetes or control). \( \& p < 0.05 \) and \( \# p < 0.01 \) (diabetes versus non-diabetes within the corresponding periodontal status).  ◆ Outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.
6.2.5 Analysis of GCF MMP-8 concentration in periodontitis patients with and without T2DM after treatment for periodontal disease

In non-diabetic controls and diabetic patients with periodontitis, the concentrations of MMP-8 in GCF were followed up after treatment for periodontal disease at 3, 6 and 12 months. GCF MMP-8 concentrations were reduced significantly after periodontal treatment in both non-diabetic controls and T2DM patients (Figure 6-5). In non-diabetic periodontitis patients, GCF MMP-8 levels declined significantly from at month 3 post-treatment ($p < 0.001$) compared with baseline value (Table 6-2). GCF MMP-8 concentrations in non-diabetic individuals at 6 and 12 month post-treatment were also significantly declined. In addition, a significant reduction in MMP-8 GCF levels in diabetic periodontitis patients at month 3 after treatment was detected. Likewise, the GCF MMP-8 levels in diabetic individuals with periodontitis at 6 and 12 months post-treatment significantly decreased compared to pre-treatment values.

6.2.6 The relationship of MMP-8 with periodontal clinical parameters

At baseline, the relationship of GCF MMP-8 concentration with CAL (mm), and PD (mm) in non-diabetics and T2DM individuals was analysed. A positive and statistically significant correlation was observed both between CAL (mm) and GCF MMP-8 ($p < 0.001$) and between PD (mm) and GCF MMP-8 levels ($p < 0.001$) (Figure 6-6). The GCF MMP-8 levels increased with increasing CAL and PD.
Figure 6.5 The effect of periodontal treatment on GCF MMP-8 concentrations in non-diabetics and T2DM patients with periodontitis

Box plots of GCF MMP-8 concentrations of patients with periodontitis and non-diabetics and of T2DM patients with periodontitis. Measurements were taken pre-treatment (non-diabetics n = 45; T2DM n = 46), and after 3 months (non-diabetics n = 41; T2DM n = 26), 6 months (non-diabetics n = 31; T2DM n = 31) and 12 months (non-diabetics n = 29; T2DM n = 25). Statistics: related samples test, Friedman, Wilcoxon Ranks test ***p < 0.001. ♦ Outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.
Figure 6-6 The relationship between GCF MMP-8 concentrations and CAL and PD in T2DM and non-diabetic patients

The graph shows Spearman correlations of GCF MMP-8 concentrations with CAL and PD in non-diabetics (n=81) and T2DM (n=59) and differentiated according to the periodontal status.
6.2.7 Comparison of clinical parameters (pre-treatment) among non-diabetics and T2DM patients

The clinical periodontal measurements for CAL and PD at GCF sampling sites were tested statistically before treatment between non-diabetic and diabetic individuals. The mean and standard deviation values of the clinical periodontal measurements are outlined in Table 6-3. No significant difference in PD values between the healthy groups in non-diabetic and diabetic individuals was observed ($p = 0.1$). The mean PD values in the gingivitis groups also showed no significant differences between non-diabetic and T2DM individuals ($p = 0.09$). The diabetic periodontitis group had significantly lower mean PD values ($3.6 \pm 1.0 \text{ mm}$) than the non-diabetic periodontitis individuals ($4.6 \pm 1.4 \text{ mm}$), however ($p < 0.001$).

No significant differences in mean CAL between healthy groups in non-diabetics and T2DM patients ($p = 0.09$) were detected. The gingivitis group in the diabetic cohort also had no significant difference in mean CAL value compared to the gingivitis group in non-diabetic individuals ($p = 0.07$). The diabetic periodontitis group exhibited a significantly lower CAL value ($3.8 \pm 0.9 \text{ mm}$) than the non-diabetic periodontitis group ($5.1 \pm 1.5$), however ($p < 0.001$).
6.2.8 Comparison of clinical parameters (pre- and post-treatment) within non-diabetic and T2DM patients with periodontitis

There was a significant improvement in PD, CAL and bleeding on probing (BOP) in both study groups at 3, 6, and 12 months after periodontal treatment compared to baseline measurement (Table 6-4). In non-diabetic individuals at 3 months, the mean PD values were significantly reduced \((p < 0.001)\) from 4.6 ± 1.4 mm pre-treatment to 3.8 ± 1.2 mm post-treatment. The mean CAL values also significantly reduced \((p < 0.001)\) from 5.1 ± 1.5 mm to 4.6 ± 1.7 mm and BOP % had significantly reduced \((p < 0.001)\) from 72.2% to 36.9%. As can be seen from Table 6-4 these clinical improvements were maintained at month 6 and 12 post treatment.

In diabetic individuals at 3 months, the mean PD values significantly reduced \((p < 0.001)\) from 3.6 ± 1.0 mm to 3.2 ± 0.9 mm. The mean CAL values also significantly decreased \((p < 0.05)\) from 3.8 ± 0.9 mm to 3.6 ± 1.0 mm and the BOP% significantly reduced \((p < 0.001)\) from 62.7% to 37.5%. Again, as shown in Table 6-4, these clinical improvements were maintained at 6 and 12 months post-treatment.
Table 6-3 The clinical periodontal parameters according to diabetic and periodontal status

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Non-diabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Health n = 15</td>
<td>Gingivitis n = 20</td>
</tr>
<tr>
<td>PD (mm) (mean±SD)</td>
<td>1.9 ± 0.2</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>CAL (mm) (mean±SD)</td>
<td>2.1 ± 0.2</td>
<td>2.3 ± 0.4</td>
</tr>
</tbody>
</table>

The table shows means ± standard deviations of the clinical parameters in non-diabetics (health n = 15, gingivitis n = 20, periodontitis n = 46) and T2DM (health n = 15, gingivitis n = 38, periodontitis n = 43) before treatment according to periodontal status. Statistics: Kruskal-Wallis, Mann-Whitney test ***p < 0.001 (diabetes versus non-diabetics within the corresponding periodontal status)
Table 6-4 The effect of periodontal treatment on periodontal clinical parameters during the study period in periodontitis individuals with and without diabetes

<table>
<thead>
<tr>
<th></th>
<th>PD (mm) mean±SD</th>
<th>CAL (mm) mean±SD</th>
<th>BOP %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-diabetic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (n=46)</td>
<td>4.6 ± 1.4</td>
<td>5.1 ± 1.5</td>
<td>72.2% ± 27.2%</td>
</tr>
<tr>
<td>Month 3 (n=41)</td>
<td>3.8 ± 1.2***</td>
<td>4.6 ± 1.7***</td>
<td>36.5% ± 28.5%***</td>
</tr>
<tr>
<td>Month 6 (n=37)</td>
<td>4.0 ± 1.4***</td>
<td>4.9 ± 1.9*</td>
<td>28.3% ± 27.7%***</td>
</tr>
<tr>
<td>Month 12 (n=26)</td>
<td>3.2 ± 0.9***</td>
<td>4.0 ± 1.2***</td>
<td>25.9% ± 31.2%***</td>
</tr>
<tr>
<td><strong>Diabetic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (n=43)</td>
<td>3.6 ± 1.0</td>
<td>3.8 ± 0.9</td>
<td>64.5% ± 31.4%</td>
</tr>
<tr>
<td>Month 3 (n=33)</td>
<td>3.2 ± 0.9***</td>
<td>3.6 ± 0.9*</td>
<td>38.6% ± 33.7%**</td>
</tr>
<tr>
<td>Month 6 (n=32)</td>
<td>3.1 ± 1.1***</td>
<td>3.5 ± 1.2*</td>
<td>33.3% ± 25.5%***</td>
</tr>
<tr>
<td>Month 12 (n=25)</td>
<td>2.9 ± 0.9***</td>
<td>3.4 ± 1.0*</td>
<td>26.0% ± 23.3***</td>
</tr>
</tbody>
</table>

The table shows mean ± standard deviations of the periodontal clinical parameters in patients with periodontitis in the diabetic and non-diabetic groups. Measurements were taken pre-treatment and post-treatment after 3, 6 and 12 months. Statistics: related samples test, Friedman, Wilcoxon Ranks test ***p < 0.001, **p < 0.01, *p < 0.05 (baseline compared to 3, 6, 12 months post treatment in non-diabetic and diabetic groups)
6.2.9 Analysis of serum MMP-9 concentrations in T2DM and non-diabetic controls with or without periodontal disease before treatment

Serum samples were obtained from T2DM patients and non-diabetic controls with or without periodontal disease as described previously (section 2.7.3). MMP-9 concentrations were determined using ELISA. A significant difference was detected in baseline serum MMP-9 concentrations in T2DM patients compared with non-diabetic controls (Figure 6-7). T2DM patients had 31% higher serum MMP-9 levels than non-diabetic individuals.

Figure 6-8 shows the MMP-9 concentration, according to the periodontal status, in non-diabetic and T2DM individuals. Significantly higher serum MMP-9 concentrations in non-diabetic individuals with gingivitis were observed compared to non-diabetic periodontally healthy individuals (Table 6-1). Non-diabetic periodontitis patients had elevated serum MMP-9 levels compared with non-diabetic healthy however after Bonferroni–Holm correction of $p$ values for multiple comparisons, the critical $p$ value was 0.025 and therefore this increase was only identified as a trend ($p = 0.03$). In diabetic patients, no significant differences in serum MMP-9 levels between periodontally healthy individuals with gingivitis or periodontitis were observed ($p = 0.4$, $p = 0.5$ respectively). A significant difference in serum MMP-9 concentrations was detected between diabetic healthy individuals and non-diabetic healthy controls. Although diabetic patients with gingivitis had higher serum MMP-9 levels than non-diabetics with gingivitis, the difference was not significant ($p = 0.2$). Likewise, diabetic patients with periodontitis showed no significant differences in MMP-9 levels compared with non-diabetics controls with periodontitis ($p = 0.09$).
Figure 6-7 Serum MMP-9 concentration in T2DM and non-diabetic controls

Box plots of serum MMP-9 concentrations in 97 T2DM patients and 83 non-diabetic controls. Statistics: Kruskal-Wallis, Mann-Whitney test **p < 0.01. • Outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.
Figure 6-8 Serum MMP-9 concentrations according to periodontal status in non-diabetics and T2DM individuals

Box plots of serum MMP-9 concentration in 83 non-diabetic controls (periodontal health n = 15; gingivitis n = 21; periodontitis n = 47) and 97 diabetic patients (periodontal health = 15; gingivitis = 39; periodontitis = 43). Statistics: Kruskal-Wallis, Mann-Whitney test **p < 0.01 (according to the periodontal status within diabetes or control). §§ p < 0.01 (diabetes versus non-diabetes within the corresponding periodontal status). ● Outlier more than 3 times the IQR from the box boundaries; ○ outlier more than 1.5 but less than 3 times the IQR from the box boundaries.
6.2.10 Analysis of serum MMP-9 concentrations in periodontitis patients with and without T2DM after periodontal treatment

In non-diabetic controls and diabetic patients with periodontitis, MMP-9 concentrations in serum were measured after treatment for periodontal disease at 3, 6 and 12 months. Serum MMP-9 concentrations were reduced significantly at 3 months after periodontal treatment in non-diabetic individuals ($p < 0.05$) compared with baseline values (Figure 6-9 and Table 6-2). However, no significant reductions compared to baseline were detected 6 and 12 months post-treatment ($p = 0.2$ and $p = 0.3$ respectively). At 3, 6 and 12 months post-periodontal treatment, no significant reductions were observed in serum MMP-9 concentrations in patients with diabetes ($p = 0.7$, $p = 0.5$, $p = 0.08$ respectively) compared to baseline levels.
Figure 6-9 The effect of periodontal treatment on MMP-9 levels in periodontitis patients with and without T2DM

Box plots of serum MMP-9 concentrations of patients with periodontitis and non-diabetics and of T2DM with periodontitis. Measurements were taken pre-treatment (non-diabetics n = 45; T2DM n = 46), and after 3 months (non-diabetics n = 41; T2DM n = 26), 6 months (non-diabetics n = 31; T2DM n = 31) and 12 months (non-diabetics n = 29; T2DM n = 25). Statistics: related samples test Friedman, Wilcoxon Ranks test \( * p < 0.05 \). ◆ Outlier more than 3 times the IQR from the box boundaries, ◆ outlier more than 1.5 but less than 3 times the IQR from the box boundaries.
6.2.11 The relationship between serum MMP-9 concentrations and clinical parameters of glycaemic control, inflammation and BMI

Blood samples of T2DM patients and non-diabetics were analysed for HbA1c and hsCRP concentrations by the Haematology and Clinical Biochemistry laboratory of the Royal Victoria Infirmary (section 2.7.3). HbA1c and hsCRP were analysed for correlations with serum MMP-9 to investigate a possible relationship of serum MMP-9 with markers of glycaemic control and inflammation. Pooled non-diabetic and T2DM groups showed a statistically significant positive correlation of serum MMP-9 with HbA1c \((p < 0.001)\) (Figure 6-10), HbA1c scores increasing with increasing serum MMP-9 concentrations. Likewise, a positive correlation between MMP-9 and hsCRP was detected with serum hsCRP concentrations increasing with increasing MMP-9 levels \((p < 0.01)\) (Figure 6-11). Correlations were also performed according to diabetic status. There were no significant correlation between serum MMP-9 and HbA1c in either the T2DM or non-diabetic groups \((p = 0.3\) and \(p = 0.2\) respectively). In addition, a positive significant correlation was detected in the non-diabetic group between serum MMP-9 and hsCRS levels \((p < 0.05)\).

Additionally, the relationship between BMI and MMP-9 was investigated to assess the possible impact of BMI on MMP-9 concentrations. A statistically significant positive correlation of BMI with serum MMP-9 was detected in pooled diabetic and non-diabetic groups \((p < 0.001)\) (Figure 6-12). The concentration of MMP-9 increased with increasing BMI. Correlations performed according to diabetic status showed a significant correlation between serum MMP-9 and BMI \((p < 0.05)\) in the non-diabetic group. Correlation analysis revealed no association between serum MMP-9 and BMI in T2DM patients \((p = 0.2)\).
Figure 6-10 The relationship between serum MMP-9 and HbA1c in T2DM and non-diabetic controls

Graph shows Spearman correlation of serum MMP-9 concentrations with percentage HbA1c in T2DM patients (n = 97) and non-diabetics (n = 83) (differentiated according to diabetic status).
Figure 6-11 The relationship between serum MMP-9 and hsCRP in T2DM and non-diabetic controls

Graph shows Spearman correlation of serum MMP-9 concentrations with serum concentrations of hsCRP in T2DM patients (n = 97) and non-diabetics (n = 83) (differentiated according to diabetic status).
Figure 6-12 The relationship between serum MMP-9 and BMI in T2DM patients and non-diabetic controls

Graph shows Spearman correlation of serum MMP-9 concentrations with BMI in T2DM patients (n = 97) and non-diabetics (n = 83) (differentiated according to diabetic status).
6.2.12 The relationship between serum MMP-9 and periodontal clinical parameters

The relationship between serum MMP-9 concentrations with CAL (mm) and PD (mm) in non-diabetics and T2DM individual was analysed. A positive and statistically significant correlation was noted both between CAL (mm) and serum MMP-9 ($p < 0.01$) and between PD (mm) and serum MMP-9 levels ($p < 0.05$). The CAL (mm) and PD (mm) values increased with increasing serum MMP-9 levels in pooled T2DM and non-diabetic groups (Figure 6-13). The correlations were split according to the diabetic status. A significant positive correlation was detected in the non-diabetic group between serum MMP-9 and PD or CAL measurements ($p < 0.01$ and $p < 0.05$ respectively). The correlation analysis in the T2DM group revealed a weak correlation between serum MMP-9 and PD or CAL ($p = 0.09$ and $p = 0.07$ respectively).
Figure 6-13 The relationship of serum MMP-9 concentrations with PD and CAL in T2DM patients and non-diabetic controls

Graph shows Spearman correlation of serum MMP-9 concentrations with PD and CAL in T2DM (n = 97) and non-diabetics (n = 83) (differentiated according to diabetic status)
6.3 Discussion

Diabetes and periodontal disease are both recognised as inflammatory conditions (Kornman et al., 1997; King, 2008) and a number of studies support diabetes as a risk factor for increased prevalence and severity of periodontal disease (Ryan et al., 2003; Heitz-Mayfield, 2005; Moles, 2006). Studies have also shown that severity of periodontitis is associated with high levels of collagenase and gelatinase (Neumann et al., 2004). A large body of evidence has shown that MMPs play major roles as key mediators of extracellular matrix degradation as well as remodelling during periodontal disease (Birkedal-Hansen, 1993). Increased development of periodontal disease in diabetic patients was linked with impaired collagen production and exaggerated MMP expression in the gingival tissues (Nishimura et al., 1998). In this study, significantly elevated GCF MMP-8 and MMP-9 levels in gingivitis and periodontitis patients compared to corresponding healthy controls in both T2DM and non-diabetic individuals were detected. GCF MMP-9 levels in gingivitis patients with T2DM were significantly higher than in individuals with gingivitis in the non-diabetic group which implies that the diabetic state could lead to an early and significant increase in MMP production in gingival inflammation. The difference between the mean GCF MMP-8 levels in gingivitis patients with T2DM and the corresponding non-diabetic individuals was not statistically significantly although a previous study has documented increased GCF MMP-8 levels in gingivitis patients with diabetes compared with healthy controls (Salvi et al., 2010). The difference between that study and the results of this study could be because the previous study used GCF samples from experimental gingivitis patients. Therefore, further study is needed to evaluate MMP-8 levels in diabetic individuals. The high GCF MMP-9 levels in this study suggest that MMP-9 is very much involved in the mechanisms of periodontal destruction in diabetic individuals.
Increased risk for progressive periodontal destruction in people with diabetes has been reported (Nelson et al., 1990; Taylor et al., 1998) and elevated salivary levels of MMP-8 were observed to be related to advanced periodontitis in diabetic patients (Collin et al., 2000). In addition, formation of AGEs was reported to play an important role in modulating pro-inflammatory mediator production (Lalla et al., 2000; Rodrigues et al., 2003; Duarte et al., 2007). Conversely, the findings from the current study identified a significant reduction in GCF MMP-9 and MMP-8 levels in periodontitis patients with T2DM when compared with periodontitis patients without diabetes. A reasonable explanation for these results may be that in this study there was no matching for periodontitis status between the diabetic and non-diabetic individuals (although this would of course be desirable in future studies). The PD and CAL levels in non-diabetic patients were significantly higher than those in diabetics and clearly, the non-diabetic patients had more advanced periodontal disease; this could be a plausible reason for the higher levels of GCF MMP-9 and MMP-8 in non-diabetic periodontitis individuals the compared to the diabetes.

Selection of GCF sampling sites was based on probing depth measurements which are indicative of past experience of periodontitis and do not necessarily reflect active periodontal destruction. It was found that the association between levels of MMP-8 and MMP-9 and the extent of periodontal disease depends on depth of the periodontal pocket (Passoja et al., 2008; Hernandez et al., 2010) which, in turn may determine the destructive potential of the inflammatory process. Thus, it is possible that periodontal status may be a good indicator of GCF collagenase and gelatinase levels in diabetic and non-diabetic individuals. Therefore, in this case it may be suggested that the increased GCF levels of MMP-9 and MMP-8 observed in non-diabetic patients are related to the
clinical parameters. The absence of matching for periodontal status between diabetic and non-diabetic patients and sampling from different pocket depth are study limitations that will need to be considered in future study design.

In this study, significant positive correlations between both probing depth and clinical attachment loss and GCF MMP-8 or MMP-9 were detected, which is in agreement with another study that showed a correlation between GCF MMP-8 and the clinical parameters of periodontitis (Buduneli et al., 2007). The findings may suggest that the increased levels of GCF MMP-8 and MMP-9 are related to increased PD or CAL in gingival inflammation leading to tissue destruction in both diabetic and non-diabetic individuals. Pro-inflammatory cytokines and MMPs are involved in tissue destruction and they may work synergistically or additionally to exaggerate the clinical appearance.

This study compared the response to conventional periodontal treatment between non-diabetic patients and T2DM patients with periodontitis. Both groups of patients showed a clinical improvement after non-surgical periodontal treatment at 3, 6 and 12 months post-treatment, confirming the widely documented clinical improvement in periodontal patients after conventional treatment (Badersten et al., 1981; Badersten et al., 1984; Kaldahl et al., 1996). A follow-up period of 12 months was chosen for the evaluation of the clinical response to the treatment, this longer period allowed the observation of any possible relapse. Studies have documented a significant decrease in mean PD (approximately 1.2 mm) in those pockets initially 4-6 mm deep and mean attachment gains of 0.5 mm following RSI. In deeper pockets of 7 mm or greater, mean probing depth reductions of around 2.1 mm and attachment gains of 1.1 mm can be expected following RSI (Cobb, 1996). In the current study, an improvement in the clinical
response was observed in both study groups at 3, 6, and 12 months, with reductions in the mean percentage of BOP and mean value of PD or CAL. These findings confirm previously published reports that diabetic and non-diabetic individuals respond well to non-surgical periodontal treatment (Tervonen et al., 1991; Christgau et al., 1998; Navarro-Sanchez et al., 2007). In addition, following treatment, a statistically significant reduction of GCF MMP-9 and MMP-8 levels was detected 3, 6, 12 months after periodontal treatment indicating reduction in gingival inflammation. These results are consistent with previous studies that observed a decrease in collagenase and gelatinase levels after non-surgical periodontal treatment and the clinical improvements in the study were similar to those reported in the literature (Buduneli et al., 2002; Tuter et al., 2002; Correa et al., 2008) and also a reduction in MMP-8 levels post-periodontal therapy with adjunctive SDD (Tuter et al., 2010). The findings from the current study demonstrate that periodontal treatment in both groups lead to a significant reduction in gingival inflammation that was accompanied by a lowering of GCF MMP-8 and MMP-9 levels in diabetic and non-diabetic individuals.

Changes in the ECM of periodontal tissue contribute to the development of periodontal disease and at the end, tooth loss. The question is: are the serum concentrations of MMP-9 potential markers for such changes? The present study suggested that circulating MMP-9 levels are increased in T2DM patients compared with non-diabetic patients regardless of periodontal inflammation, and periodontal treatment had no effect on serum MMP-9 levels. This may be because diabetics have such upregulated MMP levels as a result of their diabetes that this outweighs any reductions associated with periodontal treatment. Alterations in the immune response in diabetic patients may explain why it may not be possible to completely eradicate circulating MMP-9 after
conventional periodontal therapy. It thus seems that the increased MMP-9 levels observed in diabetic individuals are related to an altered inflammatory state. Diabetic patients show an abnormal response to pathogenic bacteria which results in an exacerbated secretion of inflammatory mediators such as TNF-α (Holzhausen et al., 2004; Southerland et al., 2006) and activated advanced glycation end product receptors might stimulate the inflammatory response resulting in increased local and systemic production of the inflammatory mediators which in turn regulate MMP production (Kadoglou et al., 2005). The high levels of serum MMP-9 observed in diabetic individuals in the present study are consistent with the results of previous studies that have shown elevated serum levels of MMP-9 (Maxwell et al., 2001; Xue et al., 2005) which have been associated with vascular complications, retinopathy, nephropathy and arteriosclerosis in diabetic patients (Xue et al., 2005).

In non-diabetic individuals, serum MMP-9 levels were higher in periodontal disease patients compared to healthy individuals suggesting that this enzyme is involved in the mechanism of periodontal inflammation. Indeed, MMP-9 has been suggested as a risk marker for cardiovascular and other chronic inflammatory diseases (Ferroni et al., 2003; Robertson et al., 2007; Welsh et al., 2008). Therefore, the increased levels of serum MMP-9 in non-diabetic individuals with periodontal disease in this study probably result from the periodontal inflammatory process and may be a major causal factor linking periodontal disease with increased susceptibility to systemic diseases. Moreover, the data from the present study showed a reduction in serum MMP-9 levels after 3 months of conventional periodontal treatment. Thus, periodontal disease generates a pro-inflammatory state, particularly in subjects without systemic disease and treatment of periodontal disease may reduce the systemic inflammatory condition as
well as the elevated levels of MMP-9 that are associated with periodontal disease. These results were consistent with those of Marcaccini et al who reported an increase in plasma MMP levels in periodontal disease patients which were reduced after periodontal treatment (Marcaccini et al., 2009). Therefore, the reduction in serum MMP-9 following periodontal treatment indicates that MMP-9 may eventually prove a useful indicator of current disease status and possibly a predictor of future disease. Thus, the relationship between serum MMP-9 concentration and systemic disease risk in subjects with periodontal disease merits further investigation.

In the current study, attempts were made to validate MMP-8 ELISA to be used for serum samples. However, the recovery measurements for serum MMP-8 using this ELISA were unacceptably low which indicated that this assay cannot be used for monitoring MMP-8 concentrations in human serum samples.

Metabolic control is highly variable among diabetics and poor control is related to the development of periodontitis (Lim et al., 2007). HbA1c has been shown to correlate with clinical indicators of gingival inflammation (CAL and PD) in diabetic patients (Cutler et al., 1999). The present study also observed a significant positive correlation between HbA1c and serum MMP-9 in diabetic and non-diabetic patients. In addition, positive correlations between serum MMP-9 and the clinical signs of periodontitis (CAL or PD) in individuals with or without diabetes were observed that support the concept of diabetes contributing to periodontal tissue breakdown through alteration of MMP production. However, no correlation of serum MMP-9 with HbA1c was detected after splitting the data according to diabetic status.
Elevated serum levels of hsCRP reflect systemic inflammation and increased risk of cardiovascular disease (Danesh et al., 2004) and studies have demonstrated a correlation between periodontitis and elevated serum hsCRP levels (Noack et al., 2000; Paraskevas et al., 2008). Likewise, serum hsCRP levels are associated with periodontal PD and CAL (Salzberg et al., 2006). In the present study, a significant positive correlation between serum MMP-9 levels and hsCRP was detected in T2DM and non-diabetic individuals. This finding is consistent with data from Jacqueminet et al, who reported a significant positive correlation between circulatory MMP-9 levels and hsCRP in diabetic patients (Jacqueminet et al., 2006). In addition, correlation analysis according to diabetic status revealed an association between serum MMP-9 and hsCRP in non-diabetic individuals but a weak correlation in T2DM patients. Therefore, the positive association that was observed in this study may reflect the inflammatory state in both groups and MMP-9 could serve as an inflammatory marker in patients with diabetes and periodontal disease.

Obesity has been related to the risk of periodontal disease (Saito et al., 1998; Wood et al., 2003; Nishida et al., 2005) and it has been reported that obesity was independently associated with increased PD and CAL after adjustment for insulin tolerance (Saito et al., 2005). The results of this study also showed a positive correlation between BMI and serum MMP-9 levels in both diabetic and non-diabetic individuals. Moreover, correlation analysis according to diabetic status revealed a significant relationship between serum MMP-9 and BMI in non-diabetic group. Since increasing BMI has been demonstrated to be associated with elevated TNF-α and IL-6 levels from adipose tissue, and these cytokines are associated with periodontal disease and diabetes (Mealey, 2006)
this could also explain the relationship between the serum MMP-9 and BMI observed in this study.

Within the limitations of the study, it can be concluded that diabetes leads to an increase in GCF MMP-9 levels in patients with gingivitis. The data also suggest that MMP-9 could be useful as a marker for the clinical severity of periodontal disease in diabetic and non-diabetic individuals. Additionally, MMPs may potentially serve as prognostic indicators of periodontal disease progression as an improvement in clinical parameters was found in both groups between baseline and 3, 6 or 12 months post-treatment. The study suggested that the significant reduction of GCF MMP-9 and MMP-8 after nonsurgical management might indicate their potential utility as markers of periodontal disease activity in diabetic and non-diabetic patients and these enzymes might serve as a surrogate marker for occurrence of periodontal disease in diabetic individuals. In addition, the study indicated that periodontal disease and diabetes may influence circulating MMP-9 levels and that periodontal treatment may affect those levels. The effects of therapy however, were observed only among non-diabetic subjects and this may indicate the complexity of the mechanisms whereby hyperglycaemia may influence circulating MMP production in periodontitis patients with diabetes.
Chapter 7  General discussion

The pathogenic processes in periodontal disease are initiated by the microbial biofilm, which activates immune and inflammatory responses leading to chronic disease progression (Kinane et al., 2001). Thus it is the host response itself that results in the tissue destruction observed. The periodontal pathogens present in the subgingival biofilm are capable of releasing a number of products including LPS and proteases which can invade the tissues and activate host defence mechanisms and activate the immune-inflammatory response (Teng, 2003; Madianos et al., 2005). The inflammatory response involves immune cells such as neutrophils, monocytes and macrophages which produce pro-inflammatory cytokines and other mediators such as IL-1β, TNF-α, IL-6 and PGE2 (Page, 1991; Madianos et al., 2005). The results presented in this thesis demonstrate that IL-1β increased the levels of IL-8, IL-32 and MMP-9 by OKF6 cells, this is consistent with other studies that have shown that cytokines such as IL-1β induce the secretion of other cytokines and pro-inflammatory mediators in positive feedback circuits and also acts in an autocrine manner to induce further IL-1β production (Chae et al., 2005; Noguchi et al., 2007). In addition, secretion of IL-1β was induced soon after bacterial exposure (Tschopp et al., 2003). Therefore, this study supports the accepted dogma that IL-1β plays an important role in periodontal disease by directly inducing production of MMPs or indirectly by induction of other inflammatory cytokines. The work presented here supports a model whereby the regulation of secondary pro-inflammatory cytokines produced by epithelial cells is modulated by IL-1β. The observations from the current study strengthen the notion that MMPs are released initially from inflammatory cells activated by bacterial plaque (Uitto et al., 2003) and also by resident periodontal cells in response to cytokines derived from the infiltrating
cells (Reynolds and Meikle, 1997). IL-1β could also amplify the immune response by inducing IL-8 and IL-32 production by oral epithelial cells. Therefore, the findings from the present study support the concept that oral epithelial cells are an integral component of the oral innate immune system. The capacity of the oral epithelium to respond to cytokine stimulation by secreting IL-8 in vitro suggests that these cells have the potential to enhance host defences against microbes and the secretion of such molecules by oral epithelial cells may lead to proliferation and activation of neutrophils as a defence mechanism to control the bacterial challenge. Levels of IL-1β have frequently been reported to be increased in GCF and gingival tissue of patients with periodontal disease (Stashenko et al., 1991) and the in vivo role of epithelial cells as a local amplifier of immune responses could be critical for recruitment of neutrophils and initiation of the inflammatory response. Given its key role in initiating inflammatory responses and stimulating inflammation, IL-1β may be a rational therapeutic target for the modulation of inflammation in the treatment of periodontal disease similar to treatment strategies already employed in the management of rheumatoid arthritis (Burger et al., 2006; So et al., 2007).

Periodontal tissue destruction has been associated with high levels of MMP-9 in GCF (Teng et al., 1992). This metalloproteinase is also highly expressed in the inflamed tissues of patients with periodontitis (Ejeil et al., 2003; Smith et al., 2004). Furthermore, there is clear consensus regarding the increased local expression of MMPs in periodontal disease and their role in the soft and hard tissue destruction that characterises the clinical symptoms of periodontal disease (Aiba et al., 1996; Kubota et al., 1996; Dahan et al., 2001; Garlet et al., 2004). The work presented in this thesis indicates that TNF-α and IL-32 stimulation induced a significant upregulation of MMP-
9 expression in the oral epithelial cells. This supports the contention that cytokines contribute directly to gingival extracellular matrix degradation by stimulating the secretion of host derived MMPs. The findings from this study confirm the role of cytokines in periodontal pathogenesis, and also that production of MMPs is prerequisite for periodontal disease progression. This study also confirms the usefulness of the OKF6 cell line as a model for examining MMP expression in periodontal disease when compared with primary cells. However the expression of TLR4 and CD14 which has been found in the OKF6 cell lines in the present study has not detected in primary cells which represents the main difference between these cells.

Inflammatory cytokines such as IL-1β and TNF-α are clearly potential therapeutic targets in inflammatory diseases such as rheumatoid arthritis and periodontal disease (Salvi and Lang, 2005; Burger et al., 2006) and anti-TNF-α therapy has already been established in the treatment of rheumatoid arthritis. Inhibition of IL-1β using IL-1Ra is also currently used as a treatment for rheumatoid arthritis (Kavanaugh, 2006). Studies have shown that using IL-1β inhibitors in combination with anti-rheumatic drugs leads to clinical improvements in the acute phase of rheumatoid arthritis (Karanikolas et al., 2008; Le Loet et al., 2008). Furthermore, it was reported that using an anti-cytokine injection in murine model arthritis reduced the incidence and severity of the disease (Khoury et al., 2008). Although, anti-IL-1R and anti-TNF-R applied to the periodontal tissues of primates with experimental periodontal disease resulted in inhibition of the pro-inflammatory response (Assuma et al., 1998), investigations using anti-cytokine treatment in the management of periodontal disease still are at a very early stage. In periodontitis, the inflammation is induced by many cytokines and the cytokine production may vary between individuals. Therefore, the patients genotype will
certainly influence the inflammatory response and immune-regulation and also the response to treatment (Taylor et al., 2004). Therefore, anti-cytokine therapies may not be ideal in the management of periodontitis, and this has directed research to the investigation of other cytokines and mediators of tissue destruction (Anderson et al., 2006). Thus, knowledge regarding the role of cytokines in the pathogenesis of periodontal disease may provide the basis for future therapeutic interventions that not only involve cytokines themselves but also other inflammatory mediators.

A characteristic response of epithelial cells to oral microbes is the synthesis and release of inflammatory mediators such as cytokines (Kagnoff and Eckmann, 1997) and many oral bacteria are able to induce IL-1β, TNF-α and IL-6 secretion by epithelial cells (Han et al., 2000; Han et al., 2003). The release of cytokines by epithelial cells in response to subgingival bacteria is considered to be an early stage of inflammation and overproduction of these cytokines can potentially cause damage to the epithelial cells themselves (Kagnoff and Eckmann, 1997). *P. gingivalis* has been reported to inhibit IL-8 release by epithelial cells due to the action of its bacterial proteases (Huang et al., 2001; Huang et al., 2004). The results presented in this study show that *P. gingivalis* LPS had no effect on IL-8 and MMP-9 induction by the epithelial cells. It is reasonable to hypothesize that the production of MMPs by epithelial cells may be dependent on IL-8 production, as IL-8 has already been shown to stimulate the secretion of MMPs (Kornman, 1997; Milner and Cawston, 2005; Zhu et al., 2007). Indeed, a general hypo-responsiveness to *P. gingivalis* LPS may be advantageous in order to limit tissue destruction that might occur if a strong inflammatory response was induced in epithelial cells exposed to *P. gingivalis*. It seems that the effect of the LPS may be more directed towards the inflammatory reaction developing in the connective tissues (Silverstein et
al., 1990) which then in turn induces inflammatory response in other resident and infiltrating cells. However, the current study showed that the pro-inflammatory cytokines are more potent than LPS in stimulating the production of inflammatory mediators such as IL-8 and MMPs by epithelial cells, which makes sense given that oral epithelial cells are continuously exposed to bacterial LPS, and that significant tissue damage may occur if inflammatory responses were continuously stimulated in epithelial tissues.

Diabetes is clearly associated with an increased incidence and severity of periodontal disease (Heitz-Mayfield, 2005; Moles, 2006). Several investigations have documented the presence of more severe periodontal disease in diabetic compared to non-diabetic individuals (Verma and Bhat, 2004) and patients with poorly controlled diabetes are particularly at higher risk for periodontal disease (Soskolne and Klinger, 2001; Taylor, 2001). Therefore, the current worldwide increases in the prevalence of diabetes likely will have an impact on the future prevalence of periodontal disease, and thus understanding the mechanistic link between the two disease becomes of increasing importance for oral health care. However, although pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 are clearly involved in the pathogenesis of both disease (Lalla et al., 1998; Nishimura et al., 2007), the role of MMPs in diabetic patients with periodontal disease needs further investigation.

The secretion of IL-1β, TNF-α, IL-6 and IL-8 by inflammatory cells, fibroblasts and epithelial cells has been shown to be altered in inflamed gingival tissue (Bartold and Narayanan, 2006). Diabetic individuals also show an abnormal response to periodontal bacteria which results in an exacerbated secretion of inflammatory mediators and
accumulation of AGEs that bind to macrophages resulting in increased secretion of inflammatory cytokines that contributes to more severe forms of periodontitis (Holzhausen et al., 2004; Southerland et al., 2006) and that lead to elevated MMP secretion (Ryan et al., 1999). Results from the in vivo study demonstrated elevated GCF and serum MMP-9 levels in diabetic patients with periodontal disease compared with non-diabetic individuals, which suggests a potential role for MMP-9 in the link between diabetes and periodontal disease and supports the hypothesis that systemic inflammatory conditions may have impact on periodontal status (Mercado et al., 2001; Perrino, 2007). Moreover, the finding of elevated MMP-9 serum levels in patients with periodontal disease compared to healthy individuals strengthens the notion that periodontal disease involves inflammatory responses that are associated with both local and systemic inflammation (Moutsopoulos and Madianos, 2006). The correlation outcomes from this study support the hypothesis that diabetes may exacerbate periodontal tissue destruction through altered of MMP-9 production. In addition, the association between hsCRP and MMP-9 that was observed in this study suggests that inflammatory responses are common in both diabetes and periodontal disease, and the association between the systemic condition and periodontitis is likely mediated by increased inflammation and possibly also induction of acute phase protein such as hsCRP (D'Aiuto et al., 2004; Bizzarro et al., 2007).

Previous studies have demonstrated that periodontal treatment including local administration of antibiotics can significantly reduce the levels of circulating TNF-α in T2DM patients (Iwamoto et al., 2001; Iwamoto et al., 2003; Nishimura et al., 2003). In addition, high GCF level of IL-1β and TNF-α has been reported at the sites of periodontal destruction (Stashenko et al., 1991), with reduced levels after periodontal
treatment (Heasman et al., 1993). The current study evaluated the effect of non-surgical periodontal treatment in diabetic and non-diabetic patients with periodontal disease and found that periodontal therapy was able to improve periodontal status as demonstrated by reductions in the clinical parameters (reduce probing depths and BOP scores) and the local enzymes. Furthermore, the study suggests that clinically successful non-surgical periodontal treatment tended to reduce inflammation through reduction of GCF MMP-9 and MMP-8 concentrations which could be important for diabetic patients with periodontal disease. Therefore, providing appropriate periodontal therapy in individuals with diabetes may contribute to reductions in other systemic inflammatory markers such as CRP and IL-6 which are known to be increased in many systemic inflammatory disease such as diabetes (Loos, 2005). Clearly this concept needs further investigation. Thus, periodontal treatment is recommended for diabetic patients who also have periodontal disease for the primary purpose of improving their periodontal health, but also potentially for contributing to improvements in systemic health, such as diabetes control, and this warrants further investigations.

Some drugs have been used as MMP inhibitors to inhibit MMP-8, MMP-9, MMP-12 and MMP-14 in the periodontal tissues (Lee et al., 2001). These drugs were found to protect collagen and other connective tissues from destruction by enhancing collagen synthesis and inhibiting collagen break down (Schneir et al., 1990) in skin, bone and other tissues (Golub et al., 1998). Although, many drugs have been developed as inhibitors for MMPs, only a few are approved for local use (Golub, 2010) and this is because MMP plays a role in pathological and physiological processes, for example processing anti-inflammatory cytokines and regulating immune responses (Sorsa et al., 2006). Minocycline, doxycycline and tetracycline have all seen shown to inhibit
collagenolytic activity (Golub et al., 1984). Doxycycline therapy results in a reduction in collagenolytic activity in GCF samples from diabetic patients with aggressive periodontitis (Golub et al., 1985). Many studies have demonstrated that elevated MMP-8 levels in periodontal disease are reduced by using SDD (20 mg doxycycline twice daily) (Golub et al., 1990; Emingil et al., 2004; Lee et al., 2004; Preshaw et al., 2004) and using combination of SDD with flurbiprofen produced a reduction in periodontal tissue MMP levels (Lee et al., 2004). Moreover, SDD can reduce the levels of a systemic bone resorption biomarker (ICTP) in the serum of postmenopausal women exhibiting mild systemic bone loss (Golub et al., 2010). SDD can reduce both pathological and physiological MMP-8 levels in the periodontal tissues (Brown et al., 2004; Sorsa et al., 2004; Sorsa et al., 2006; Golub et al., 2008) and in addition, SDD can reduce systemic serum and plasma levels of many inflammatory biomarkers including MMP-8, MMP-9, and CRP (Tuter et al., 2007; Tuter et al., 2010). Therefore, the beneficial effects associated with the clinical improvements after periodontal treatment could potentially be monitored by assaying MMP-8 levels in the periodontal tissues (Emingil et al., 2004; Lee et al., 2004).

Further clarification of the expression patterns and levels of MMPs in periodontal tissues and oral fluids such as GCF and saliva is essential for future development of MMP diagnostic tools (Mancini et al., 1999; Sorsa et al., 1999) to monitor the periodontal disease progression and for the effect of treatment. Furthermore, quantifying inflammatory biomarkers in oral fluids may serve as a useful tool to predict an individual’s susceptibility to periodontitis, to provide information regarding periodontal disease activity and to monitor the efficacy of periodontal therapy (Ito et al., 2008; Pellegrini et al., 2008). The results from the present study indicate that MMPs
may be an intriguing target for future research, including investigating their potential prognostic value for periodontal treatment outcomes and/or as drug target (Abraham et al., 2005). This can hopefully lead to a better understanding of the pathogenesis of periodontal disease and in turn, result in more effective screening, prevention and treatment of this common and distressing condition.

In conclusion, the findings of the present study contribute to the knowledge of cytokine-induced MMP production in periodontal disease and the role of MMPs in diabetic patients with periodontal disease. Moreover, the current study highlights the importance of studying MMPs in both periodontal disease and diabetes, and more specifically, MMP-9 shows potential as a contributor to pathogenic links between diabetes and periodontal disease, with the consideration of MMPs as potential prognostic markers and future therapeutic targets for periodontal treatment in individuals with and without diabetes.
Future work

A number of findings in the present study revealed opportunities for further research and these could be addressed in future studies.

1. Further investigation is required to extend knowledge regarding the role of the bacterial biofilm, such as including whole bacteria (*P. gingivalis*) and different strains of periodontal pathogens.

2. Further study of the effect of cytokines on production of MMPs using other periodontal cell type such as fibroblasts is warranted.

3. It would be very worthwhile to analyse the effect of cytokines on novel MMPs such as MMP-28 and MT-MMP using TLDAs to investigate the expression levels of these genes and their contribution in periodontal tissue destruction. It would also be relevant to investigate these MMPs in saliva and GCF samples from patients with periodontal disease and compare them with levels in healthy subjects.

4. Investigation of the effect of different IL-32 isoforms on MMP expressions would be interesting as very little is known about the functional roles of these isoforms.

5. Investigation of the activity of MMP-9 using gelatine zymography would be interesting because MMP is produced as a pro-enzyme and requires cleavage to become active. This assay would clarify the effects of cytokine on OKF6 cells by increasing MMP activation.
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