Epidemiology, prognosis and treatment of aggressive non-Hodgkin lymphomas

Dr. Michal Konrad Sieniawski

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Newcastle University

UK

Academic Haematology

Northern Institute for Cancer Research

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Moim pacjentom

Betegeimnek

To my patients

ABSTRACT

The concept of aggressive non-Hodgkin lymphoma (NHL) originates in the Working Formulation classification of NHL and describes a heterogeneous group of NHLs. Despite the introduction of the WHO classification the term 'aggressive non-Hodgkin lymphoma' still remains in clinical use. This work will focus on some important aspects of epidemiology, clinical presentation, prognosis, treatment and treatment outcome in two main subtypes of aggressive lymphoma: diffuse large B-cell lymphoma (DLBCL) and peripheral T-cell lymphoma (PTCL).

DLBCL is the most common NHL occurring more frequently in older patients and is characterised by heterogeneous histology. The current standard treatment is immunochemotherapy with cyclophosphamide, doxorubicine, vincristine, prednisone (CHOP) and anti-CD20 antibody rituximab. Approximately 50-60% of patients eventually die from DLBCL. Depending on the patient's age, two different approaches can be defined in order to improve the outcome in DLBCL. In younger patients, the better risk stratification and prediction of response to standard therapy can help to define the high-risk groups suitable for new, more intensive therapy approaches. In elderly patients, where already delivery of standard chemotherapy is difficult, the introduction of the targeted therapies and its combination with new less toxic chemotherapy regimens could be seen as a future approach. Targeted therapies can be also adopted in younger patients.

It was aimed to describe DLBCL in a population-based setting in order to obtain the realistic picture of disease, define the needs of patients and develop a prognostic model based on expression of molecular factors in routine formalin fixed paraffin embedded (FFPE) tissue samples using quantitative polymerase chain reaction (qPCR) for identification of high risk patients.

A population-based study on 1863 DLBCL patients described picture of disease, therapy and outcome. Importantly, it revealed that a significant number of patients (30%) who did not receive standard chemotherapy were characterised by a very bad prognosis. Additionally, the duration of first remission was one of the most important factors for patient survival, suggesting first-line treatment should be escalated in suitable high-risk patients.

The model based on expression of *MYC*, *HLA-DRB* and variant 2 of the C13orf25 locus (*v2-transcript*) using qPCR was successfully established in FFPE tissue. Unfortunately, we could not confirm its predictive value in patient cohort. The preliminary work on expression of microRNA encoded in *v2-transcript* as predictive biomarker was performed in FFPE tissue and delivered promising results.

PTCL is characterised by dismal outcome when treated with standard CHOP. The rarity and heterogeneity of the PTCL, lack of prospective trials in homogenous types of the PTCL as well as hesitation to use high intensity treatments can contribute to the poor outcome.

It was aimed to collect prospective data in a population-based setting on clinical presentation, treatment and outcome of patients with enteropathy associated T-cell lymphoma (EATL), a subtype of PTCL, and to assess the role of a novel high-dose chemotherapy with ifosfamide, epirubicin, etoposide/methotrexate and autologous stem cell transplantation (IVE/MTX-ASCT) in EATL and other PTCL subtypes.

The incidence of EATL in the studied population was 0.14/100 000 per year. The detailed picture of the disease at presentation was obtained. The prognosis was dismal when treated with conventional treatment with 5-years PFS and OS of 18% and 20%, respectively. The new IVE/MTX–ASCT regimen improved the patient outcome with the 5-years PFS and OS of 52% and 60%, respectively. The results of IVE/MTX-ASCT in other PTCL were also promising with 3-years PFS and OS of 65% and 72%, respectively.

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List of abbreviations

aaIPI = age adjusted International Prognostic Index
ABC = activated B-cell like
ABI = Applied Biosystem Incorporated
AITL = angioimmunoblastic T-cell lymphoma
ALCL = anaplastic large cell lymphoma
ALK = anaplastic lymphoma kinase
ASCT = autologous stem cell transplant
ATLL = adult T-cell leukaemia and lymphoma
BCL2 = B-cell lymphoma 2 protein
BCL6 = B-cell lymphoma 6 protein
BCR = B-cell receptor
BIC = B-cell integration cluster
BM = bone marrow
BL = Burkitt lymphoma
β2M = beta-2-microglobulin
C/EBP = Ccaat-enhancer-binding protein
c13orf25 = chromosome 13 open reading frame 25
CCND2 = cyclin D2
CCR = chemokine (C-C motif) receptor
CD = cluster of differentiation
CHL = classic Hodgkin lymphoma
CHOEP = cyclophosphamide, doxorubicine, vincristine, etoposide, prednisolone
CHOP = cyclophosphamide, doxorubicine, vincristine, prednisolone
CKD = cyclin dependent kinase
CKI = cyclin dependent kinase inhibitor
CLL = chronic lymphocytic leukaemia
CNOP = cyclophosphamide, mitoxantrone, vincristine, prednisone
CR = complete remission
CS = clinical stage
Ct = cycle threshold
CXCR = chemokine (C–X-C motif) receptor
c-MYC = v-myc avian myelocytomatosis oncogene homolog

DFS = disease free survival
DLBCL = diffuse large B-cell lymphoma
DNA = deoxyribonucleic acid
DGRC8 = DiGeorge syndrome critical region 8
DMEM = Dulbecco's minimal essential medium
DSHNHL = The German High Grade non-Hodgkin's Lymphoma Study Group
DSMZ = German Collection of Microorganisms and Cell Cultures
EATL = enteropathy associated T-cell lymphoma
EBV = Ebstein-Barr virus
ECOG = Eastern Cooperative Oncology Group
EFS = event free survival
EGF = epidermal growth factor
EGFR = epidermal growth factor receptor
FFS = failure free survival
FFTF = freedom from treatment failure
FISH = fluorescence in situ hybridisation
FL = follicular lymphoma
FN1 = fibronectin 1
FOXP1 = fork head box protein P1
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GC = germinal centre
GCB = germinal centre B-cell like
GCET1 = serpin peptidase inhibitor, clade A
G-CSF = granulocyte colony stimulating factor
GELA = Group of Adult Lymphoma
GEP = gene expression profiling
GMP = guanosine monophosphate
GTP = guanosine triphosphate
Hb = haemoglobin
HCV = hepatitis C-virus
HD = high-dose
HDCT = high dose chemotherapy
HHV8 = human herpesvirus-8
HIV = human virus immunodeficiency virus

HL = Hodgkin lymphoma
HLA = human leukocyte antigen
HTLV-I = human T-cel leukaemia / lymphoma virus I
ICAM-1 = intercellular adhesion molecule-1
IDH = isocitrate dehydrogenase
IMP = inosine monophosphate
IFN = interferon
Ig = immunoglobulin
IHC = immunohistochemistry
ILSG = The international Lymphoma Study Group
IPI = International Prognostic Index
IRF4/MUM1 = interferon regulatory factor-4 / multiple myeloma oncogene 1
ISH = in situ hybridisation
ITLP = International T-cell Lymphoma Project
JAK = Janus kinase
LDH = lactate dehydrogenase
LMO2 = LIM domain only 2
MALT = mucosa associated lymphoid tissue
MAX = c-myc-associated factor X
MB = c-Myc box
MCL = mantel cell lymphoma
MEM = minimal essential medium
MHC = major histocompatibility complex
MInT = MabThera International Trial Group
miRNA = microRNA
MZL - = marginal zone lymphoma
MMP = matrix metalloproteinase
NF-κβ = nuclear factor kappa-light-chain-enhancer of activated B-cells
NHL = non-Hogdkin lymphoma
NK cell = natural killer cell
NOS = not otherwise specified
nt = nucleotides
ORR = overall response rate
OS = overall survival

PAX5 = paired box 5

PB = peripheral blood

PCR = polymerase chain reaction

PD = progressive disease

PDGRF = platelet derived growth factor receptor

PET = positron emission tomography

PGK1 = phosphoglycerate kinase 1

PFS = progression free survival

PI3K = phosphatidylinositol 3-kinase

PKC = protein kinase

PMLBL = primary mediastinal large B-cell lymphoma

PR = partial remission

PTCL = peripheral T-cell lymphoma

PTLD = post-transplant lymphoproliferative disorders

qPCR = quantitative polymerase chain reaction

PTNP = protein tyrosine phosphatase, receptor

p53 = tumor protein p53

REAL = Revised European-America Classification of Lymphoid Neoplasm

RISC = RNA-induced silencing complex

RNA = ribonucleic acid

RPMI = Roswell Park Memorial Institute

ROC = receiver operating characteristic

RT = reverse transcription

RT-PCR = real time polymerase chain reaction

R-CHOP = rituximab, cyclophosphamide, doxorubicine, vincristine, prednisolone

R-IPI = revised International Prognostic Index

SCT = stem cell transplantation

SD = standard deviation

siRNA = short interfering RNA

SNLG = Scotland and Newcastle Lymphoma Group

STAT = signal transducer and activator of transcription

TBP = TATA box binding protein

TCR = T-cell receptor

TDT = deoxynucleotidyl transferase

TET-2 = Tet methylcytosine dioxygenase 2

Th = helper T-cell

TLR = toll like receptor

TMA = tissue microarray

TP53 = tumour protein 53

TRBP = trans-activating response RNA-binding protein

Treg = regulatory T-cells

TRM = transplant related mortality

v2 = version 2 of chromosome 13 open reading frame 25 transcript

VACOP-B = etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin

VAPEC-B = vincristine, doxorubicin, etoposide, prednisolone, cyclophosphamide, bleomycin

VEGF = vascular growth factor

VEGFR = vascular growth factor receptor

WBC = white blood cell

WF = Working Formulation

WHO = World Health Organisation

5-FdUMP = 5-fluoro-deoxyuridine-monophosphate

5-FdUTP = 5-fluoro-deoxyuridine-triphosphate

5-FUTP = 5-fluoro-uridine-triphosphate

Chapter 1. Introduction

1.1 Lymphomas

1.1.1 *Definition and classification of lymphomas*

Lymphomas and leukaemias are both malignancies of the haemopoietic and lymphoid system. Traditionally, in accordance with histopathological findings, lymphoma can be divided into two main groups; Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) (Cannelos et al., 2006). The World Health Organisation (WHO) classification of tumours of haematopoietic and lymphoid tissues introduced the new term "lymphoid neoplasms" covering both lymphoma and leukaemia of lymphoid origin (Swerdlow et al., 2008). This new term overcomes the artificial separation between leukaemia and lymphoma used in previous classifications. Lymphoid neoplasms are subdivided into two classes; B-cell neoplasms and natural killer (NK) / T-cell neoplasms. The previously separate group of HL is now included with the B-cell neoplasms, after the recent discovery of its cell of origin. In both classes two separate groups are defined: precursor and mature neoplasms. This new definition is a significant step forwards in the classification of lymphoid neoplasms; however, the old classification is still in use particularly in clinical and epidemiological studies.

Looking backwards, the first description of what we now recognize as a lymphoma is attributed to Thomas Hodgkin in 1832 (Hodgkin, 1832). Subsequently thirty years passed until the term lymphosarcoma was first introduced by Rudolf Virchow in 1863 (Jaffe et al., 2008) and another forty years before in 1898 and 1902 Carl Sternberg and Dorothy Reed described independently Reed-Sternberg cells in Hodgkin lymphoma (Dawson, 1999). Several years later, Ewing, Oberling and Roulet introduced the term "reticulum cell sarcoma" for tumours of large cells and postulated their origin in the supporting fibrous reticulum of lymphoid tissue. This was in contrast to "lymphosarcoma" a term, which was applied to small cell neoplasms with their origin in lymphocytes (Jaffe et al., 2008). The next important step in the history of lymphoma classification was the description of patients with lymphadenopathy and splenomegaly characterized pathologically by a proliferation of lymphoid follicles. Brill et al. in 1925 (Brill et al., 1925) and Symmers et al in 1927 (Symmers, 1927) both described this entity and its progression to a large cell neoplasm (Symmers, 1938).

The first proposed morphological classification of lymphoma was introduced in 1941 in the United States by E.A. Gall and T.B. Mallory (pathologists from the Massachusetts General Hospital), more than 100 years after the discovery of Thomas Hodgkin (Gall and Mallory, 1942). This classification recognized follicular lymphoma (FL) as a distinctive morphologic and clinical entity and included Hodgkin's disease as a separate type of lymphoma.

The modern era in lymphoma classification began with the introduction of the Rappaport classification by the Armed Forces Institute of Pathology in 1966 (the initial version was published in 1956) (Hicks et al., 1956). Despite its revolutionary character, in some areas this classification was a step backwards. Rappaport questioned the idea that FL could arise from reactive follicles and replaced the term follicular with nodular. Rappaport's classification was based on the stratification of tumours either in a nodular or diffuse pattern; the former usually had a better prognosis. Consequently the previously well-defined FL was split into four separate categories. The origin of tumours with large cells was thought to be from non-lymphoid stromal or other cells despite the recent discovery of lymphocyte transformation. Despite these drawbacks the classification was highly clinically useful and widely employed in the United States, mostly due to the high frequency of FL in that country.

In the same era, a new classification of Hodgkin's disease was published by Lukes and Butler (Lukes et al., 1966a). It was subsequently modified in 1974 (Lukes et al., 1966b). This classification distinguishes between four types of Hodgkin's disease. In the following 50 years it remained in common use undergoing only a few changes.

In the 1960s several discoveries contributed to a better understanding of the immune system and its neoplasms; firstly the potential of lymphocytes to transform into large proliferating cells in response to mitogens and antigens (Nowell, 1960) secondly the recognition of the existence of several distinct lymphocyte lineages (B, T and NK) that could be not predicted by morphology but have distinctive functions and physiologies (Papermaster and Good, 1962) (Cooper et al., 1965). Additionally, in the early 1970's lymphoid cells were found to express surface antigens or receptors that could be exploited to identify the lineage of both normal and neoplastic cells (Shevach et al., 1973) (Jaffe et al., 1977). This led to the recognition that i) lymphomas were tumours of the immune system, ii) nodular lymphoma is the same as FL, iii) "reticulum cell sarcomas" are of B-cell origin and iv) the cells of Sezary syndrome and most lymphoblastic lymphomas are of T-cell origin (Jaffe et al., 2008).

Pathologists were quick to apply this new information to further refine the classification of lymphoma. The first and most important work came from Karl Lennert from Kiel (Lennert, 1978). His classification was based on ultrastructural studies and linkage of lymphoma cells with the hypothetical scheme of lymphocyte differentiation, particularly with cells within the lymphoid follicle. He separated the neoplasms into two cell lineages (B- and T-cell), and grouped them into low and high-grade malignancies. Despite employing the recent discoveries in the field of immunology the classification was lacking a practical approach in everyday clinical practice and became widely used in Europe only. It never replaced the Rapapport classification in the United States.

In parallel clinicians were improving their knowledge of treatments and staging of lymphomas. The heterogeneity between different types of lymphoma became increasingly obvious in clinical practice. However use of different classification systems made it difficult to compare published results from different centres. This prompted clinicians and pathologists to seek a consensus in a new classification of lymphoma. Despite several meetings, (the last was held in Airline House in Warrenton, Virginia in 1975), such a consensus was never reached (Jaffe et al., 2008). As a compromise the Working Formulation (WF) for NHL was developed (NationalCancerInstitute, 1982). The WF stratified lymphomas according to clinical outcome, based on the survival of patients in clinical trials conducted in the 1970s. The neoplasms were divided into ten major types (A – J) and a miscellaneous group (see table 1.1). The ten major types could be subdivided into three groups: low grade lymphomas (types A – C), intermediate grade lymphomas (D – G) and high-grade lymphomas (H – J). This was intended to provide a clinical grade to aid patient management. The categories closely followed those of the Rappaport classification. Immunophenotyping was not applied and since the system attempted to cover all entities in only a few groups the categories were heterogeneous and were not reproducible by pathologists. In many respects the WF was a step backwards (Jaffe et al., 2008). It did not recognize well-defined entities such as centrocytic lymphoma from the Kiel classification (now known as mantle cell lymphoma). Diffuse large B-cell lymphoma (DLBCL) was divided into two categories: large cell and large cell immunoblastic. Finally no use was made of widely available immunologic techniques. Therefore both T-cell lymphomas and many DLBCL were lumped together in a single category (diffused mixed small and large cell).

Low grade lymphoma group	
Type	**Name**
A	Malignant lymphoma; small lymphocytic, consistent with CLL, plasmocytoid
B	Malignant lymphoma; follicular. Predominantly small cleaved cell, diffuse areas, sclerosis
C	Malignant lymphoma; follicular. Mixed, small cleaved and large cell, diffuse areas, sclerosis
Intermediate grade lymphoma group	
Type	**Name**
D	Malignant lymphoma; follicular. Predominantly large cells, diffuse areas, sclerosis
E	Malignant lymphoma; diffuse. Small cleaved cells, sclerosis
F	Malignant lymphoma; diffuse. Mixed, small and large cell, sclerosis, epitheloid cell component
G	Malignant lymphoma; diffuse. Large cell, cleaved cells, noncleaved cell, sclerosis
High grade lymphoma group	
Type	**Name**
H	Malignant lymphoma; large cell, immunoblastic, plasmacytoid, clear cell, polymorphous, epitheloid cell component
I	Malignant lymphoma; lymphoblastic, convulted cell, nonconvulted cell
J	Malignant lymphoma; small noncleaved cell, Burkitt's, follicular areas
Miscelaneous group	
• Composite • Mycosis fungoides • Histiocytic • Extramedullary plasmocytoma • Unclassifiable • Other	

Table 1.1 Classification of non-Hodgkin lymphoma according to Working Formulation (NationalCancerInstitute, 1982).

Despite all these controversies the WF gained a positive response from many clinicians and became very popular in the U.S. particularly for clinical trials where it replaced the Rappaport classification. Unfortunately it was employed in several important trials and biased their results, which has consequences until the present day. In Europe and in Asia the Kiel classification remained the leading tool for the classification of lymphoma.

With so many different classifications in use it remained difficult for both pathologists and clinicians to interpret the results of published studies. Furthermore in the 1980s and 1990s numerous new diseases were defined that were not included in any classification e.g. anaplastic large cell lymphoma (ALCL), lymphomas of mucosa associated lymphoid tissues (MALT) and adult T-cell lymphoma (Jaffe et al., 2008). Additionally fundamental discoveries were being made in immunology and genetics with potential applications in lymphoma research and disease classification. One of these discoveries was the development of monoclonal antibodies (Kohler and Milstein, 1975). This led on to the discovery of a whole range of cell-markers so called "clusters of differentiation (CD)". Monoclonal antibodies were employed in immunohistochemistry (IHC) studies first in cell suspension or cryostat sections and later on formalin-fixed paraffin embedded (FFPE) sections (Taylor and Mason, 1974) (Mason et al., 1982). Simultaneously a dramatic increase in the understanding of the genetics of normal lymphocytes and lymphoma happened. Recurrent cytogenetic abnormalities were identified including the translocations in FL, Burkitt lymphoma (BL) and Mantle Cell Lymphoma (MCL) (Zech et al., 1976) (Fukuhara et al., 1979) (Dalla-Favera et al., 1982) (Taub et al., 1982) (Yunis et al., 1982) (Tsujimoto and Croce, 1984). The process of rearrangement of immunoglobulin (Ig) and T-cell receptors (TCR) during normal lymphoid cell development was discovered and described (Arnold et al., 1989) (Kuppers et al., 1993) (Kuppers et al., 1999). Techniques like fluorescence in situ hybridisation (FISH) and polymerase chains reaction (PCR) were developed and allowed the analysis of antigen receptor genes and oncogene rearrangements on FFPE sections together with genetic alterations at a single-cell level (Deane and Norton, 1990) (Ngan et al., 1989) (Wlodarska et al., 1995). The latter was employed in studies on Hodgkin's disease samples and led to the demonstration of Ig heavy chain gene rearrangements in Reed-Sternberg cells thus defining the origin of the malignant cell as a B-cell. Consequently Hodgkin's disease was renamed as HL (Kuppers et al., 1994).

To try and overcome this stalemate and promote a better understanding between European and American haematopathologists Peter Isaacson and Harald Stein formed an international group of pathologist in 1991 naming it The International Lymphoma Study Group (ILSG) (Jaffe et al., 2008). During the first meeting a broad consensus was reached and a first common paper on mantle cell lymphoma was published (Banks et al., 1992). This initial success was followed by a series of frequent meetings and the

group worked through the long list of lymphoid neoplasms. The outcome of these meetings formed the basis of a new classification called the Revised European-American Classification of Lymphoid Neoplasms (REAL), published in 1994 (Harris et al., 1994). The ILSG defined all individual entities by a combination of morphology, immunophenotype, genetic anomalies and characteristic clinical profile including the site of presentation. The inclusion of clinical criteria was one of the novel aspects of the REAL classification. The clinical criteria played an important role particularly in the classification of T-cell neoplasms or MALT and primary mediastinal large B-cell lymphoma (PMLBL). The REAL classification focused on identification of "real" diseases rather than a global theoretical framework such as survival in WF, or cellular differentiation in the Kiel and Lukes-Collins classifications (Jaffe et al., 2008).

Recognizing that HL and plasma cell myeloma are both lymphoid in origin, and that lymphoid neoplasms can present in solid and circulating phases the classification included all lymphoid neoplasms: NHL, HL, lymphoid leukaemias (chronic and acute) and plasma cell disorders (Harris et al., 1994). The tumours were stratified according to lineage; B-cell vs. T/NK cell and further into precursor and mature neoplasms. Hodgkin lymphoma remained a separate category. The REAL classification recognized that because of limitations in current knowledge some broad categories of disease could not be further subdivided, for example, DLBCL or peripheral T-cell lymphoma (PTCL) NOS. These categories should be the subject of future studies. Importantly the REAL classification did not attempt to stratify lymphomas according to a histologic or clinical "grade", as was the case in the WF or Kiel classifications. It was recognized that lymphomas are a heterogeneous group of distinct diseases mostly unrelated to one another, and not a single disease with a spectrum of histologic grade and clinical behavior. In the REAL classification grading was used within some disease entities such as FL but not across a range of different diseases. The main goals of the REAL classification were proven in an international study by James Armitage et al and the conclusion of the study affirmed its principles (The Non-Hodgkin's Lymphoma Classification Project, 1997).

Briefly, after the publication of the REAL classification the WHO published an update of its classification of tumours of hematopoietic and lymphoid tissues (Jaffe et al., 2008). A common steering committee of the Society of Haematology and of the European Association of Haematopathology was appointed and work started under its supervision (Jaffe et al., 1999). The new WHO classification was developed over 7

years. Its development was intensively discussed in the international arena in order to achieve worldwide acceptance. In general the WHO classification was based on the REAL classification. Importantly it was again concluded that clinical grouping for either protocol treatment or routine clinical practice is not feasible. Each disease needs to be treat individually when new therapies are introduced. The basis of the classification was the fact that B-cell and NK/T-cell neoplasms in many respects appear to replicate normal B-cell or NK/T-cell differentiation. However, some common neoplasms do not clearly correspond to a currently recognized normal differentiation stage. Other tumours exhibit lineage heterogeneity or plasticity. Obviously in these cases the normal counterparts cannot be the sole basis for the classification. The WHO classification was published in 2001 (Jaffe et al., 2001). In order to include continuous developments in immunology, genetics and haematology the WHO classification has been reviewed and updated; the new version was published in 2008, see table 1.2 (Swerdlow et al., 2008).

Precursor lymphoid neoplasm
• B lymphoblastic leukaemia / lymphoma
• B lymphoblastic leukaemia / lymphoma, NOS
• B lymphoblastic leukaemia / lymphoma, with recurrent genetic abnormalities
• B lymphoblastic leukaemia / lymphoma with t(9;22)(q34;q11.2); *BCR-ABL1*
• B lymphoblastic leukaemia / lymphoma with t(v; 11q23); MLL rearranged
• B lymphoblastic leukaemia / lymphoma with t(12;21)(p13;q22); *TEL-AML1 (ETV6-RUNX1)*
• B lymphoblastic leukaemia / lymphoma with hyperdiploidy
• B lymphoblastic leukaemia / lymphoma hypodiploidy (hypodiploid ALL)
• B lymphoblastic leukaemia / lymphoma with t(5;14)(q31;q32); *IL3-IGH*
• B lymphoblastic leukaemia / lymphoma with t(1;19)(q23;p13.3); *E2A-PBX1 (TCF3-PBX1)*
• T lymphoblastic leukaemia / lymphoma

Table 1.2 2008 WHO classification of lymphoid malignancies (Swerdlow et al., 2008).

Mature B-cell neoplasm
• Chronic lymphocytic leukaemia / small lymphocytic lymphoma
• B-cell prolymphocytic leukaemia
• Splenic marginal zone lymphoma
• Hairy cell leukaemia
• Splenic B-cell lymphoma / leukaemia, unclassifiable
Splenic diffuse red pulp small B-cell lymphoma
Hairy cell leukaemia - variant
• Lymphoplasmacytic lymphoma
• Waldenstroem macroglobulinaemia
• Heavy chain diseases
Apha heavy chain disease
Gamma heavy chain disease
Mu heavy chain disease
• Plasma cell myeloma
• Solitary plasmacytoma of bone
• Extraosseous plasmacytoma
• Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
• Nodal mariginal zone lymphoma
• Paediatric nodal mariginal zone lymphoma
• Follicular lymphoma
• Paediatric follicular lymphoma
• Primary cutaneous follicle centre lymphoma
• Mantel cell lymphoma
• Diffuse large B-cell lymphoma (DLBCL), NOS
T-cell / histiocyte rich large B-cell lymphoma
Primary DLBCL of the CNS
Primary cutaneous DLBCL, leg type
EBV positive DLBCL of the elderly
• DLBCL associated with chronic inflammation
• Lymphomatoid granulomatosis
• Primary mediastinal (thymic) large B-cell lymphoma
• Intravascular large B-cell lymphoma
• ALK positive large B-cell lymphoma
• Plasmablastic lymphoma

Table 1.2 2008 WHO classification of lymphoid malignancies (Swerdlow et al., 2008).

Mature B-cell neoplasm (continuation)
• Large B-cell lymphoma arising in HHV8—associated multicentre Castelman disease • Primary effusion lymphoma • Burktitt lymphoma • B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma • B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma
Mature T-cell and NK-cell neoplasm
• T-cell prolymphocytic leukaemia • T-cell large granular lymphocytic leukaemia • Chronic lymphoproliferative disorders of NK cells* • Aggressive NK-cell leukaemia • Systemic EBV-positive T-cell lymphoproliferative disorders of childhood • Hydroavacciniforme-like lymphoma • Adult T-cell lymphoma/leukaemia • Extranodal NK/T-cell lymphoma, nasal type • Enteropathy-associated T-cell lymphoma • Hepatosplenic T-cell lymphoma • Subcutaneous panniculitis-like T-cell lymphoma • Mycosis fungoides • Sezary syndrome • Primary cutaneous CD30+ lymphoproliferative disorder • Lymphomatoidpapulosis • Primary cutaneous anaplastic large cell lymphoma • Primary cutaneous gamma-delta T-cell lymphoma* • Primary cutaneous CD8 positive aggressive epidermotropic cytotoxic T-cell lymphoma • Primary cutaneous CD4 positive small/medium T-cell lymphoma • Peripheral T-cell lymphoma, not otherwise specified • Angioimmunoblastic T-cell lymphoma • Anaplastic large cell lymphoma, ALK-positive • Anaplastic large cell lymphoma, ALK-negative*

Table 1.2 2008 WHO classification of lymphoid malignancies (Swerdlow et al., 2008).

Hodgkin lymphoma
• Nodular lymphocyte predominant Hodgkin lymphoma
• Classical Hodgkin lymphoma
Nodular sclerosis classical Hodgkin lymphoma
Lymphocyte-rich classical Hodgkin lymphoma
Mixed cellularity classical Hodgkin lymphoma
Lymphocyte-depleted classical Hodgkin lymphoma
Posttranspantation lymphoproliferative disorders (PTLD)
• Early lesions
• Plasmacytic hyperplasia
• Infectious mononucleosis-like PTLD
• Polymorphic PTLD
• Monomorphic PTLD (B- and T/NK-cell types)
• Classical Hodgkin lymphoma type PTLD

Table 1.2 2008 WHO classification of lymphoid malignancies (Swerdlow et al., 2008).

During the updating process special attention was paid to heterogeneous and ambiguous categories e.g. FL and DLBCL. One of the major changes included the introduction of provisional borderline categories: i) B-cell lymphoma unclassifiable, with features intermediate between DLBCL and BL and ii) B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical HL. The other alteration was the identification of diseases characterized by involvement of specific anatomic sites, or by other clinical features e.g. age. Both B-cell and T-cell lymphomas involving cutaneous or other extranodal sites such as the central nervous system (CNS), paediatric marginal zone lymphomas, paediatric FLs and Epstein-Barr virus (EBV) positive DLBCL in elderly persons are now recognised as distinct entities. One unresolved issue is the difficulty of pathological prognostication in big, heterogeneous categories like FL or DLBCL. Despite the inclusion of specific new types of DLBCL, a heterogeneous category of DLBCL NOS remains for which pathologic features are lacking to further stratify them for predicting prognosis or response to therapy. Development of targeted therapies and recognition of additional markers of clinical behaviour will likely result in additional modification to this category in the future. Furthermore the new WHO classification emphasises other future areas of research such as studies on early events in lymphomagenesis, the recognition of small clonal lymphoid populations or lineage plasticity in hematopoietic cells and their neoplasms.

1.1.2 *Epidemiology of lymphomas*

The American Cancer Society estimated there were 71,380 new cases of lymphoma in 2007, including 8,190 cases of HL and 63,190 cases of NHL (AmericanCancerSociety, 2007). The survey from the Surveillance, Epidemiology and End Results program in the United States indicated an incidence rate per 100,000 persons per year of 33.65 for all lymphoid neoplasms, 26.13 for B-cell neoplasms, 1.79 for T-cell neoplasms and 2.67 for HL (Morton et al., 2006). According to these data the incidence rates for NHL have nearly doubled since the early 1970s. Although some of this increase is due to AIDS-related NHL in general it remains unexplained, (see more in the aetiology section) (AmericanCancerSociety, 2007). According to epidemiological data precursor and mature lymphoid neoplasms comprise approximately 10% and 90% of lymphoid neoplasms respectively (Armitage and Weisenburger, 1998) (The Non-Hodgkin's Lymphoma Classification Project, 1997). Precursor lymphoid neoplasms are primarily diseases of children (approximately 75% of cases are diagnosed in children aged <6 years) and more common in males (Swerdlow et al., 2008). A B-cell origin is more common in leukemic variants and a T-cell origin in lymphoma variants (Swerdlow et al., 2008). The vast majority of mature neoplasms are of B-cell origin (approximately 90%) with NK/T-cell neoplasms making up the remaining 10% (Armitage and Weisenburger, 1998) (The Non-Hodgkin's Lymphoma Classification Project, 1997). DLBCL and FL are the most common variants of mature B-cell neoplasms, together accounting for more than 60% of all cases (see figure 1.1) (Anderson et al., 1998) (The Non-Hodgkin's Lymphoma Classification Project, 1997).

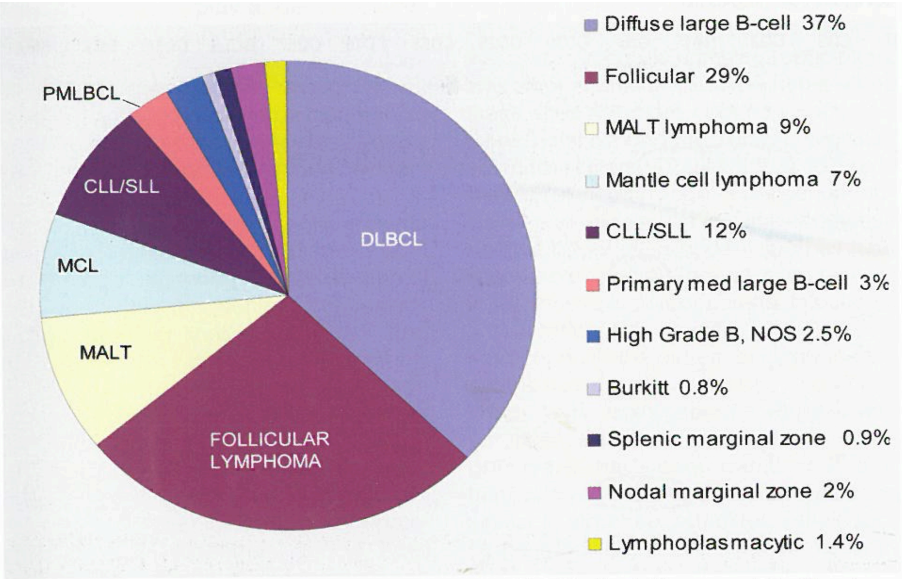

Figure 1.1 Relative frequencies of B-cell lymphoma subtypes in adults (The Non-Hodgkin's Lymphoma Classification Project, 1997).

PTCL NOS and angioimmunoblastic T-cell lymphoma (AITL) are the main subtypes of mature T-cell neoplasms (see figure 1.2) (Vose et al., 2008).

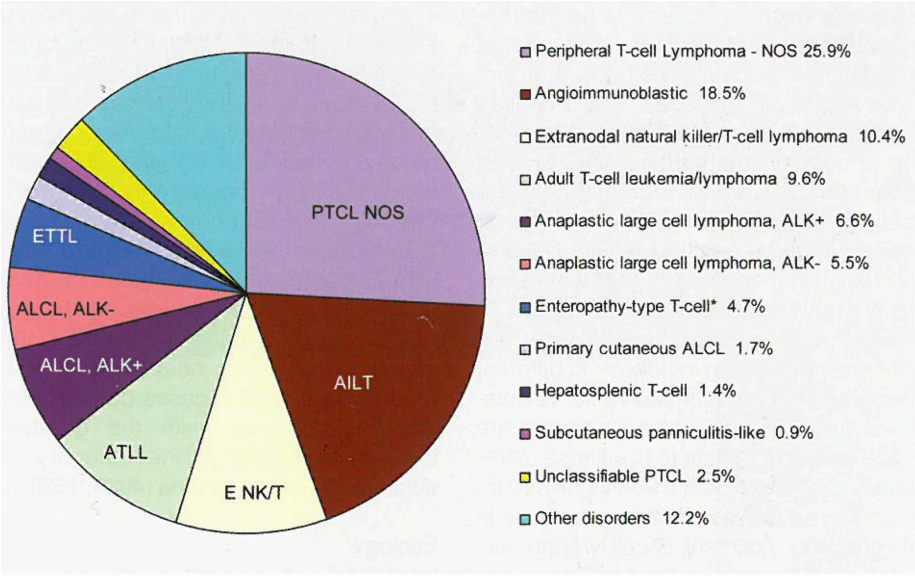

Figure 1.2 Relative frequencies of T-cell lymphoma subtypes in adults (Vose et al., 2008).

There are significant international differences in frequency of individual lymphoma types. As a rule T-cell lymphomas are more frequent in Asia, and B-cell lymphomas in developed western countries (United States, Australia, New Zealand and Western Europe) (Swerdlow et al., 2008). DLBCL shows a more or less equal distribution worldwide whereas FL is more common in the United States and Western

Europe and is uncommon in South America, Eastern Europe, Africa or Asia. By contrast BL is very common in Africa (particularly in the equatorial part where the disease is endemic), but very rare in Europe and North America (Swerdlow et al., 2008). For NK/T-cell lymphomas, adult T-cell leukaemia/lymphoma (ATLL) is the most common lymphoma in Japan and the Caribbean basin. EBV associated NK/T-cell neoplasms are much more common in Asians as compared with other races e.g. in Hong-Kong extranodal NK/T-cell lymphoma nasal type is one of the most common subtypes, whereas in other regions it is an extremely rare disease (Vose et al., 2008). By contrast type 1 enteropathy associated T-cell lymphoma (EATL) is more common in northwest Europe than anywhere else in the world, particularly in individuals of Welsh and Irish descent.

Generally there is a male predominance amongst patients with B-cell lymphoma, however, there are types with a female predominance: FL or even more significantly PMLBL (Swerdlow et al., 2008). B-cell neoplasms are tumours of elderly people with a median age at diagnosis of 60 – 70 years with the exception of HL and PMLBL The median age at diagnosis is lower in patients with NK/T-cell neoplasms.

Survival rates in patients with lymphoma vary depending on the type and stage of disease. The average five-year survival for HL is 86% and for NHL 63%. It had been predicted that in 2007 an estimated 19,730 deaths will occur in the USA due to lymphoma; HL 1,070 and NHL 18,660 (AmericanCancerSociety, 2007).

1.1.3 *Aetiology of lymphomas*

The main cause of the majority of lymphomas is still unknown. The most studied are infectious agents and among them EBV is the most common (Swerdlow et al., 2008). It is present in 100% of endemic BL cases and 15-35% of sporadic and HIV-associated BL cases (Hamilton-Dutoit et al., 1993). Additionally EBV is involved in the pathogenesis of many other B-cell lymphomas such as post-transplant lymphoproliferative disorders (PTLD), plasmablastic lymphomas, EBV+ large B-cell lymphoma of the elderly, extranodal NK/T-cell lymphoma and HL (Kanegane et al., 1998) (Quintanilla-Martinez et al., 2000). Human T-cell leukaemia/lymphoma virus-I (HTLV-1) is the causative agent of ATLL (Takatsuki, 1995) and human herpesvirus-8 (HHV8) is found in primary effusion lymphoma and the lymphomas associated with multicentric Castelman's disease (Cesarman et al., 1995). Infection with hepatitis C virus (HCV) may be linked to the development of lymphoplasmacytic lymphoma

associated with type II cryoglobulinaemia, splenic marginal zone lymphoma, nodal marginal zone lymphoma or DLBCL (Agnello et al., 1992) (Swerdlow et al., 2008) (de Sanjose et al., 2008). The HCV does not seem to directly infect neoplastic B-cells but appears to influence the lymphoma development through activation of a B-cell immune response (Swerdlow et al., 2008). Infections with *Helicobacter pylori* are associated with the development of gastric MALT lymphoma (Hussell et al., 1993) (Wotherspoon et al., 1991) (Wotherspoon et al., 1993), *B. burgdorferi* with cutaneous MALT lymphoma (Swerdlow et al., 2008), *Clamydia psitttaci*, *C. pneumoniae* and *C. trachomatis* in ocular adnexal MALT lymphomas (Chanudet et al., 2006) (Ruiz et al., 2007) and *Campylobacter jejuni* with intestital MALT lymphoma associated with alpha heavy chain disease (Price, 1990).

Other known risk factors include severely reduced immune function like immunosuppression after organ transplant which predisposes not only to hepatosplenic T-cell lymphoma and PTLD but also primary cutaneous and mucosa associated B-cell lymphomas. Other severe autoimmune conditions and infections with human immunodeficiency virus (HIV) are associated DLBCL, BL and other HIV associated lymphomas (Swerdlow et al., 2008). A family history, as well as exposure to certain chemicals; like herbicides or chlorinated organic compounds are further risk factors particularly for the development of follicular lymphoma (Colt et al., 2006).

1.1.4 *Pathobiology of lymphomas*

The mechanisms involved in the pathogenesis of B-cell and NK/T-cell neoplasms in many respects are based on the physiology of lymphoid cells. Likewise the clinical manifestations of lymphomas reflect the normal function of lymphoid cells in vivo. The former is particularly the case in B-cell neoplasms and the latter more significant for NK/T-cell neoplasms (Jaffe et al., 2008).

1.1.4.a *Pathobiology of B-cell lymphomas*

Normal B-cell differentiation begins in bone marrow (BM) with precursor B-cells known as progenitor B-cells (B lymphoblasts). These cells undergo immunoglobulin *VDJ* gene rearrangement (first heavy chain gene rearrangement in pre-B cells and subsequently heavy and light chain gene rearrangement in immature B-cells), and finally differentiate into mature surface immunoglobulin positive (IgM+ and IgD+) naïve B-cells (Inghirami et al., 1991). Neoplasms with their origin in the above

cells include B lymphoblastic leukaemia / lymphoma and they are called precursor B-cell neoplasms. Naïve B-cells are small resting lymphocytes, that circulate in the peripheral blood (PB) and also occupy primary lymphoid follicles and follicle mantle zones. Most cases of mantle cell lymphoma have their origin in CD5+ naïve B-cells; they belong to the group of pre-germinal centre (GC) neoplasms (Hummel et al., 1994). During further differentiation processes in the interfollicular area naïve B-cells undergo transformation, proliferation and ultimately maturation into short lived plasma cell or memory B-cells when encountering antigen that fits their surface Ig receptors. Alternatively they migrate into the centre of a primary follicle, proliferate and fill the follicular dendritic cell meshwork, forming a GC (Liu et al., 1991) (MacLennan, 1994). In the GC naïve B-cells transform into centroblasts expressing low sIg and switch off the expression of B-cell lymphoma 2 (BCL2) protein, thus becoming susceptible to death through apoptosis (Reed, 2008). Centroblasts also express CD10 and B-cell lymphoma 6 (BCL6) proteins, which are characteristics of GC cells (Pittaluga et al., 1996). Somatic hypermutation in the Ig heavy and light chain variable region genes and switch of Ig type from IgM to IgG or IgA also occurs in the germinal centre (MacLennan et al., 1990). *BCL6* also undergoes somatic hypermutation, albeit with a lower frequency (Pasqualucci et al., 1998). *IGV* region gene mutations and *BCL6* mutations serve as markers of cells which have been through the GC. Centroblasts mature to centrocytes, and these cells are seen predominantly in the light zone of the GC. Centrocytes express the altered sIg and those with mutations resulting in increased affinity re-express BCL2 and escape from apoptosis (MacLennan, 1994). Through the interaction with the follicular dendritic cells (Cattoretti et al., 1995) (Pittaluga et al., 1996) and through the overexpression of interferon regulatory factor-4 / multiple myeloma oncogene 1 (IRF4/MUM1) (Falini et al., 2000) (Saito et al., 2007) centroblasts switch off BCL6 protein and differentiate into post-GC B-cells: either memory B-cells or plasma cells (MacLennan, 1994). Neoplasms having their origin in GC B-cells are FL, BL, some DLBCL and HL (Swerdlow et al., 2008). Post-germinal memory cells circulate in the PB and constitute at least some of the cells in the follicular marginal zones of lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT). These cells usually express pan-B antigens surface IgM, and at low level, IgD and are negative for CD5 and CD10 (van den Oord et al., 1989). Plasma cells produced in the germinal centre enter the PB and home to the BM. These cells contain IgG or IgA, they lack soluble Ig and CD20, but express IRF4/MUM1, CD79a, CD38 and

CD138 (Swerdlow et al., 2008). Both cells have mutated *IGV* regions. Neoplasms derived from post-germinal centre cells are: MZL of MALT, splenic and nodal lymphoplasmacytic lymphoma, chronic lymphocytic leukaemia / small lymphocytic leukaemia (CLL/SLL), DLBCL and plasma cell myeloma (Swerdlow et al., 2008). A simplified diagram of normal B-cell differentiation is shown in figure 1.3.

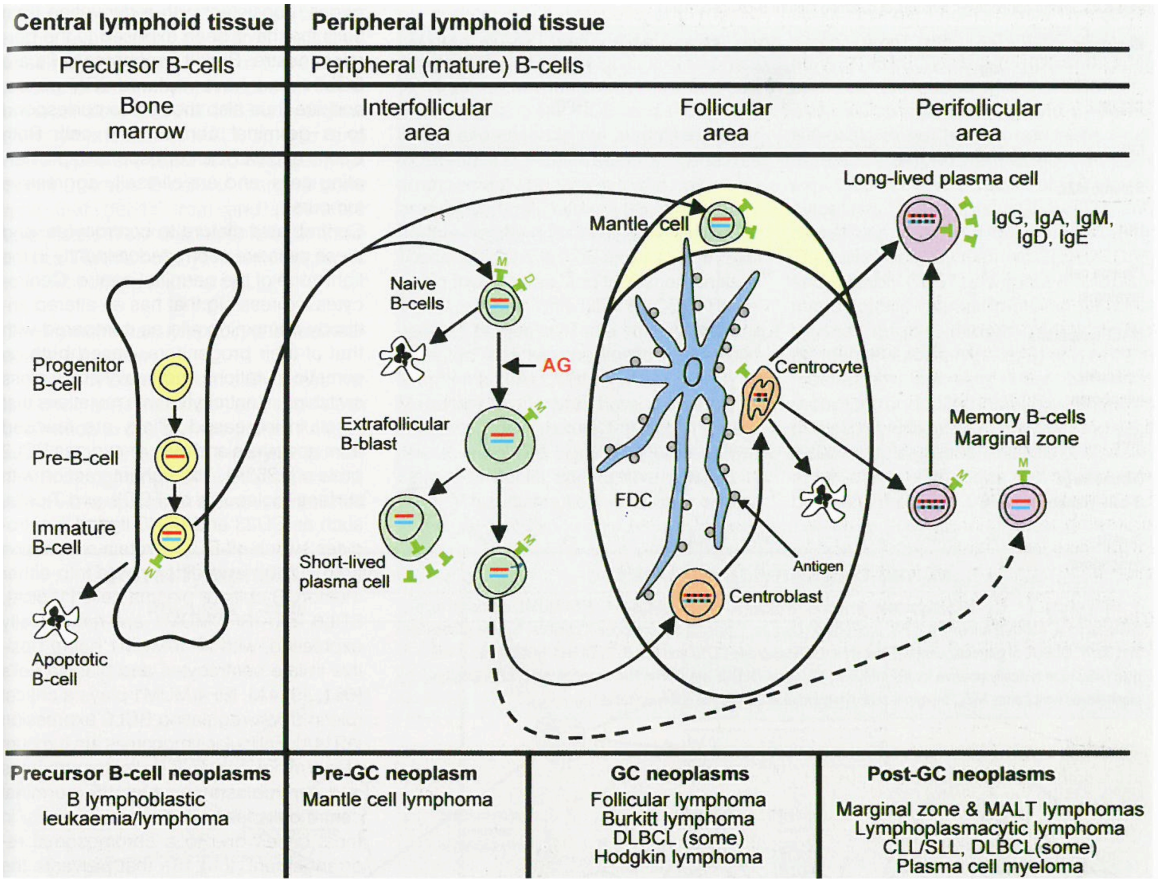

Figure 1.3 Diagrammatic representations of B-cell differentiation and relationship to major B-cell neoplasms (Swerdlow et al., 2008).

1.1.4.b *Pathobiology of T-cell lymphomas*

By contrast to the current classification of B-cell neoplasms the classification of NK/T-cell neoplasms is based more on clinical characteristics of the disease including involved site and clinical course. Nevertheless, some aspects of normal NK/T-cell development and differentiation are important and helpful for understanding of their classification and pathology. The characteristic feature of T-cells is expression of the TCR. Depending on the structure of the TCR, there are two classes of T-cells; α/β and γ/δ (Brown et al., 2002). By contrast NK cells do not express TCR, CD3 or BCR. They usually express the surface markers CD16 (FcγRIII) and CD56 in humans.

T-cells arise from a BM precursor progenitor T-cell (prothymocyte). The progenitor T-cells subsequently migrate into the thymus gland and settle down in the thymic cortex as subcapsular cortical thymocytes. Antigen specific α/β T-cells mature from cortical thymocytes. The cortical thymocytes have an immature T-cell phenotype and express terminal deoxynucleotidyl transferase (TDT), CD1a, CD3, CD5 and CD7 and are initially double negative for both CD4 and CD8. Subsequently these antigens are co-expressed in maturing thymocytes. The T lymphoblastic / leukaemias have their origin in the immature T-cells described above (Swerdlow et al., 2008). The cortical thymocytes develop into medullary thymocytes, which express only CD4 or CD8 like mature α/β T-cells. Medullary thymocytes give rise to naïve α/β T-cells and all these cells have a similar phenotype. Naive α/β T-cells leave the thymus and upon antigen stimulation they may undergo blast transformation (T-blast) and develop further into CD4+ and CD8+ effector and memory cells; components of an adaptive immune system. The CD4+ T cells have mostly a regulatory function and can be divided into two major classes: helper T-cell 1 and 2 (Th1 and Th2). Th1 provide the help to other T-cells and macrophages and Th2 to the B-cells (Brugieres et al., 1998). Recently two new classes of CD4+ cells have been described; follicular T-helper cells (FTH) are present in germinal centres and provide help to B-cells in the context of germinal centre reaction (Grogg et al., 2006) and regulatory T cells (Treg) that play an important role in preventing autoimmunity (Roncador et al., 2005). The FTH are postulated to be the cell of origin for angioimmunoblastic T-cell lymphoma (Dupuis et al., 2006) and Treg for ATLL (Roncador et al., 2005). The CD8+ cells have a cytotoxic function. The precise pathway of maturation of γ/δ T-cells is not fully understood. It is postulated that these cells also originate from a cortical thymocyte. The mature γ/δ T-cells do not express CD5, CD4 or usually CD8. They comprise about 5% of all normal T-cells and are mostly present in the splenic red pulp and epithelial tissue (Swerdlow et al., 2008). The γ/δ T-cells recognize a limited range of antigens, mostly heat shock proteins (Brown et al., 2002).

NK cells have their origin in progenitor T-cells and they do not undergo thymic differentiation. Mature NK cells express CD2, CD7, sometimes CD8 but not surface CD3. NK cells and γ/δ T-cells are major component parts of the innate immune system and are present in the spleen, intestine, other epithelial sites and skin. Figure 1.4 presents a simplified diagram of normal NK/T-cell differentiation.

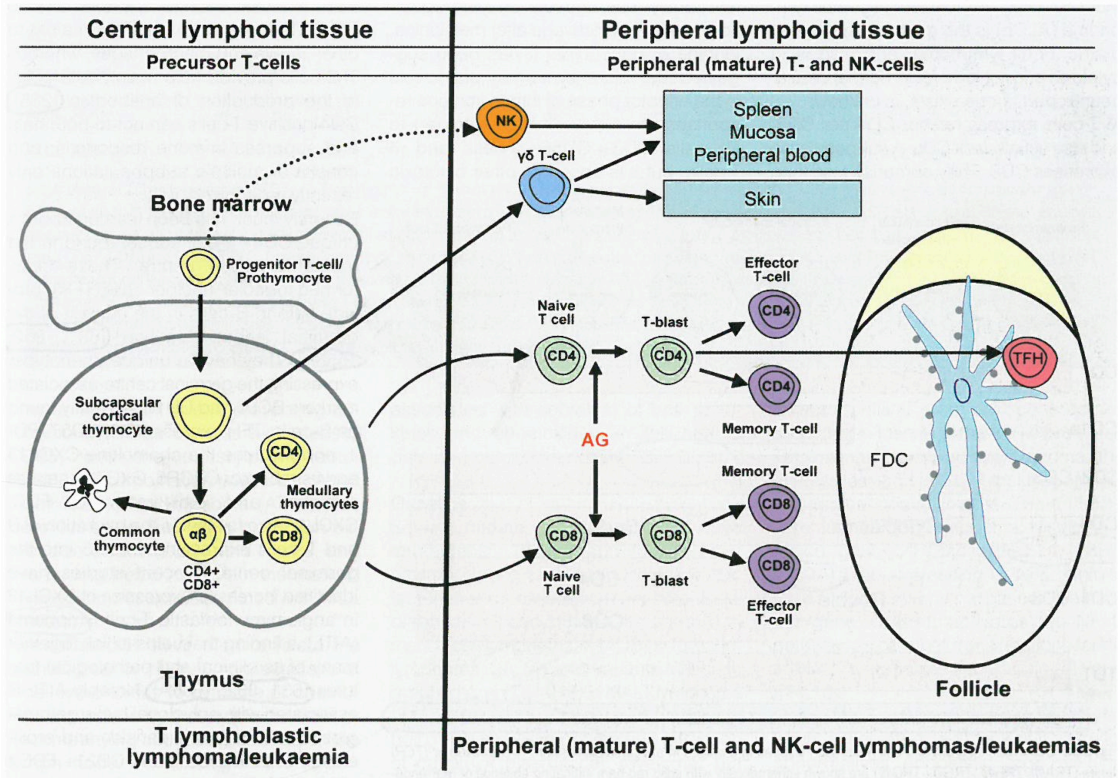

Figure 1.4 Diagrammatic representation of T-cell differentiation and relationship to major T-cell neoplasms (Swerdlow et al., 2008).

Mature NK/T-cell lymphomas can be divided into lymphomas of the innate immune system and lymphomas of the adaptive immune system. The former are predominantly extranodal in presentation and are observed in paediatric and young adult age groups and originate in NK or γ/δ T-cells, e.g. aggressive NK-cell leukaemia, systemic EBV-positive T-cell lymphoproliferative disease of childhood, most hepatosplenic T-cell lymphomas (γ/δ – origin) and γ/δ T-cell lymphomas affecting cutaneous and mucosal sites (Jaffe, 2006). By contrast the latter are mostly nodal lymphomas of adult age groups, e.g. PTCL NOS, AILT, anaplastic large cell lymphoma (ALCL), anaplastic lymphoma kinase (ALK) positive or negative (Jaffe, 2006).

1.1.5 *Genetics of lymphomas*

In general lymphoid neoplasms lack universal genetic abnormalities. However, some can be found in several lymphomas of B-cell origin. The most widespread are: t(11;14) in MCL, t(14;18) in FL, t(8;14) and its variants in BL and t(11; 18) in MALT lymphoma (de Boer et al., 1995) (Kanungo et al., 2006) (Levine et al., 1989). The most common paradigm of these translocations is involvement of the Ig heavy chain gene on 14q. A cellular proto-oncogene comes under the influence of an Ig heavy chain gene enhancer. By contrast only two T-cell neoplasms have been associated with specific

genetic abnormalities: ALCL with a translocation involving the ALK gene on chromosome 5: t(2;5) and hepatosplenic T-cell lymphoma with isochromosome 7q (Swerdlow et al., 2008). However, the next generation whole genome / exome sequencing characterised several new genetic changes in PTCL, they need more re-valuation, for more details see section 1.4.4.b *Biological and molecular prognostic factors.*

1.1.6 Basic of pharmacological therapy of lymphoma

The systemic anticancer drugs can be classified into two main groups: conventional cytostatic drugs and molecular targeted drugs. Multiple drugs are often combined in a treatment regimen. The combined drugs usually have different mechanisms of action and toxicity profiles, so they can be given close to their individual maximal dose. In this way the combination of drugs may overcome tumour resistance to an individual drug and at least in theory is more effective. Below we will briefly characterize the mechanism of action of the most important drugs and possible resistance mechanisms. We will focus on the drugs used in the treatment of lymphoproliferative disorders.

1.1.6.a *Conventional cytostatic drugs*

The conventional cytostatic drugs are traditionally divided into several groups This division can sometimes be confusing, as individual groups are defined by different criteria like mechanism of action (eg. alkylating agents, antimetabolites, topoisomerase inhibitors or antimicrotubular agents) or structure (antibiotics or platinating agents). The following groups are usualy described: alkylating agents, platinating agents, antimetabolites, topoisomerase inhibitors, antimicrotubular agents, antibiotics and miscellaneous cytotoxic drugs

Alkylating agents

Alkylating agents were the first cytostatic drugs introduced in cancer treatment (Tannock et al., 2013). The group is heterogenous chemically but all the substances include alkyl groups (eg. $–CH_2Cl$) and cause alkylating of biological molecules via the nucleophile substitution reaction (Forth et al., 2001). In detail, alkyl groups generate highly reactive, electrophile (positively charged) intermediates, which then react with nucleophile (negatively charged) groups like amino, phosphate, sulfhydryl, or

hydroxymoietes on intracellular macromolecules like DNA. Alkylating agents may contain one or two reactive groups and respectively are described as mono- or bi-functional. The major mechanism of action of bi-functional agents is building the crosslinks between DNA strands. This inter-strand linking prevents cell replication. By contrast the mono-functional agents causes single-strand brakes in DNA or damage to bases. The vast majority of alkylating agents used in clinical practice are bi-functional. The most common side of alkylation of DNA by nitrogen is N-7 or O-6 position (dacarbazine) on the guanine. As alkylating agents bind directly to DNA they lack cell cycle specificity, however the proliferating cells are more vulnerable. The resistance mechanisms to alkylating agents include: decrease transport across the cell membrane, increased intracellular thiol concentration, increased enzymatic detoxification of reactive intermediates and alternations in DNA repair enzymes.

Alkylating agents include a big family of nitrogen mustard derivatives e.g. nitrogen mustard (mechlorethamine), cyclophosphamide, trophosphamide, ifosfamide, melphalan, chlorambucil and bendamustine. Cyclophosphamide is the most used drug from this group. It requires activation by hepatic microsomal enzymes to 4-hydroxycylclophospahmide, which exists in equilibrium with its acyclic isomer aldophosphamide. 4-hydroxycyclophosphamide after entering the cells spontaneously decomposes to active phosphoramid mustard and acrolein. Two other cyclophosphamide derivatives: ifosfamide and trofosfamide undergo a similar hepatic activation process. Another group of alkylating agents are nitrosourea derivates: carmustine (BCNU) and lomustin (CCNU). Thiotepa, procarbazine, dacarbazine, temozolomide and busulphan are other components with alkylating potential with diverse chemical structure, e.g. busulphan is a bi-functional alkyl alkane sulfonate and reacts with thiol groups of aminoacids and proteins, but its ability to crosslink the DNA is uncertain.

Platinating agents

Platinating agents are planar platinum complexes. The central atom of the complexe is a platinum atom in 2+ oxidation state (Forth et al., 2001). It has four linking bonds to attached groups. Two of these groups are considered carrier groups and two active–leaving groups. In cisplatin two carrier groups are amine ligands and two leaving groups are chloride ligands. In carboplatin the leaving groups are replaced by a bidentate: ciclobutan dicarboxylic acid. By contrast in oxaliplatin both carer and leaving

groups were replaced by bidenates: 1,2-diaminocyclohexan and oxalate, respectively. During the activation process the active groups after dissociation from the platinum atom leave a positively charged electrophile complex, which reacts in nucleophilic substitution mechanism with nucleophile groups of molecules similar to mustard nitrogen. The preferred sites of the substitution are N-7 atoms of guanine and adenosine. Cisplatin binds in two sites of DNA, in 95% causing intra-strand cross linkages between two adjacent guanine bases or guanine and adenosine and in the remaining 5% inter-strand guanine linkages. Carboplatin and oxaliplatin produce the same type of adducts like cisplatin, however oxaliplatin more often causes cell death, most likely due to the tertiary structure of substitution products. Cisplatin and carboplatin have similar activity profile and show cross resistance and have different toxicity profiles. The resistance mechanisms are decreased uptake and increased binding to intracellular scavengers e.g. gluthiation or SH-positive metalloproteins and also increased activity of DNA repair.

Antimetabolites

The antimetabolites are cytotoxic agents, which interfere with the synthesis of DNA (Forth et al., 2001). Usually their structure resembles the structure of physiological elements of DNA e.g. purines and pyrimidines. Currently used antimetabolites can be divided into three categories: i) anti-folates, ii) purine and purine nucleoside analogues and iii) pyrimidine and pyrimidine nucleoside analogues. Antimetabolites are usually cell cycle specific in S-phase. As they do not interact directly with DNA, they do not cause late cancerogenesis problems.

i) Antifolates. Methotrexate is the first and the most commonly used anti-metabolite (Forth et al., 2001). It is an analogue of the vitamin folic acid. It binds and inhibits the enzyme di-hydrofolate reductase, which catalyses a reduction of di-hydrofolate to tetra-hydrofolate. Tetra-hydrofolate is used as donor of active carbon groups in reaction of conversion of deoxyuridine monophosphate to thymidine monophosphate catalysed by thymidil synthase. In this reaction the tetra-hydrofolate is binge oxidised to di-hydrofolate. The di-hydrofolate reductase guarantees the appropriate concentration of tetra-hydrofolate in the cell and its inhibition stops synthesis of DNA. Methotrexate passively crosses the cell membrane and undergoes polyglutamation, this prevents the efflux of the drug and polyglutamed derivatives are also more potent (Tannock et al., 2013). The cytotoxic action of methotrexate depends

not only on the dose but also on the time of exposure. The toxicity of methotrexate can be reversed by administration of thymidine and exogenous purines or sources of reduced folate. Methotrexate can be given in standard doses and in high doses with leucovorin cover (reduced form of folate). It can be given orally, intramuscularly, intravenously and intrathecally. Methotrexate crosses the blood–brain barrier but achieves the cytotoxic concentration in cerbro-spinal fluid only if given in high concentration or intrathecally. The toxic profile includes myelosupression and mucositis, damage to liver, lung and central nervous system.

Pemetrexed is another antifolate, a derivative of folic acid, which inhibits directly thymidylate synthase, di-hydrofolate transferase and glycinamid rybocucleotide formytransferase (enzyme involved in purine synthesis) (Tannock et al., 2013). The main toxicities are myelosupression, mucositis and skin rash. Ralitrexed is another inhibitor of thymidyl synthase, similar to pemetrexed.

ii) Purine and purine nucleoside analogues. 6-mercaptopurine and 6-thioguanin are analogues of hypoxantines and guanine. Both drugs are metabolized by hypoxantine-guanin-phosphoribosyltranspherase to active metabolites 6'-thioinosin-5'-phosphate (thio-IMP) and thioguanosin-5-phosphate (thio-GMP) (Tannock et al., 2013). Thio-IMP and thio-GMP inhibit the conversion of IMP to AMP and GMP and are feedback inhibitors of purine-biosynthesis. Further, thio-GTP and desoxy-thio-GTP are incorporated into RNA and DNA as false nucleotides. The main toxicities are myelosupression and hepatotoxicity.

Fludarabine and cladribine are purine nucleoside analogues, specifically adenosine analogues (Tannock et al., 2013). Fludarabine after administration is dephosphorylated to 2-fluoro-ara-A which is thereafter transferred into the cells and converted to active triphosphate derivative. The mechanism of action is inhibition of DNA polymerase and termination of DNA and RNA synthesis. The majority of the drug is eliminated renally. The common toxicities are myelosupression and immunosuppression. Rarely fludarabine can cause autoimmune disorders like haemolytic anaemia, autoimmune thrombocytopenia or CNS toxicity. Cladribine has a similar mechanism of action and toxicological profile to fludarabine.

iii) Pyrimidine and pyrimidine nucleoside analogues. 5-fluorouracil is an analogue of pyrimidine bases uracil and thymine. It penetrates quickly into cells where is metabolized in nucleosides and nucleotides to 5-fluoro-uridine-triphospate (5-FUTP), 5-fluoro-deoxyuridine-triphosphate (5-FdUTP) and 5-fluoro-deoxyuridine-

monophosphate (5-FdUMP) (Tannock et al., 2013). 5-FUTP and 5-FdUTP are built as false nucleotides into RNA and DNA causing inhibition of the nuclear processing. The 5-FdUMP together with the cofactor 5,10-methyl-N-tetrahydrofolate binds to and irreversibly inhibits thymidylate synthase. The excess of 5,10-methyl-N-tetrahydrofolate stabilizes this complex. 5-fluorouracil is administered parenterally as the oral route cannot guarantee predictable, appropriate drug levels. The most common toxicities of 5-flurouracil are myelotoxicity and mucositis, followed by skin rashes, conjunctivitis, neuro and cardio-toxicities. Approximately 80% of 5-fluorouracil is metabolized to carbondioxide, urea and alfa-fluoro-beta-alanine by di-hydropyrimidine dehydrogenase. Patients with partial or complete deficiency of the enzyme are at risk of severe toxicities.

Capecitabine is an oral fluoropyrimidine derivative which is metabolized in liver to 5'-deoxy-5-fluorocytidine by carboxyesterase and then to to 5'-deoxyfluorouridine by cytidine deaminase (Tannock et al., 2013). 5'-deoxyfluorouridine is finally converted to 5-fluoruracil by thymidine phosphorylase. This enzyme is present in higher concentrations in tumour cells, where the latter reaction usually takes place. The toxicities of capecitabine are similar to those of 5-fluorouracil with lower frequencies of mylotoxicities and mucositis and higher of palmar-plantar syndrome.

Cytosine arabinoside (ara-C) and gemcitabine are purine nucleotide analogues (Forth et al., 2001). Cytosine arabinoside following the conversion to arabinoside-CTP by kinases is incorporated into DNA, which stops DNA-polymerase. Additionally arabinoside-CTP is a direct inhibitor of DNA-polymerase. The availability of the active form of the drug depends on activity of kinases activating the pro-drug and aminazes which deactivate it. Cytosine arabinoside is highly efficient in tumours with high kinase activity e.g. AML. The main toxicity is myelosupression, nausea and vomiting, diarrhoea, mucositis and fever. Gemcitabine (2',2'-difluorodeoxycitidine) is a cytosine analogue which like Cytosine arabinoside requires intracellular activation to triphosphate derivatives dFdCTP which is then incorporated into DNA and inhibits further DNA synthesis. Gemcitabine is less active in DNA chain elimination as compared with cytosine arabinoside, however it shows other intracellular effects contributing to its cytotoxicity e.g. inhibition of ribonucleotide reductase, stimulation of deoxycytidine kinase and inhibition of cytidine deaminase. Gemcitabine is effective in a larger number of tumours as compared with cytosine arabinoside. Toxicity is primarily myelosupression with thrombocytopenia.

Topoisomerase inhibitors

DNA topoisomerases are enzymes that introduce or eliminate supercoils in double DNA strand. Type I topoisomerases catalyse the relaxation of supercoiled DNA and type II topoisomerases add negative supercoils to DNA (Tannock et al., 2013). Torsional strain occurring during the processes described above is relieved by the formation of a single-strand nick (topoisomerase I) or double-strand nick (topoisomerase II). Topoisomerase inhibitor binds to the DNA/topoisomerase cleavable complex preventing the relegation of DNA strands. Irreversible damage results when an advancing replication fork encounters topoisomerase inhibitor/DNA/topoisomerase stabilized cleavable complex leading to lethal double strand breaks and cell death.

Topoisomerase I inhibitors are derivatives of campothecin, an extract from the wood of the Chinese tree Campotheca acuminate. They selectively bind to topoisomerase I/DNA complex, mostly in S phase. Currently there are two topoisomerase I inhibitors in clinical use; topotecan and irinotecan. Topotecan in contrast to irinotecan does not undergo any metabolic changes and it is eliminated in unchanged form via the kidneys. The dose limiting toxicity is myelosupression. Irinotecan requires esterification to its active metabolite SN38, which is subsequently inactivated by glucorinidation by the uridine diphosphate (UDP) – glucuronosyltransferase 1A1 (UGT1A1) and it is eliminated with bile. A dose limiting toxicity is myelosupression and diarrhoea. Irinotecan can cause two different types of diarrhoea – one with an early onset associated with cholinergic syndrome which can be treated / prevented with the dose of atropine and second type with later onset. The latter one is associated with polymorphism of UGT1A1 resulting in slower metabolisms of SN38 causing damage to interstitial mucosa and profound myelosupression and can be life threatening.

Two topoisomerase II inhibitors currently in clinical use: etoposide and teniposide are glycoside derivatives of Podophylotoxines, an antimitotic agent derived from the mandrake plant. The resistance mechanism is associated with overexpression of P170-glycoprotein and changes in topoisomerase II. Both agents are administered parenterally, however etoposide can be given orally. The limiting toxicity is myelosupression and etoposide is associated with development of secondary AML usualy 2 -3 years post etoposide application.

Antimicrotubular agents

This group of cytotoxic agents consists of two separate subgroups i) vinca alkaloids and ii) taxanes.

i) Vinca alkaloids. The naturally occurring in peri-winkle plants vinblastine and vincristine and their semisynthetic derivatives belong to this group (Tannock et al., 2013). All these components have same basic structure and are characterized by the same mechanism of action. They all bind to protein tubulin and prevent its polymerisation and building microtubules. The main function of microtubules are formatting of the mitotic spindle responsible for separating of chromosomes during metaphase of mitosis or meiosis and structural and transport functions in cells, particularly they are crucial for the function of nerve axons. Hence vinca alkaloids interrupt the metaphase and the lethally damaged cell undergoes apoptotic death. They belong to cell cycle specific cytostatic drugs with the action time-point of mitosis or S-phase. All vinca-alkaloids with the exception of vinorelbin have to be administered i.v. They are lipofilic and have a large volume of distribution, undergo hepatic metabolism and biliary secretion. The resistance mechanism is an over-expression of P170-Glykoprotein and changes in tubulin structure. Despite their similarities in the structure and mechanism of action, the vinca alkaloids differ in their clinical spectra and their toxicity profiles. Vincristine is characterized by neurotoxiciy to peripheral nerves, by contrast the dose limiting toxicity of vinblastine is myelotoxicity and neurotoxicity to the autonomic nervous system. Vinorelbine has a toxicity profile similar to vinblastine with a difference to neurotoxicity which affects more peripheral nerve system (this doesn't quite make sense, but not quite sure what you are saying).

ii) Taxanes. Taxanes are a second group of antimicrotubular agents (Tannock et al., 2013). Two drugs belonging to this group are currently in clinical use: Paclitaxel originally delivered from the needles of the Pacific Yew tree Taxus brevifolia and its semi-syntetic derivative Docetaxel. Both drugs bind to polymerised tubulin inhibiting microtubular dissemble and causing aborted mitosis leading to apoptotic cell death. Taxanes have a large distribution volume with extensive tissue building and undergo hepatic metabolism through the cytochrome P450 enzyme complex. The resistance mechanism is like in vinca-aloids an overexpression of P-170 protein. The common toxicities are myelosupression, conduit disturbances in the heart and peripheral neuropathies. Doxetaxel is often associated with fluid retention and peripheral oedema. Both drugs can cause severe allergic reactions.

Antibiotics

This group is very heterogenous in structure and mechanism of action of its components with the exception of the fact that all these drugs are antibiotics with cytotoxic activity. The group includes a subgroup of cytostatic agents anthracyclines, and individual drugs like mitoxantrone, dactinomycin, amsacrin, mitomycin C and bleomycin (Tannock et al., 2013).

Anthracyclines are ones of the most common used cytostatic drugs. daunorubicin and doxorubicin are antibiotics originally isolated from Streptomyces peucetius. Epirubicin and indarubicine are semi-synthetic derivatives. All drugs have tetracyclic ring attached via glycoside bound to aminosugar daunosamin. The mechanism of action is similar in all anthracyclines and includes: DNA intercalation, interactions with topoisomerase II and building of free radicals. Anthracyclines are S-phase cell cycle specific drugs. All anthracyclines with exception of idarubicin are given parenterally and undergo hepatic metabolism. The resistance mechanism is increased concentration and activity of radical scavenger system (e.g. gluthation) and increased drug efflux caused by P-170 glycoprotein. The toxicity profile includes myelosupression, cardiotoxicity (most likely due to free radicals), gastrointestinal side effects and local tissue necrosis.

Mitoxantrone is a syntetic antrhacenedione derivate. It has a tricyclic structure with two secondary amine groups. Mitoxantrone has a mechanism of action similar to that of anthracyclines including DNA intercalation, interactions with topoisomerase II and synthesis of free radicals. It is however less potent, but is also characterized by a milder toxicity profile.

Dactinorubicine is a cytotoxic drug isolated from Streptomycess spp. It consists of a tricyclic phenoxasone ring which is connected to two identical pentapeptid rings. The mechanism of action is a DNA intercalation, which results in RNA and protein synthesis. It is administered parenterally and is eliminated in unmetabolised form in bile and urine. The common toxicities are myelosupression, nausea and vomiting and mucositis.

Amsacrine is an acridine derivative, which causes DNA intercalation and interferes with topoisomerase II. The common side effects are myelosupression and cadiotoxicity.

Mitomycin C is an antibiotic isolated form Streptomyces caespitosus with a bifunctional DNA alkylating potential. Mitomycin C is an indol derivative and is

charachterized by an aziridine ring. It requires activation by reductive metabolism. It is usually used locally in treatment of bladder or anal canal cancers. It can cause myelosupression, gastric side effects and rarely but importantly a severe, often letal microangiopathy with haemolitic uraemic syndrome or interstitial lung fibrosis.

Bleomycin consists of a family of metal binding glycoproteins isolated from Streptomyces verticullis. The dominant active components are Bleomycin A2 and B2. The mechanism of action includes DNA intercalation and consequently fragmentation of DNA, and aborts DNA synthesis. Bleomycin is a G2 phase cell cycle specific molecule; however it shows also an action in plateau phase as well. Because of its large size bleomycin crosses the cell membrane very slowly and subsequently is activated by bleomycin hydrolase, this enzyme is also responsible for breaking down the drug. Bleomycin is usually administered intravenously, but can be given intramuscularly and subcutaneously. Approximately 60% of Bleomycin is eliminated in the urine. The most important toxicities are fever, headache, allergic reactions including common skin reactions and interstitial lung fibrosis.

Miscellaneous cytotoxic drugs

Asparginase is an enzyme deaminating asparagine to aspartic acid (Tannock et al., 2013). It is isolated from the bacterium Erwinia-chrisanthemi. For normal, healthy cells asparagine is not an essential amino acid; however it is for the fast dividing leukaemia and lymphoma cells. The side effects are severe allergic reactions, coagulopathies, hyperglycemia, pancreatitis and encephalopathy.

Mitelfosin is a phospholipid derivative with an unclear cytostatic mechanism. It is an inhibitor of membrane phospholipids, and interferes with phospholipid dependent singnal pathways and induces apoptosis. Its clinical use is limited.

1.1.6.b *Molecular-targeted agents*

Molecular-targeted agents are a new class of anticancer drugs that target changes in molecular pathways rather than in DNA, as the conventional drugs do. They have a better toxicological profile as they are more specific. At the moment there are approximately 50 different drugs in clinical use. They usually belong to the mononoclonal antibody group or small molecule group. Here we describe closer those which are important for the general understanding of the group (inhibitors of angiogenesis, epidermal growth factor inhibitor and tyrosine kinases or proteasome

inhibitors) or are important in the treatment of lymphoproliferative disorders (anti CD20 or anti CD52 antibodies or brentuximab vedotin).

Inhibitors of angiogenesis

One of the first and the most important group of the molecular-targeted drugs are inhibitors of angiogenesis, a necessary process for tumour growth (Tannock et al., 2013). The crucial factor stimulating the angiogenesis is vascular endothelial growth factor (VEGF) which binds to its receptors (VEGFR) and activates the intercellular tyrosine kinases. There are currently two groups of drugs inhibiting the angiogenesis – the monoclonal antibodies directed against VEGF (e.g. bevacizumab) and inhibitors of intracellular kinases associated with VEGFR pathways (sunitinib, sorafenib and pazopanib). The mechanism of action of thalidomide and lenalidomide is also associated with angiogenesis, however is not fully understood.

Epidermal growth factor inhibitors

Other important group of molecular- targeted drugs are associated with epidermal growth factor (EGF) (Tannock et al., 2013). The epidermal growth factor is polypeptide that binds to its cell surface receptor. There are four subgroups of EGF receptors (EGFR): EGFR, HER 2/c-neu, Her 3 and Her 4. The activation of EGFR initiates cascades of intracellular RAS-RAF-MAPK and PI3K-AKT pathways which stimulate transcription of genes that promote cell proliferation and survival including tumour growth, angiogenesis, invasion and metastasis and also apoptosis. There are two groups of drugs which interfere with the EGF pathway: antibodies against cell surface receptors (cetuximab and panitumumab – against EGFR and trastuzumab against HER 2/c-neu) and small molecules inhibiting the kinases associated with EGFR (erlotinib and gefitinib) or with HER 2/c-neu (lapatinib).

Tyrosine kinase inhibitors

Tyrosine kinase inhibitors are the first successfully used drugs targeting signalling pathways. The first agent was imatinib inhibiting the tyrosine kinase activity of the constitutively active fusion protein BCR-ABL arising from the Philadelphia chromosome of CML (Tannock et al., 2013). Imatinib also inhibits c-KIT tyrosine kinase, which is overexpressed in 80% of GISTs. Resistance to imatinib results from mutations that alter amino acid sequences at the drug binding side or prevent

achievement of the inactive confirmation of the kinase, which is necessary for binding of the drug. Imatinib is well tolerated with common side effects including mild nausea, diarrhoea, fluid retention, muscle cramps and fatigue. The other two tyrosine kinases are dasatinib and nilotinib, which can be successfully used in most of patients who have developed resistance to imatinib.

Proteasome inhibitors

This is a new group of anti-cancer agents. The oldest representative of the group is bortezomib (Velcade) (Tannock et al., 2013). Proteasomes are intracellular organelles, responsible for degradation of cellular protein including ubiquitylated molecules, damaged or misfiled or regulatory proteins. During the cell-cycle multiple proteins are produced in cells in a synchronized way and they have to be switched off at appropriate moments in order to allow the progression of the cell to the next stage. The proteasomes are responsible for appropriate turnover of these molecules and their inhibition can lead to cell death. The inhibition of proteasomes can lead to stress reaction of endoplasmic reticulum, inhibition of NF-kB, inflammatory pathway, increased generation of reactive oxygen species and activation of Caspase 8 and apoptosis. In the tumour cells bortezomib may prevent degradation of pro-apoptotic factors, permitting activation of apoptosis pathways. Bortezomib is an N-protected dipeptide (pyrazinoic acid - phenylalanine – leucine with bronic acid instead of carboxylic acid). Its boron atom binds covalently but reversible to catalytic site of the 26S ribosome. It can be administered subcutaneously of intravenously. It is approved to be used in multiple myeloma and mantle cell lymphoma. The adverse events are neuropathy, myelosupression, immunosuppression or acute interstitial nephritis. New proteasome inhibitors are in development e.g. carfizomib which inhibits the active site of the proteasome irreversibly and as a more powerful agent can overcome resistance to bortezomib.

Anti-CD20 antibodies

The CD20 is a target of several monoclonal antibodies used in treatment of lymphoproliferative disorders, rheumatology or autoimmundiseases (Lim et al., 2010). The CD20 is an activated-glycosylated phosphoprotein expressed on the surface of normal and malignant B-cells (Lim et al., 2010). In normal B-cells it begins to be expressed at the stage of late pro-B cells through all developmental stages till memory

cells. It is not expressed on plasma cells. CD20 does not have a natural ligand and it acts as a calcium channel in the cell membrane. Its function is to enable optimal B-cell immune response against T-independent antibodies, it also plays a role in microenvironmental interaction of B-cells.

The CD20 is the first antigen which has been targeted clinically. Currently there are several non-conjugated anti CD20 antibodies in clinical use: chimeric e.g. rituximab and humanized antibodies e.g. ofatumumab and obinutuzumab or conjugates with radioactive molecules e.g. ibritumomab tiuxetan and tositumomab.

The anti-CD20 antibodies can be divided into two groups: type I and type II antibodies depending on their in vivo activities in various assays like: ability of antibodies to redistribute CD20 in Triton X-100-insoluble lipid rafts, induce homotypic adhesion, evoke complement dependent cytotoxicity or direct cell death (Lim et al., 2010). In vivo studies of Type I antibodies (rituximab or ofatumumab) are more powerful at translocating CD20 into lipid rafts and inducing complement dependent cytotoxicity and Type II antibodies (obinutuzumab or tositumomab) are more potent at inducing homotypic adhesion and direct cell death induction (Lim et al., 2010).

Rituximab was the first approved chimeric anti-CD20 monoclonal antibody. Currently it is being used as monotherapy in the treatment of B-cell lymphoma, autoimmune disorders including multiple sclerosis and rheumatological disorders. In haemato-oncology is also used in combination with chemotherapy. The mechanism of action includes: i) Fc:FcR dependent mechanism including antibody directed cellular cytotoxicity and antibody directed cellular phagocytosis), ii) complement dependent cytotoxicity, iii) programed cell death and iiii) through T-cell mediated immunity (Lim and Levy, 2014). The resistance mechanism to rituximab include i) trygocytosis, a process of selective cleavage of antigen/antibody complexes from the surface of the target cells by phagocytic cells, ii) loss of expression of CD20 following the treatment with rituximab iii) internalization of antigen/antibody complexes (Lim and Levy, 2014).

The success of rituximab stimulated the development of new antiCD20 monoclonal antibodies. The new drugs can belong to 2nd generation antibodies where the IgG1 monoclonal antibody is humanized or fully human in order immunogenicity (not sure what you are saying) (eg ocrelizumab, veltuzumab or ofatumumab) or to 3rd generation antibodies which are humanized and additionaly have an engineered FC region in order to improve the affinity to FcR (e.g. obinutuzumab, ocaratuzumab and

Pro13192) (Lim and Levy, 2014). Among the new substances ofatumumab and obinutuzumab are clinically the most advanced.

Ofatumumab is a new type I, humanized anti CD20 antibody. In contrast to rituximab it recognizes a distinct epitope of CD20 and has a slower dissociation rate from CD20 resulting in greater complement dependent cytotoxicity activity and ability to lyse the tumour cells (Lim and Levy, 2014). Ofatumumab is characterized by more effective induction of antibody directed cellular phagocytosis by macrophages.

Obinutuzumab is a type II, humanized anti CD20 antibody. It has a sugar residue removed from its Fc domain resulting in higher affinity to the Fc receptor (Lim and Levy, 2014). The antibody is known for its high effectiveness in inducing lysosomal-mediated cell death. Obinutuzumab is superior at enhancing NK cell activation and antibody directed cellular cytotoxicity.

Anti CD52 antibodies

Alemtuzumab targets CD52 antigen, a glycoprotein, which in humans is encoded by the CD52 gene and is present on the surface of mature lymphocytes, monocytes and dendritic cells. It is a peptide of 12 aminoacids, is highly negatively charged and its function is anti-adhesion. Altemtuzumab, after binding to its antigen, causes a cell death via antibody-dependent cell-mediated cytotoxicity.

Brentuximab vedotin

Brentuximab vedotin is an antibody drug conjugate consisting of a chimeric monoclonal anti CD30 antibody brentuximab linked to an antimitotic agentmonomethyl auristatin E molecule – vedotin. CD30 (TNFRSF8) is a cell membrane protein of the tumour necrosis factor receptor family and tumour marker. It is expressed on activated B-cells and T-cells. During the pathological state it is expressed on ALCL, embryonal carcinoma and HL Reed-Sterberg cells. TNF receptor-associated factor 2 and 5 are natural ligands of CD30. Their binding to the receptor activates NF-κβ pathway and apoptosis. Brentuximab vedotin is approved for the treatment of relapsed and refractory HL and ALCL.

1.2 *A concept of aggressive non-Hodgkin lymphomas (NHL)*

The concept of “aggressive / high grade NHL” as opposed to “indolent / low grade lymphoma NHL” has its origin in the WF classification of NHLs. The WF

stratified NHL according to differences in survival and other clinical parameters e.g. age and curability (NationalCancerInstitute, 1982). Unfortunately the formulation ignored some already well-described pathological features and did not include the immunophenotypes of tumours. Despite its limitations the WF gained broad clinical acceptance, particularly in North America, and numerous crucial clinical trials in NHL lymphomas included patients according to the WF. Probably one of the most important trials was the Intergroup trial on aggressive lymphomas (WF D-H and J) comparing the efficacy of new generation regimens with conventional chemotherapy with cyclophosphamide, doxorubicine, vincristine, prednisolone (CHOP) (Fisher et al., 1993). Further important clinical trials were designed for aggressive lymphomas including studies of the German High Grade non-Hodgkin Lymphoma Study Group (DSHNHL) (Pfreundschuh et al., 2004a) (Pfreundschuh et al., 2004b) and French Adult Lymphoma Study Group (GELA) (Gisselbrecht, 2002). The term aggressive lymphoma covers a number of currently individual entities with different survival rates e.g. PMLBL, DLBCL NOS or almost all subtypes of PTCL. It is likely that the inclusion of different categories of lymphomas in the same trials has biased the results.

This thesis aims to give some solutions for the most significant problems in the management of patients and some direction for future translational research in patients with certain types of aggressive NHLs (DLBCL and PTCL). In the following part of this introduction there will be a closer focus on these types.

1.3 *Diffuse large B-cell lymphoma (DLBCL)*

1.3.1 *Definition and classification of DLBCL*

According to the current WHO classification of tumours of haematopoietic and lymphoid tissues DLBCL is a neoplasm of large B lymphoid cells with a nuclear size equal to or exceeding normal macrophage nuclei, or more than twice the size of a normal lymphocyte, and is characterized by a diffuse growth pattern (Swerdlow et al., 2008). The postulated normal counterpart of DLBCL NOS tumour cells is a peripheral B-cell of either GC or post-GC (activated B-cell) origin (Swerdlow et al., 2008).

In the recent classification several distinct disease entities have been re-defined and selected from the former category of DLBCL (see table 1.3) (Swerdlow et al., 2008). For the remaining large number of cases there are no clear and accepted criteria

for subdivision, although they appear to be biologically heterogeneous. These cases are currently classified as DLBCL NOS (Swerdlow et al., 2008).

New defined entities selected for former category of DLBCL
• Diffuse large B-cell lymphoma associated with chronic inflammation
• Lymphomatoid granulomatosis
• Primary mediastinal (thymic) large B-cell lymphoma
• Intravascular large B-cell lymphoma
• ALK positive large B-cell lymphoma
• Plasmablastic lymphoma
• Large B-cell lymphoma arising in HHV8 associated multicentre Castelman disease
• Primary effusion lymphoma
• B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkit lymphoma
• B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma

Table 1.3 Other subtypes of large B-cell lymphomas according to 2008 WHO classification of lymphoid malignancies (Swerdlow et al., 2008).

Among DLBCL NOS already several subtypes have been described and defined (see table 1.4). In the section on epidemiology of DLBCL in this thesis the definition of DLBCL from the previous classification was used as the new one was not available at the time of publication of the quoted papers. By contrast in the laboratory-based research section patients with a diagnosis of DLBCL NOS according to the 2008 WHO classification were included.

Morphologic variants
Common morphologic variants • Centroblastic • Immunoblastic • Anaplastic Rare morphologic variants
Molecular subgroups
• Germinal centre B-cell-like (GCB) • Activated B-cell-like (ABC)
Immunohistochemical subgroups
• CD5-positive DLBCL • Germinal centre B-cell-like (GCB) • Non-germinal centre B-cell-like (non-GCB)

Table 1.4 DLBCL NOS, variants, subgroups and DLBCL subtypes according to 2008 WHO classification of lymphoid malignancies (Swerdlow et al., 2008).

1.3.2 *Epidemiology of DLBCL*

DLBCL is the most common NHL with an annual incidence of over 25,000/year and it accounts for about 40% of all cases worldwide. DLBCL NOS accounts for approximately 25-30% of adult NHL in western countries and an even a higher percentage in developing countries (Swerdlow et al., 2008).

More than half of patients with DLBCL and DLBCL NOS are over 60 years of age (median age at presentation is in mid-60s – 70s) (Feugier et al., 2005) (Shipp, 1993) (The Non-Hodgkin's Lymphoma Classification Project, 1997), are male and present with advance-stage disease (American Cancer Society, 2007).

1.3.3 *Clinical presentation of DLBCL*

The disease presents in lymph nodes or in extranodal sites with up to 40% being at least initially confined to extranodal sites (Harris et al., 1994). The most common extranodal site is the gastrointestinal tract (stomach and ileocoecal region), other sites include: bone, testis, spleen, Waldeyer's ring, salivary gland, thyroid, liver, kidney, adrenal gland and lung (Swerdlow et al., 2008). BM involvement is reported in 11 – 27% of cases (Chung et al., 2007). Approximately one third of patients with BM involvement has evidence of malignant cells in PB smears.

Patients usually present with a rapidly enlarging mass at single or multiple nodal sites with or without extranodal involvement. Approximately half of the patients have stage 1 or 2 disease. The majority of patients are asymptomatic but when symptoms are present then they are highly dependent on the site of involvement (The Non-Hodgkin's Lymphoma Classification Project, 1997) (Armitage and Weisenburger, 1998).

The extent of the disease is evaluated using the Ann Arbor staging system, which is based on the results obtained from physical examination, BM biopsy, and imaging studies. The criteria for staging are given in table 1.5.

Patients are further sub staged into A or B according to absence or presence of systemic symptoms (fever >38˚C, night sweats and/or weight loss >10% of body weight in the 6 months preceding presentation) respectively.

Stage	Definition
I	Involvement of a single lymph node or of a single extranodal organ or site (IE)
II	Involvement of two or more lymph node regions on the same side of diaphragm, or localized involvement of an extranodal site or organ (IIE) and one or more lymph node regions on the same side of the diaphragm
III	Involvement of lymph node regions on both sides of the diaphragm, which may also be accompanied by localized involvement of an extranodal organ or site (IIIE) or spleen (IIIS), or both (IIISE)
IV	Diffuse or disseminated involvement of one or more distant extranodal organs with or without associated lymph node involvement

Table 1.5 Ann Arbor staging system (Carbone et al., 1971).

1.3.4 *Aetiology of DLBCL*

The aetiology of DLBCL NOS is unknown. The tumour usually arises de novo but some of the cases are transformed from a less aggressive lymphoma: CLL/SLL, FL, MZL or nodular lymphocyte predominant HL. Immunodeficiency is a well-known risk factor particularly for EBV positive variants. EBV positive cases account for approximately 10% of DLBCL NOS in patients without immunodeficiency (Hummel et al., 1995) (Park et al., 2007).

1.3.5 *Pathology of DLBCL*

1.3.5.a *Morphology*

DLBCL is an aggressive NHL of B-cell origin characterised by a diffuse growth pattern of large lymphoid cells with a high proliferation fraction. The architecture of the involved nodes is totally (more common pattern) or at least partially (interfollicular and/or sinusoidal) affected. Perinodal tissue is often infiltrated (Swerdlow et al., 2008). The morphology of DLBCL is diverse and three common (centroblastic, immunoblastic and anaplastic) and other rare morphological variants of DLBCL have been defined (Swerdlow et al., 2008). These are illustrated in table 1.4. The morphological division of DLBCL NOS has neither therapeutic nor prognostic implication.

1.3.5.b *Immunophenotype*

The tumour cells express pan-B-cells markers such as CD19, CD20, CD22 and CD79a, but may lack some of them (Swerdlow et al., 2008). 50-75% of cases are positive for surface and/or cytoplasmic immunoglobulin (IgM>IgG>IgA) (Loddenkemper et al., 2004). CD30 expression is usually present in the anaplastic variant (Piris et al., 1990) and approximately 10% of tumours are CD5 positive (Tagawa et al., 2005). The reported expression of CD10, BCL6 and IRF4/MUM1 varies between studies (Amen et al., 2007) (Berglund et al., 2005) (Colomo et al., 2003) (de Leval and Harris, 2003) (Muris et al., 2006). Importantly co-expression of IRF4/MUM1 and BCL6, which is mutually exclusive in normal GC B-cells, was found in 50% of DLBCL (Falini et al., 2000). The proliferation fraction measured by Ki67 expression is high (usually >40%) but may be greater than 90% in some cases (Miller et al., 1994). p53 is expressed in 20-60% of cases (Chilosi et al., 1996) (Koduru et al., 1997) (Young et al., 2007).

The subdivision of DLBCL NOS by immunophenotyping into germinal centre-like (GCB) and non-germinal centre-like (non GCB) subgroups has been proposed by several groups and will be introduced in a section on prognostic factors in DLBCL, see table 1.4.

1.3.5.c *Genetics*

As described previously, diffuse large B-cell lymphoma arises from the clonal expansion of B cells in the GC (Klein and Dalla-Favera, 2008). During their

development in GC, naïve B-cells are the subject of complex processes of somatic hypermutation and class switch recombination in order to develop antibodies with higher affinity and capable of distinct functions (Muramatsu et al., 2000). These processes are associated with single and double DNA strands breaks and can succeed successfully only with switched off DNA damage responses, pro-apoptotic mechanisms and inhibition of activation and differentiation. Additionally, during the GC reaction B-cells undergo very rapid proliferation with <12 hours doubling time and are at constant risk of mutations (Victora and Nussenzweig, 2012). If the class-switch recombinations affect not only constant immunoglobulin genes, they can cause chromosomal translocations. Furthermore if the somatic hypermutations are not limited to variable regions of immunoglobulin genes, they can cause aberrant hypermutations in other genes including oncogenes and tumour suppressor genes and contribute to lymphomagenesis (Pasqualucci and Dalla-Favera, 2015).

Clonally rearranged Ig heavy and light chains genes are detectable. They show somatic hypermutations in the variable regions (Swerdlow et al., 2008).

Approximately 50% of DLBCL cases show aberrant somatic hypermutations targeting multiple genetic loci, including *PIMI*, *c-MYC* (v-myc avian myelocytomatosis oncogene homolog), *RHOH/TTF* (ARHH) and *PAX5*. These mutations can contribute to the oncogenesis of this lymphoma (Pasqualucci et al., 2001).

The translocation involving the BCL6 gene is the most common in DLBCL NOS, accounting for up to 30% of cases (Offit et al., 1994) (Ohno and Fukuhara, 1997). Translocation of the BCL2 gene occurs in 20-30% of cases (Weiss et al., 1987) (Lipford et al., 1987) and a *c-MYC* re-arrangement was observed in approximately 10% of cases in an unselected series (Yunis et al., 1989). The *c-MYC* break partner is an Ig gene in 60% and a non Ig gene in 40% of cases (Hummel et al., 2006). Approximately 20% of cases with a *c-MYC* translocation have a concurrent *BCL2* and or a *BCL6* translocation (Hummel et al., 2006). These cases are characterised by very high proliferation rates (>90% Ki67), and it is suggested that they can be better categorized as “B-cell lymphomas, unclassifiable with features intermediate between DLBCL and BL” (Swerdlow et al., 2008).

Gene expression profiling (GEP) identified two distinctive subgroups in DLBCL: germinal centre B-cell-like (GCB) and activated B-cell like (ABC) (Alizadeh et al., 2000) (Rosenwald et al., 2002). The GCB subgroup has the gene expression profile of GC B-cells and the ABC subgroup has the profile of activated peripheral B-

cells. Initially a third group was defined (termed type 3) but it has turned out to be a collection of undefined cases and not a distinct group (Hummel et al., 2006) (Wright et al., 2003). The two defined groups are characterised by different chromosomal aberrations. The ABC cases have frequent gains of 3q, 18q21-q22 and losses of 6q21-q22 and by contrast GCB cases show frequent gains at 12q12 (Bea et al., 2005) (Tagawa et al., 2005) and *BCL2* rearrangements (Huang et al., 2002).

The recent expansion of sequencing technologies, allowing studies on the whole genome / exome, has delivered a new comprehensive and unbiased view of the genetics basis of DLBCL (Pasqualucci and Dalla-Favera, 2015). The above described known abnormalities have been confirmed and put in the context of oncogenic pathways and new alternations have been described. Importantly, it has been revealed that the coding genome has approximately 50 – 100 lesions/case and great variability as compared with the genomes of other B-cells malignancies like CLL or ALL (Schneider et al., 2011).

Using next generation sequencing methods the genetic abnormalities in DLBCL, NOS can be classified in the context of the cell of origin theory in: i) those commonly affecting both GCB- and ABC-DLBCL e.g. genes involved in epigenetic mechanisms like acetylation/deacetylation and methylation/demethylation of histones and DNA, *BCL6*, changes affecting genes involved in immune surveillance mechanism or *TP53*; ii) the genetic lesions characteristic to GCB-DLBCL e.g. *MYC* and *BCL2*, *PTEN* and mir-17-92 micro RNA cluster and iii) genetic lesions to ABC-DLBC e.g. activation of *NF-κβ* or block in terminal B cell differentiation (Pasqualucci and Dalla-Favera, 2015).

i) Genetic abnormalities affecting both GCB- and ABC-DLBCL. Up to 30% of DLBCL, NOS cases have mutations in acetyltransferase *CREBBP* and rarely in acetyltransferase *EP300* (Goodman and Smolik, 2000). Both enzymes modify lysine residues by acetylation on both histone and non-histone nuclear proteins (Goodman and Smolik, 2000). This can result in both activation and deactivation of the modified protein. It has been postulated that acetylotransferases contribute to lymphomagenesis via impaired acetylation of *BCL6* and *p53*, which leads to constitutive activation of the oncoprotein and inhibition of tumour suppressor (Bereshchenko et al., 2002). The changes affecting acetyltransferases are important as they can be targeted by a new group of drugs called inhibitors of deacetylases.

Another group of affected proteins involved in epigenetic mechanisms are methyltransferases (Pasqualucci and Dalla-Favera, 2015). These enzymes transfer methyl groups on histones and other nuclear proteins. The most commonly affected

methyltransferase is MLL2. Mutations to the *MLL2* gene can potentially lead to broad effects on chromatin regulations, which may contribute to lymphomagenesis. Importantly, the *MLL2* mutations have been also found in FL cases (Morin et al., 2011).

Alternations deregulating *BCL6* are the main mechanism of transformation in DLBCL, NOS. The chromosomal translocations involving the *BCL6* locus are present in approximately 35% of DLBCL cases, more frequently involving ABC-DLBCL (Iqbal et al., 2007). The *BCL6* locus is also a target for point mutations observed in >70 cases (Shen et al., 1998). The mutations in the first noncoding exon are found exclusively in DLBCL and they deregulate *BCL6* expression by disruption of the auto-regulatory loop by which BCL6 inhibits and regulates its own transcription (Wang et al., 2002) and by inhibition of binding of IRF4 and transcriptional repression (Saito et al., 2007). The activity of BCL6 can be also increased by mutation in the MEF2B transcription factor, a very potent *BCL6* transcriptional activator (Ying et al., 2013). These mutations promote the function of MEF2B. In 5% of DLBCL cases the loss of function mutations of *FBX011* inhibit proteosomal degradation of BCL6 (Duan et al., 2012).

The loss of the immune surveillance mechanism is affecting a significant number of DLBCL cases (Pasqualucci and Dalla-Favera, 2015). The beta-2-microglobuline (β2M) is a non-variable unit of the HLA class I complex and is not expressed on the tumour cells in approximately 60% of DLBCL cases. In 29% of DLBCL cases the β2M is lost because of mutation affecting directly its gene and in the remaining 30% due to another mechanism involving its expression (Challa-Malladi et al., 2011). The other gene involved in immune surveillance of DLBCL is CD58, a member of the immunoglobulin superfamily and a ligand of the CD2 receptor on T-cells participating in their adhesion and activation (Pasqualucci and Dalla-Favera, 2015).

Among other lesions affecting DLBCL the mutations of the Tumour Protein 53 (TP53) gene are important and common (found in approximately 20% of DLBCL cases) (Monti et al., 2012). Tumour Protein 53 is a protein which is crucial in preventing cancer formation in multicellular organisms and *TP53* is classified as a tumour suppressor gene. The role of TP53 in prevention of oncogenesis is associated with its function in i) activation of DNA repair, ii) arresting the cells at the G1/S point due to DNA damage, iii) initiation of apoptosis and iv) response to short telomeres. Mutations of *FOXO1* transcription factor are also of importance and are present in all subtypes of DLBCL (Trinh et al., 2013).

ii) Genetic abnormalities affecting GCB-DLBCL. The genetic lesions characteristic to GCB-DLBCL were very poorly characterized until recently. The majority of research focused on chromosomal translocations of *MYC*, *BCL2*, *PTEN* and mir-17-92 micro RNA cluster (Trinh et al., 2013) (Saito et al., 2009) (Lenz et al., 2008).

As described above, in 35% of DLBCL the translocation (14;18) leads to ectopic expression of *BCL2* (a key anti-apoptotic molecule) by juxtaposing the *BCL2* in direct neighborhood of the Ig locus and disturbing negative suppression by *BCL6* (Saito et al., 2009). Additionally, 40% of DLBCL cases without (14;18) translocation co-express BCL2 and BCL6 as a result of i) deregulation of Miz1 (a molecule connecting *BCL6* to *BCL2*), ii) aberant somatic hypermutation to *BCL2* promotor side and iii) mutations to *BCL2* coding sequence (Saito et al., 2009).

Approximately 15% of DLBCL cases show a translocation t(8;14) involving the *c-MYC* transcription factor gene and the Ig gene locus (Kramer et al., 1998). Amplification of the MIGH1 region encoding the microRNA 17-92 cluster is present in around 12% of GCB-DLBCL (Lenz et al., 2008). The microRNAs from this cluster enhance the lymphomagenetic function of c-MYC (He et al., 2005). The cluster also promoted the oncogenesis by inhibition of tumour suppressor gene *PTEN* and the pro-apoptotic protein BIM (He et al., 2005). The deletion of *PTEN* leads to the activation of AKT. The AKT can be also activated by phosphatidylinositol 3 kinase (PI3K) leading to inhibition of apoptosis, promotion of cell growth, cell motility and angiogenesis (Abubaker et al., 2007).

The mutations involving the histone methyltransferase *EZH2* gene have been reported in 22% of GCB DLBCL patients (Morin et al., 2010). These mutations usually cause the increased activity of the enzyme resulting in increased GCB hyperplasia and induction of DLBCL in cooperation with BCL2 (Béguelin et al., 2013). Importantly the *EZH2* inhibitors just entered the clinical trials (Roschewski et al., 2014).

Approximately 20% of GCB-DLBCL show structural damaging mutations in various components of G-proteins e.g. GNA12, SIPR2, ARHGEF1 and P2RY8 resulting in increased GC B cells survival and dissemination (Muppidi et al., 2014).

iii) Genetic abnormalities affecting ABC-DLBCL. Genetic lesions to ABC-DLBCL are better characterized and they include two main groups: activation of *NF-κβ* and block in terminal B cell differentiation (Pasqualucci and Dalla-Favera, 2015). Additionally, the recurrent lesions involve the *BCL2* locus as describe above and the *CDKN2A/2B* locus (Lenz et al., 2008).

Genetic lesions contributing to constitutive activation of *NF-κβ* are usually caused by mutations activating continuously the BCR (B-cell receptor) signaling pathway via Ig superfamily members CD79B and CD79A or CARD11, a BCR signalosom complex (Davis et al., 2010). BCR signaling is responsible for canonical activation of *NF-κβ*. Mutations of myeloid differentiation primary response gene 88 (*MYD88*) gene, a universal activating protein responsible for *NF-κβ* activating in the TLR (toll like receptor) pathway, can lead to constitutive activation of *NF-κβ* and JAK/STAT transcriptional responses (Ngo et al., 2011). Finally the activation of *NF-κβ* can follow the mutation, inactivating negative regulators of *NF-κβ* like mutation to *TNFAIP3* gene encoding A20 a dual function ubiquitin modification enzyme involved in the *NF-κβ* responses triggered by BCR and TLR stimulations (Ngo et al., 2011). The bi-allelic mutations of *TNFAIP3* inhibit the function of A20 leading to inappropriately prolonged activation of *NF-κβ*.

The second group of genetic aberrations characteristic to ABC-DLBCL are genetic lesions preventing terminal differentiation of B-cells into plasma cells. The B-cell requires for this final step PRMD1, a sequence-specific transcriptional repressor (Shapiro-Shelef et al., 2003). The mutations to *PRMD1* prevent this differentiation step. Approximately 25% of ABC-DLBCL have lost of PRDM1 gene owning to truncating mutations, missense mutations and / or genomic deletions (Pasqualucci et al., 2006).

1.3.6 *Prognostic factors in DLBCL*

As DLBCL remains a very heterogeneous diagnosis with variable outcomes, a number of different studies have been performed in recent years in order to identify clinical and biological parameters, which could then be used to select patients with high-risk disease in order to assign the most efficient treatment to them. Furthermore, it is important to recognize that risk assessment is a moving target. The introduction of a more effective new therapy rituximab, cyclophosphamide, doxorubicine, vincristine, prednisolone (R-CHOP) has significantly altered the predictive power of the currently used prognostic indicators (Sehn et al., 2007).

1.3.6.a *Clinical prognostic factors*

The treatment of patients with DLBCL is traditionally dictated by their Ann Arbor stage (Lossos and Morgensztern, 2006). This system was originally developed for patients with Hodgkin lymphoma (Carbone et al., 1971) and is based on the premise

that the disease spreads contiguously to adjacent lymph nodes (Rosenberg, 1977). This assumption is less accurate for NHL, particularly aggressive types, that are characterised by more unpredictable behaviour including early systemic dissemination and involvement of extranodal sites (Freeman et al., 1972).

Currently the most widely accepted prognostic scoring system for patients with DLBCL is the International Prognostic Index (IPI) (Sehn, 2006). Originally the index was developed in patients with WF categories F, G and H (intermediate grade lymphoma) (Shipp, 1993). This cohort of patients was very heterogeneous and included not only lymphomas of B-cell origin other than DLBCL but also lymphomas with T-cell phenotypes which are characterised by a significantly worse outcome. Subsequently with the introduction of the pathological diagnosis of DLBCL the IPI was reassessed in patients with the specific diagnosis of DLBCL (Nicolaides et al., 1998) (Wilder et al., 2002). The IPI is based on the number of negative prognostic clinical features present at the time of diagnosis (age >60 years, clinical stage (CS) III and IV, elevated serum lactate dehydrogenase (LDH) level, Eastern Cooperative Oncology Group (ECOG) performance status ≥2 and involvement of more than one extranodal site); see table 1.6 (Shipp, 1993). The IPI identifies four risk groups with predicted five-year survival rates of 73% - low risk group, 51% - low intermediate risk group, 43% - high intermediate risk group and 26% - high-risk group (Shipp, 1993). It was subsequently shown that patients ≤60 years had significantly different disease outcomes to patients >60years and an age-adjusted IPI (aaIPI) model for younger patients was developed (table 1.6) (Shipp, 1993). The aaIPI also identifies four risk groups with predicted five-years overall survival (OS) rates of 83% (low risk group), 69% (low intermediate risk group), 46% (high intermediate risk group), and 32% (high-risk group) (Shipp, 1993).

The development of the IPI and aaIPI was based on studies that included patients treated with CHOP or CHOP-like therapies. The recent addition of rituximab to CHOP has led to marked improvement in patient survival and has called into question the prognostic significance of the IPI. Recently a retrospective study on a homogeneous group of patients with DLBCL treated with R-CHOP has been performed to re-assess the predictive value of the IPI in the era of immunochemotherapy (Sehn et al., 2007).

IPI
Variables • Age >60 years • Poor performance status (ECOG ≥2) • Advanced Ann Arbor status (III – IV) • Extranodal involvement (≥2 sites) • High serum LDH (>normal) Risk groups • Low: 0 - 1 variable • Low intermediate: 2 variables • High intermediate: 3 variables • High: 4 - 5 variables
Age adjusted IPI (patients ≤60 years)
Variables • Poor performance status (ECOG ≥2) • Advanced Ann Arbor status (III – IV) • High serum LDH (>normal) Risk groups • Low: 0 variable • Low intermediate: 1 variable • High intermediate: 2 variables • High: 3 variables
Revised IPI (for rituximab treated patients)
Variables • Age >60 years • Poor performance status (ECOG ≥2) • Advanced Ann Arbor status (III – IV) • Extranodal involvement (≥2 sites) • High serum LDH (>normal) Risk groups • Very good: 0 variable • Good: 1 - 2 variables • Poor: 3 - 5 variables

Table 1.6 International prognostics index and its variants (Shipp, 1993).

Initially the outcome of patients was predicted using the standard IPI. Although the IPI remained predictive, it now distinguished only two rather than four risk groups. The first group incorporated the “low” and “low-intermediate” risk groups and the

second the "high" and "high intermediate" risk groups. When outcome was plotted according to the number of individual IPI factors present at diagnosis three risk groups were identified: a "very good" group with zero risk factors, a "good" group with 1 – 2 factors and a "poor" group with 3 – 5 factors (table 1.6). This gave rise to a new revised International Prognostic Index (R-IPI). If the two indices are compared the R-IPI is a better predictor of outcome for patients with DLBCL treated with R-CHOP.

Based on the information derived from clinical trials elevated β2M levels (>3mg/L) and the presence of more than one extranodal site of disease have been found to be negative prognostic indicators in terms of OS (Feugier et al., 2005). Bulky disease and the presence of at least one aaIPI factor were significant prognostic parameters for event-free survival (EFS) on a multivariate analysis in the MabThera International Trial Group (MInT) trial (Pfreundschuh et al., 2006). Table 1.7 represents the clinical prognostic models identified.

Author	Risk factor
Shipp 1)	Performance status, bulky disease, extranodal sites
Jagannath 2)	LDH, tumour burden
Velasquez 3)	Age, tumour burden, LDH
Swan 4)	LDH, ß$_2$-microglobulin
Coiffier 5)	LDH, stage, extranodal sites, tumour bulk
Rodriguez 6)	ß$_2$-microglobulin, LDH, stage, bulky disease, B-symptoms
Shipp 7)	Age, stage, performance status, LDH, extranodal sites
Conconi 8)	IPI, ß$_2$-microglobulin

Table 1.7 Additional clinical prognostic models in DLBCL.
1) (Shipp et al., 1986) 2) (Jagannath et al., 1986) 3) (Velasquez et al., 1989) 4) (Swan et al., 1989) 5) (Coiffier, 1991) 6) (Rodríguez et al., 1992) 7) (Shipp et al., 2002) 8) (Conconi et al., 2000)

These indices have important limitations. They rely on a small number of dichotomised predictive variables, and patients are assigned to one of a small number of risk groups rather than giving a direct prediction, such as a predictive survival probability or a predictive median lifetime on a continuous scale for each patient.

1.3.6.b *Biological prognostic markers*

The new high-throughput genomic technologies have yielded many potential biomarkers or biomarker models for prediction of survival in DLBCL. Their potential is

enormous and it is possible that in the near future they will become an integral part of our daily practice. Here the most important biomarkers and models will be described.

Individual biomarkers for prediction of survival

The heterogeneity of cancer including DLBCL is highlighted by the variable expression of molecular aberrations, which contribute to aggressive tumour behaviour namely: the capacity to sustain proliferation, evade apoptosis, inhibit differentiation and the ability to invade and promote angiogenesis (Lossos and Morgensztern, 2006). Multiple studies have assessed the relationship between patient survival and expression of specific biological markers by the malignant cells. These factors include: cell-cycle regulatory molecules (TP53, $p27^{KIP1}$, Cyclin D, Ki-67), apoptosis-related proteins (survivin, BCL2, Caspases), proteins involved in B-cell differentiation (BCL6, HGAL, CD10, CD5, fork head box protein P1 [FOXP1], protein kinase ß [PKC-ß], CD21), cell adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], CD44), angiogenesis factors (endostatin, vascular growth factor [VEGF], matrix metalloproteinase 2 and 6 [MMP-2 and MMP-6]) and others factors (IL-10, hepatocyte growth factor, major histocompatibility complex [MHC] molecules) (Lossos and Morgensztern, 2006). Table 1.8 provides an overview on selected molecular prognostic markers and their effect on patients outcome.

Prognostic marker	Effect on outcome	Mechanism
BCL2	Unfavourable	Anti-apoptosis
BCL6	Favourable	Transcriptional repressor
CD10	Favourable?	Neutral endopeptidase
CD5	Unfavourable?	B-cell differentiation
HGAL	Favourable	Germinal center phenotype
FOXP1	Unfavourable	Transcription factor
MUM1	Unfavourable	Transcription factor
Mutation p53	Unfavourable	Cell cycle regulation
Cyclin D2/D3	Unfavourable	Cell cycle regulation
Skp2	Unfavourable	Cell cycle progression
Survivin	Unfavourable	Anti-apoptosis
PKC-ß	Unfavourable	B-cell signalling
CD21	Favourable	B-cell differentiation
ICAM-1	Unfavourable	Lymphocyte trafficking
Endostatin	Unfavourable	Angiogenesis
sVEGF	Unfavourable	Angiogenesis
MMP-9	Unfavourable	Promotes metastases
Caspase 8 inhibition	Favourable	Apoptosis signalling
Caspase 9 inhibition	Unfavourable	Apoptosis signalling
Nm23-H1	Unfavourable	B-cell differentiation
sIL-10	Unfavourable	Immune response regulator
Loss MHC class II	Unfavourable	Immune surveillance

Table 1.8 Molecular prognostic markers in DLBCL (Lossos and Morgensztern, 2006).

BCL2, BCL6, CD10 and Ki-67 are the most studied molecular markers in patients with DLBCL treated with chemotherapy and immunochemotherapy.

BCL2 is an anti-apoptotic protein that is important in normal B-cell development and differentiation (Hockenbery et al., 1990). Approximately 47% to 58% of DLBCL cells express BCL2 protein (Hill et al., 1996) (Gascoyne et al., 1997). The 14;18 (q32;q21) translocation juxtaposes the *BCL2* gene from position 18q21 to the immunoglobulin heavy chain locus, resulting in BCL2 overexpression (Bakhshi et al., 1985) (Cleary and Sklar, 1985). However, this translocation is not the only mechanism that results in the overexpression of BCL2 protein as amplification of the *BCL2* gene also induces its overexpression (Monni et al., 1997) (Rao et al., 1998). Several studies have shown that *BCL2* translocation status does not correlate with patient survival

(Lossos and Morgensztern, 2006). By contrast a number of studies have reported that the overexpression of BCL2 protein in patients with DLBCL is significantly associated with poorer survival (Hill et al., 1996) (Gascoyne et al., 1997) (Kramer et al., 1996) (Hermine et al., 1996). The significance of BCL2 overexpression was re-evaluated in patients treated with CHOP and R-CHOP in the GELA trial (Mounier et al., 2003). In the group of patients treated with CHOP BCL2 protein overexpression was associated with poorer disease outcome. This was not the case in patients treated with R-CHOP. This finding was in concordance with other studies (Gutierrez-Garcia et al., 2011a) (Johnson et al., 2012). By contrast significant numbers of subsequent studies confirmed the prognostic value of BCL2 in patients treated with immunochemotherapy (Fu et al., 2008) (Salles et al., 2011). As BCL2 staining is acceptably reproducible different cut-off rates for reporting are the most likely reason for this phenomenon. Recently published studies propose that expression of BCL2 assessed with IHC may be a strong indicator of prognosis when assessed in conjunction with other molecules like Ki-67 (Salles et al., 2011) or c-MYC (Johnson et al., 2012) (Green et al., 2012).

BCL6 is a proto-oncogene involved in chromosomal translocations affecting band 3q27, the most common translocation detected in DLBCL (Chang et al., 1996) (Kerckaert et al., 1993) (Seyfert et al., 1996). BCL6 is necessary for GC formation (Ye et al., 1997) and it is almost exclusively expressed in GC lymphocytes or lymphomas originating at the GC differentiation stage (Cattoretti et al., 1995). The expression of *BCL6* may be deregulated by translocations that affect the *BCL6* locus, and also by mutations in the 5' non-translated regulatory region of the gene (Pasqualucci et al., 2003) (Wang et al., 2002). Although initial studies reported better disease outcomes for DLBCL patients with *BCL6* gene rearrangements (Offit et al., 1994), this could not be confirmed in subsequent studies (Bastard et al., 1994) (Pescarmona et al., 1997) (Jerkeman et al., 2004). By contrast, mRNA or protein expression of BCL6 as a marker of GC origin of malignant cells predicted better survival in patients with DLBCL (Hans et al., 2004) (Lossos et al., 2001). The predictive power of BCL6 mRNA and protein expression was independent of the IPI in a multivariate analysis in a study performed by Lossos et al. (Lossos et al., 2001). The US Intergroup Trial examined the prognostic value of BCL6 protein expression in patients treated with CHOP and R-CHOP (Winter et al., 2006). Whilst BCL6 positive patients receiving CHOP experienced better outcomes, BCL6 status did not influence the outcomes in R-CHOP treated patients. Further reports in patients treated with R-CHOP are inconclusive (Salles et al., 2011)

(Copie-Bergman et al., 2009) (Natkunam et al., 2008) (Seki et al., 2009) (Winter et al., 2006). The reason for this may be due to the varying expression thresholds employed and the fact that IHC staining patterns are reported to be unstable with poor reproducibility, particularly when assessed using tissue microarrays (TMAs) (de Jong et al., 2007) (Linderoth et al., 2007).

Another molecular prognostic factor for DLBCL evaluated in a number of trials is the expression of the common acute lymphoblastic leukaemia antigen (CD10), a cell membrane-associated neutral endopeptidase. CD10 appears twice during lymphocyte differentiation, firstly on pro-B-cells, then it is lost during cell maturation to naïve B cells, and secondly it is re-expressed during antigen-dependent GC maturation. Twenty to thirty per cent of DLBCL tumours express CD10 protein (Colomo et al., 2003) (Fabiani et al., 2004). Expression of CD10 as a marker of GC origin has been assessed for prediction in patients with DLBCL and has revealed conflicting findings. While some authors reported increased OS in patients expressing CD10 protein (Ohshima et al., 2001), others were not able to confirm this correlation (Colomo et al., 2003) (Fabiani et al., 2004). The studies on prognostic impact of CD10 in patients treated with immunochemotherapy delivered controversial results (Salles et al., 2011) (Seki et al., 2009).

Ki-67 is a nuclear antigen expressed by dividing cells. It is another molecule with controversial prognostic value in DLBCL (Lossos and Morgensztern, 2006). Miller et al reported in two separate studies a significant decrease in overall survival in patients with high expression of Ki-67 protein (Miller et al., 1994). By contrast the Nordic Lymphoma Study and other additional studies failed to confirm the prognostic value of Ki-67 protein expression (Colomo et al., 2003) (Jerkeman et al., 2004) (Zhang et al., 1999) (Saez et al., 2004). These conflicting findings may partly be due to different cut off points of positivity / negativity used by the various groups (Lossos and Morgensztern, 2006). In two recent papers Ki-67 expression had significantly predictive power for survival estimations in patients treated with immunochemotherapy (Salles et al., 2011) (Culpin et al., 2013). In the study of Sales it was a basic part of the predictive model together with expression of BCL2 and the IPI (Salles et al., 2011).

Gene expression profiling for prediction of survival

Although the studies on individual biomarkers in DLBCL provide additional prognostic information to the IPI and have enriched our knowledge on the

pathophysiology of DLBCL, their results were often conflicting and inconclusive. This was probably due to the retrospective nature of the various studies, small sample size and lack of uniformity in experimental design (Sehn, 2006). The role of individual biomarkers in clinical practice may be limited by the complexity of biological processes, the involvement of multiple genes, signalling pathways, and regulatory mechanisms (Lossos and Morgensztern, 2006). Genome-scale biomarker expression profiles assessed by DNA microarrays (Shipp et al., 2002) (Alizadeh et al., 2000) (Rosenwald et al., 2002) or real-time polymerase chain reaction (RT-PCR) (Lossos et al., 2004) have been used recently to assess the correlation between prognosis and molecular features of tumour cells in DLBCL.

The pivotal study of microarrays in DLBCL was performed by Alizadeh et al. using spotted DNA microarrays made by mechanical deposition of double-stranded cDNA fragments (approximately 500 base pairs) or long oligonucleotides (50-70 mer) on glass slides (Alizadeh et al., 2000). Among 42 patients with DLBCL treated with CHOP-like chemotherapy two molecularly distinct types of tumour were identified; one expressing genes characteristic of germinal centre B cells - "germinal centre B-cell-like DLBCL (GCB)", and the second type expressing genes normally induced during in vitro activation of peripheral blood B-cells "activated B-cell like DLBCL (ABC)". The 5-year overall survival for patients with GCB was 76% and for those with ABC only 16%. This difference was statistically significant ($p<0.01$). Rosenwald et al. confirmed these observations using the same methodology on 240 patients with DLBCL treated with CHOP-like chemotherapy (Rosenwald et al., 2002). This later study identified a third phenotype called "type 3 DLBCL". Patients with GCB had a 5 –year survival rate of 60%, compared with a rate of 39% for patients with type 3 DLBCL and 35% for those with ABC ($p<0.001$) (Rosenwald et al., 2002). The subgroups differed in that the (14;18) translocation involving the *BCL2* gene and the amplification of the *c-rel* locus on chromosome 2p occurred only in patients with GCB, suggesting the involvement of a different oncogenic mechanism in each subgroup. Shipp et al using oligonucleotide microarrays in tumour biopsies of 58 patients with DLBCL treated with CHOP (Shipp et al., 2002) analyzed the expression of 6,817 genes. In this study, two groups were identified: the group predicted to be cured and those predicted to have fatal/refractory disease. The 5-year overall survival was 70% and 12%, respectively ($p=0.00004$).

Additionally, Rosenwald et al and Shipp et al on the basis of a small number of previously identified predictive genes were able to construct survival models that could

be used routinely in the clinical assessment of patients with DLBCL (Shipp et al., 2002) (Rosenwald et al., 2002). The 13-gene IPI-independent model of Shipp et al included the following genes: *dystrophin related protein 2*, *protein kinase C gamma*, *MINOR*, *5-hydroxytryptamine 2B receptor*, *H731*, *transducin-like enhancer protein 1*, *PDE4B*, *protein kinase C-beta-1*, *oviductal glycoprotein*, *zinc-finger protein C2H2-150*, and three expressed sequence tags (Shipp et al., 2002). The model derived by Rosenwald et al. was based on the expression of 17 genes: *BCL6*, *HGAL*, *clone 1334260*, *HLA-DPα*, *clone 1334260*, *HLA-DPα*, *HLA-DQα*, *HLA-DRα*, *HLA-DRβ*, *α-actin*, *collagen type III α1, connective-tissue growth factor*, *fibronectin 1*, *KIAA0233*, *urokinse plasminogen activator*, *c-MYC*, *E21G3*, *NPM3*, and *BMP6* (Rosenwald et al., 2002). Interestingly, there was no overlap of predictive genes between the two models.

Using the RT-PCR technique Lossos et al. performed a study on 36 genes whose expression had been previously reported to predict survival in patients with DLBCL. The strongest predictors were found to be *LIM domain only 2* (*LMO2*), *BCL6*, *fibronectin 1* (*FN1*), *CCND2*, *SCYA3* and *BCL2*. In multivariate analysis the expression of these genes was independent of the IPI and increased its predictive power. This model identified three independent risk groups with 5-year OS rates of 65%, 49% and 15% for the low, intermediate and high-risk groups respectively (p=0.004) (Lossos et al., 2004).

Immunohistochemical models for prediction of survival

Although gene expression profiling by DNA microarrays identified biologically distinct subtypes of DLBCL, this kind of profiling is not a practical tool for risk assessment in routine patient care. This is due to the lack of standardized, commercially available tests, to the requirement for fresh or fast frozen tissue specimens and to the high costs. The semi-quantitative assessment of protein expression in paraffin-embedded tissue can be performed by IHC, which is used routinely for histological evaluation in lymphoma diagnostics. In several recent studies the information derived from gene-array investigations has been used to create immunohistochemical models that predict the cell of origin and / or survival.

Barrans et al and Colomo et al assessed the prognostic value of the expression of proteins associated either with GCB phenotype or ABC phenotype. However these studies did not use hierarchical staining and never gained general acceptance (Barrans et al., 2002) (Colomo et al., 2003).

Subsequently models based on the assessment of expression of GC or ABC related proteins were developed (Hans et al., 2004) (Choi et al., 2009) (Muris et al., 2006) (Nyman et al., 2009) (Meyer et al., 2011) (Visco et al., 2012). Some of these models were originally developed on patients treated with chemotherapy only (Hans et al., 2004) (Muris et al., 2006), whilst others included patients treated with immunochemotherapy (Choi et al., 2009) (Nyman et al., 2009) (Meyer et al., 2011) (Visco et al., 2012). All of them were recently reassessed on representative cohorts of patients treated with immunochemotherapy (Culpin et al., 2013) (Fu et al., 2008) (Meyer et al., 2011). Only the models of Hans and Choi used samples with completed GEP. Others investigators will not have this data until revalidation studies are completed (Hans et al., 2004) (Choi et al., 2009) (Muris et al., 2006) (Nyman et al., 2009) (Meyer et al., 2011) (Visco et al., 2012). Table 1.9 provides a list of the most important IHC models for prediction of survival in DLBCL and subsequent studies which revaluated their predictive value. Figure 1.5 shows the design of the most important models.

Model	Proved CHOP-era	Prediction	Proved R-CHOP-era	Prediction
Hans 1)	Berglund 7)	Yes	Meyer 5)	Yes
	Sjo 8)	Yes	Fu 17)	Yes
	Haarer 9)	Yes	Visco 6)	Yes
	Chang 10)	Yes	Culpin 18)	Yes
	van Imhoff 11)	Yes	Nyman 4)	No
	Nyman 12)	Yes	Nyman 12)	No
	Amara 13)	Yes	Natkunam 14)	No
	Natkunam 14)	No		
	Veelken 15)	No		
	Dupuis 16)	No		
Muris 2)	Sjo LD 8)	Yes	Nyman 4)	Yes PFS / No OS
			Meyer 5)	Yes
			Culpin 18)	No
Choi 3)	NA	NA	Meyer 5)	Yes
			Visco 6)	Yes
			Culpin 18)	Yes PFS / No OS
Nyman 4)	NA	NA	Meyer 5)	Yes
			Culpin 18)	No
Modified Hans 5)	NA	NA	Culpin 18)	Yes
Modified Choi 5)	NA	NA	Culpin 18)	Yes PFS / No OS
Tally 5)	NA	NA	Visco 6)	Yes
Visco 6)	NA	NA	Culpin 18)	No

Table 1.9 Immunohistochemical models for prediction of survival in DLBCL.
1) (Hans et al., 2004); **2)** (Muris et al., 2006); **3)** (Choi et al., 2009); **4** (Nyman et al., 2009); **5)** (Meyer et al., 2011); **6)** (Visco et al., 2012); **7)** (Berglund et al., 2005); **8)** (Sjo et al., 2007); **9)** (Haarer et al., 2006); **10** (Chang et al., 2004); **11** (van Imhoff et al., 2006); **12** (Nyman et al., 2007); **13)** (Amara et al., 2008); **14)** (Natkunam et al., 2008); **15)** (Veelken et al., 2007); **16)** (Dupuis et al., 2007) **17)** (Fu et al., 2008) and **18)** (Culpin et al., 2013)

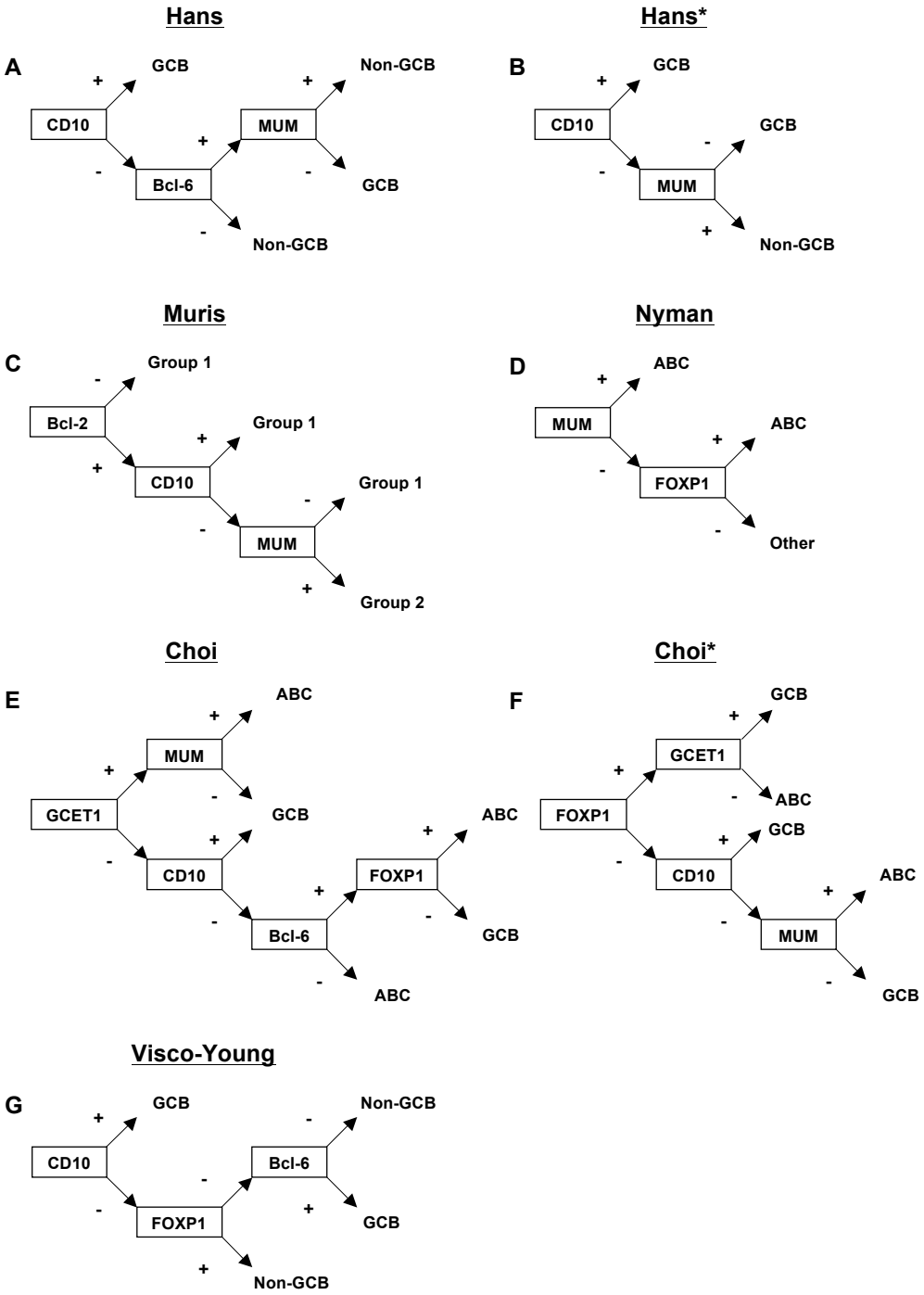

Figure 1.5 The design of the most important IHC algorithms in DLBCL.
GCB, Germinal centre B-cell-like DLBCL; ABC, Activated B-cell-like DLBCL. **(A)** The Hans model (Hans et al., 2004); **(B)** The modified Hans model (Meyer et al., 2011); **(C)** The Muris model (Muris et al., 2006); **(D)** The Nyman model (Nyman et al., 2009); **(E)** The Choi model (Choi et al., 2009); **(F)** The modified Choi model (Meyer et al., 2011); **(G)** The Viso-Young model (Visco et al., 2012)

Hans et al developed a prognostic model based on the expression of three biomarkers; CD10, BCL6 and MUM1/IRF4 to assign patients to GCB and ABC

phenotype in patients with de novo DLBCL treated with CHOP (Hans et al., 2004). MUM1/IRF4 is a lymphoid-specific member of the interferon (IFN) regulatory factor family of transcription factors (Mamane et al., 1999) (Tsuboi et al., 2000) (Falini et al., 2000), and it is a reliable marker of "late-stage GC" or "post-GC" B cells (Tsuboi et al., 2000). There were statistically significant differences in overall survival between patients with GCB and ABC phenotype ($p<0.001$) (Hans et al., 2004). Additionally GEP analysis using cDNA microarrays were performed in all patients. Eight-seven per cent of patients assigned to GCB by the IHC model had the GCB signature in cDNA microarray. In patients with ABC 73% had the same signature in cDNA microarray. The Hans model was the first published IHC based model and was developed on a cohort of patients treated with chemotherapy only. It has subsequently been tested in several studies on patients treated with chemotherapy only (Berglund et al., 2005) (Sjo et al., 2007) (Haarer et al., 2006) (Chang et al., 2004) (van Imhoff et al., 2006) (Nyman et al., 2007) (Natkunam et al., 2008) (Veelken et al., 2007) (Dupuis et al., 2007) and later on patients treated with immunochemotherapy (Meyer et al., 2011) (Fu et al., 2008) (Culpin et al., 2013) (Visco et al., 2012) (Nyman et al., 2007) (Natkunam et al., 2008) (Nyman et al., 2009). Some of these studies confirmed its predictive power (Berglund et al., 2005) (Sjo et al., 2007) (Haarer et al., 2006) (Chang et al., 2004) (van Imhoff et al., 2006) (Nyman et al., 2007) (Meyer et al., 2011) (Fu et al., 2008) (Culpin et al., 2013) (Visco et al., 2012) whilst others did not (Natkunam et al., 2008) (Veelken et al., 2007) (Dupuis et al., 2007) (Nyman et al., 2007) (Nyman et al., 2009). These partially contradictory results prompted the researcher to investigate development of other models.

Choi et all developed other IHC models based on the expression of GCET1, MUM1, CD10, BCL6 and FOXP1 in patients with DLBCL treated with immunochemotherapy (Choi et al., 2009). The GCET1 transcript is highly associated with the GCB subtype of DLBCL, and the GCET1 protein was later found to be restricted to B-cell neoplasms with a presumed GC B-cell origin (Choi et al., 2009). The FOXP1 gene on 3p14.1 encodes a member of the FOX family of transcription factors. Two alternatively spliced *FOXP1* mRNA iso-forms are highly expressed in ABC-DLBCL, and some studies showed that uniform high expression of FOXP1 protein is associated with an inferior survival in DLBCL patients (Choi et al., 2009). The algorithm closely approximated to the GEP classification with 93% concordance. The differences in OS for GCB and ABC were also significant ($p<0.001$). The predictive

value of the Choi algorithm was successfully confirmed by the work of Meyer et al, Visco et al and Culpin et al (for progression free survival [PFS] only) (Culpin et al., 2013) (Meyer et al., 2011) (Visco et al., 2012).

Recently, Meyer et al developed a simplified version of both models by the removal of BCL6 expression as a factor (Meyer et al., 2011). This step should improve the reproducibility of the models as anti-BCL6 staining is very sensitive and several laboratories reported difficulties in reproducibility. The modified version shows the same efficacy but a much simplified methodology (Meyer et al., 2011). This was confirmed by the study of Culpin et al. (Culpin et al., 2013).

The models developed by Muris et al and Nylman et al deserve special attention as neither of them include the BCL6 protein and both are based on the expression of molecules associated with the activated B-cell phenotype (Muris et al., 2006) (Nyman et al., 2009). The Muris model based on expression of BCL2, CD10 and MUM1 was able to stratify patients into low and particularly high clinical risk groups ($p=0.04$ and $p<0.0001$; respectively) (Muris et al., 2006). The model was designed using a DLBCL cohort treated with chemotherapy only, but was subsequently reassessed in patients treated with immunochemotherapy with contradictory results (Culpin et al., 2013) (Meyer et al., 2011) (Nyman et al., 2009). In the study of Meyer et al the algorithm maintained its predictive power (Meyer et al., 2011) but in the study of Culpin et al it lost it (Culpin et al., 2013). In Nyman's study prediction was retained for PFS and lost for OS. The Nyman model is based on the expression of MUM1 and FOXP1. Both proteins are associated with the ABC phenotype (Nyman et al., 2009). Tumours with high expression of MUM1 or FoxP1 were defined as ABC phenotype and they have a significantly shorter failure-free survival (FFS) ($p=0.04$) and OS ($p=0.110$) compared with other lymphomas. The model was developed on patients treated with immunochemotherapy and was subsequently re-evaluated by Meyer et al where it retained its predictive value (Meyer et al., 2011) and Culpin et al where it did not (Culpin et al., 2013).

The most recent model based on sequential evaluation of immunohistochemical markers is the Visco-Young model, which is based on expression of CD10, FOXP1 and BCL6 (Visco et al., 2012). This model could distinguish between cases with GCB and non-GCB phenotype. The OS and PFS for GCB were significantly longer than for non-GCB ($p=0.003$ and $p=0.002$; respectively). The algorithm was reported to have an extraordinary concordance with GEP results of 92.6%.

In addition to all the models described above Meyer et al designed the "Tally – model" which deserves particular attention. In contrast to other IHC models it does not rely on a particular sequence of applied staining but on the sum of two GC-markers (CD10 and GCET1), and two ABC-markers (Mum1 and FoxP1) with one "stratifying marker" – LMO2 (Meyer et al., 2011). Although the model was able to precisely assign the patient either to the GCB or ABC group with different OS and PFS, it also had some drawbacks. It uses antibodies which are not commonly used in the laboratories and which are difficult to use, and it predicts OS slightly less precisely compared with the Choi algorithm.

There are only a few studies that compare all the important models on the same group of patients (Meyer et al., 2011) (Culpin et al., 2013) (Gutierrez-Garcia et al., 2011a). Their results demonstrate that there is no consensus regarding the best immunohistochemical model for prediction of survival in DLBCL. Meyer et al reassessed most important IHC models (Hans, modified Hans, Choi, modified Choi, Muris, Nyman and Tally) using TMA in an unselected population of DLBCL patients treated with immunochemotherapy and with established GEP origin (Meyer et al., 2011). The algorithms of Tally, Choi, modified Choi, Hans and modified Hans were the best at predicting cell of origin as defined by GEP. By contrast the Muris algorithm was the least accurate at predicting tumour cell origin being too specific for ABC-type DLBCL. All tested algorithms were able to separate the patients into two groups with significantly different survivals. All algorithms predicted EFS and OS independent of the IPI with the exception of the Nyman algorithm and OS. The Muris algorithm was the best at predicting OS and EFS. However the authors concluded that its application in everyday practice is limited because of its poor effectiveness at predicting cell of origin. The Choi, Hans and Tally algorithms were the others most accurate at predicting OS and PFS. Recently our group also published data on reassessment of IHC algorithms (Hans, modified Hans, Choi, modified Choi, Muris, Nyman and Visco-Young) in an unselected population of DLBCL patients treated with immunochemotherapy using whole IHC sections (Culpin et al., 2013). Unfortunately we were not in possession of GEP results, thus the data is limited to the assessment of prediction of survival only. The Hans, modified Hans and Muris were predictive for OS and PFS and Choi and modified Choi for PFS only. The Visco-Young and Nyman were not predictive. Gutiérrez-Garcia assessed the predictive value of the following algorithms: Colmo (was not described here in detail), Hans, Muris, Choi and Tally using TMA in patients with

DLBCL uniformly treated with immunochemotherapy (Gutierrez-Garcia et al., 2011a). The study revealed very low concordance of GCB / non GCB cases compared with the results of GEP and surprisingly none of the assessed algorithms was able to retain the prognostic impact of the GCB / non GCB groups.

Despite the advantages of IHC models in diagnosis of DLBCL their value in stratification of the treatment of DLBCL is still limited. There are several issues, which could contribute to the disparity in results observed to date. Firstly, the cohort of patients employed in the study needs to be representative of the whole population and the patients need to be treated with identical treatments. In several studies on individual markers or IHC models the patients were recruited from highly preselected clinical trials, many received chemotherapy without the addition of rituximab and sometimes were not treated with uniform regimens. Secondly, different studies used different materials; TMA vs. whole tissue sections, or applied different antibodies with different fixation and staining procedures and used different cut-offs. Finally IHC is an examiner dependent, semi-quantitative method and thus will always have a certain degree of subjectivity. In the study on validation of immunohistochemical prognostic markers in DLBCL de Jong et al. concluded that IHC for subclassification of DLBCL is feasible and reproducible, but exhibits varying rates of concordance for different markers (de Jong et al., 2007). These findings may explain the wide variation of biomarker prognostic impact reported in the literature. Harmonization of techniques and centralized consensus review appears mandatory when using immunohistochemical biomarkers for treatment stratification.

1.3.7 *Treatment of DLBCL*

DLBCL has an aggressive natural history but most patients have a relatively good response to chemotherapy at least initially. It is maintenance of achieved remissions that is the bigger therapeutic problem. Although overall response rate (ORR) with modern therapeutic regimens vary between 75% and 80%, only 40% - 50% of patients achieve long-term remission (Coiffier, 2001).

Chemotherapy with six – eight cycles of CHOP has been the mainstay of therapy since its development in the 1970s (McKelvey et al., 1976). The treatment outcome for patients with DLBCL varies considerably based on initial stage of disease. For patients with early-stage disease (stages I and II non-bulky), chemotherapy with “abbreviated CHOP”, 2 – 4 cycles plus involved field radiation therapy seems to be effective. 5-year

event free survival reaches 73% and overall survival is 84% (Horning et al., 2004). By contrast the outcome of patients with advanced disease treated with CHOP-like therapy is not satisfactory. This therapy can induce complete responses in only 44 % of patients, with three-year EFS and OS rates of 43% and 55%, respectively (Fisher et al., 1993).

In order to try and improve treatment outcomes new and more complex regimens were developed in the 1970s and 1980s. Some are based on dosage escalation, others are multidrug chemotherapies that include additional drugs. Initial results from single-institution studies using new multidrug regimens were promising and suggested that the number of patients cured with those regimens might be doubled compared with results achieved with CHOP-like regimens. However, these findings were not confirmed by multicentre prospective randomised trials carried out by the Southwest Oncology Group (SWOG) and the ECOG (Fisher et al., 1993). Results showed that the new regimens were more toxic and the achieved remissions rates and OS were not improved compared with those achieved using CHOP alone (Fisher et al., 1993).

The role of chemotherapy intensification was re-investigated in the late 1990s with the availability of granulocyte colony stimulating factors (G-CSF) or erythropoietin, which were supposed to reduce chemotherapy induced toxicities. The addition of etoposide to CHOP with or without time intensification improved complete remission (CR) and 5-year EFS for patients < 60 years but not for those > 60 years (Pfreundschuh et al., 2004a) (Pfreundschuh et al., 2004b).

High-dose chemotherapy (HDCT) with up-front autologous stem-cell transplantation (ASCT), was another therapy option investigated in order to try and improve treatment responses in patients with poor-prognosis DLBCL. A variety of regimens were used but conflicting results were reported. A recent meta-analysis analysing efficacy of HDCT compared to conventional therapy in DLBCL patients found no evidence that HDCT improved OS and EFS in good risk DLBCL patients and the evidence for poor risk patients was inconclusive (Greb et al., 2007). Inadequate selection of patients and use of inappropriate induction regimens were postulated as possible reasons for the results. Therefore, the role of ASCT as initial therapy for patients judged to be at high-risk of treatment failure with conventional therapy remains to be assessed in prospective clinical trials.

Further attempts to improve the outcome of CHOP treatment have been made by combining it with immune-factor-based therapy such as the infusion of B-cell specific monoclonal antibodies. The CD20 antigen is expressed on normal and malignant B-

lymphocytes and it is known to regulate the cell cycle and cell differentiation (Maloney et al., 1994). Activation of CD20 by binding to its antibodies induces apoptosis of CD20 expressing lymphocytes. Rituximab (MabThera, Rituxan Roche, Neuillyu-sur-Seine, France) is a chimeric human/murine immunoglobulin G1 monoclonal antibody that binds specifically to CD20. Activation of CD20 on lymphoma cells by rituximab results in complement mediated lysis of tumour cells, antibody dependent cell mediated cytotoxicity and induction of apoptosis (Golay et al., 2000) (Hofmeister et al., 2000) (Shan et al., 1998) (Cartron et al., 2004) (Eisenbeis et al., 2003). Recent clinical studies have shown that rituximab acts synergistically with chemotherapy in patients with B cell lymphoma. In phase II trials Rituximab demonstrated efficacy in patients with DLBCL either alone or in combination with CHOP (Sonkoly et al., 2007) (Vose et al., 2001a). In a randomized study carried out by the GELA on elderly untreated patients with DLBCL, the addition of rituximab to CHOP significantly increased the CR rate (76% vs. 63%, $p=0.005$), and prolonged EFS and OS without a significant increase in toxicity (Coiffier et al., 2002). The five-year follow-up study showed that EFS, PFS, disease free survival (DFS), and OS remained statistically significant in favour of the combination R-CHOP ($p=0.00002$, $p<0.00001$, $p<0.00031$ and $p<0.0073$, respectively) (Feugier et al., 2005). Since the publication of the GELA Study R-CHOP has become the new standard in the treatment of elderly patients with DLBCL. Similarly, a retrospective study from British Columbia showed that the addition of rituximab to CHOP-like treatment improved EFS and OS in a group of previously untreated patients with DLBCL of all ages (Sehn et al., 2005). Additionally the MInT revealed that patients receiving CHOP or CHOP-like therapy with rituximab had a significantly higher 3-year EFS and OS compared with those who received chemotherapy alone (79% vs. 59%, $p<0.0001$ and 93% vs. 84%, $p=0.0001$; respectively). Importantly, there were no differences between the patients treated with R-CHOP and those given the more-intensified regimen cyclophosphamide, doxorubicine, vincristine, etoposide, prednisolone (CHOEP) (Pfreundschuh et al., 2006). Furthermore, data analysis showed that R-CHOP was an effective treatment for the younger patients with good-prognosis DLBCL. Although the results of prospective multicentre clinical trials appear to be very promising, the experience of clinicians working with unselected populations of patients with DLBCL seems to be less optimistic. In particular the treatment results for patients with poor-prognosis disease remain unsatisfactory.

More recent trials have focussed on improving the treatment of DLBCL on the basis of time intensification of the R-CHOP regimen with the support of growth factors (Halaas et al., 2005). Initial results are not promising nor are the results of the studies on the role of intermediate positron emission tomography (PET) scanning to aid treatment decisions.

Another new approach in the therapy of DLBCL is the combination of R-CHOP with new less-toxic cytostatic agents, antibodies or small molecules (Zaja et al., 2006). To date findings of the first clinical trials which combine R-CHOP and antibodies to VEGF (bevacizumab), or anti-CD22 antibodies (epratuzumab) are very promising but require further study (Micallef et al., 2006) (Ganjoo et al., 2006). Targeting the affected pathways in GCB and ABC DLBCL with genasence and bortezomib is other new therapeutic option.

In conclusion, the treatment of patients with DLBCL, particularly those of high-risk, remains an important unresolved issue. New therapeutic concepts need to be developed particularly in the area of risk stratification. The above statements are of particularly high importance in view of the very disappointing results in relapsed/refractory patients with DLBCL. Patients with relapsed or progressive disease (PD) still have a very poor prognosis. High dose chemotherapy followed by ASCT is the treatment of choice for these patients (Verdonck et al., 1992) (Vose et al., 2001b) (Prince et al., 1996) (Saez et al., 1994) (Nademanee et al., 2000) (Kewalramani et al., 2000) (Philip et al., 1995). The most compelling evidence for the superiority of HDCT compared with conventional-dose salvage therapy in relapsed and progressive NHL is based on the randomized “Parma trial” (Philip et al., 1995). In this study all patients received two cycles of conventional chemotherapy. Responders were randomized to receive either four cycles of conventional chemotherapy or HDCT followed by ASCT. Analysis at five years revealed that patients treated with HDCT had superior outcomes as measured by freedom from treatment failure (FFTF) (12% vs. 46%) and by OS (32% and 53%). However, in the “Parma trial” more than 50% of patients treated in the HDCT-arm relapsed and most of them died. Recent published results of second-line treatments based on high-dose (HD) protocols and ASCT are very limited (Vellenga et al., 2008) (Sieniawski et al., 2007) (Josting et al., 2005).

1.4 Peripheral T-cell lymphoma (PTCL)

1.4.1 *Definition of PTCL*

PTCL is a heterogeneous group of malignancies derived from mature (post-thymic) T-cells and NK-cells. PTCLs are characterised by a broad range of cellular composition. Some subtypes lack identified immunophenotypic profiles and defined cellular origin (de Leval and Gaulard, 2008). With the exception of ALCL, ALK positive tumours there are no known molecular markers in PTCL (de Leval and Gaulard, 2008). Although the current WHO 2008 Classification of Tumours of Haematopoietic and Lymphoid Tissues has included several new biological-genetic features, there is still a great emphasis on clinical features and anatomic location in the classification of PTCL (Swerdlow et al., 2008). There are approximately 23 defined types of lymphoid malignancies with T-cell origin, see table 1.2.

1.4.2 *Epidemiology of PTCL*

PTCLs are rare diseases, altogether accounting for less than 15% of NHL (Savage, 2007). In an evaluation of NHL performed worldwide on 1403 patients by the International T-cell Lymphoma Project (ITLP), PTCL was found in only 7% of cases, and anaplastic large T/null-cell lymphoma in 2.4% (Vose et al., 2008). The study revealed that the most common subtype is PTCL NOS with 25.9%, followed by AILT with 18.5% and natural killer/T-cell lymphoma with 10.4%,.

Taking into consideration the fact that the total number of PTCLs is rather small then some of the lymphoma subtypes are extremely rare. The disease also shows a geographic variation; in Western countries it accounts for 5% - 10% of all NHL and 15% - 20% of aggressive lymphomas. By contrast, PTCL is more common on the Asian continent accounting for 15% - 20% of all lymphomas, (21% in Hong-Kong). Subtypes of PTCL also show a geographic variation in incidence, (see table 1.10). In both North America and Europe PTCL NOS was the most common subtype with 34.4% and 34.3%, respectively. Next in incidence were AITL and ALCL, ALK positive, both with 16% in North America. In Europe AITL accounted for 28.7% and EATL (9.1%). In contrast ATLL was at 25% the most common subtype in Asia, followed by NK/T-cell lymphoma (22.4%), PTCL NOS (22.4%) and AITL (17.9%) (Vose et al., 2008). The presence or absence of pathogenetic factors in different regions may be at least partially

responsible for these differences e.g. HTLV-1 for ATLL, EBV for NK/T-cell lymphoma or coeliac disease for EATL (Vose et al., 2008).

	North America (%)	Europe (%)	Asia (%)
PTCL – NOS	34.4	34.3	22.4
Angioimmunoblastic	16.0	28.7	17.9
Anaplastic large cell lymphoma, ALK positive	16.0	6.4	3.2
Anaplastic large cell lymphoma, ALK negative	7.8	9.4	2.6
Natural killer/T-cell lymphoma	5.1	4.3	22.4
Adult T-cell leukaemia/lymphoma	2.0	1.0	25.0
Entheropathy Associated T-cell Lymphoma	5.8	9.1	1.9
Hepatosplenic T-cell lymphoma	3.0	2.3	0.2
Primary cutaneous anaplastic large cell lymphoma	5.4	0.8	0.7
Subcuteneos panniculitis-like	1.3	0.5	1.3
Unclassifiable T-cell	2.3	3.3	2.4

Table 1.10 Major Lymphoma Subtypes by Geographic Region (Vose et al., 2008).

1.4.3 *Histopathology and classification of PTCL*

The classification of T-cell lymphoma is based on both morphologic and clinical criteria. WHO 2008 classification includes approximately 22 entities (six of them provisional), which can be classified according to their presentation, see table 1.11. (Swerdlow et al., 2008).

More detailed characteristics of those subtypes included in our studies are provided here for: PTCL NOS; ALCL, ALK positive; ALCL, ALK negative, extranodal NK/T-cell lymphoma, nasal type, EATL and hepatosplenic T-cell lymphoma.

Leukaemic or disseminated
• T-cell prolymphocytic leukaemia • T-cell large granular lyphocytic leukaemia • Chronic lymphoproliferative disordes of NK cells* • Aggressive NK-cell leukaemia • Adult T-cell lymphoma/leukaemia (HTLV1 – positive) • Systemic EBV-positive T-cell lyphoproliferative disorders of childhood
Nodal
• Angioimmunoblastic T-cell lymphoma (AILT) • Anaplastic large cell lymphoma, ALK-positive • Anaplastic large cell lymphoma, ALK-negative* • Peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS)
Extranodal
• Extranodal NK/T-cell lymphoma, nasal type • Enteropathy-associated T-cell lymphoma • Hepatosplenic T-cell lymphoma
Extranodal – cutaneous
• Mycosis fungoides • Sezary syndrome • Primary cutaneous CD30+ lymphoproliferative disorder • Primary cutaneous anaplastic large cell lymphoma • Lymphomatoid papulosis • Subcutaneous panniculitis-like T-cell lymphoma • Primary cutaneous gamma-delta T-cell lymphoma* • Primary cutaneous aggressive epidermotropic CD8+ cytotoxic T-cell lymphoma* • Primary cutaneous small/medium CD4+ T-cell lymphoma*

Table 1.11 Classification of mature T/NK-cell neoplasms according to 2008 WHO classification of lymphoid malignancies (Swerdlow et al., 2008). *) designates provisional entities

1.4.3.a *PTCL, not otherwise specified*

This is a heterogeneous category of nodal and extranodal mature T-cell lymphomas that do not correspond to any of the specifically defined subtypes of mature T-cell lymphoma in the current classification (Swerdlow et al., 2008).

PTCL NOS accounts for approximately 30% of all mature T-cell neoplasms. Most patients are adults and predominantly male. The disease is rare in children (Rizvi et al., 2006).

The most common disease sites are peripheral lymph nodes. However any site may be affected and generalized disease with involvement of BM, liver, spleen and extranodal tissue is often seen. The involvement of PB can be seen but the leukemic variant is uncommon. Primary extranodal involvement may also occur with gastrointestinal tract being the most often involved (Rizvi et al., 2006). Most patients present with lymphadenopathy and have advanced stage disease with B-symptoms (Rizvi et al., 2006). Other paraneoplastic syndromes may be seen, for example, eosinophilia, pruritus or haemophagocytic syndrome (Rizvi et al., 2006).

In the lymph nodes PTCL NOS are characterised by paracortical or diffuse growth patterns with destruction of lymph node architecture (Swerdlow et al., 2008). The cytological spectrum is extremely broad. Most cases consist of numerous medium- or large-sized cells with irregular, pleomorphic, hyperchromatic or vesicular nuclei, prominent nucleoli and many mitotic figures (Harris et al., 1994) (Jaffe, 2006). Clear cells and Reed-Sternberg-like cells can also be seen. An inflammatory background is often present with small lymphocytes, eosinophils, plasma cells and large B-cells (Warnke et al., 2007). There are three defined morphological variants of PTCL NOS: 1) lymphoepithelioid (Lennert lymphoma), 2) follicular and 3) T-zone. Extranodal involvement has a diffuse infiltration pattern composed of similar cells.

PTCL NOS is characterized by an aberrant T-cell phenotype with frequent down regulation of CD5 and CD7 (Went et al., 2006). Most nodal cases are CD4+/CD8-, although double positivity and negativity is also seen, as is CD8, CD56 and cytotoxic granule expression (Went et al., 2006). T-cell receptor beta-chain is usually expressed and CD52 is absent in 60% (Chang et al., 2007) (Piccaluga et al., 2007b) (Rodig et al., 2006). CD30 can be expressed, exceptionally with CD15 (Barry et al., 2003) (Went et al., 2006). PTCL NOS usually lacks a follicular T helper phenotype with the exception of the follicular variant (Attygalle et al., 2007) (Dorfman et al., 2006) (Went et al., 2006). The proliferation index is usually high and Ki67 rates >70% are associated with a worse prognosis (Went et al., 2006).

TCR genes are clonally rearranged in most cases (Rizvi et al., 2006). PTCL NOS is a highly aberrant neoplasm and complex karyotypic changes are common (Rizvi et al., 2006). Recurrent chromosomal gains have been observed in chromosomes 7q, 8q, 17q and 22q and recurrent losses in 4q, 5q, 6q, 9p, 10q, 12q and 13q (Zettl et al., 2004) (Thorns et al., 2007). Genetic imbalances and gene expression signatures differ from those of AILT or ALCL.

1.4.3.b *Anaplastic large cell lymphoma, anaplastic lymphoma kinase positive*

ALCL, ALK positive is a T-cell tumour consisting of large cells with abundant cytoplasm, pleomorphic, often horseshoe-shaped nuclei, with a translocation involving the ALK gene and expression of CD30 (Swerdlow et al., 2008).

The tumour is most frequent in the first 3 decades of life and accounts for approximately 10% - 20% of childhood lymphomas and 3% of adult NHL. It shows a male predominance (Stein et al., 2000) (Benharroch et al., 1998) (Falini et al., 1999a).

ALCL, ALK positive can involve both nodal and extranodal sites. The most common extranodal sites are: skin, bone, soft tissue, lung and liver (Benharroch et al., 1998) (Falini et al., 1999a). The involvement of gut or CNS is rare. The number of patients with BM involvement differs depending on the method of assessment (Fraga et al., 1995). The majority of patients present with advanced stage disease with peripheral or abdominal lymphadenopathy, often associated with extranodal involvement including BM. B-symptoms are common (Brugieres et al., 1998) (Falini et al., 1999a).

ALCL, ALK positive is characterised by a broad morphologic spectrum from small cell neoplasms to tumours composed of large cells (Benharroch et al., 1998) (Falini et al., 1998) (Harris et al., 1994). However, all cases contain so-called hallmark cells. These cells are mostly large, (small variants are also seen), and have eccentric horseshoe- or kidney-shaped nuclei (Benharroch et al., 1998). There are five morphologic patterns of ALCL, ALK positive: 1) common pattern (60%), 2) lymphohistiocytic pattern (10%), 3) small cell pattern (5-10%), 4) Hodgkin-like pattern (3%) and 5) “composite pattern” when more than one pattern is observed in the tissue sample (Swerdlow et al., 2008).

The tumour cells are positive for CD30 on the cell membrane and in the Golgi region. CD 30 immunostaining is dependent on cell size with the strongest staining on large cells (Benharroch et al., 1998) (Stein et al., 1985). The cells are positive for ALK staining with a nuclear and / or cytoplasmic pattern depending on the translocation partner (Benharroch et al., 1998) (Falini et al., 1998) (Pulford et al., 2004). The cells are positive for EMA (Benharroch et al., 1998). ALCL, ALK positive tumours are mostly negative for pan T cell antigens CD3 and CD8 but positive for CD2, CD5 and CD4 (Benharroch et al., 1998) (Bonzheim et al., 2004). Most cases are also positive for cytotoxic associated antigens: TIA1, granzyme B and / or perforin (Foss et al., 1996)

(Krenacs et al., 1997). ALCL, ALK positive tumours are consistently negative for BCL2 and EBV (Costes-Martineau et al., 2002) (Brousset et al., 1993).

Clonal rearrangements of the *TCR* are present in approximately 90% of cases. The remaining 10% of tumours show no rearrangement of *TCR* or *Ig* genes (Foss et al., 1996). The characteristic feature of ALCL, ALK positive tumours is a genetic mutation involving *ALK,* which lead to its expression. The *ALK* gene encodes a tyrosine kinase receptor belonging to the insulin receptor superfamily, normally absent in postnatal human tissue including lymphoid cells. It can be expressed in rare cells in the brain (Morris et al., 1994) (Pulford et al., 1997). The most frequently observed translocation is t(2;5)(p23;q35) between the *ALK* gene on chromosome 2 and the nucleophosmin gene on chromosome 5 (Lamant et al., 1996) (Mason et al., 1990). *ALK* can also have different translocation partner genes on chromosome 1, 2, 3, 17, 19, 22 and X (Cools et al., 2002) (Falini et al., 1999b) (Hernandez et al., 1999) (Lamant et al., 1999) (Lamant et al., 2003) (Mason et al., 1998) (Rosenwald et al., 1999) (Stein et al., 2000) (Tort et al., 2001) (Trinei et al., 2000) (Wlodarska et al., 1998). Additionally it has been shown that ALCL, ALK positive tumours carry secondary chromosomal imbalances: losses of chromosome 4, 11q and 13q and gains of 7, 17p and 17q (Salaverria et al., 2008). In gene expression analysis studies among 117 genes over-expressed in ALCL, ALK positive tumours as compared with ALCL, tumours, the four most common were: *BCL6, PTPN12, serpinA1* and *C/EBP* (Lamant et al., 1999).

1.4.3.c *Anaplastic large cell lymphoma, anaplastic lymphoma kinase negative*

ALCL, ALK negative was introduced as a provisional entity in the current WHO lymphoma classification. It is a CD30 positive T-cell tumour, which is not reproducibly distinguishable morphologically from ALCL, ALK positive but lacks expression of the ALK protein (Swerdlow et al., 2008). Most cases express T-cell-associated markers and cytotoxic granule associated proteins.

The tumour occurs mostly in adults aged 40 – 65 years, (unlike those that are ALCL, ALK positive), and has a slight male predominance (Falini, 2001) (Stein et al., 2000).

ALCL, ALK negative involves both lymph nodes and extranodal tissue, although not as often as ALCL, ALK positive. The commonest extranodal sites include bone, soft tissue and skin. Most patients present with advanced stage disease, with peripheral and abdominal lymphadenopathy and B symptoms (ten Berge et al., 2000).

The usual morphological presentation is with solid cohesive sheets of neoplastic cells affecting the architecture of the lymph nodes or other involved tissue (Swerdlow et al., 2008). If the normal lymph node architecture is preserved the lymphocytes infiltrate the sinuses and mimic carcinoma metastases (Swerdlow et al., 2008). Generally the cells in ALCL, ALK negative are larger, more pleomorphic and with a higher nuclear: cytoplasmic ratio compared with conventional ALCL, ALK positive. "Hallmark" cells with eccentric horseshoe or kidney-shaped nuclei are seen to a variable degree (Falini et al., 1999a) (Nelson et al., 2008).

All tumour cells are strongly positive for CD30. The loss of T-cell markers can occur, however more than half of all cases express at least one or more T-cell markers. CD2 and CD3 are found more often than CD5, and CD43 is almost always expressed (Swerdlow et al., 2008). CD4 is positive in the majority of cases and CD8-positive cases are rare. Cytotoxic proteins are present in many cases. EMA is expressed in a minority of cases in contrast to ALCL, ALK positive where it is always seen. ALCL, ALK positive are consistently negative for EBV (Swerdlow et al., 2008).

TCR genes show clonal rearrangements despite the expression status of T-cell antigens. There are no characteristic, recurrent, primary cytogenetic abnormalities acknowledge in the recent 2008 WHO classification (Swerdlow et al., 2008), however the next generation sequencing characterised several translocation but they need re-validation in larger cohort of cases. Several studies have reported differences between ALCL, ALK negative; PTCL NOS and ALCL, ALK positive in terms of genes gained and lost (Salaverria et al., 2008) (Zettl et al., 2004) and gene expression (Ballester et al., 2006) (Lamant et al., 2007).

1.4.3.d *Extranodal NK/T-cell lymphoma, nasal type*

This entity is a predominantly extranodal lymphoma characterized by vascular damage and destruction, prominent necrosis, cytotoxic phenotype and association with EBV (Swerdlow et al., 2008). Most cases appear to be genuine NK-cell neoplasms. Some cases show a cytotoxic T-cell phenotype, thus are called NK/T-cell tumours.

The disease occurs mostly in Asians and in the Native American population of Mexico, Central and South America (Au et al., 2005) (Chan, 1998). Like other subtypes of PTCL, there is a predominance of male and adult patients (Swerdlow et al., 2008).

The aetiology of the disease is unknown but there is a very strong correlation with EBV infection irrespective of the ethnic origin of the patient (Arber et al., 1993)

(Chan et al., 1994). The circulating number of EBV-DNA copies correlates directly with the extent of disease, unfavourable response to therapy and poor survival (Au et al., 2004). Additionally the disease can occur in immunosuppressed patients including the post-transplant setting (Hoshida et al., 2001) (Kwong et al., 2000).

The nasal cavity, nasopharynx, paranasal sinuses and palate are the most frequently involved sites (Swerdlow et al., 2008). However, extranodal NK/T-cell lymphoma can also occur in extranasal sites such as skin, soft tissue, gastrointestinal tract and testis. Both secondary (Chan, 1998) (Chan et al., 1997) (Kern et al., 1992) and primary nodal involvement is also possible (Chim et al., 2005) (Kagami et al., 1999). Patients with nasal involvement usually present with symptoms of nasal obstruction and epistaxis, or with extensive midfacial destructive lesions (Swerdlow et al., 2008). The disease is primarily restricted to the upper aerodigestive tract but can spread rapidly to various sites: skin, gastrointestinal tract, testis or cervical nodes. BM involvement is uncommon (Wong et al., 2001) but some patients suffer from haemophagocytic syndrome (Cheung et al., 1998) (Kwong et al., 1997). Patients with extranodal NK/T-cell lymphomas of non-nasal type usually present with advanced stage disease, with involvement of extranodal sites, lymph nodes and with B-symptoms (Chan et al., 1997) (Kern et al., 1992). Symptoms from the primary extranodal site will vary depending on localisation, for example skin ulcers or nodules or perforation in intestinal cases (Swerdlow et al., 2008).

The histopathological features of NK/T-cell lymphoma are similar despite the site of involvement. The mucosa often shows ulceration. Tumour cells infiltrate the mucosa in diffuse, angiodestructive and angiocentric patterns (Swerdlow et al., 2008). Coagulative necrosis and admixed apoptotic bodies are common findings (Swerdlow et al., 2008). The tumour cells are different sizes from small/ medium to large and anaplastic. The cells have irregular folded and elongated nuclei, which can be vesicular in large cells. The chromatin is granulated. The cytoplasm is moderate in amount and often pale or clear. The tumour can contain a large admixture of inflammatory cells (Chan, 1998) (Hasserjian and Harris, 2007).

Typical cells are positive for CD2, CD56 and cytoplasmic CD3, and negative for surface CD3. CD56 is not specific for NK tumours and can be found in other PTCL (Chan, 1998) (Jaffe, 1995) (Tsang et al., 1996). Cytotoxic molecules e.g. perforin, granzyme B or TIA1 are positive (Elenitoba-Johnson et al., 1998). The common NK/T-cell markers like CD4, CD5, CD8, TCRδ, CD16 and CD57 are usually negative, and

CD43, CD45RO, HLA-DR, CD25, FAS and FAS ligand are commonly expressed (Ng et al., 1997) (Ohshima et al., 1997). A diagnosis of extranodal NK/T-cell lymphoma is less likely if EBV is negative (Swerdlow et al., 2008).

TCR and Ig genes are in germline configuration in most cases (Swerdlow et al., 2008). Although different cytogenetic aberrations have been reported, so far no specific abnormalities have been found. The commonest aberrations are: del(6)(q21q25) and i(6)(p10) (Siu et al., 2000) (Siu et al., 1999) (Tien et al., 1997) (Tien et al., 1997). Aberrant methylation of the promoter CpG regions of multiple genes is common (e.g. *p73*) (Siu et al., 2002). The studies also showed that the most affected genes are *FAS, TP53, β-catenin, KRAS or KIT* (Hongyo et al., 2005) (Quintanilla-Martinez et al., 2001) (Shen et al., 2002).

1.4.3.e *Enteropathy associated T-cell lymphoma (EATL)*

EATL is an intestinal tumour with origins in intraepithelial T lymphocytes. In the 2008 WHO Classification of Tumours of Hematopoietic and Lymphoid Tissues, two types of EATL were defined; type I EATL (tumour composed of large lymphoid cells) and type II EATL (tumour composed of monomorphic medium-size cells – monomorphic variant) (Swerdlow et al., 2008). The relationship between type I EATL and coeliac disease is well established (Diamanti et al., 2006) (Green and Cellier, 2007) (Cheung et al., 1998) (Isaacson, 1985) (Howdle et al., 2003) (Salmi et al., 2006). By contrast, any association of type II EATL with coeliac disease is not proven (Deleeuw et al., 2007).

EATL occurs primarily in patients in the sixth or seventh decade of life. The disease is uncommon in most parts of the world. However type I EATL is more common in areas with a high prevalence of coeliac disease (Northern Europe). By contrast type II EATL has a broader geographic distribution and is also encountered in Asia and other regions where coeliac disease is rare (Swerdlow et al., 2008).

In type I EATL the association with coeliac disease is characterised by positive serological tests, HLA-DQ2 and HLA-DQ8 expression, and other clinical findings associated with coeliac disease like dermatitis herpetiformis or hyposplenism (Diamanti et al., 2006) (Salmi et al., 2006) (Van Overbeke et al., 2005). In type II EATL an association with coeliac disease and other risk factors is yet not proven.

The commonest presentation of type I EATL is reappearance of malabsorption, accompanied by abdominal pain, and frequently associated with intestinal perforation in

patients with a history of coeliac disease or refractory coeliac disease (Isaacson and Du, 2005). In some patients the diagnosis of EATL and coeliac disease is made at the same time. The presentation of type II EATL is very similar except that patients usually do not have evidence of coeliac disease.

EATL may occur in any part of the gastrointestinal tract but predominantly arises in the jejunum or ileum. Macroscopically, in most patients the tumour is multifocal (Diamanti et al., 2006), forming ulcers, nodules, plaques, strictures or less commonly large masses, often infiltrating the mesentery and mesenteric nodes (Isaacson and Du, 2005). Microscopically the picture of type I EATL is characterised by an infiltration of relatively monotonous medium-sized to large cells with round or angulated nuclei, prominent nucleoli and moderate to abundant, pale-staining cytoplasm (Swerdlow et al., 2008). In some cases the tumour cells exhibit marked pleomorphism with multinucleated anaplastic cells. Most of the tumours show an inflammatory infiltrate with abundant histiocytes and eosinophils, which may obscure the small numbers of tumour cells (Isaacson and Du, 2005). The intestinal mucosa adjacent to the tumour usually shows enteropathic features i.e. villous atrophy, crypt hyperplasia, increased lamina propria lymphocytes and plasma cells, and intraepithelial lymphocytosis (Chott et al., 1998) (Chott et al., 1999). In type II EATL the neoplastic cells are medium-sized and have round darkly staining nuclei with a rim of pale cytoplasm. Intestinal crypt epithelia show florid infiltration, and adjacent intestinal mucosa exhibit villous atrophy and crypt hyperplasia with intraepithelial lymphocytosis of crypt and surface epithelium. An inflammatory component is absent and necrosis is usually less evident than in type I (Swerdlow et al., 2008).

In most type I EATL cases the tumour cells are positive for CD3, CD7, and CD103, negative for CD5 and positive / negative for CD8 and TCRß. A varying proportion of the tumour cells express CD30 (Spencer et al., 1985) (Wright, 1997). Intraepithelial lymphocytes in the adjacent enteropathic mucosa may show an abnormal immunophenotype, usually CD3+, CD5-, CD8- and CD4- (Spencer et al., 1988) (Wright, 1997). The immunophenotype of type II EATL is distinctive, and the tumour cells are CD3+, CD4-, CD8+, CD56+, TCRß+ (Swerdlow et al., 2008). Intraepithelial lymphocytes in the adjacent mucosa share the identical immunophenotype (Chott et al., 1998).

TCR beta and gamma chains genes are clonally rearranged in both types (Isaacson, 1985) (Murray et al., 1995). *HLA-DQA1*0501* and *HLA-DQB1*0201*

genotypes characteristic for coeliac disease are also present in patients with type I EATL (Howell et al., 1995). Both types of EATL harbour complex segmental amplification of the 9q31.3 chromosome region, or show deletions in the 16q12.1 chromosome region, in contrast to primary nodal PTCL (Swerdlow et al., 2008). Type I EATL frequently displays gains in chromosomes 1q and 5q. By contrast type II EATL is more often characterized by 8q24 (*c-MYC*) amplifications (Deleeuw et al., 2007) (Zettl et al., 2002).

1.4.3.f *Hepatosplenic T-cell lymphoma*

Hepatosplenic T-cell lymphoma is an extranodal and systemic neoplasm derived from cytotoxic T-cells, usually of γ/δ TCR type (Swerdlow et al., 2008). It usually involves spleen, liver and BM.

This is a very rare type of lymphoma. The reported incidence is <1% of all NHL. It usually presents in adolescents and young adults males.

It is associated with chronic immunosuppression; most commonly after long-term immunosuppressive therapy for organ transplant or prolonged antigenic stimulation (Belhadj et al., 2003) (Gaulard et al., 2003) (Vega et al., 2007) (Wu et al., 2000). It is also seen in patients treated with azathioprine and infliximab for Crohn's disease (Mackey et al., 2007) (Rosh et al., 2007).

As its name suggests it commonly presents with involvement of spleen and liver. BM is almost always involved, (Belhadj et al., 2003) (Cooke et al., 1996) (Vega et al., 2007), however patients have no lymphadenopathy (Farcet et al., 1990). Usually patients present with hepato-splenomegaly associated with B-symptoms. They can suffer from thrombocytopenia, anaemia and leukopenia. Peripheral blood involvement is uncommon at presentation but may appear during the course of the disease (Belhadj et al., 2003) (Cooke et al., 1996) (Vega et al., 2007).

The tumour cells are monotonous with medium-sized nuclei, loosely condensed chromatin and a rim of pale cytoplasm (Cooke et al., 1996). They diffusely infiltrate the cords and sinuses of the splenic red pulp with atrophy of the white pulp. The liver shows sinusoidal and BM intrasinusoidal infiltration (Swerdlow et al., 2008).

The cells are positive for CD3 and are usually TCRδ1+, TCRαβ-, CD56+/-, CD4-, CD8-/+ and CD5-. A minority of cases appear to be of α/β type (Macon et al., 2001) (Suarez et al., 2000). They express cytotoxic granule-associated proteins TIA1

and granzyme M, but they are usually negative for granzyme B and perforin (Cooke et al., 1996) (Felgar et al., 1997) (Krenacs et al., 2003).

TCR is rearranged in both γ/δ and α/β types. There is no characteristic single genetic mutation. The most common genetic mutations include: isochromosome 7q, ring chromosome 7, trisomy 8 and loss of a sex chromosome (Alonsozana et al., 1997) (Wlodarska et al., 2002) (Tamaska et al., 2006).

1.4.4 *Prognostic factors in PTCL*

Compared with other lymphomas e.g. DLBCL or FL prognostic factors are not as extensively studied in PTCL. This is most probably due to the heterogeneity, and consequent rarity of the individual entities. The definition and classification system of PTCL is also relatively new and has undergone several major changes in the last two decades. Another reason may be the generally poor outcome of patients diagnosed with PTCL, with only minimal differences in survival between subtypes. Thus it would be difficult to find a place for prognostic models in everyday clinical practice. Small patient numbers and differences in survival between individual patients also makes it very difficult from a research point of view. Despite these problems clinical prognostic indices have been used in several PTCL studies, and recently some novel models have been developed including some work on biomarkers.

1.4.4.a *Clinical prognostic factors*

The most frequently used prognostic index in PTCL is the IPI. This is despite the fact that the IPI was primarily developed in a cohort of patients with aggressive lymphomas including both B-cell and T-cell subtypes, with a significant majority of the former (Shipp, 1993). There are, however, different opinions on the usefulness of the IPI in PTCL. In some studies the IPI worked very well and could distinguish between group of patients with different outcomes (Suzumiya et al., 2009) (Lopez-Guillermo et al., 1998) (Chim et al., 2004); in others it could not (Savage et al., 2004) (Sieniawski et al., 2010).

This confusion prompted the “Intergruppo Italiano Linfomi” to develop a new index called the PIT designed especially for patients with PTCL (Gallamini et al., 2004). The index was developed in a cohort of 385 patients with PTCL NOS. Most patients had received chemotherapy with anthracyclines (78%), others were treated with high-dose chemotherapy and ASCT (12%) or with non-anthracycline regimens (8%),

and 2% of patients received no therapy. The score included the following factors: age (≤60 years vs. >60 years), performance status with ECOG (≤1 vs. >1), serum LDH (≤normal vs. >normal) and BM involvement (yes vs. no). Four separate groups with different risks were distinguished: group 1: no adverse factors – 5-year and 10-year OS of 62.3% and 54.9%, respectively; group 2: one factor – 52.9% and 38.8%, respectively; group 3: 2 factors – 32.9% and 18.0%, respectively and group 4: with 3 or 4 factors – 18.3% and 12.6%. It should be noted however that the index was not developed in a group of uniformly treated patients.

Subsequently, the index was modified (mPIT) by removing BM involvement and adding proliferation index as assessed by Ki-67 immunostaining in a cohort of 193 patients: 148 PTCL NOS and 45 AILT (Went et al., 2006). Patients again received different treatments modalities: combination chemotherapy with anthracyclines (84%), single-agent chemotherapy (4%), combination chemotherapy without anthracyclines (3%) and 9% of patients received no chemotherapy. The mPIT could differentiate 3 groups with different outcomes: group I (0 or 1 prognostic factor), median survival 37 months; group II (2 prognostic factors), median survival 23 months and group III (3 or 4 prognostic factors), median survival 12 months. However this index has several features which may lead to bias, namely: inclusion of two different histological entities with different prognoses: PTCL NOS and AILT, inclusion of patients receiving different treatment modalities and finally inclusion of approximately 50% of patients who were positive for HIV.

The latest prognostic score (IPTCLP) was published by the ITLP using a cohort of patients with PTCL and AITL. It is based on the presence or absence of 3 factors: age (≤60 years vs. >60 years), ECOG performance status (≤1 vs. >1), and platelet count (≤150 x 10^9/l vs. >150 x 10^9/l) (Vose, 2005). The index identifies 4 different groups: group 1 (0 risk factor), 5 year FFS and OS of 21% and 42% (respectively), group 2 (1 risk factor), 19% and 27%, respectively; group 3 (2 risk factors), 10% and 19%, respectively and group 4 (3 risk factors), 5% and 12%, respectively. Again however, the index was produced using a cohort of patients with different histological subtypes and treated with different regimens.

Recently Gutierrez-Garcia evaluated these four indices in a cohort of 121 patients (Gutierrez-Garcia et al., 2011b). The IPI and PIT allocated patients equally among 4 risk groups. The IPTCLP was characterised by higher numbers of low-intermediate risk patients and the mPIT by higher numbers of low risk patients. Total

concordance across three scores was 52% for low-risk, 27% for low-intermediate-risk, 20% for high-intermediate-risk and 14% only for high-risk groups. Three from four tested indices, (IPI, PIT, and IPTCLP) predicted statistically significantly differences in CR, with IPI being the best predictor in logistic regression. All four indices predicted statistically significantly differences in PFS and OS with PIT being the best predictor for PFS and IPTCLP for OS (Gutierrez-Garcia et al., 2011b).

Generally all four published indices are characterised by similar limitations: inclusion of different histological subtypes, and use of different treatment modalities. They rely on a small number of dichotomised predictive variables. Additionally as all of them use cox-regression multivariate analysis patients are assigned to one of a small number of risk groups, rather than being given a direct prediction, such as predictive survival probability, or predictive median lifetime on a continuous scale. Table 1.12 shows factors included in all the described clinical indices for PTCL.

Factor	IPI	PIT	mPIT	IPTCLP
Age (≤60 years vs. >60 years)	✓	✓	✓	✓
ECOG (≤1 vs. >1)	✓	✓	✓	✓
LDH (normal vs. high)	✓	✓	✓	
CS by Ann Arbor (I/II vs. III/IV)	✓			
Extranodal involvement (<2 vs. ≥2 sites)	✓			
BM involvement (negative vs. positive)		✓		
Platelet count (≤150 x 10^9 vs. >150 x 10^9)				✓
Ki 67 (%) (≤75 vs. >75)			✓	

Table 1.12 Variables used for calculation of the different prognostic indicies for PTCL.

1.4.4.b *Biological and molecular prognostic factors*

Biological and molecular prognostic factors are less frequently studied in PTCL compared with other lymphomas. However, some interesting studies have been recently published. In particular, there are new studies evaluating selected subgroups of disease and these deliver more reliable results than older reports, which include cohorts of patients with different diagnoses.

Taking into consideration the fact that the vast majority of PTCL are CD4+ / CD8- several studies assessed the impact of T-helper phenotype on patient outcome. This factor was proposed as a potential indicator of the better outcome characteristics of PTCL NOS. Tsuchiya et al assessed the expression of chemokine receptors associated

either with the Th1 phenotype: chemokine (C–X-C motif) receptor 3 and 5 (CXCR3 and CXCR5) or with the Th2 phenotype: ST2(L) and activated TCR OX40/CD134 in patients with PTCL (Tsuchiya et al., 2004). Specific subtypes of PTCL had different expression patterns. In AILT, almost all cases were immunoreactive for OX40/CD134 (96%) and for CXCR3 (89%). By contrast, in ALCL, all cases were immunonegative for OX40/CD134, and only a few cases (24%) were immunoreactive for CXCR3. Almost all cases (94%) were positive for ST2(L). Importantly, when cases of PTCL NOS were divided into 2 groups; "positive activity group" with cases positive for at least one of the markers: CXCR3, CXCR5 or ST2(L), and "negative activity group" with cases negative for all markers, the patients from the first group had a statistically significant better OS. In a study by Ishida et al. the expression of chemokine receptors: CXCR3 (Th1 marker) and chemokine (C-C motif) receptor 4 (CCR4) (Th2 marker) have been assessed in samples of patients with PTCL (Ishida et al., 2004). The Th1 phenotype was predominantly expressed in AITL and Th2 in mycosis fungoides in transformation and ALCL, ALK negative. As suspected PTCL NOS was characterised by a heterogeneous expression pattern. In an analysis of OS CXCR3 appeared to be a significantly favourable factor and CCR4 a significantly unfavourable factor.

Several studies have assessed the role of apoptotic pathways and their inhibitors and outcomes of patients with PTCL, particularly ALCL. Ten Berge et al assessed the expression of caspase3 an apoptosis execution enzyme, BCL2 an inhibitor of the mitochondrial apoptotic pathway, and PI9 an inhibitor of granzyme B induced apoptosis in patients with ALCL (ten Berge, 2002). The expression of caspase3 in >5% of tumour cells was a negative prognostic factor. Expression of BCL2 in >50% of tumour cells and expression of PI9 were positive prognostic factors for PFS and OS in the evaluation of all patients and in ALK negative patients. Additionally, there were significant differences in expression between ALK positive and ALK negative cases – the latter was characterised by lower expression of caspase3 and higher expression of both inhibitors.

Schlette et al assessed the expression and prognostic value of an anti-apoptotic molecule survivin in ALCL, ALK positive and negative (Schlette et al., 2004). Survivin was expressed in approximately half of the patients and was a negative prognostic factor for PFS and OS in both ALCL, ALK positive and negative.

Cytogenetic studies have revealed a high number of genetic aberrations in PTCL. Some could distinguish between the different subtypes of PTCL e.g. between

AILT and PTCL NOS (Thorns et al., 2007) or between AILT, PTCL NOS and ALCL, ALK negative (Nelson et al., 2008). Nelson's study could find no association between specific chromosomal abnormalities and OS (Nelson et al., 2008). However, the cases with complex karyotypes, most frequently observed in ALCL, ALK negative and PTCL NOS had a significantly shorter OS. The only chromosomal abnormality in PTCL with a definite impact on treatment outcome and survival remains the translocation of ALK in ALCL.

Recently new studies on gene expression analysis in PTCL have been published following on from the success of the studies in DLBCL. Martinez-Delgado et al. (Martinez-Delgado et al., 2004) using the CNIO-OncoChip containing 6386 cancer-related genes found significant differences between peripheral and lymphoblastic T-cell lymphomas, including deregulation and over-expression of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κβ1) signalling pathway in PTCL NOS. Additionally, two sets of genes, which differentiate between PTCL and normal T-cells as well as activated lymph nodes were found.

In another study using cDNA microarray, the expression profiling could distinguish between AITL, ALCL and T-LBL (Ballester et al., 2006). By contrast, PTCL NOS had no common signature and three different molecular subgroups called U1, U2 and U3 could be distinguished. The U1 signature included genes associated with aggressive behaviour and poor outcome in several tumours like DLBCL (e.g. *CCND2*). The U2 genes were associated with translation, signal transduction and apoptosis (e.g. *NF-κβ1* and *BCL2*) and the U3 genes with the *IFN* / *Janus kinase* (*JAK*) / *signal transducer and activator of transcription* (*STAT*) pathway. Importantly, patients with U2 and U3 signatures had similar outcomes that were better than patients with the U1 signature.

Recent gene expression profiling studies using Affymetrix arrays were published by ITLP (Iqbal et al., 2010). In an unsupervised analysis each major defined subtype of PTCL forms 2 – 3 main clusters. However PTCL NOS was characterised by strong heterogeneity and some minor PTCL subtypes formed distinct clusters, but because of low numbers of cases were not studied further. AITL was characterised by 3 distinctive signatures: (i) B-cell signature, (ii) follicular dendritic cell signature and (iii) cytokine signature. AITL cases had over-expression of a set of genes involved in TCR signalling / activation, HTLV1 associated genes and a set of genes associated with melanoma. In ALCL, ALK positive the expression of Th17-associated molecules was noted. In PTCL

NOS, (despite its heterogeneity), a molecular subgroup with features of cytotoxic T lymphocytes and a poorer survival was identified.

The recent developments in molecular biology and genetics have opened new perspectives in the assessment of pathobiology of PTCL. The studies on whole exon or whole genome sequencing revealed new prognostic markers and pathways (Iqbal et al., 2016).

Tet methylcytosine dioxygenase 2 (TET-2) is an enzyme converting methylcytosine to 5-hydroxymethylcytosine and is involved in epigenetic processes (Couronné et al., 2012). The enzyme is inhibited by 2-hydroxyglutarate an oncogenic metabolite by the mutated forms of isocitrate dehydrogenase isotype 1 and 2 (IDH1 and IDH2) (Couronné et al., 2012). The mutations involving *TET-2* were first identified in myeloproliferative neoplasm and myelodysplastic syndromes (Delhommeau et al., 2009). The mutations include deletions, missense, nonsense and frameshift mutations resulting in loss-of function and reduction in the level of intracellular 5-hydroxymethylcytosine. Recently the *TET2* mutations have been reported in T-cell lymphoid malignancies: AITL, "Th follicular like" PTCL-NOS and ATLL (Couronné et al., 2012). The concordant occurrence of *TET2* mutations and mutations in *DNMT3A* was observed in a series of T-cell lymphomas. *DNMT3A* is a gene frequently showing mutations in myeloid cancers and its product is involved in cytosine methylation. The patients with mutations in *TET2* and *DNMT3A* can be potentially treated with demethylating agents.

IDH is an enzyme that catalyzes the oxidative of isocitrate producing alpha-ketoglutatrate and CO_2. In humans, the enzyme is naturally present in 3 isoforms: IDH1-3 (Cairns et al., 2012). The IDH3 isoform catalyzes the third step in the citric acid cycle. The IDH1 and IDH2 isoforms catalyze the same reaction but outside the citric acid cycle. The mutations of *IDH1* and *IDH2* have been described in grade 2 and 3 gliomas, secondary glioblastomas and acute myeloid leukaemias. Mutations in *IDH2* have been described in two separate sets of AILT cases, in 20% and 45% of assessed cases respectively (Cairns et al., 2012). The mutations involved *IDH2* only and were largely confined to alternations resulting in a R172 substitution. The mutated enzyme converts 2-oxoglutarate to D-2-hydroxyglutarate. D-2-hydroxyglutarate can play a role of oncometabolite by its interaction with numerous enzymes involved in hypoxia signaling, histones and DNA methylation and inhibits the activity of TET2 enzyme. There was no association found between the *IDH2* mutation status and survival in

AILT. In the re-sequencing performed on a new series of AILT the frequency of *IDH* mutation was 32.8% and it was limited to the *IDH2* at R172 locus. Interestingly, the *IDH2*^{R172} mutation defines a unique subgroup of PTCL with a distinct T_{FH} gene expression and can induce DNA and histone methylation in AILT (Wang et al., 2015).

Recurrent translocation t(6;7)(p25.3;q32.3) was recently described in ALCL ALK negative cases (Feldman et al., 2011). This translocation was associated with increased expression of DUSP22, a dual specificity phosphatase that inhibits T-cell antigen receptor signaling in reactive T-cells by inactivation of MAPK and ERK2. DUSP22 is a putative tumour repressor in T-cell malignancies. The translocation was also associated with increased expression of miR29A, a microRNA with potentially oncogenic function in T-cell lymphomas (Feldman et al., 2011).

CD28 is the major TCR co-stimulatory receptor in T-cells. After binding its ligand and simultaneous activation of TCR, CD28 induces sustained T-cell activation and cytokine production. Recently the recurrent mutations in two residues of *CD28* have been identified in patients with AILT (Rohr et al., 2016). These mutations lead to increased affinity to ligand CD86 and intracellular adaptor proteins GRB2 and GADS/GRAP2. The over-expression of the CD28 responsive genes *CD226* and *TNFA* was found in cells with T195 mutation and increased activation of NF-κβ in cells with both mutations.

The changes to p53 pathway were not recognized in PTCL for a long time. The study on identification of rearrangements across the entire genome identified five mutations involving p53 pathway in *TP53*, *TP63*, *CDKN2A*, *WWOX* and *ANKRD11* (Vasmatzis et al., 2012). Mutations in *TP63* were particularly interesting as the product of TP63 is known to have oncogenic properties and it inhibits the p53 pathway by a dominant-negative mechanism. Indeed the patient with *TP63* mutations have worse outcome. TP63 mutations were seen in 12.5% ALCL ALK negative, 10.5% ALCL primary cutaneous and 9.4% PTCL NOS.

Recent whole exon sequencing combined with RNAseq analysis and targeted deep sequencing identified known recurrent epigenetic factor mutations in *TET2*, *DNMT3* and *IDH2* but also identified a new highly prevalent *RHOA* p.Gly17Val mutation present in 67% AILT and 18% PTCL NOS (Palomero et al., 2014). RHOA is a small GTPase from the Rho family and it is responsible for linking of cell-surface receptors to different intracellular signaling proteins (Bar-Sagi and Hall, 2000). GTPases can be present in active (GDP bound) or inactive form (GTP-bound) (Bar-Sagi

and Hall, 2000). The active RHOA is responsible for the control of the structure and dynamics of the actin cytoskeleton and the formation of stress fiber. The gene encoding a p.Gly17Val alternation leads to inhibition of the signaling pathway (Palomero et al., 2014). The subsequent study found that the *RHOA* was mutated in 14.6% of PTCL and 34.2% of AITL (Palomero et al., 2014). Hence, the mutations of *RHOA* were associated with activation of several cellular pathways e.g. pathways related to CD4 T-cell and AILT signature, p38 mitogen-activated protein kinase, phosphatidylinositol 3-kinase and KRAS – the alternative NF-κβ pathway and RAC1 pathway. The *RHOA* status can be helpful in directing targeted therapies. The mutational status of *RHOA* had no prediction value for patient survival.

Additionally, the whole exon sequencing identified mutations in *FYN*, *ATM*, *β2M* and *CD58* implicating SCR signaling, an impaired DNA damage response and an escape from the immune surveillance mechanism (Palomero et al., 2014). The FYN tyrosine kinase is with LCK the predominant kinase found in T lymphocytes. It plays an important role in TCR signaling. The *FYN* mutations accounted in PTCL result in increased activation of the enzyme. This deserves more attention as it could be potentially targeted with SRC kinase inhibitors.

1.4.5 *Treatment of PTCL*

The standard therapy for PTCL remains CHOP and other anthracycline-based regimens despite modest outcomes (Vose et al., 2008). Reported OS rates at 5-years for PTCL treated with CHOP range between 25-35%. According to the data presented by the ITLP, the outcome varies between individual subgroups with 5-year PFS & OS from 60% & 70% in ALCL, ALK positive; 36% & 49% in ALCL, ALK - negative to 20% & 32% in PTCL NOS, 18% & 32% in AITL, 4% & 20% in EATL and 0% & 7% in hepatosplenic T-cell lymphoma. Interestingly the authors could find no advantage to anthracycline-based chemotherapy compared with non-anthracycline regimens in PTCL NOS and angioimmunoblastic lymphoma (Vose et al., 2008). The idea of using anthracycline-based regimens in the treatment of PTCL has its origins in the conventional inclusion of the majority of entities, currently known as PTCL, into the intermediate or high-grade lymphoma groups according to the WF (National Cancer Institute, 1982). Consequently, the recommendation to use an anthracycline-based regimen applied to the whole group irrespective of immunophenotype. The recommendation of CHOP as a specific standard anthracycline-based therapy comes

from the results of the large intergroup phase III trial which demonstrated non-inferiority of CHOP compared with third generation combination regimens in intermediate subgroups of lymphoma according to the WF (Fisher et al., 1993). As previously discussed accepting the results of this study as a standard could be extremely misleading because it used the International WF, did not use immunophenotyping and included lymphomas with B-cell, T-cell and NK-cell phenotype. With current knowledge, it is accepted that all these tumours have different characteristics and outcomes and eventually will likely require different therapies (Vose, 2008).

Several attempts have been made subsequently to try and improve the outcome of aggressive lymphomas including PTCL e.g. shortening of the chemotherapy intervals (CHOP-14 vs. 21), incorporation of additional drugs into the CHOP regimen (CHOEP-21 and VACEP) (Karakas et al., 1996) or both approaches (CHOEP-14) (Pfreundschuh et al., 2004a) (Pfreundschuh et al., 2004b). The value of published results is limited mostly because of the fact that studies were performed in all aggressive lymphomas and subanalysis of PTCL was performed later. Importantly, the number of ALK positive cases included was unknown (Nickelsen et al., 2009).

The prognostic impact of immunophenotype on the outcome of aggressive lymphomas was first evaluated by the GELA group who discovered that PTCL are characterised by a worse outcome than B-cell lymphomas treated with the same protocols for aggressive lymphomas: LNH84 and LNH87 (Gisselbrecht et al., 1998) (Coiffier et al., 1990).

At the beginning of the 21st century the treatment of B-cell aggressive lymphomas moved towards the addition of anti-CD20 antibodies (Coiffier et al., 2002) and consequently the first studies separately designed for PTCL were introduced. The development of first-line treatment for PTCL moved in two different directions – the first was to try and find the analogue of "rituximab" in B-cell lymphomas, a monoclonal antibody (e.g. anti CD52) (Zinzani et al., 2005) (Enblad et al., 2004) or a cytostatic drug (e.g. gemcitabine or bleomycin) (Sung et al., 2006) (Kim et al., 2006), which could be added to the existing gold standard of CHOP. The second looked at the role of intensification of treatment by adding consolidation with ASCT. This approach was studied mostly in small cohorts of patients (Mercadal et al., 2008) (Rodriguez et al., 2007) (Corradini et al., 2006) with only two big prospective trials assessing ASCT as consolidation after induction with CHOP or its derivatives (Reimer et al., 2009) (d'Amore et al., 2009). The preliminary results are promising, however the number of

patients with primary PD during induction remains high as expected using CHOP–like regimens (Reimer et al., 2009) (d'Amore et al., 2009) (Mercadal et al., 2008).

Recently several phase I and II studies on new drugs in the treatment of PTCL have been published. The most advanced works have focused on a new antifolate – pralatrexate (O'Connor et al., 2009) (O'Connor et al., 2011), histone deacetylase inhibitors - romidepsin (Piekarz et al., 2008), an immunomodulatory drug – lenalidomide (Dueck et al., 2010) and a purine nucleoside phosphorylase inhibitor forodesine (Furman et al., 2006). Additionally, with the increasing knowledge of molecular pathology in PTCL, there is more hope of targeting individual aberrant pathways like the apoptotic pathway with drugs targeting BCL2 (ABT – 263), the NF-κβ pathway with proteasome inhibitors (bortezomib) (Zinzani et al., 2007) or the platelet derived growth factor receptor (PDGRFα) and a tyrosine kinase receptor with the tyrosine kinase inhibitor (imatinib) (Piccaluga et al., 2007a).

1.5 Aims of the thesis

The aims of the thesis are:

1. To describe patient characteristic at presentation, treatment and outcome of patients with DLBCL in a population-based setting in order to obtain the realistic picture of disease on unselected cohort patient in pre- and post-rituximab era representative for the UK.
2. To develop a prognostic model based on expression of molecular factors (*c-MYC* and *HLA-DRβ* – genes previously assessed in GEP studies) in FFPE tissue samples of nodal DLBCL using quantitative PCR (qPCR) in order to identify the high-risk patients suitable for more intense treatment on better base than standard clinical based IPI
3. To assess whether version 2 of chromosome 13 open reading frame 25 transcript *(v2-transcript)* can be amplified by qPCR in lymphoma cell lines and FFPE tissue sections from lymph node biopsies of patients with DLBCL and eventually include it in prognostic model.
4. Asses weather the microRNAs (miRNAs) encoded in *v2-transcript* can be amplified and measured by qPCR in lymphoma cell line and FFPE tissue section of nodal DLBCL and potentially be used as a biomarker.

5. To collect prospectively data in population-based setting on clinical presentation, treatment and outcome of patients with EATL, one of the subtypes of PTCL.
6. To assess the role of a novel high-dose chemotherapy with ifosfamide, epirubicin, etoposide/methotrexate IVE/MTX and ASCT in first line treatment of EATL and subsequently in other subtypes of PTCL.

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Chapter 2. Population-based studies on epidemiology, clinical presentation, treatment and outcome in DLBCL

2.1 Introduction

The published results of randomized clinical trials on the treatment of DLBCL, particularly since the advent of rituximab, give the impression that the disease outcome has improved and is now satisfactory. The monoclonal anti CD20 antibody, rituximab has been seen as a panacea for all difficulties in the treatment of DLBCL, but the reality is rather different. Regarding any drug as a panacea is dangerous as it turns off our vigilance. There is no doubt that rituximab significantly improves the outcome of patients with DLBCL but it is still not satisfactory in all groups of patients (Coiffier et al., 2002) (Pfreundschuh et al., 2006) (Pfreundschuh et al., 2008) (Habermann et al., 2006). So it remains true that professionals involved in the everyday treatment of DLBCL have an impression that despite recent progress the optimal treatment of DLBCL remains an unsolved issue.

There are several reasons for this. On the one hand based on recent studies DLBCL is now known to be a heterogeneous tumour (Swerdlow et al., 2008). This knowledge is already at least partially reflected in the new WHO classification of lymphoproliferative disorders, but has not yet been employed in clinical trials or everyday clinic practice (Swerdlow et al., 2008). The current impression is that there is an enormous gap between progress in pathobiology and clinical management of DLBCL. On the other hand there are outstanding treatment results coming from clinical trials performed on highly selected groups of patients (mostly in early stage disease who anyway have a more favourable prognosis) (Pfreundschuh et al., 2008). Additionally many studies do not include clearly defined risk groups of patients (Coiffier et al., 2002) (Pfreundschuh et al., 2004a) (Pfreundschuh et al., 2004b) (Habermann et al., 2006). The IPI and aaIPI seem to be universal keys for selection of patients entering clinical trials. However, unfortunately they are not applied uniformly in all studies, some of which use additional inclusion criteria e.g. the presence of bulky disease or increased LDH in addition to the IPI, making it then very difficult to compare results (Pfreundschuh et al., 2008). Due to the lack of other sources of information the results of these trials are then applied to treatment algorithms for the whole population of patients where outcomes may be very different. This situation resembles very much that seen in pathology of

lymphoma before the introduction of the REAL classification. The best solution to these problems would be to gather a true picture of the disease from broad population-based studies and then subsequently design clinical trials to successfully target unsolved areas of disease management.

In this chapter data on epidemiology, disease picture, treatment and outcome of population-based studies of patients with nodal origin DLBCL registered in the Scotland and Newcastle Lymphoma Group (SNLG) will be presented. Additionally the data from a recent population-based study on DLBCL treated with R-CHOP in the Northern region of England will be shown. This will allow to show a true picture of disease as it presents in the clinic. Furthermore, these data will be discussed in the context of other population-based studies and recent clinical trials performed in DLBCL. Particular attention will be paid to the feasibility of patients to receive curative treatment, the choice of appropriate age limits for studies on elderly and very elderly patients, the application of the IPI in population-based cohorts of patients, the role of the duration of first remission in survival of patients, and finally the impact of the inclusion of rituximab in the treatment of DLBCL on the population of the UK treated within the NHS.

2.2 Material and methods

2.2.1 *Scotland and Newcastle Lymphoma Group (SNLG)*

The SNLG was established in 1979 to create a forum for interested clinicians to design and conduct trials in the field of lymphoma and to maintain a database of patients diagnosed with lymphoma. Beginning in 1990, with the assistance of hospital pathology departments, the group aimed for total registration of all patients with lymphoma within the population of Scotland and the Northern Region of England. The project allowed accurate incidence and outcome measures for every patient with lymphoma and not just those entered into randomised clinical trials – a process known as population adjusted clinical epidemiology PACE (Proctor and Taylor, 2000). The SNLG worked with 27 regional hospitals and covered a population of circa 7.6 million, see figure 2.1. The creation of such a high quality clinical database had numerous other benefits, such as sub-group analysis and the possibility of studying rare disorders or interventions, which a single institution could not undertake alone.

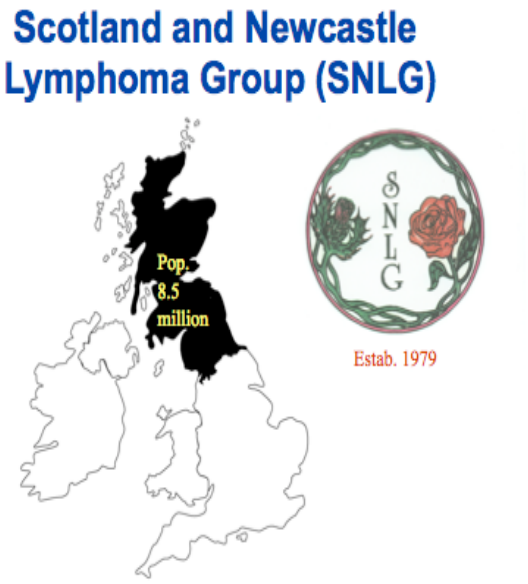


Figure 2.1 Map of Scotland and Newcastle Lymphoma Group's activity area.

2.2.2 *Data collection and response assessment in SNLG*

Data was collected using standardised SNLG forms and subsequently checked for accuracy before being entered onto the SNLG computer database. The SNLG forms are presented in Appendix 1. At baseline evaluation patient demographics including date of birth, diagnosis, date of diagnosis of underlying malignancy, gender, and ECOG performance status were documented. The results of pathological examination and extent of disease as assessed by clinical examination, laboratory tests, biopsy, endoscopy and imaging were also recorded. Additionally the presence of general symptoms, calculated CS according to Ann Arbor criteria and IPI were entered. The first evaluation of patient treatment and outcome was performed at one year from diagnosis, or if patient was deceased at the time of death. Further follow-up was carried at one-year intervals or at the time of death. CR, partial remission (PR) and PD were defined according to the criteria reported by Cheson et al (Cheson et al., 2007).

The following demographic and disease related data collected by the SNLG were used in this study. The format of the data is given in brackets.

- Date of birth (dd/mm/yyyy)
- Date of diagnosis (dd/mm/yyyy)
- Gender (male or female)
- CS according to Ann Arbor criteria (I - IV)
- B – Symptoms (present or absent)

- Performance status measured by ECOG (0 - 5)
- Extranodal involvement (yes or no)
- Type of organ involved (name of organ)
- Bulk (yes or no)
- Haemoglobin (Hb) level (normal and abnormal and absolute level in g/dl)
- White blood cells (WBC) (normal and abnormal and absolute level in g/dl)
- LDH serum level (normal and abnormal and absolute level in U/l)
- Albumin serum level (normal or abnormal)
- Urea serum level (normal or abnormal)
- Alkaline phosphatase (AP) serum level (normal or abnormal)
- Treatment (chemotherapy, radiotherapy or combined modality)
- Type of given chemotherapy (name of regimen)
- The disease outcome at 12 months (remission / failure)
- Date last seen / date of death (dd/mm/yyyy)
- Remission status at date last seen or date of death (remission or relapse)
- Cause of death

2.2.3 *Inclusion and exclusion criteria in SNLG*

In order to evaluate the clinical presentation, therapy and outcome of patients with DLBCL of primary nodal origin in a population-based setting; the SNLG database was searched for patients using the following inclusion and exclusion criteria.

Inclusion criteria:

- first diagnosis of DLBCL
- primary nodal origin of disease
- age at diagnosis ≥18 years
- time of diagnosis between 1990 and 2003
- known CS of disease
- known outcome of disease

Exclusion criteria:

- unknown treatment
- treatment with rituximab

2.2.4 *Inclusion / exclusion criteria, data collection and response assessment in evaluation of patients treated with immunochemotherapy*

In order to obtain a picture of current treatment and outcome of patients with DLBCL in the rituximab era records of patients diagnosed with the disease were retrospectively and prospectively searched in four NHS Trusts in the Northern Cancer Research Network. The selected patients had to fulfil the following inclusion criteria:

- First diagnosis of DLBCL
- No previous history of chemotherapy, immunotherapy or radiotherapy
- Age at diagnosis ≥18 years
- Started treatment with CHOP or CNOP (cyclophosphamide, mitoxantrone, vincristine and prednisolone) polychemotherapy in combination with rituximab (at least one cycle)
- No previous or concurrent diagnosis of other malignant disorders including leukaemia and lymphoma
- No extranodal origin of disease in CNS, testis or stomach
- No known history of HIV positivity

The data collected at baseline included the same parameters collected in the SNLG forms. Data on treatment and outcome was slightly modified as follows:

- Treatment (chemotherapy, radiotherapy or combined modality)
- Type of given chemotherapy (name of regimen)
- Number of cycles given
- Status of scheduled therapy (completed or not)
- Reason for premature discontinuation of scheduled chemotherapy if applicable (progression, infection, organ failure, other)
- Disease outcome after the end of first line treatment (CR, PR or failure)
- Date last seen / date of death (dd/mm/yyyy)
- Patients status at date last seen / date of death (alive or dead)
- Remission status at date last seen or date of death (remission or relapse)

The information was registered manually in a specially developed questionnaire (see Appendix 2) during an on-site visit and then transcribed into an electronic database, checked for accuracy and evaluated. This data was then compared with data of patients from an historical SNLG cohort treated with the same anthracycline-based regimen without the addition of rituximab.

2.2.5 *Statistics*

Demographics and disease characteristics were summarized using descriptive statistics. The χ^2-test and Fisher's exact test were used as appropriate to investigate differences on proportions and t-test, Mann-Whitney-test or Krushkai-Walis test on means. OS and PFS rates were estimated according to the method of Kaplan and Meier and comparison between the groups was with log-rank test. OS was calculated from date of diagnosis to the date of death, or if no death occurred, to the last documented follow up for the patient. PFS was calculated from date of diagnosis to the first documentation of progression during therapy, failure at the end of therapy, or further relapse or death from any cause during or after the end of treatment. Due to the method of data collection if disease progression occurred during first line therapy the date of the event was assigned as the date of pathological diagnosis. All tests were performed with a confidence interval of 95 % and statistical significance was defined as $p \leq 0.05$ using SPSS Version 13.0 for MAC OS X (SPSS Incorporated, Chicago, IL).

2.3 Results

2.3.1 *Patient selection*

A total number of 2025 patients with a first diagnosis of DLBCL of primary nodal origin were registered on the SNLG database between 1990 and 2003. Forty-five patients were excluded after assessment of baseline data: nine were younger than 18 years at diagnosis and 36 had no data on CS. A further 67 patients were excluded after assessment of treatment data: in 46 patients there was no data on treatment and 21 patients received rituximab as part of their first line chemotherapy. Finally 50 patients were excluded because of missing outcome data; the remission status was not available in 49 patients and survival status in one patient. Thus 1863 patients fulfilled all criteria and proceed to further evaluation, see figure 2.2.

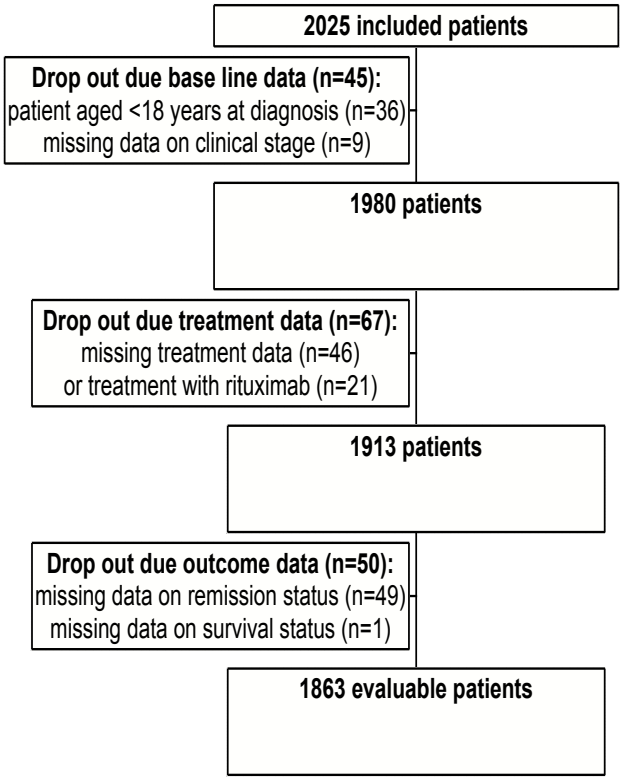


Figure 2.2 Flow diagram for patient with DLBCL of nodal origin registered with SNLG between 1990 and 2003.

2.3.2 *Evaluation of all patients*

2.3.2.a *Patient characteristics*

The median age of the 1863 evaluable patients at diagnosis was 66 years (range, 18–98 years), 711/1863 (38.2%) patients were aged ≤60 years and 1152/1863 (61.8%) >60 years. Figure 2.3 shows the age histogram for all patients and demonstrates a maximum incidence between 60 and 80 years. Age distribution remains the same for patients in early CS (CS I and II) and advanced CS (CS III and IV), except for a slightly higher number of patients aged 20 – 40 years in the early stage group. However the difference was not statistically significant (p=0.297) figure 2.4.

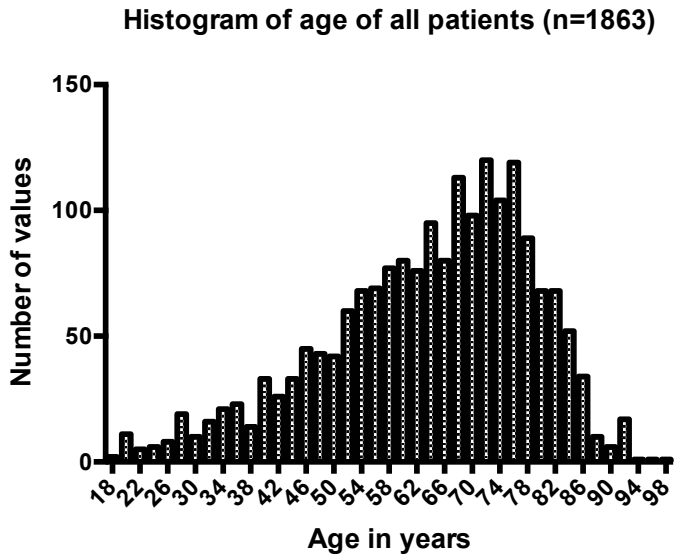


Figure 2.3 Age histogram for all patients (n=1863).

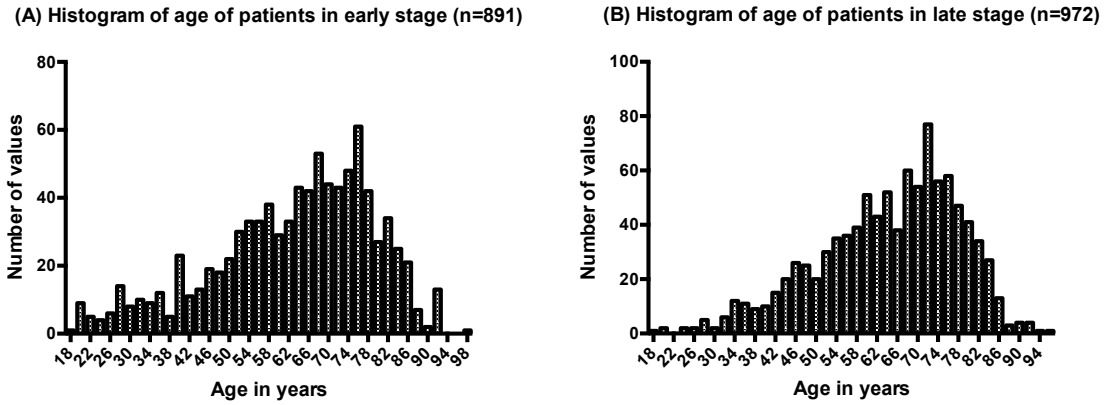


Figure 2.4 Age histogram for patients with early and advanced stage disease, evaluation of all patients (n=1863). **(A)** patients in early stage (n=891) and **(B)** patients in advanced stage (n=972); (p=0.297, Mann-Whitney Test).

The patients were equally distributed by sex with 953/1863 (51.2%) females and by CS with 891/1863 (47.8%) early stage cases and 972/1863 (52.2%) advanced stage cases see table 2.1. B-symptoms were assessed in 1834 patients and 41.7% had B-symptoms at diagnosis. Performance status measured by ECOG was recorded in 1673 patients and the majority of patients (73.9%) had ECOG equal to or less than one. Bulky disease was diagnosed in 895/1705 (52.5%) patients and 787/1635 (48.1%) patients had extranodal involvement. BM was the most frequently involved extranodal site in 242/1477 (16.4%) patients.

Clinical parameter	N (%)
Median age, range	66, 18 – 98
Patients aged	
≤ 60 years	711/1863 (38.2)
> 60 years	1152/1863 (61.8)
Female gender	953/1863 (51.2)
ECOG >1	436/1673 (26.1)
CS	
I	403/1863 (21.6)
II	488/1863 (26.2)
III	441/1863 (23.7)
IV	531/1863 (28.5)
B-symptoms	765/1834 (41.7)
Bulk disease	895/1705 (52.5)
BM involvement	242/1477 (16.4)
Extra nodal involvement	787/1635 (48.1)
IPI Group	
Low	403/1057 (38.1)
Low intermediate	280/1057 (26.5)
High intermediate	217/1057 (20.5)
High	157/1057 (14.9)
Abnormal Hb	624/1787 (34.9)
Abnormal WBC	370/1794 (20.6)
Abnormal albumin	439/1707 (25.7)
Abnormal urea	507/1763 (28.8)
Abnormal AP	440/1729 (25.4)
Abnormal LDH	694/1166 (59.5)

Table 2.1 Patient characteristics, all patients (n=1863).

Hb, WBC, albumin, urea and AP were measured in more than 90% of patients at diagnosis and the majority of patients had normal results. LDH was measured in 1166 patients and was elevated in 59.5% of patients. The IPI could be calculated for 1057 patients. Most patients (38.1%) were in the low risk group, followed by 26.5% of patients in the low intermediate risk group, 20.5% of patients in the high intermediate risk group and 14.9% of patients in the high-risk group.

2.3.2.b *Treatment*

The majority of patients 1391 (74.7%) were treated with anthracycline-based chemotherapy, followed by 142 (7.6%) patients who received no chemotherapy and no radiotherapy, and 132 (7.1%) patients who were treated with radiotherapy only. Chemotherapy not including anthracyclines was given to 107 (5.7%) patients and 91 (4.9%) patients were treated with ASCT in first CR after successful treatment with anthracycline-based chemotherapy, see table 2.

Clinical parameter	Patients N (%)
Treatment modality	
Ctx + anthracycline	1391/1863 (74.7)
ASCT in 1st remission	91/1863 (4.9)
Ctx w/o anthracycline	107/1863 (5.7)
Rtx only	132/1863 (7.1)
No Ctx, no Rtx	142/1863 (7.6)
Treatment group	
Curative treatment	1482/1863 (79.6)
Palliative treatment	381/1863 (20.4)
Anthracycline-based regimens	
CHOP	963/1391 (69.2)
CNOP	155/1391 (11.1)
VACOP-B	98/1391 (7.0)
Other	175/1391 (12.6)
Radiotherapy in anthracycline-based regimens	
Chemotherapy alone	936/1391 (967.3)
Combined modality	455/1391 (32.7)

Table 2.2 Patient treatment, all patients (n=1863).

If any anthracycline-based regimen with or without consolidation with ASCT is considered as a curative treatment and other treatment modalities including alternative chemotherapy, radiotherapy alone, no chemotherapy and no radiotherapy are considered palliative treatments, then the vast majority of patients were treated with curative intent 1482 (79.6%). The number of patients treated with palliative intention rose with increasing age of patients. Among the patients aged ≤60 years it was less than or about 10% rising to 16.9% among patients aged 60 – 70 years, 28.8% among patients aged 70 – 80 years and >50% for patients aged >80 years (figure 2.5).

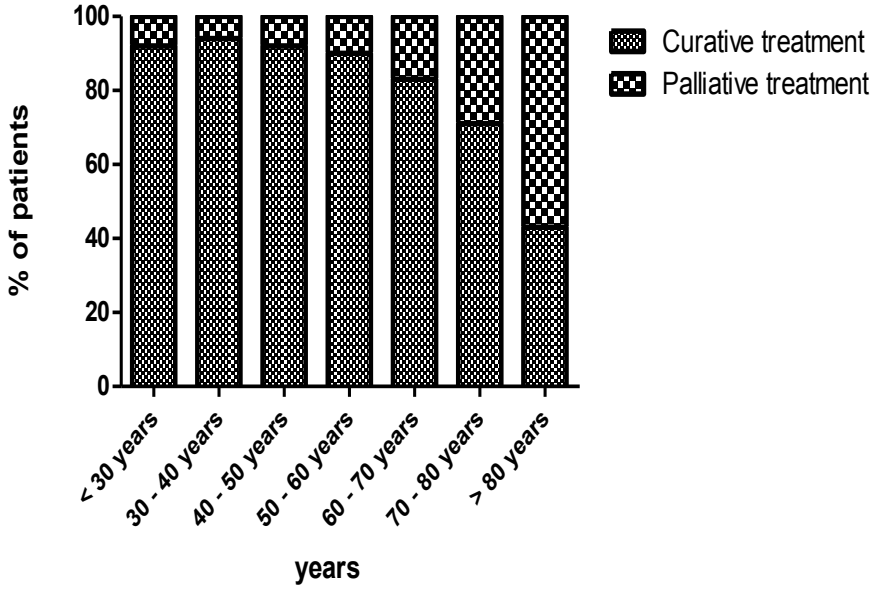


Figure 2.5 Curative and palliative treatment over age-decade groups, evaluation of all patients (n=1863).

For patients treated with anthracycline-based chemotherapy, CHOP was the most frequently given regimen in 963/1391 (69.2%) patients, followed by CNOP in 155/1391 (11.1%) patients and etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone and bleomycin (VACOP-B) in 98/1391 (7.0%) patients. The remaining patients received different regimens, table 2. In patients treated with anthracycline-based regimes, chemotherapy was combined with radiotherapy in 455/1391 (32.7%) patients, and given alone in 936/1391 (67.3%) patients, table 2.2.

The treatment groups are statistically significantly different in terms of patient characteristics in all assessed factors except for serum albumin levels. In order to obtain more detailed results the individual treatment groups were compared with patients treated with standard anthracycline-based chemotherapy, see table 2.3 and 2.4.

Clinical parameter	Ctx + anthracycline N (%)	ASCT in 1st remission N (%)	Ctx w/o anthracycline N (%)	Rtx only N (%)	No Ctx, no Rtx N (%)
Median age, range	64, 18 – 92	45, 20 – 64	75, 42 – 92	73, 24 – 98	76.5, 33 – 96
Patient aged >60 years	831/1391 (21.4)	3/91 (3.3)	94/107 (87.9)	100/132 (75.8)	124/142 (87.3)
Female gender	687/1391 (49.4)	41/91 (45.1)	67/107 (62.6)	73/132 (55.3)	85/142 (59.9)
ECOG >1	266/1245 (21.4)	17/82 (20.7)	51/101 (50.5)	19/120 (15.8)	83/125 (66.4)
Clinical stage 3/4	750/1391 (52.2)	64/91 (70.3)	58/107 (54.2)	16/132 (12.1)	84/142 (59.1)
B-symptoms	584/1378 (42.4)	46/90 (51.1)	49/105 (46.7)	15/130 (11.5)	71/131 (54.2)
Bulk disease	678/1282 (52.9)	67/84 (79.8)	51/98 (52.0)	35/125 (28.0)	64/116 (55.2)
BM involvement	191/1195 (16.0)	16/86 (18.6)	15/62 (24.2)	3/83 (3.6)	17/51 (33.3)
Extra nodal involvement	584/1273 (45.9)	46/91(50.5)	40/107 (37.4)	21/132 (15.9)	72/142 (50.7)
Low IPI	305/1057 (38.0)	22/65 (33.8)	10/60 (16.7)	56/78 (71.8)	10/51 (19.6)
Low intermediate IPI	212/1057 (26.4)	25/65 (38.5)	15/60 (25.0)	19/78 (24.4)	9/51 (17.6)
High intermediate IPI	174/1057 (21.7)	13/65 (20.0)	22/60 (36.7)	2/78 (2.6)	6/51 (11.8)
High IPI	112/1057 (13.9)	5/65 (7.7)	13/60 (21.7)	1/78 (1.3)	26/51 (51.0)
Abnormal Hb	455/1339 (34.0)	31/90 (34.4)	46/103 (44.7)	22/123 (17.9)	70/132 (53.0)
Abnormal WBC	280/1345 (20.8)	15/90 (16.7)	22/102 (21.6)	18/124 (14.5)	35/133 (26.3)
Abnormal albumin	308/1294 (23.8)	19/86 (22.1)	29/100 (29.0)	9/108 (8.3)	74/119 (62.2)
Abnormal urea	341/1340 (25.4)	18/86 (20.9)	47/99 (47.5)	36/114 (31.6)	65/124 (52.4)
Abnormal AP	330/1313 (25.1)	22/87 (25.3)	36/102 (35.3)	9/105 (8.6)	43/122 (35.2)
Abnormal LDH	521/879 (59.3)	57/70 (81.4)	46/72 (63.9)	22/84 (26.2)	48/61 (78.7)

Table 2.3 Patient characteristics in different treatments modalities – all patients (n=1863).

Clinical parameter	Ctx + anthracycline vs. ASCT in 1st remission	Ctx + anthracycline vs. Ctx w/o anthracycline	Ctx + anthracycline vs. Rtx only	Ctx + anthracycline vs. No Ctx, no Rtx
Median age, range	<0.001**	<0.001**	<0.001**	<0.001**
Patient >60 years	<0.001*	<0.001*	<0.001*	<0.001*
Female gender	0.423*	0.008*	0.194*	0.017*
ECOG >1	0.892*	<0.001*	0.154*	<0.001*
CS III / IV	0.002*	0.954*	<0.001*	0.233*
B symptoms	0.105*	0.392*	<0.001*	0.009*
Bulk disease	<0.001*	0.872*	<0.001*	0.637*
BM involvement	0.524*	0.089*	0.002*	0.001*
Extranodal involvement	0.013*	0.056*	<0.001*	<0.001*
IPI Group	0.151*	0.002*	<0.001*	<0.001*
Abnormal Hb	0.928*	0.028*	<0.001*	<0.001*
Abnormal WBC	0.345*	0.857*	0.095*	0.140*
Abnormal albumin	0.718*	0.242*	<0.001*	<0.001*
Abnormal urea	0.349*	<0.001*	0.152*	<0.001*
Abnormal AP	0.974*	0.024*	<0.001*	0.015*
Abnormal LDH	<0.001*	0.443*	<0.001*	0.003*

Table 2.4 Patient characteristics, comparison between different treatments modalities, all patients (n=1863).*) χ^2 – test or Fisher exact test, **) Mann-Whitney-Test

Patients treated with ASCT in first remission were characterised by a statistically significant lower median age, more advanced disease stage (fewer patients in CS I and more in CS IV), increased incidence of bulky disease, extranodal involvement and elevated LDH. By contrast patients treated with chemotherapy not including anthracyclines were statistically significantly older, were more likely to be female, have a higher ECOG status, higher IPI score, and abnormal values of Hb, urea and AP. Patients treated with radiotherapy alone had a higher median age and more localised disease with more patients in CS I. This group was also characterised by lower numbers of patients with B-symptoms, bulky disease, extranodal and BM involvement. There was predominance of the low risk IPI group and a low number of abnormal laboratory tests except for urea levels. Finally, the group of patients offered no anti-cancer treatment differed significantly statistically from those patients treated with standard anthracycline-based chemotherapy in all factors except for CS, bulky disease and abnormal WBC values (table 2.3 and 2.4).

A separate evaluation to compare patients treated with curative and palliative intent was performed, see table 2.5. In the group given palliative treatment there were more patients aged >60 years, more females, patients with ECOG >1, with early CS, extranodal involvement, low and intermediate low IPI and patients with abnormal values of albumin and urea. By contrast there were fewer patients with B-symptoms, bulky disease and elevated LDH.

Clinical parameter	Curative treatment N (%)	Palliative treatment N (%)	p-values
Median age, range	63, 18 – 92	75, 24 – 98	<0.001**
Patient aged > 60 years	834/1482 (56.2)	318/381 (83.5)	<0.001*
Female gender	728/1482 (49.1)	225/381 (59.1)	0.001*
ECOG >1	283/1327 (21.3)	153/346 (44.2)	<0.001*
CS III / IV	814/1482 (54.9)	158/381 (41.5)	<0.001*
B symptoms	630/1468 (42.9)	135/366 (36.9)	0.038*
Bulk disease	745/1366 (54.5)	150/339 (44.2)	0.001*
BM involvement	207/1281 (16.2)	35/196 (17.9)	0.535*
Extra nodal involvement	638/1364 (46.8)	149/271 (55.0)	0.014*
IPI Group			
Low	327/1122 (29.1)	76/189 (40.2)	0.021*
Low intermediate	237/1122 (21.1)	43/189 (22.8)	
High intermediate	187/1122 (16.7)	30/189 (15.9)	
High	117/1122 (10.4)	40/189 (21.2)	
Abnormal Hb	486/1429 (34.0)	138/358 (38.5)	0.108*
Abnormal WBC	295/1435 (20.6)	75/359 (20.9)	0.884*
Abnormal albumin	327/1380 (23.7)	112/327 (34.3)	<0.001*
Abnormal urea	359/1426 (25.2)	148/337 (43.9)	<0.001*
Abnormal AP	352/1400 (25.1)	88/329 (26.7)	0.574*
Abnormal LDH	578/949 (60.9)	116/217 (53.5)	0.046*

Table 2.5 Patient characteristics, comparison of patients with curative and palliatives treatment, all patients (n=1863).*) χ^2 – test or Fisher exact test and **) Mann-Whitney Test

2.3.2.c *Responses and outcome*

At the evaluation of response to treatment 12 months after diagnosis 920/1863 (49.4%) patients were in CR or PR. Remission rates were higher in patients with early disease stage (CS I and II) 556/1863 (62.4%) than those with advanced stage

(CS III and IV) 364/1863 (37.4%). This difference was statistically significant, p<0.001 (table 2.6). The highest rate of remission was observed in patients treated with radiotherapy only with 77/132 (58.3%), followed by patients treated with anthracycline-based chemotherapy with 759/1391 (54.6%) and ASCT in first remission with 48/91 (52.7%). By contrast the remission rates among patients treated with other chemotherapy or with no anti-caner therapy were significantly lower with only 18/107 (16.8%) and 18/142 (12.7%), respectively, the differences were statistically significant (p<0.001), see table 2.6. In the comparative evaluation of patients treated with curative and palliative intent, as expected, the highest response rates were observed in patients given curative treatment 807/1482 (54.5%) vs. 113/381(29.7%), see table 2.6. The difference was statistically significant with p<0.001.

Patient group	CR and PR N (%)	Death N (%)
All patients (n=1863)	920 (49.4)	1071 (57.5)
CS I/II (n=891)	556 (62.4)	417 (46.8)
CS III/IV (n=972)	364 (37.4)	654 (67.3)
p-value	<0.001*	<0.001*
Ctx + anthracycline (n=1391)	759 (54.6)	738 (53.1)
ASCT in 1st remission (n=91)	48 (52.7)	37 (40.7)
Ctx w/o anthracycline (n=107)	18 (16.8)	88 (82.2)
Rtx only (n=132)	77 (58.3)	81 (61.4)
No Ctx and no Rtx (n=142)	18 (12.7)	127 (89.4)
p-value	<0.001*	<0.001*
Curative treatment (n=1482)	807 (54.5)	775 (52.3)
Palliative treatment (n=381)	113 (29.7)	296 (77.7)
p-value	<0.001*	<0.001*

Table 2.6 Patient outcome in all patients and according to disease stages, treatment modality and treatment group – all patients (n=1863). *) χ^2 – test or Fisher exact test

During the study more than half the patients died 1071/1863 (57.5%). In the patients with early stage disease the number of deaths was statistically significantly lower compared with patients with advanced stage disease: 417/891 (46.8%) vs. 654/972 (67.3%), p<0.001 (table 2.6). The highest mortality was observed in patients who did not receive any anti-neoplastic treatment – 127/142 (89.4%) and patients treated with other chemotherapy – 88/107 (82.2%), followed by patients treated with radiotherapy only – 81/132 (61.4%). The lowest death rates were observed among

patients treated with anthracycline-based chemotherapy – 738/1391 (53.1%) and ASCT in first remission – 37/91 (40.7%), see table 2.6. In the evaluation of patients treated with curative treatment compared with palliative treatment the death rate was lower in patients treated with curative treatment at 775/1482 (52.3%) versus patients treated with palliative intent 296/381 (77.7%). The difference was statistically significant with $p<0.001$ (table 2.6).

The median PFS and OS were 11.0 and 32.8 months, respectively. The 5-years PFS and OS were 33% and 41% respectively (table 2.7). In the analysis of PFS and OS of patients in early and advanced stage disease, there were significant differences between both patients groups. The 5-years PFS in patients with early stages vs. patients with advanced stages were 43% vs. 22%, respectively ($p<0.001$) and 5-years OS 51% and 30%, respectively ($p<0.001$) – see table 2.7.

Patient group	5-years PFS	5-years OS
All patients (n=1863)	33%	42%
CS I/II (n=891)	43%	52%
CS III/IV (n=972)	23%	32%
p-value*	<0.001	<0.001
Ctx with anthracyclines (n=1391)	37%	46%
ASCT in 1st remission (n=91)	36%	61%
Ctx without anthracyclines (n=107)	12%	16%
Rtx only (n=132)	29%	41%
No Ctx and no Rtx (n=142)	9%	10%
p-value*	<0.001	<0.001
Curative treatment (n=1482)	37%	46%
Palliative treatment (n=381)	16%	21%
p-value*	<0.001	<0.001

Table 2.7 5-years OS and PFS in all patients and according to disease stages, treatment modality and treatment group, all patients (n=1863),*) Log Rank test

Regarding the 5-years PFS and OS rates in different treatment groups the highest rates were observed in patients treated with ASCT in first remission (36% and 61%, respectively), followed by anthracycline-based chemotherapy (37% and 46%, respectively), and radiotherapy alone (29% and 41%, respectively). Outcomes were significantly lower in patients treated with other chemotherapy (12% and 16%, respectively) and in the group not given any anti-neoplastic treatment (9% and 10%,

respectively). The differences were statistically significant with p values of p<0.001 and p<0.001, (table 2.7). In an evaluation of patients treated with curative intent versus palliative treatment, the 5-years PFS and OS in the curative treatment group was 36.8% and 45.9%, respectively and in the palliative treatment group 16.2% and 21.2%, respectively. The differences were statistically significant for both PFS and OS (p<0.001 and p<0.001, respectively), see table 2.7.

2.3.3 *Age in DLBCL*

Currently the majority of clinical trials for DLBCL are designed separately for young and elderly patients. Additionally, there are individual studies for patients with different disease stages in both age groups, particularly in younger patients. Thus age remains an important factor in the choice of therapy and subsequent outcome of patients with DLBCL. A separate evaluation was performed for young and elderly patients with additional stratification for early and advanced stages. In the evaluation we used the age limits ≤60 years for the younger group of patients and >60 years for elderly patients. We used the Ann Arbor classification to separate early and advanced stages. Early stage was defined as Ann Arbor stages I and II and advanced stage as stages III and IV. The reason for this was the fact that such separation is used in every day decisions in our centre. Additionally an age evaluation of patients decade by decade was performed in order to prove or dispute the correctness of currently used age limits for clinical trials.

2.3.3.a *Evaluation of young and elderly patients*

Patient characteristics

A total number of 711 patients aged ≤60 years and 1152 aged >60 years were evaluated. In younger patients, median age was 50 years; range 18 – 60 years, 46.3% were female and 15.7% had ECOG >1. Patients were equally distributed over CS, 41.9% of patients presented with B-symptoms and 55.5% had bulky disease. Extranodal involvement was found in 43.2% of patients with BM being infiltrated in 13.5%. Most patients were in low or low intermediate IPI risk groups (84.6%) and only 15.4% were in high intermediate and high IPI groups. Laboratory tests were normal in the majority of patients except for LDH. See table 2.8 for patient characteristics.

Clinical parameter	≤ 60 years N (%)	> 60 years N (%)	p-value*
Median age, range	50, 18 - 60	72, 61 - 98	n.a.
Female gender	329/711 (46.3)	624/1152 (54.2)	0.002
ECOG >1	103/658 (15.7)	333/1015 (32.8)	<0.001
CS			
I	157/711 (22.1)	246/1152 (21.4)	0.688
II	195/711 (27.4)	293/1152 (25.4)	
III	165/711 (23.2)	276/1152 (24.0)	
IV	194/711 (27.3)	337/1152 (29.3)	
B-symptoms	295/704 (41.9)	470/1130 (41.6)	0.922
Bulk disease	360/649 (55.5)	535/1056 (50.7)	0.058
BM involvement	85/628 (13.5)	157/849 (18.5)	0.013
Extra nodal involvement	287/664 (43.2)	500/971 (51.5)	<0.001
IPI Group			
Low	252/442 (57.0)	151/615 (24.6)	<0.001
Low intermediate	122/442 (27.6)	158/615 (25.7)	
High intermediate	50/442 (11.3)	167/615 (27.2)	
High	18/442 (4.1)	139/615 (22.6)	
Abnormal Hb	196/680 (28.8)	428/1107 (38.7)	<0.001
Abnormal WBC	129/684 (18.9)	241/1110 (21.7)	0.150
Abnormal albumin	114/659 (17.3)	325/1048 (31.0)	<0.001
Abnormal urea	103/674 (15.3)	404/1089 (37.1)	<0.001
Abnormal AP	164/671 (24.4)	276/1058 (26.1)	0.462
Abnormal LDH	275/478 (57.5)	419/688 (60.9)	0.250

Table 2.8 Patient characteristics, comparison of younger and elderly patients - all patients (n=1863).*) χ^2 – test or Fisher exact test.

Among elderly patients the median age was 72 years (range 61 – 98 patients), there were more females than males (54.8% vs. 45.8%), 32.8% patients had ECOG >1 and 41.6% B-symptoms. The patients were also equally distributed over CS. Bulky disease was diagnosed in 50.7% and 51.5% had extranodal involvement with 18.5% of patients with BM involvement. The elderly patients were equally distributed over IPI risk groups with 50.3% of patients in low and low intermediate risk groups and 49.7% in high intermediate and high-risk groups. The majority of patients had normal laboratory tests except for LDH but more patients had abnormal Hb levels, albumin and urea as compared to younger patients (table 2.8).

In the comparison of patients aged ≤60 years and those aged >60 years; in the younger group there were statistically significantly more male patients (p=0.001), patients with ECOG equal to or less than one (<0.001), and fewer patients with extranodal and BM involvement, p=0.001 and p=0.013, respectively. Significantly more younger patients were in IPI low risk and low intermediate risk groups (<0.001), and had normal Hb (p=0.001), albumin (p<0.001) and urea values (p<0.001), see table 2.8.

Treatment

The majority of younger patients received anthracycline-based chemotherapy 560 patients (74%), followed by a group treated with ASCT in 1st remission 88 patients (12.4%), radiotherapy only 32 patients (4.5%), no chemotherapy and no radiotherapy 18 (2.5%) and other chemotherapy 13 (1.8%). In the comparison of early and advanced stage patients, there were more patients treated with ASCT in 1st remission in the advanced stage group (27/352 – 7.7% vs. 61/359 – 17%) and fewer patients treated with radiotherapy only (4/359 – 1.1% vs. 28/352 – 8.0%), see figure 2.6.

In patients aged >60 years a majority were also treated with anthracycline-based chemotherapy 831/1152 (72.1%), followed by the group of patients given no chemotherapy or radiotherapy 124/1152 (10.8%), radiotherapy only 100/1152 (8.7%), and other chemotherapy 94/1152 (72.1%). Only 3 patients received ASCT in 1st remission. Also in this age group, there were statistically significant differences in the treatment given to patients in early and advanced stage disease. In patients with early stage disease more patients were treated with radiotherapy only, whereas the advanced stage patients received anthracycline-based chemotherapy (figure 2.6).

In the comparison between young and elderly patients, there were more patients treated with anthracycline-based chemotherapy and ASCT in 1st remission among younger patients. By contrast among elderly patients, there were more patients treated with radiotherapy alone, no chemotherapy or radiotherapy or other chemotherapy. The difference was statistically significant, p<0.001, for details see figure 2.6.

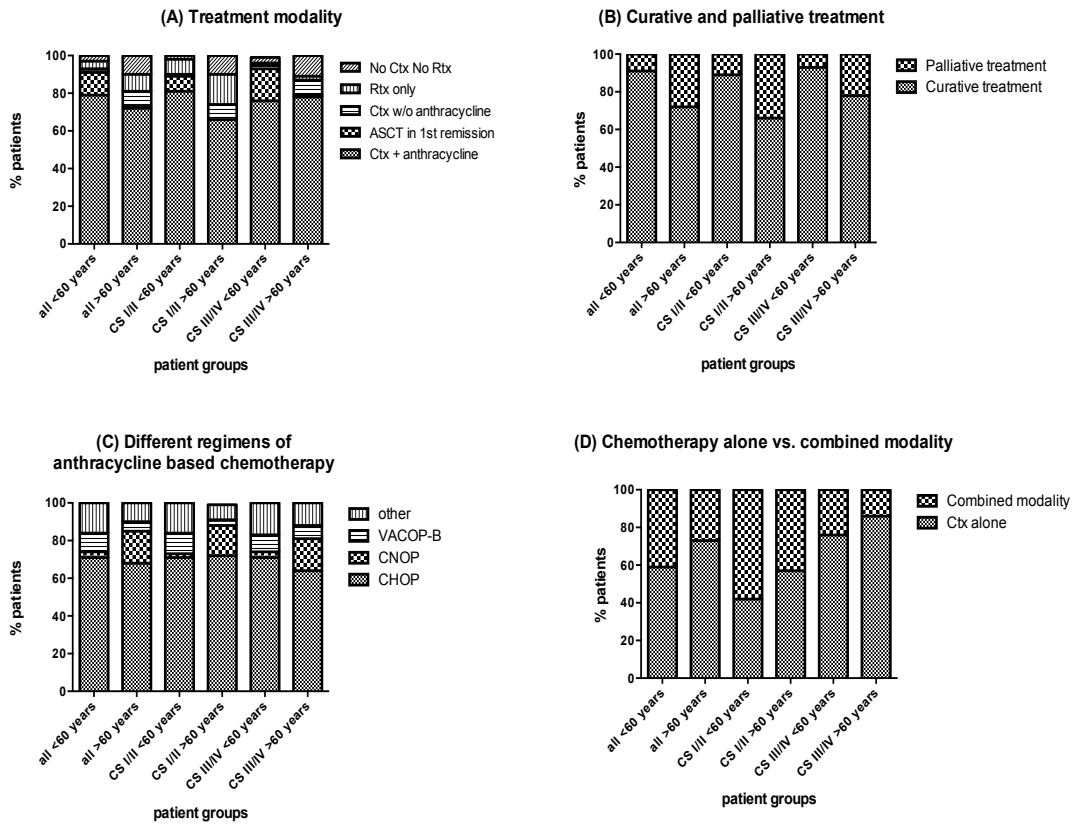


Figure 2.6 Treatment in younger and elderly patients in early and advanced disease stages evaluation of all patients (n=1863). **(A)** treatment modality: ≤60 years vs >60 years CS, (p=0.000)* and ≤60 years: CS I/II vs CS III/IV (p=0.000)* and >60 years CS I/II vs CS III/IV (p=0.000)* **(B)** Curative and palliative treatment: ≤60 years vs >60 years CS, (p=0.000)* and ≤60 years: CS I/II vs CS III/IV (p=0.086)* and >60 years CS I/II vs CS III/IV (p=0.000)* **(C)** Different regimens of anthracycline-based chemotherapy: ≤60 years vs >60 years CS, (p=0.000)* and ≤60 years: CS I/II vs CS III/IV (p=0.752)* and >60 years CS I/II vs CS III/IV (p=0.030)* **(D)** Chemotherapy alone vs. combined modality in anthracycline-based chemotherapy ≤60 years vs >60 years CS, (p=0.000)* and ≤60 years: CS I/II vs CS III/IV (p=0.000)* and >60 years CS I/II vs CS III/IV (p=0.000)*, *)Mann-Whitney test

In the evaluation of groups treated with curative versus palliative intent, the vast majority of younger patients received the curative option: 648/711 (91.1%) and only 63/711 patients (8.9%) received palliative treatment (figure 6). There were no significant differences in evaluation of early and advanced stages diseases (p=0.086). Among elderly patients the number of patients in the palliative group was statistically significantly higher as compared with younger patients: 318/1152 (27.6%), p=0.000. In the comparison of early vs. advanced stages there were more early stage patients in in the palliative treatment group (p=0.000).

In the evaluation of patients treated with anthracycline-based chemotherapy CHOP was the most frequently given treatment to 398/560 (71%) of younger patients

and 565/831 (68%) of elderly patients. In younger patients this was followed by groups of patients treated with VACOP-B 56/560 (10%) and patients treated with CNOP 16/560 (2.9%). In elderly patients CNOP 139/831 (16.7%) and VACOP-B 42/831 (5.1%) were the next most commonly utilised regimens after CHOP. The difference was statistically significant (p=0.000). In an evaluation comparing early and advanced stages the difference was statistically significant in elderly patients, where patients in CS III and IV received significantly less CHOP and more CNOP (p=0.030). This finding was not seen in younger patients (p=0.752), figure 2.6.

Among 560 patients aged ≤60 years treated with anthracycline-based chemotherapy, 232 (41.4%) received additional radiotherapy. In the group of elderly patients the figure was 223/831 (26.8%), a statistically significant lower number (p=0.000). In both age groups there were more early stage patients treated with combined modality treatment compared with patients with advanced stage disease. The differences were statistically significant for younger and for elderly patients, both p<0.001, for details see figure 2.6.

Responses and outcome

At the evaluation 12 months after the day of diagnosis 430/711 (60.5%) of younger patients and 490/1152 (42.5%) of elderly patients were in CR/PR, the difference was statistically significant, p=0.000 (table 2.9). In a comparison of early and advanced stage disease we observed a significantly higher number of CR/PR in patients with early stage disease in both age groups; 262/352 (74.4%) vs. 168/359 (46.8%); p=0.000 in younger patients, and 294/539 (54.5%) vs. 196/613 (32%); p=0.000 in elderly patients (table 2.10).

As previously mentioned this study was not designed to assess the difference between treatment groups or reach a conclusion on superiority of one over another. Hence this is the only description of the outcomes in each group. In the group of younger patients remission rates were highest in patients treated with anthracycline-based chemotherapy 356/560 (63.6%) followed by radiotherapy only 20/32 (71.4%) and ASCT in first remission 47/88 (55.6%) (table 2.9). By contrast among elderly patients the highest remission rates were observed in patients treated with radiotherapy only 57/100 (57%), followed by patients receiving anthracycline-based chemotherapy 403/831 (48.5). The number of patients treated with ASCT in first remission is too small to make any conclusions (table 2.9). The lowest remission rates were found in

both age groups in patients receiving chemotherapy without anthracyclines 3/13 (23.1%) and 15/94 (16.0%) or no chemotherapy and no radiotherapy 4/18 (22.2%) and 14/124 (11.3%) (table 2.9).

Patient group	CR / PR N (%)		Death N (%)	
	Younger (n=711)	Elderly (n=1152)	Younger (n=711)	Elderly (n=1152)
All patients (n=1863)	430 (60.5)	490 (42.5)	297 (41.8)	774 (67.2)
p-value*	<0.000		<0.000	
Ctx +anthracyclines (n=1391)	356 (63.6)	403 (48.5)	226 (41.0)	512 (61.6)
ASCT in 1st remission (n=91)	47 (53.4)	1 (33.3)	36 (40.9)	1 (33.3)
Ctx w/oanthracyclines (n=107)	3 (23.1)	15 (16.0)	7 (53.8)	81 (87.1)
Rtx only (n=132)	20 (62.5)	57 (57.0)	14 (45.2)	67 (67.0)
No Ctx and no Rtx (n=142)	4 (22.2)	14 (11.3)	14 (82.4)	113 (91.1)
p-value*	<0.000	<0.000	0.014	<0.000
Curative treatment (n=1482)	403 (62.2)	404 (48.4)	262 (40.4)	513 (61.5)
Palliative treatment (n=381)	27 (42.9)	86 (27.0)	35 (55.5)	261 (82.1)
p-value*	0.004	<0.000	0.021	<0.000

Table 2.9 Patient outcome in younger and elderly patients, all patients, in different treatment modality and treatment groups - all patients (n=1863).*) χ^2 – test or Fisher exact test

In the evaluation of early versus advanced stage disease in both age groups the standard treatment with anthracycline-based chemotherapy produced the highest and the second highest number of remissions in early stage disease: in younger patients 223/287 (77.7%) and 216/354 (54.5%) in elderly patients. In younger patients it was followed and in elderly preceeded by the radiotherapy alone group with 20/28 (71.4%) and 54/88 (61.4%), respectively. In advanced stage disease the highest remission rates were among the patients treated with ASCT in first remission in younger patients 32/61 (52.5%) followed by anthracycline-based chemotherapy 133/277 (48.7%); the latter regimen also gave the highest responses in elderly patients 187/377 (39.2%). The patients treated with chemotherapy without anthracyclines and with no chemotherapy or no radiotherapy had the lowest response rates in both age cohorts and disease stages (table 2.10).

(A)

Patient group	CR / PR N (%)		Death N (%)	
	CS I/II (n=352)	CS III/IV (n=359)	CS I/II (N=352)	CS III/IV (N=359)
All patients (n=711)	262 (74.4)	168 (46.8)	101 (28.7)	196 (54.6)
p-value*	<0.001		0.000	
Ctx + anthracyclines (n=560)	223 (77.7)	133 (48.7)	77 (26.8)	149 (54.6)
ASCT in 1st remission (n=88)	15 (55.6)	32 (52.5)	10 (37.0)	26 (42.6)
Ctx w/o anthracyclines (n=13)	2 (50.0)	1 (11.1)	0 (0.0)	7 (77.8)
Rtx only (n=32)	20 (71.4)	0 (0.0)	10 (35.7)	4 (100.0)
No Ctx and no Rtx (n=18)	2 (33.3)	2 (16.7)	4 (66.6)	10 (83.3)
p-value*	0.008	0.006	0.237	0.011
Curative treatment (n=648)	238 (75.8)	165 (49.4)	87 (27.7)	175 (52.4)
Palliative treatment (n=63)	24 (63.2)	3 (12.0)	14 (36.8)	21 (84)
p-value*	0.114	<0.001	0.251	0.001

(B)

Patient group	CR / PR N (%)		Death N (%)	
	CS I/II (N=539)	CS III/IV (N=613)	CS I/II (N=539)	CS III/IV (N=613)
All patients (n=1152)	294 (54.5)	196 (32.0)	316 (58.6)	458 (74.7)
p-value*	<0.001		0.008	
Ctx + anthracyclines (n=831)	216 (61.0)	187 (39.2)	184 (52.0)	328 (68.8)
ASCT in 1st remission (n=3)	0 (0.0)	1 (33.3)	0 (0.0)	1 (33.3)
Ctx w/o anthracyclines (n=94)	13 (28.9)	2 (4.1)	32 (71.1)	49 (100.0)
Rtx only (n=100)	54 (61.4)	3 (25.0)	57 (64.9)	10 (83.3)
No Ctx and no Rtx (n=124)	11 (21.2)	3 (4.2)	43 (82.7)	70 (97.2)
p-value*	<0.001	<0.001	<0.001	<0.001
Curative treatment (n=834)	216 (61.0)	188 (39.2)	184 (52.0)	329 (68.5)
Palliative treatment (n=318)	78 (42.2)	8 (6.0)	132 (71.4)	129 (97.0)
p-value*	<0.001	<0.001	<0.001	<0.001

Table 2.10 Patient outcome according to CS in younger and elderly patients, all patients and in different modality and treatment groups. **(A)** younger patients (n=711) and **(B)** elderly patients (n=1152). *) χ^2 – test or Fisher exact test

In the evaluation of patients treated with curative versus palliative intent we observed statistically significantly higher levels of CR/PR in patients treated with

curative intent: 403/648 (62.2%) vs. 27/63 (42.3%); p=0.004 in younger patients and 404/834 (48.4%) vs. 86/318 (27.0%); p=0.000 in elderly patients (table 2.9). This trend was also seen in the evaluation of early and late stages of the disease among younger and elderly patients (table 2.10).

The number of patients who died during the study was significantly higher among the elderly patients and patients with advanced disease, the latter was true for both age groups (table 2.9 and 2.10). The highest death rates were observed in patients receiving no chemotherapy or radiotherapy, followed by the group treated with chemotherapy without anthracyclines and the group treated with radiotherapy only. This was the case for both age groups and all stages except for early stage disease in younger patients (table 2.10). The death rates were statistically significantly higher in patients treated with palliative treatment in both age cohorts for all disease stages. In the group of elderly patients with advanced disease almost all patients died (97%).

The 5-years PFS and OS were both significantly higher among younger patients compared with elderly patients: 44.6% vs. 57.7% and 24.7% vs. 29.8% respectively (p=0.000 for both), table 2.11.

Patient group	5-years PFS %		5-years OS %	
	Younger (N=711)	Elderly (N=1152)	Younger (N=711)	Elderly (N=1152)
All patients (n=1863)	44.6	24.7	57.7	29.8
p-value*	<0.001		<0.001	
Ctx + anthracyclines (n=1391)	47.4	29.4	58.7	35.2
ASCT in 1st remission (n=91)	35.6	33.3	60.1	50.0
Ctx w/o anthracyclines (n=107)	0.0	10.2	49.5	8.8
Rtx only (n=132)	45.8	23.1	58.1	33.2
No Ctx and no Rtx (n=142)	0.0	7.1	15.7	8.5
p-value*	<0.001	<0.001	<0.001	<0.001
Curative treatment (n=1482)	45.8	29.4	58.9	35.3
Palliative treatment (n=381)	31.9	12.9	44.5	16.3
p-value*	0.017	<0.001	0.002	<0.001

Table 2.11 5-years PFS and OS in younger and elderly patients, all patients, in different treatment modality and treatment groups - all patients (n=1863).*) Log Rank test

(A)

	5-years PFS (%)		5-years OS (%)	
	CS I/II (N=352)	CS III/IV (N=359)	CS I/II (N=352)	CS III/IV (N=359)
All patients (n=711)	59.8	29.6	71.5	45.7
p-value*	<0.001		<0.001	
Ctx + anthracyclines (n=560)	63.9	29.8	73.0	44.9
ASCT in 1st remission (n=88)	32.6	36.8	62.5	59.8
Ctx w/o anthracyclines (n=13)	50.0	0.0	ND	27.8
Rtx only (n=32)	52.4	0.0	67.2	0.0
No Ctx and no Rtx (n=18)	33.3	6.7	33.0	16.7
p-value*	0.004	0.003	<0.001	<0.001
Curative treatment (n=648)	61.2	31.3	71.8	46.9
Palliative treatment (n=63)	48.4	8.0	63.8	14.3
p-value*	0.111	0.001	0.234	<0.001

(B)

Patient group	5-years PFS (%)		5-years OS (%)	
	CS I/II (N=539)	CS III/IV (N=613)	CS I/II (N=539)	CS III/IV (N=613)
All patients (n=1152)	31.1	19.0	37.8	23.9
p-value*	<0.001		<0.001	
Ctx + anthracyclines (n=831)	37.6	23.2	43.7	29.8
ASCT in 1st remission (n=3)	ND	33.3	ND	50.0
Ctx w/o anthracyclines (n=94)	19.1	20.0	16.6	20.0
Rtx only (n=100)	23.6	16.7	37.4	16.7
No Ctx and no Rtx (n=124)	13.3	28.0	17.6	21.0
p-value*	<0.001	<0.001	<0.001	<0.001
Curative treatment (n=834)	37.6	23.2	43.3	29.3
Palliative treatment (n=318)	19.3	3.8	25.9	3.5
p-value*	<0.001	<0.001	<0.001	<0.001

Table 2.12 5-years PFS and OS according to CS in younger and elderly patients, all patients and in different modality and treatment groups. **(A)** younger patients (n=711) and **(B)** elderly patients (n=1152). *) Log Rank test

This was the case for all treatment groups. The highest 5-years PFS and OS rates were observed in patients treated with standard anthracycline-based chemotherapy with

or without consolidation with ASCT and in the group treated with radiotherapy only. By contrast the lowest rates were observed in patients treated with chemotherapy without anthracyclines, and those receiving no chemotherapy and no radiotherapy, table 2.11. In early stage patients the highest 5-years PFS and OS rates were observed in patients treated with anthracycline-based chemotherapy or radiotherapy alone in both age groups. In patients with advanced stage disease the best survival rates were achieved in the standard chemotherapy group and the group receiving ASCT in first remission (younger and elderly patients). In the evaluation of curative and palliative groups, as expected, the 5-years PFS and OS were significantly lower for palliative treatment compared with curative treatment in both age groups and for all disease stages (table 2.11 and 2.12).

2.3.3.b ***Evaluation of age limits***

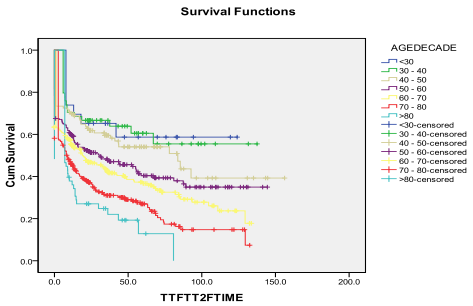
In order to evaluate the influence of patient age on PFS and OS more accurately, the patients were assigned to eight different age groups:

- <30 years
- 30 – 40 years
- 40 - 50 years
- 50 - 60 years
- 60 – 70 years
- 70 - 80 years
- >80 years

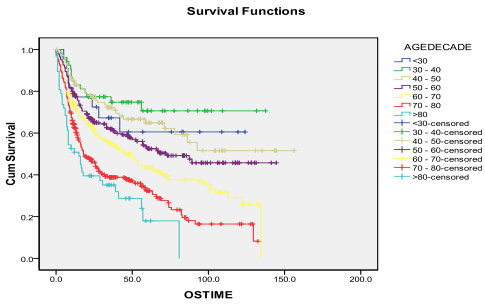
All groups were evaluated for PFS and OS. This evaluation was performed for all patients treated with CHOP or CNOP and separately for patients in early and advanced stages. The large number of patients made it possible to perform such an analysis

Figures 2.7 – 2.9 show Kaplan-Meyer plots and corresponding 5-years PFS and OS for all patients, patients with early, and patients with advanced stage disease. In the log-rank test calculated over all age groups, there were significant differences for both PFS and OS ($p<0.001$ for all tests). Tables in figures show the p-values for the log-rank test run pairwise between all age groups for PFS and OS in all patients, patients with early and patients with advanced stage disease.

(A)



(B)



(C)

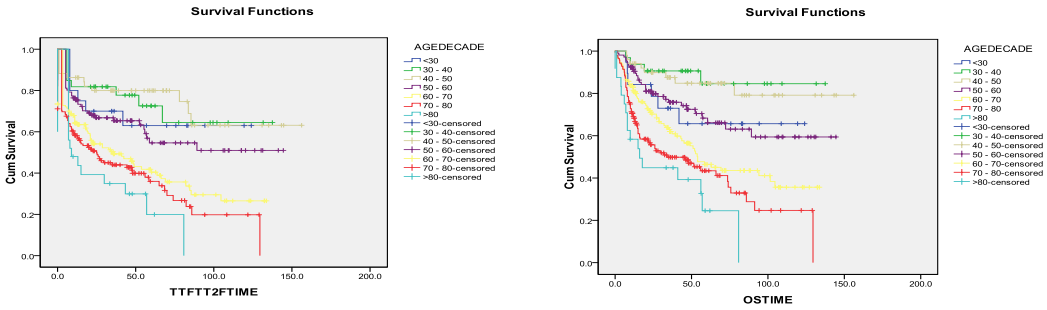
Age group	5-years PFS	5-years OS
≤30 years	58.7	60.5
30 - 40 years	60.5	70.6
40 - 50 years	54.1	64.9
50 - 60 years	40.4	53.4
60 - 70 years	36.7	43.4
70 - 80 years	27.0	32.4
>80 years	12.9	18.0

(D)

PFS						
Years	**30 – 40**	**40 – 50**	**50 – 60**	**60 – 70**	**70 – 80**	**>80**
≤30	0.992	0.502	0.111	0.030	<0.001	<0.001
30 – 40		0.351	0.023	0.002	<0.001	<0.001
40 – 50			0.073	0.002	<0.001	<0.001
50 – 60				0.137	<0.001	<0.001
60 – 70					0.002	<0.001
70 – 80						0.075
OS						
Years	**30 – 40**	**40 – 50**	**50 – 60**	**60 – 70**	**70 – 80**	**>80**
≤30	0.410	0.956	0.423	0.930	0.003	<0.001
30 – 40		0.336	0.027	0.001	<0.001	<0.001
40 – 50			0.079	<0.001	<0.001	<0.001
50 – 60				0.020	<0.001	<0.001
60 – 70					<0.001	<0.001
70 – 80						0.053

Figure 2.7 Kaplan-Meier plots for PFS and OS in all CHOP/CNOP treated patients according to age decades (n=1118). **(A)** PFS (p<0.001)*, **(B)** OS (p<0.001)* **(C)** 5-years PFS and OS **(D)** p-values* for pairwise comparison of PFS &OS in different age decades, all patients treated with CHOP/CNOP (n=1118). *) Log Rank Test

(A) **(B)**



(C)

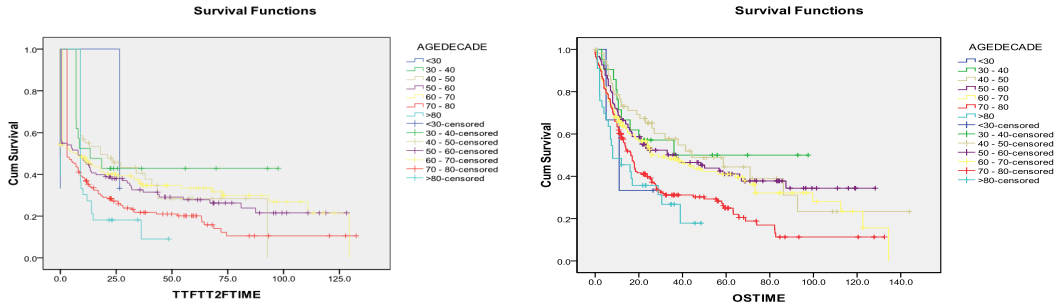
Age group	5-years PFS	5-years OS
≤30 years	63.0	65.7
30 - 40 years	72.5	84.6
40 - 50 years	80.0	84.8
50 - 60 years	54.6	68.3
60 - 70 years	40.4	46.5
70 - 80 years	36.0	43.4
>80 years	19.9	24.5

(D)

PFS						
Years	**30 – 40**	**40 – 50**	**50 – 60**	**60 – 70**	**70 – 80**	**>80**
≤30	0.575	0.385	0.622	0.050	0.014	0.004
30 – 40		0.778	0.174	0.002	<0.001	<0.001
40 – 50			0.053	<0.001	<0.001	<0.001
50 – 60				0.005	<0.001	<0.001
60 – 70					0.200	0.033
70 – 80						0.145
OS						
Years	**30 – 40**	**40 – 50**	**50 – 60**	**60 – 70**	**70 – 80**	**>80**
≤30	0.103	0.152	0.984	0.146	0.023	0.005
30 – 40		0.726	0.054	0.001	<0.001	<0.001
40 – 50			0.05	0.000	<0.001	<0.001
50 – 60				0.004	<0.001	<0.001
60 – 70					0.030	0.008
70 – 80						0.110

Figure 2.8 Kaplan-Meier plots for PFS and OS in early CS CHOP/CNOP treated patients according to age decades (n=524). **(A)** PFS (p<0.001)*, **(B)** OS (p<0.001)* **(C)** 5-years PFS and OS **(D)** p-values* for pairwise comparison of PFS &OS in different age decades. *) Log Rank Test

(A) **(B)**



(C)

Age group	5-years PFS	5-years OS
≤30 years	33.3	33.3
30 - 40 years	42.9	50.0
40 - 50 years	28.5	44.4
50 - 60 years	27.8	41.1
60 - 70 years	33.4	41.0
70 - 80 years	20.1	25.0
>80 years	9.1	17.9

(D)

PFS						
Years	**30 – 40**	**40 – 50**	**50 – 60**	**60 – 70**	**70 – 80**	**>80**
≤30	0.588	0.603	0.785	0.768	0.890	0.666
30 – 40		0.589	0.309	0.461	0.040	0.029
40 – 50			0.552	0.887	0.020	0.004
50 – 60				0.575	0.031	0.025
60 – 70					0.005	0.010
70 – 80						0.231
OS						
Years	**30 – 40**	**40 – 50**	**50 – 60**	**60 – 70**	**70 – 80**	**>80**
≤30	0.380	0.197	0.439	0.568	0.827	0.939
30 – 40		0.920	0.563	0.447	0.037	0.025
40 – 50			0.529	0.220	<0.001	<0.001
50 – 60				0.513	<0.001	0.003
60 – 70					0.006	0.008
70 – 80						0.211

Figure 2.9 Kaplan-Meier plots for PFS and OS in advanced CS according to age decades (n=594). **(A)** PFS (p<0.001)*, **(B)** OS (p<0.001)*, **(C)** 5-years PFS and OS, **(D)** p-values* for pairwise comparison of PFS &OS in different age decades. *) Log Rank Test

In the PFS evaluation of all patients and patients with advanced stage disease statistically significant differences were observed between “60 – 70 years” vs. “70 – 80 years” groups. In patients with early stage disease the significance difference was observed between “50 - 60 years” vs. “60 – 70 years” groups.

In the OS evaluation of all patients and patients with early stage disease statistically significant differences were observed for OS between following groups: “50 - 60 years” vs. “60 – 70 years” and “60 – 70 years” vs. “70 – 80 years”. By contrast significant differences for OS in patients with advanced stage disease were seen between: “60 – 70 years” vs. “70 – 80 years” only. Using these data the following new age groups could be built for evaluation of PFS:

1) All patients and patients with advanced stages:

- “18 – 70 years”
- “>70 years”

2) Patient with early stages:

- “18 – 60 years”
- “>60 years”.

And for evaluation of OS:

1) All patients and patient with early stage:

- “18 – 60 years”
- “60 – 70 years”
- “>70 years”

2) Patient with advanced stages:

- “18 – 70 years”
- “>70 years”

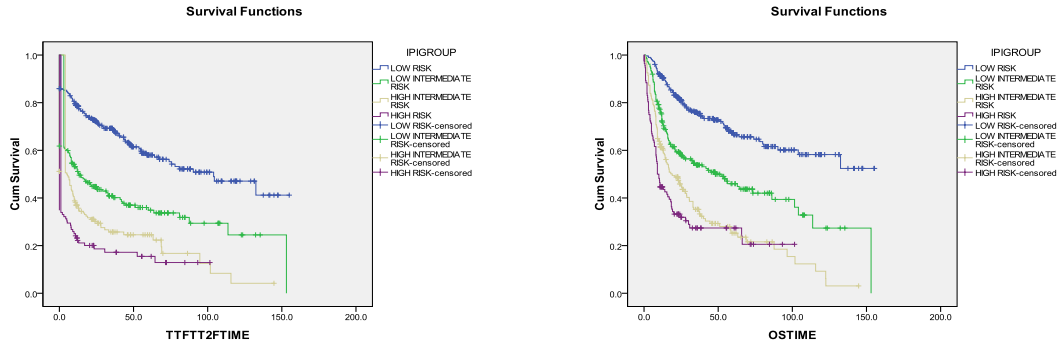
2.3.4 *Evaluation of International Prognostic Index*

An additional separate analysis of the prognostic value of IPI and aaIPI were performed in patients treated with anthracycline-based chemotherapy. The IPI was calculated for all patients and the aaIPI additionally in younger patients. The IPI could be calculated in 803/1391 (57.7%) patients treated with anthracycline-based chemotherapy and the aaIPI was additionally calculated in 353/560 (63.0%) younger patients. In the evaluation of all patients according to IPI most patients belonged to the low risk group 305/803 (38.0%), followed by low intermediate group 212/803 (26.4%) and high intermediate group 174/803 (21.7%). Only 112/803 (13.9%) belonged to the high-risk group. By contrast the distribution of risk groups by aaIPI in younger patients was different with the low intermediate risk group being the most frequent 114/353

(32.3%), followed by high-intermediate risk group 109/353 (30.9%) and low risk group 101/353 (28.6%). The high-risk group was the least frequent at 29/353 (8.2%).

For all patients the 5-year PFS and OS were: 58.0% and 67.3% in the low risk group, 34.9% and 46.0% in the low intermediate group, 24.5% and 25.2% in the high intermediate group and 15.5% and 27.4% in the high risk group; p<0.001 (figure 2.10). In the pairwise evaluation, the IPI was able to distinguish between all risk groups for OS except for no difference between low risk and low-intermediate risk groups. For PFS there were also no differences between low risk and low intermediate groups or between low intermediate and high intermediate risk groups (figure 2.10).

(A) **(B)**



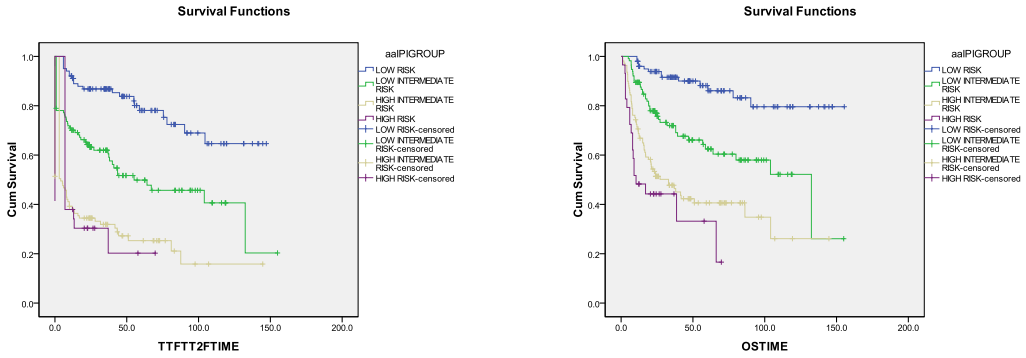
(C)

PFS			
IPI group	**Low intermediate**	**High intermediate**	**High**
Low risk	0.275	0.015	<0.000
Low intermediate		0.153	<0.000
High intermediate			0.012
OS			
IPI group	**Low intermediate**	**High intermediate**	**High**
Low risk	0.433	0.004	<0.000
Low intermediate		0.023	<0.000
High intermediate			0.033

Figure 2.10 Kaplan-Meier plots for PFS and OS according to IPI risk groups, population-based evaluation, all patients treated with anthracycline-based chemotherapy with calculated IPI (n=803). **(A)** 5-years PFS: low risk group 58.0%, low intermediate risk group 34.9%, high intermediate risk group 24.5% and high risk group 15.5%; (p=0.000)*, **(B)** 5-years OS: low risk group 67.3%, low intermediate risk group 46.0%, high intermediate risk group 25.2% and high risk group 27.4%; (p=0.000)* and **(C)** p-values for pairwise comparison of PFS & OS in different IPI groups. *) Log Rank Test

In younger patients the 5-year PFS and OS according to aaIPI were as follows: 78.1% and 88.2% in the low risk group, 49.9% and 62.5% in the low intermediate group, 25.3% and 40.6% in the high intermediate group and 20.2% and 33.2% in the high risk group; p<0.001 (Figure 2.11). In contrast to the IPI the aaIPI was able to distinguish between all risk groups in a pairwise comparison for PFS and OS with the exception of high intermediate and high-risk groups, see figure 2.11.

(A) **(B)**



(C)

PFS			
Risk group	**Low intermediate**	**High intermediate**	**High**
Low risk	<0.001	<0.001	<0.001
Low intermediate		<0.001	<0.001
High intermediate			0.609
OS			
Risk group	**Low intermediate**	**High intermediate**	**High**
Low risk	<0.001	<0.001	<0.001
Low intermediate		<0.001	<0.001
High intermediate			0.112

Figure 2.11 Kaplan-Meier plots for PFS and OS according to aaIPI risk groups, population-based evaluation, patients aged ≤60 years treated with anthracycline-based chemotherapy with calculated aaIPI (n=353). **(A)** 5-years PFS: low risk group 78.1%, low intermediate risk group 49.9%, high intermediate risk group 25.3% and high risk group 20.2%; (p=0.000)*: **(B)** 5-years OS: low risk group 88.2%, low intermediate risk group 62.5%, high intermediate risk group 40.6% and high risk group 33.2%; (p=0.000)* and **(C)** p-values for pairwise comparison of PFS & OS in different aaIPI groups. *) Log Rank Test

2.3.5 *Prognostic value of duration of first remission on overall survival*

In order to assess the impact of duration of first remission on OS of patients treated with anthracycline-based chemotherapy, four groups were defined: “refractory

group" for patients who did not respond to first line treatment or relapsed within 9 months from diagnosis, "early relapse group" for patients who relapsed within 9 – 18 months from diagnosis, "late relapse group" for patients who relapsed after 18 months from diagnosis and "no relapse group" for patients who did not relapse. These groups were also assessed for possible differences between them in clinical features or IPI at presentation.

Of 1391 patients treated with anthracycline-based chemotherapy 1375 patients were evaluable. 16 patients were excluded from analysis due to short follow-up. Most patients belonged to the "no relapse group" – 597/1375 (43%), followed by a comparable number 566/1375 (41%) in the "refractory group", 120/1375 (9%) in the "late relapse group" and finally 92/1375 (7%) patients in the "early relapse group", see figure 12

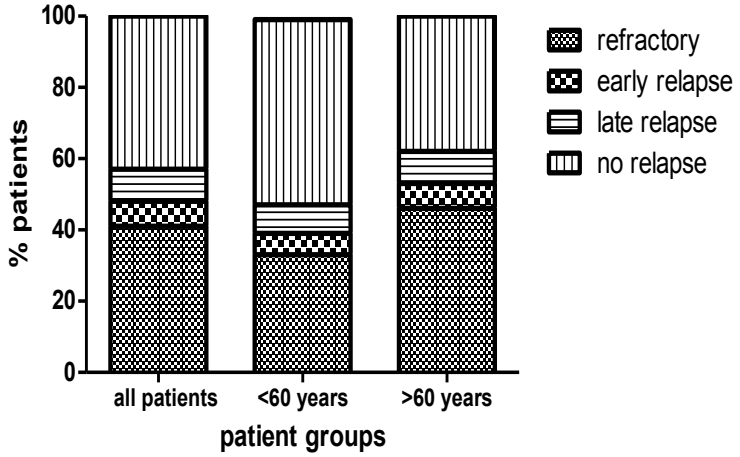


Figure 2.12 Different group according to duration of remission, all patients treated with anthracycline-based chemotherapy (n=1375), patients ≤60 years (n=557) and patients >60 years (n=818), p=0.001 - χ^2 – test or Fisher exact test.

557/1375 patients were aged ≤60 years and their distribution pattern was comparable with that described above and was as follows: 290/557 (52%) patients in the "no relapse group", 185/557 (33%) patients in the "refractory group", 46/557 (8%) patients in the "late relapse group" and 36/557 (7%) patients in the "early relapse group" (see figure 2.12). By contrast for patients aged >60 years most patients 381/818 (46%) belonged to the "refractory group", followed by 307/818 (38%) patients in the "no relapse group", 74/818 (9%) patients in the "late relapse group" and 56 patients (7%) in the "early relapse group" (figure 2.12).

Detailed patient characteristic for each group is provided in table 2.13. Generally the "refractory group" was characterised by the highest median age of patients and by contrast the "no relapse group" by the lowest. The female/male ratio varied about 1.0 with the highest value in the "no relapse group" and the lowest in the "early relapse group". The "refractory group" was characterised by the highest number of patients with advanced disease as measured by Ann Arbor CS, IPI, higher ECOG score values, B-symptoms, bulky disease, extranodal and BM involvement, and abnormal laboratory. By contrast fewer patients with the above features were in the "late relapse group" or in the "no relapse group".

Clinical parameter	Refractory (n=566)	Early Relapse (n=92)	Late relapse (n=120)	No relapse (n=597)
	N (%)	N (%)	N (%)	N (%)
Median age (range)	68 (18 - 91)	66 (26 – 85)	63 (28 – 92)	61 (18 – 92)
Age >60 years	381/566 (67)	56/92 (61)	74/120 (62)	307/597 (51)
Female gender	272/566 (48)	39/92 (42)	60/120 (50)	308/597 (52)
CS III and IV	393/566 (69)	61/92 (67)	59/120 (50)	129/597 (39)
B-symptoms	318/556 (57)	37/92 (40)	46/119 (39)	177/595 (30)
ECOG >1	162/495 (33)	15/80 (19)	9/106 (8)	75/549 (14)
Bulky disease	330/517 (64)	40/87 (46)	50/118 (42)	248/545 (45)
BM involvement	114/477 (24)	17/84 (20)	17/103 (17)	41/518 (8)
Extranodal involvement	249/566 (44)	30/91 (33)	29/120 (24)	152/597 (25)
Low IPI	53/323 (16)	16/47 (34)	27/53 (51)	205/369 (56)
low intermediate IPI	93/323 (29)	11/47 (23)	15/53 (28)	90/369 (24)
High intermediate IPI	98/323 (30)	15/47 (32)	8/53 (15)	52/369 (14)
High IPI	79/323 (25)	5/47 (11)	3/53 (6)	22/369 (6)
Abnormal Hb	245/543 (45)	33/88 (37)	34/116 (29)	138/577 (24)
Abnormal WBC	148/542 (27)	15/87 (17)	24/116 (21)	92/586 (16)
Abnormal albumin	187/529 (35)	15/85 (18)	14/103 (14)	89/561 (16)
Abnormal urea	190/545 (35)	17/88 (19)	19/112 (17)	110/579 (19)
Abnormal AP	176/539 (33)	20/87 (23)	24/106 (23)	109/565 (19)
Abnormal LDH	287/362 (79)	26/49 (53)	25/56 (45)	176/400 (44)

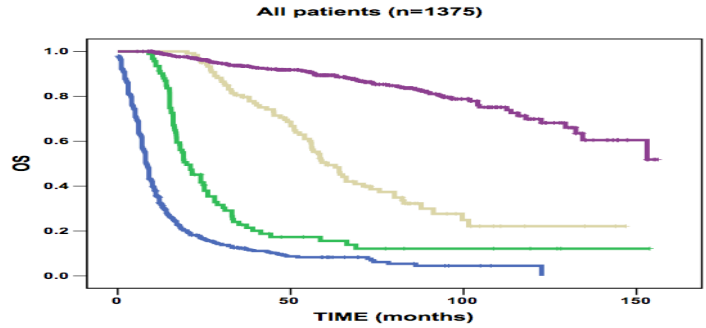
Table 2.13 Patient characteristics, different group according to duration of remission, in patients treated with anthracycline-based chemotherapy (n=1375).

In an analysis of OS there were statistically significant differences between the groups. The 5-year OS was 8% in the "refractory group", 16% in the "early relapse

group", 50% in the "late relapse group" and 90% in the "no relapse group"; p<0.001 (figure 2.13). These results were confirmed in patients aged ≤60 years: 5-years OS was 13% in the "refractory group", 31% in the "early relapse group", 66% in the "late relapse group" and 94% in the "no relapse group"; p<0.001 (figure 2.13) and in the evaluation of patients aged >60 years: 4%, 6%, 40% and 85%, respectively, p<0.001 (figure 2.13). Importantly the differences remained statistically significant, (all p-values <0.001), in a pairwise analysis of all patients, patients ≤60 years and >60 years, see figure 2.13.

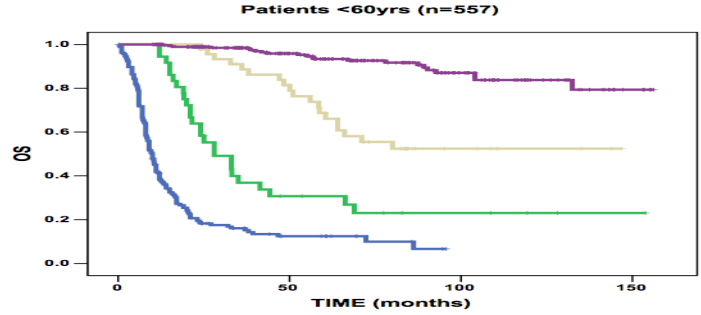
To assess potential differences in clinical features at presentation between patients in different groups, the groups were compared pair-wise: "refractory group" vs. "early relapse group", "refractory group" vs. "late relapse group", "refractory group" vs. "no relapse group", "early relapse group" vs. "late relapse group", "early relapse group" vs. "no relapse group" and "late relapse group" vs. "no relapse group" for all patients and then separately in young and elderly patients. The following 13 clinical factors were included in the evaluation: age, sex, CS, B-symptoms, ECOG, extranodal and BM involvement, bulky disease, Hb, leukocytes, albumin, urea and AP serum level. In the evaluation of all patients, the "refractory group" and "no relapse group" differed by the largest number of factors (12 of 13), the exception being gender. The "refractory group" and "late relapse" group varied by all factors (10 from 13 factors) except for gender, BM involvement and WBC The "refractory group" and "early relapse group" varied by 8 from 13 factors. Age, gender, CS, BM involvement, abnormal Hb and AP serum levels were the exceptions. The "early relapse group" differed from "no relapse group" by age, CS, B-symptoms, IPI-group, BM involvement and Hb levels. The "early relapse group" differed from the "late relapse group" by one factor only, ECOG status, and the "late relapse group" varied from the "no relapse group" by two factors: CS and BM involvement, see table 2.14.A. A similar pattern of differences between the groups was observed in both age cohorts (see table 2.14.A and B). Importantley, the IPI could not distinguish between all individual groups in a pair-wise comparison of the remission groups. For all patients, younger and elderly patients there were no differences between the "early relapse group" vs. "late relapse group" and "late relapse group" vs. "no relapse group". Additionally, in evaluations of younger and elderly patients there were no significant differences between "early relapse group" vs. "no relapse group" and for elderly patients only no significant differences between "refractory group vs. "early relapse group" (table 2.14).

(A)



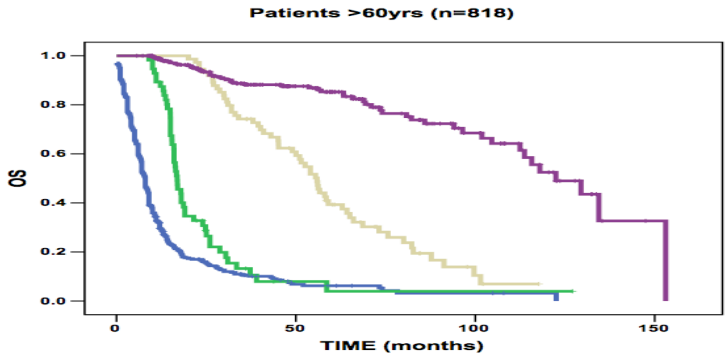
Patient group	Early relapse	Late relapse	No relapse
Refractory	>0.000	>0.000	>0.000
Early relapse		>0.000	>0.000
Late relapse			>0.000

(B)



Patient group	Early relapse	Late relapse	No relapse
Refractory	>0.000	>0.000	>0.000
Early relapse		>0.000	>0.000
Late relapse			>0.000

(**C**)



Patient group	Early relapse	Late relapse	No relapse
Refractory	>0.000	>0.000	>0.000
Early relapse		>0.000	>0.000
Late relapse			>0.000

Figure 2.13 Kaplan-Meier plot for OS in different group according to duration of remission in patients treated with anthracycline-based chemotherapy. Refractory (blue line), early relapse (red line), late relapse (green line) and no relapse (purple line). **(A)** all patients (n=1391). 5-years OS for refractory 8%, early relapse 16%, late relapse 50% and no relapse 90%; (p=0.001)*, **(B)** patients ≤60 years (n=560): 5-years OS for refractory 13%, early relapse 31%, late relapse 66% and no relapse 94%; (p=0.001)* and **(C)** patients >60 years (n=831): 5-years OS refractory 4%, early relapse 6%, late relapse 40% and no relapse 85%; (p=0.001)* *)Log Rank Test

(A)

Clinical parameter	All	Refractory vs Early relapse	Refractory vs Late relapse	Refractory vs No relapse	Early relapse vs Late relapse	Early relapse vs No relapse	Late relapse vs No relapse
Age	<0.001	n.s.	0.011	<0.001	n.s.	0.007	n.s.
Gender	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Clinical stage	<0.001	n.s.	<0.001	<0.001	n.s.	<0.001	0.019
B-symptoms	<0.001	0.002	<0.001	<0.001	n.s.	0.044	n.s.
ECOG	<0.001	<0.001	<0.001	<0.001	0.043	n.s.	n.s.
IPI - group	<0.001	0.013	<0.001	<0.001	n.s.	0.004	n.s.
Extranodal involvement	<0.001	0.048	<0.001	<0.001	n.s	n.s.	n.s.
BM involvement	<0.001	n.s.	n.s.	<0.001	n.s.	<0.001	0.006
Bulk	<0.001	0.002	<0.001	<0.001	n.s.	n.s.	n.s.
Haemoglobin	<0.001	n.s.	0.002	<0.001	n.s.	0.007	n.s.
WBC	<0.001	0.047	n.s.	<0.001	n.s.	n.s.	n.s.
Albumin serum level	<0.001	0.001	<0.001	<0.001	n.s.	n.s.	n.s.
Urea serum level	<0.001	0.004	<0.001	<0.001	n.s.	n.s.	n.s.
AP serum level	<0.001	n.s.	0.042	<0.001	n.s.	n.s.	n.s.

Table 2.14 P-values of Mann-Whitney test or χ^2 – test / Fisher exact test for pairwise comparison of different group according to duration of remission. **(A)** all patients treated with anthracycline-based chemotherapy (n=1391) **(B)** patients ≤60 years treated with anthracycline-based chemotherapy (n=560) and **(C)** - patients >60 years treated with anthracycline-based chemotherapy (n=831).

(B)

Clinical parameter	All	Refractory vs Early relapse	Refractory vs Late relapse	Refractory vs No relapse	Early relapse vs Late relapse	Early relapse vs No relapse	Late relapse vs No relapse
Age	n.s.	n.s.	n.s.	0.021	n.s.	n.s.	n.s.
Gender	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Clinical stage	n.s.	n.s.	0.004	<0.001	0.028	<0.001	0.021
B-symptoms	<0.001	0.002	0.004	<0.001	n.s.	n.s.	n.s.
ECOG	<0.001	<0.001	<0.001	<0.001	n.s.	n.s.	n.s.
IPI - group	<0.001	0.029	<0.001	<0.001	n.s.	n.s.	n.s.
Extranodal involvement	<0.001	n.s.	0.006	<0.001	n.s.	0.028	n.s.
BM involvement	<0.001	n.s.	n.s.	<0.001	n.s.	n.s.	0.002
Bulk	<0.001	n.s.	<0.001	<0.001	0.003	0.021	n.s.
Haemoglobin	<0.001	n.s.	0.007	<0.001	n.s.	n.s.	n.s.
WBC	0.002	n.s.	n.s.	<0.001	n.s.	n.s.	n.s.
Albumin serum level	<0.001	<0.001	0.013	<0.001	n.s.	n.s.	n.s.
Urea serum level	0.026	n.s.	n.s.	0.005	n.s.	n.s.	n.s.
AP serum level	<0.001	n.s.	n.s.	<0.001	n.s.	n.s.	n.s.

Table 2.14 P-values of Mann-Whitney test or χ^2 – test / Fisher exact test for pairwise comparison of different group according to duration of remission. **(A)** all patients treated with anthracycline-based chemotherapy (n=1391) **(B)** patients ≤60 years treated with anthracycline-based chemotherapy (n=560) and **(C)** - patients >60 years treated with anthracycline-based chemotherapy (n=831).

(C)

Clinical parameter	All	Refractory vs Early relapse	Refractory vs Late relapse	Refractory vs No relapse	Early relapse vs Late relapse	Early relapse vs No relapse	Late relapse vs No relapse
Age	0.002	n.s.	0.008	0.003	0.019	0.038	n.s.
Gender	n.s.	n.s.	n.s.	0.045	n.s.	n.s.	n.s.
Clinical stage	<0.001	n.s.	0.001	<0.001	0.018	0.006	0.017
B-symptoms	<0.001	n.s.	0.014	<0.001	n.s.	0.025	n.s.
ECOG	<0.001	0.016	<0.001	<0.001	n.s.	n.s.	n.s.
IPI - group	<0.001	n.s.	<0.001	<0.001	n.s.	n.s.	n.s.
Extranodal involvement	0.001	n.s.	0.003	0.001	n.s.	0.953	n.s.
BM involvement	<0.001	n.s.	0.089	<0.001	n.s.	0.003	n.s.
Bulk	<0.001	<0.001	0.112	0.002	n.s.	n.s.	n.s.
Haemoglobin	0.002	n.s.	n.s.	<0.001	n.s.	n.s.	n.s.
WBC	0.024	n.s.	n.s.	0.004	n.s.	n.s.	n.s.
Albumin serum level	<0.001	<0.001	0.001	<0.001	n.s.	n.s.	n.s.
Urea serum level	<0.001	0.013	0.002	<0.001	n.s.	n.s.	n.s.
AP serum level	0.006	n.s.	n.s.	0.001	n.s.	n.s.	n.s.

Table 2.14 P-values of Mann-Whitney test or χ^2 – test / Fisher exact test for pairwise comparison of different group according to duration of remission. **(A)** all patients treated with anthracycline-based chemotherapy (n=1391) **(B)** patients ≤60 years treated with anthracycline-based chemotherapy (n=560) and **(C)** - patients >60 years treated with anthracycline-based chemotherapy (n=831).

2.3.6 *Evaluation of addition of rituximab to anthracycline-based chemotherapy*

2.3.6.a *Patients characteristic*

The addition of rituximab to anthracycline-based chemotherapy (CHOP or CNOP) was introduced routinely in the Northern Region in 2004. The data of 214 patients treated in 5 regional haematological departments were available for the present evaluation.

Median age at diagnosis was 68 years (range, 18–91years) and the patients were equally distributed by sex with 105/214 (49.0%) females and by CS with 102/205 (49.7%) early and 103/205 (50.3%) advanced CS (table 2.15). The majority of patients 38/209 (18.2%) had ECOG ≤1 and B-symptoms were present at diagnosis in 72/197 (36.5%) patients. Bulky disease was present in 93/193 (48.2%) patients and 92/206 (44.7%) patients had extranodal disease with BM being involved in 12/173 (6.9%) patients. At diagnosis Hb, WBC, albumin, urea and AP were measured in more than 90% of patients and the majority of patients had normal results. By contrast, LDH was elevated in most patients 108/152 (71.1%). The IPI and revised IPI could be calculated for 131 patients; most patients 40/131 (30.5%) were in the low risk group, followed by 36/131 (27.5%) patients in the high intermediate risk group, 33/131 (25.2%) of patients in the low-intermediate risk group and 22/131 (16.8%) patients in the high-risk group, table 28. For the revised IPI, 10/131 (7.6%) patients were in the very low risk group, 63/131 (48.1%) in the good risk group and 58/131 (44.3%) in the poor risk group. Patient characteristics at baseline were compared to those of 1118 patients treated with CHOP/CNOP from our population-based cohort and were generally comparable (table 2.15). There were no statistically significant differences between the groups of patients according to age, sex, and features at presentation including IPI, except for a lower number of patients with BM involvement: 12/173 (6.9%) vs. 132/956 (13.8%); p=0.013.

Clinical parameter	Chemotherapy with rituximab n (%)	Chemotherapy without rituximab n (%)	p-value
Median age, range	68, 18 – 91	66, 18 – 92	0.614**
Age group			
≤ 60 years	61/214 (28.5)	414/1118 (37.0)	0.017*
> 60 years	153/214 (71.5)	704/1118 (63.0)	
Female gender	105/214 (49.0)	557/1118 (49.8)	n.s.*
ECOG >1	38/209 (18.2)	222/997 (22.3)	n.s.*
CS			
I	40/205 (19.5)	216/1118 (19.3)	n.s.*
II	62/205 (30.2)	308/1118 (27.5)	
III	56/205 (27.3)	296/1118 (26.5)	
IV	47/205 (22.9)	298/1118 (26.7)	
B symptoms	72/197 (36.5)	452/1108 (1108)	n.s.*
Bulk disease	93/193 (48.2)	533/1023 (52.1)	n.s.*
BM involvement	12/173 (6.9)	132/956 (13.8)	0.013*
Extranodal involvement	92/206 (44.7)	451/1024 (44.0)	n.s.*
IPI Group			
Low	40/131 (30.5)	238/640 (37.2)	n.s.*
Low intermediate	33/131 (25.2)	181/640 (28.3)	
High intermediate	36/131 (27.5)	131/640 (20.5)	
High	22/131 (16.8)	90/640 (14.1)	
Abnormal Hb	79/210 (62.4)	353/1080 (32.7)	n.s.*
Abnormal WBC	49/210 (23.3)	226/1081 (20.9)	n.s.*
Abnormal albumin	46/197 (23.4)	244/1037 (23.5)	n.s.*
Abnormal urea	69/199 (34.7)	292/1079 (27.1)	n.s.*
Abnormal AP	54/197 (27.4)	254/1054 (24.1)	n.s.*
Abnormal LDH	108/152 (71.1)	411/702 (58.5)	n.s.*

Table 2.15 Characteristics of patients treated with immunochemotherapy (n=214) and chemotherapy only (n=1118).*) χ^2 – test or Fisher exact test and **) Mann-Whitney Test

2.3.6.b *Treatment*

Of the 214 patients in the immunochemotherapy group the majority 190 (88.8%) received CHOP and 24 (11.2%) patients CNOP with substitution of mitoxantrone for doxorubicin. All except one patient given CNOP were aged >60 years. When patients from the immunochemotherapy group were compared with patients treated with

CHOP/CNOP there were no statistical differences in the number of patients treated with both regimens in the evaluation of all patients, patients aged ≤60 years and >60 years, see figure 2.14.

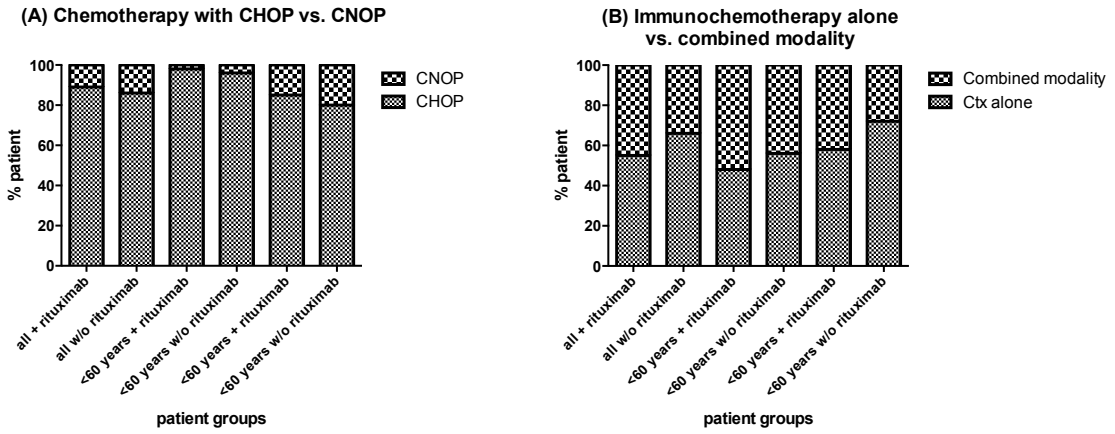


Figure 2.14 Type of anthracycline-based chemotherapy and combined modality in patients treated with immunochemotherapy vs. chemotherapy only, in all patients, younger and elderly patients. **(A)** CHOP and CNOP regimens in immunochemotherapy (n=214) vs chemotherapy only group (n=1118) – all patients (p = n.s.*), patients ≤60 years (p=n.s.*) and >60 years (p=n.s.*) **(B)** Chemotherapy alone vs combined modality in immunochemotherapy (n=214) vs chemotherapy only group (n=1118) – all patients (p=0.003*), patients ≤60 years (p=n.s.*) and >60 years (p=0.001*); *) χ^2 – test or Fisher exact test

Data on additional radiotherapy was available in 210 patients from the immunochemotherapy group, 116 (55.2%) received chemotherapy alone and 94 (44.8%) combined modality treatment. There were more patients treated with combined modality aged ≤60 years than patients aged >60 years; 32/61 (52.5%) vs. 62/149 (41.6%). When compared with the control group of patients treated with CHOP/CNOP, the number of patients treated with combined modality increased significantly in the evaluation of all patients (44.8% vs. 34.2%, respectively; p=0.003) and patients aged >60 years (41.6& vs. 28.3%, respectively; p=0.001). There was no significant difference for patients aged ≤60 years (52.5% vs. 44.2%, respectively), see figure 2.14.

3.3.6.c *Responses and outcome*

All patients were evaluable at 12 months for treatment response. In order to assess the impact of the addition of rituximab the response rates were compared with the control group treated with the same regimens (CHOP/CNOP) without rituximab. The majority of patients in immunochemotherapy group were in CR or PR at 12

months, 160/214 (74.8%). For patients with early stage disease CR/PR response rates were 85/102 (83.3%) versus 69/103 (67.0%) for advanced stage patients. When compared with patients treated with CHOP/CNOP, there were significant advantages for immunochemotherapy for all groups, see table 2.16.

Patient group		CR / PR N (%)	p-value*	Death N (%)	p-value*
All patients	R-CHOP / R-CNOP (n=214)	160 (74.8)	<0.001	75 (35)	<0.001
	CHOP / CNOP (n=1118)	615 (55)		575 (51.8)	
	R-CHOP (n=190)	146 (76.8)	<0.001	62 (32.6)	<0.001
	CHOP (n=963)	557 (57.8)		462 (48.0)	
Early stages	R-CHOP / R-CNOP (n=102)	85 (83.3)	0.002	26 (25.5)	0.005
	CHOP / CNOP (n=524)	357 (68.1)		209 (40.3)	
	R-CHOP (n=90)	75 (83.3)	<0.026	23 (25.6)	0.068
	CHOP (n=459)	330 (71.9)		163 (35.5)	
Late stages	R-CHOP / R-CNOP (n=103)	69 (67.0)	<0.001	45 (43.7)	<0.001
	CHOP / CNOP (n=594)	258 (43.4)		36.6 (62.0)	
	R-CHOP (n=91)	65 (71.4)	<0.001	35 (38.5)	<0.001
	CHOP (n=504)	227 (45.0)		299 (59.3)	

Table 2.16 Patient outcome in all patients treated with immunochemotherapy vs. chemotherapy only. R-CHOP/R-CNOP (n=214) vs. CHOP/CNOP (n=1118) and R-CHOP (n=190) vs CHOP (n=963).*) χ^2 – test or Fisher exact test

The same comparisons were performed for patients aged ≤ 60 years and > 60 years treated with immunochemotherapy and chemotherapy alone. In younger patients overall CR/PR rates were 56/61 (86.9%); early stage 30/33 (90.9%) and advanced stage 21/25 (84%). Compared with patients treated with CHOP/CNOP, the statistically significant differences were for all and advanced stage patients, but not early stage patients, see table 2.17. In contrast response rates for elderly patients were lower at 69.9% (55/69) overall; 79.7% (48/78) for early stage patients and 61.5% (48/78) for advanced stage patients. However, the response rates for all rituximab treated patient groups were statistically significant higher compared with the no rituximab groups (table 2.17).

In evaluation of patients treated with CHOP chemotherapy with and without addition of rituximab the outcome reminded same with the exemption that the differences in death rates in patients with early stage disease lost their significance in evaluation of all patient and younger and elderly patients.

(A)

Patient group		CR / PR N (%)	p-value*	Death N (%)	p-value*
All patients	R-CHOP / R-CNOP (n=61)	53 (86.9)	<0.001	9 (14.8)	<0.001
	CHOP / CNOP (n=414)	267 (64.5)		158 (38.4)	
	R-CHOP (n=60)	52 (86.7)	<0.001	9 (15.0)	<0.001
	CHOP (n=398)	259 (65.1)		149 (37.4)	
Early stages	R-CHOP / R-CNOP (n=33)	30 (90.9)	n.s	2 (6.4)	0.025
	CHOP / CNOP (n=210)	167 (79.5)		48 (23.1)	
	R-CHOP (n=33)	30 (90.9)	n.s	2 (6.1)	n.s.
	CHOP (n=203)	164 (80.8)		43 (21.2)	
Late stages	R-CHOP / R-CNOP (n=25)	21 (84.0)	<0.001	6 (24.0)	0.004
	CHOP / CNOP (n=204)	100 (49.0)		110 (54.2)	
	R-CHOP (n=24)	20 (83.3)	0.002	6 (25.0)	0.008
	CHOP (n=195)	95 (48.7)		106 (54.6)	

(B)

Patient group		CR / PR N (%)	p-value*	Death	p-value*
All patients	R-CHOP / R-CNOP (n=153)	107 (69.9)	<0.001	66 (43.1)	<0.001
	CHOP / CNOP (n=704)	348 (49.4)		41.7 (59.7)	
	R-CHOP (n=130)	94 (72.3)	<0.001	53 (40.8)	0.002
	CHOP (n=565)	298 (52.7)		313 (55.4)	
Early stages	R-CHOP / R-CNOP (n=69)	55 (79.7)	0.003	24 (34.8)	0.011
	CHOP / CNOP (n=314)	190 (60.5)		161 (51.8)	
	R-CHOP (n=57)	45 (78.9)	0.043	21 (36.8)	n.s.
	CHOP (n=256)	166 (64.8)		120 (46.9)	
Late stages	R-CHOP / R-CNOP (n=78)	48 (61.5)	<0.001	39 (50.0)	0.007
	CHOP / CNOP (n=390)	158 (40.5)		256 (66.1)	
	R-CHOP (n=67)	45 (67.2)	<0.001	29 (43.3)	0.004
	CHOP (n=309)	132 (42.7)		193 (62.5)	

Table 2.17 Patient outcome in younger and elderly patients treated with immunochemotherapy and chemotherapy only. **(A)** patients aged ≤60 years treated with R-CHOP/R-CNOP (n=61) vs. CHOP/CNOP (n=414) and R-CHOP (n=60) vs CHOP (n=398) and **(B)** patients aged >60 years treated with R-CHOP/R-CNOP (n=153) vs. CHOP/CNOP (n=704) and R-CHOP (n=130) vs CHOP (n=565) *) χ^2 – test or Fisher exact test

The median observation time of all patients treated with CHOP-R/CNOP-R was 24.3 months and for living patients 34.5 months. For patients from the control group

treated with CHOP/CNOP it was 21.8 months and 48.15 months, respectively. For all CHOP-R/CNOP-R patients the 3-years PFS and OS were 57.6% and 64.6% and this was statistically significantly higher than the 43.1% and 54.3%, for CHOP/CNOP patients, (p=0.000 and 0.014, respectively). However in a sub-evaluation of patients with early stage disease the 3-years PFS and OS were 67.7% and 74.2% in the immunochemotherapy group and 57.0% and 66.1% in the chemotherapy group with no statistically significant differences. For advanced stage disease patients the 3-years PFS and OS were lower at 47.7% and 56.0% in the immunochemotherapy group and 30.3% and 43.8% in the chemotherapy group with statistically significant difference for PFS but not for OS (table 2.18).

Patient group		3-years PFS (%)	p-value*	3-years OS (%)	p-value*
All patients	R-CHOP / R-CNOP (n=214)	57.6	<0.001	64.6	0.014
	CHOP / CNOP (n=1118)	43.1		54.3	
	R-CHOP (n=190)	59.2	<0.001	66.8	0.031
	CHOP (n=963)	45.8		57.7	
Early stages	R-CHOP / R-CNOP (n=102)	67.7	0.064	74.2	0.159
	CHOP / CNOP (n=524)	57.0		66.1	
	R-CHOP (n=90)	66.6	0.187	73.1	0.512
	CHOP (n=459)	60.5		70.1	
Late stages	R-CHOP / R-CNOP (n=103)	47.7	<0.001	56.0	0.054
	CHOP / CNOP (n=594)	30.3		43.8	
	R-CHOP (n=91)	52.3	<0.001	61.9	0.022
	CHOP (n=504)	31.8		46.3	

Table 2.18 3-years PFS and OS in all patients treated with immunochemotherapy vs. chemotherapy only. R-CHOP/R-CNOP (n=214) vs. CHOP/CNOP (n=1118) and R-CHOP (n=190) vs CHOP (n=963).*) Log Rank test

Subsequently, separate evaluations were performed for younger patients (aged ≤60 years) and elderly patients (aged >60 years); table 2.19. As suspected, generally the PFS and OS rates were higher among younger patients. For all patients, the 3-years PFS and OS were 78.7% and 87.6%, for CHOP-R/CNOP-R and 54.9% and 67.4%, for CHOP/CNOP; p=0.002 and p=0.005, respectively.

(A)

Patient group		3-years PFS %	p-value*	3-years OS %	p-value*
All patients	R-CHOP / R-CNOP (n=61)	78.7	0.002	87.6	0.005
	CHOP / CNOP (n=414)	54.9		67.4	
	R-CHOP (n=60)	78.4	0.002	87.4	0.006
	CHOP (n=398)	55.2		67.8	
Early stages	R-CHOP / R-CNOP (n=33)	90.8	0.062	93.8	0.082
	CHOP / CNOP (n=210)	72.8		80.9	
	R-CHOP (n=33)	90.8	0.071	93.8	0.097
	CHOP (n=203)	73.5		81.9	
Late stages	R-CHOP / R-CNOP (n=25)	63.5	0.013	81.0	0.025
	CHOP / CNOP (n=204)	36.2		53.4	
	R-CHOP (n=24)	62.3	0.016	80.7	0.029
	CHOP (n=195)	35.7		52.8	

(B)

Patient group		3-years PFS %	p-value*	3-years OS %	p-value*
All patients	R-CHOP / R-CNOP (n=153)	48.5	0.001	54.9	0.081
	CHOP / CNOP (n=704)	35.6		46.3	
	R-CHOP (n=130)	49.4	0.003	56.7	0.183
	CHOP (n=565)	38.5		50.2	
Early stages	R-CHOP / R-CNOP (n=69)	55.7	0.114	64.1	0.234
	CHOP / CNOP (n=314)	46.0		55.8	
	R-CHOP (n=57)	50.7	0.415	60.0	0.815
	CHOP (n=256)	49.6		60.1	
Late stages	R-CHOP / R-CNOP (n=78)	42.4	0.002	47.8	0.236
	CHOP / CNOP (n=390)	27.2		38.6	
	R-CHOP (n=67)	48.6	0.001	55.0	0.122
	CHOP (n=309)	29.2		42.1	

Table 2.19 3-years PFS and OS in younger and elderly patients treated with immunochemotherapy vs. chemotherapy only. **(A)** patients ≤60 years treated with R-CHOP/R-CNOP (n=61) vs. CHOP/CNOP (n=414) and R-CHOP (n=60) vs CHOP (n=398) **(B)** patients >60 years treated with R-CHOP/R-CNOP (n=153) vs. CHOP/CNOP (n=704) and R-CHOP (n=130) vs CHOP (n=565) *) Log Rank test

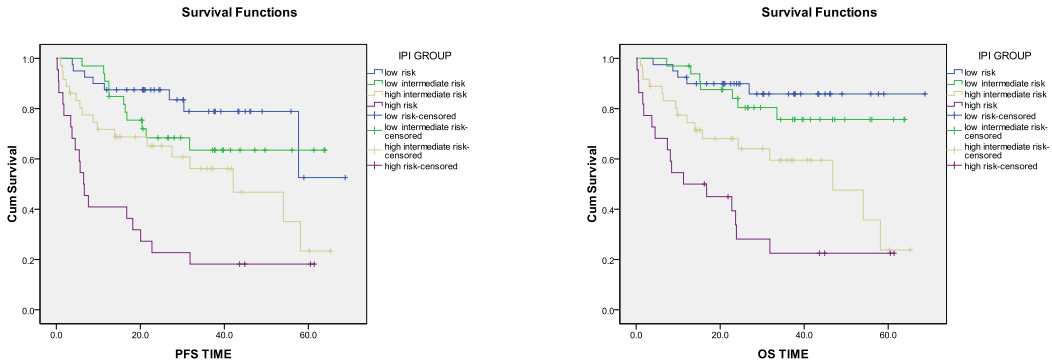
In younger patients, in a sub-analysis of early stage patients, the 3-years PFS and OS were 90.8% and 93.8%, respectively in the immunochemotherapy group and 72.8% and 80.9%, respectively in the chemotherapy group with no statistically significant differences between them. For patients with advanced stage disease, the 3-years PFS and OS were 63.5% and 81.0%, respectively for CHOP-R/CNOP-R and 36.2% and 53.4% for CHOP/CNOP; p=0.013 and p=0.025, respectively (table 2.19). In elderly patients the 3-years PFS and OS in all patients were 48.5% and 54.9% in the immunochemotherapy group and 35.6% and 46.3% in the chemotherapy group (p=0.001 and p=0.081; respectively), see table 32. The 3-years PFS and OS were higher in patients with early stage disease at 55.7% and 64.1% in the immunochemotherapy group and 46.0% and 55.8% in the chemotherapy group, with no statistically significant differences for both PFS and OS. In patients with advanced stage disease PFS and OS were lower at 42.4% and 47.8% in the immunochemotherapy group and 27.2% and 38.6% in the chemotherapy alone group with significant differences for PFS but not for OS (table 2.19).

An additional evaluation of patients treated with CHOP, with or without the addition of rituximab revealed similar results with one difference; in evaluation of all patients with advanced stage disease, where the previously not statistically significant difference became significant (table 2.18).

2.3.6.d ***Clinical prognostic indices in patients treated with anthracycline-based chemotherapy with rituximab***

The 3-year PFS and OS for IPI risk groups were as follows: 78.95 and 85.8% in the low risk group, 63.5% and 75.7% in the low intermediate risk group, 56.1% and 59.5% in the high intermediate risk group and 18.2% and 22.5% in the high risk group with p=0.000 for both PFS and OS (figure 2.15). In the more detailed pairwise evaluation, the IPI was able to distinguish for PFS between all groups except between “low risk group” vs. “low intermediate risk group” and “low risk group” vs. “high intermediate risk group”, see figure 2.15. For OS the IPI was additionally able to distinguish between “low intermediate risk group” and “high intermediate risk group” but failed to differentiate between “low risk group” vs. “low intermediate risk group”.

(A) **(B)**



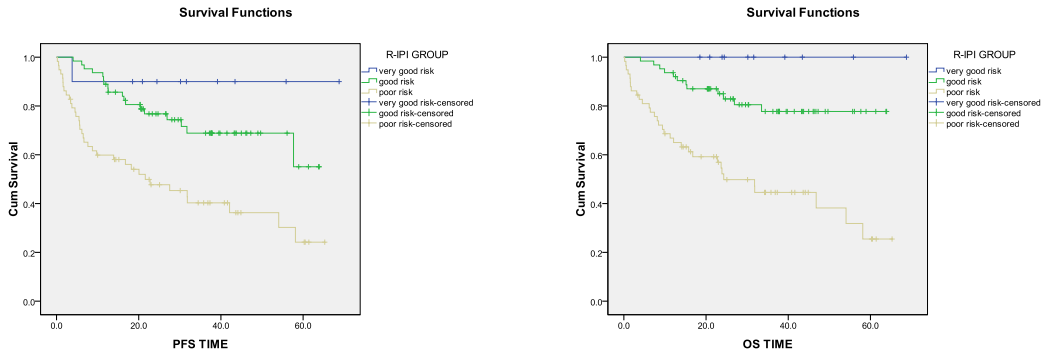
(C)

PFS - all patients			
Group	**Low intermediate**	**High intermediate**	**High**
Low risk	0.275	0.015	0.000
Low intermediate		0.153	0.000
High intermediate			0.012
OS - all patients			
Group	**Low intermediate**	**High intermediate**	**High**
Low risk	0.433	0.004	0.000
Low intermediate		0.023	0.000
High intermediate			0.033

Figure 2.15 Kaplan-Meier plots for PFS and OS according to IPI in population-based evaluation of all patients treated with R-CHOP/R-CNOP (n=131). **(A)** 3-years PFS: low risk group 78.9%, low intermediate risk group 63.5%, high intermediate risk group 56.1% and high risk group 18.2% (p=0.000)*, **(B)** 3-years OS: low risk group 85.8%, low intermediate risk group 75.7%, high intermediate risk group 59.5% and high risk group 22.5% (p=0.000)* and **(C)** p-values* of pairwise comparison of PFS & OS in different IPI group. *)Log Rank Test.

The 3 year PFS and OS for revised IPI (R-IPI) risk groups were as follows: 90% and 100% for the very good risk group, 68.9% and 77.7% for the good risk group and 40.3% and 44.6% for the poor risk group. Differences between the groups were statistically significant for both PFS and OS (figure 2.16). However, in the more detailed pairwise comparison for both PFS and OS, the differences were statistically significant between all groups except for “very good risk group” vs. “good risk group”, see figure 2.16.

(A) **(B)**



(C)

PFS - all patients		
Group	**Good**	**Poor**
Very good	0.261	0.014
Good		<0.000
OS - all patients		
Group	**Good**	**Poor**
Very good	0.153	0.005
Good		<0.000

Figure 2.16 Kaplan-Meier plots for PFS and OS according to R-IPI in population-based evaluation of all patients treated with R-CHOP/R-CNOP (n=131). **(A)** 3 years PFS: very good risk group 90%, good risk group 68.9%, poor risk group 40.3% (p=0.000)*, **(B)** 3-years OS: very good risk group 100%, good risk group 77.7% and poor risk group 44.6% - (p=0.000)* and **(C)** p-values* of pairwise comparison of PFS & OS in different R-IPI groups. *)Log Rank Test.

2.4. Discussion

Evaluation of all patients

The majority of data on DLBCL comes from clinical trials performed mostly on highly selected patients. Clinical trials are obviously useful, however it should always be kept in mind that they are not representative of all patients but only those fulfilling the inclusion criteria. Thus the conclusions may be biased and not always representative for patients presenting in every day practice. In contrast the epidemiology, clinical features, treatment and outcome of DLBCL have not yet been studied extensively in a population-based setting. Ideally the general picture of the disease should be assessed in population-based studies and then selected, specific problems should be further assessed in clinical trials.

There are few available publications on population-based studies in DLBCL. Unfortunately most of them are based on the results of regional / national cancer registries and are not specifically designed studies. Also most evaluate the entire group of lymphomas and the data on DLBCL are presented as a sub-analysis only. Some of the reports focus on aspects of DLBCL e.g. treatment of elderly patients. The vast majority of these data come from the pre-rituximab era and thus their value in the rituximab era is limited.

Among the currently published general population-based studies on DLBCL the studies of Hasselblom et al and Krol et al are the most important and the results of our study will be discussed in their context (Hasselblom et al., 2007) (Krol et al., 2003). Hasselblom et al conducted a retrospective study on adult patients with a de novo diagnosis of DLBCL registered with the population-based Regional Lymphoma Registry of the Western Sweden Health Care Region (Hasselblom et al., 2007). The evaluation included epidemiology, clinical presentation, treatment and outcome including prognostic factors. In the Netherlands, Krol et al. performed a population-based study on epidemiology of NHL using the resources of the population-based registry of the Comprehensive Cancer Centre West (Krol et al., 2003). The registry covered the west region of the Netherlands and was based on pathological data; only patients with a confirmed diagnosis of lymphoma could enter the registry. The study was similar to that of Hasselblom et al., however all cases with NHL were included, with additional evaluations of DLBCL and FL (Hasselblom et al., 2007). Recently the data on lymphoma incidence, survival and prevelance were published by Haematological Malignancy Network from the UK {Smith et al., 2015, #30493}. The studies of Møller et al. and Luminari et al. are two other studies frequently citied in the literature. Both of them however suffered from major limitations (Moller et al., 2004) (Luminari et al., 2007). Møller et al. assessed the clinical implications of an extranodal vs. nodal presentation of DLBCL in a population-based study using the capacity of the LYFO registry of the Danish Lymphoma Group covering western Denmark (Moller et al., 2004). The study compared both groups of patients in terms of clinical presentation, treatment, prognosis and survival. Unfortunately since it included patients with complete IPI staging only its relevance as a population-based study is nearly lost. Luminari et al performed a population-based study on incidence, clinical characteristics, treatment and survival of all lymphoma in the province of Modena in northern Italy (Luminari et al., 2007). This study suffers from two issues, which limit its value. The

presented data on treatment are rather sparse and surprisingly BL and DLBCL were evaluated as a one group. Thus any detailed discussion of results solely relating to DLBCL is difficult.

The Scotland and Newcastle Lymphoma Group operated in Scotland and the Northern region of England, which can be regarded as a representative population not only for the UK but also for Western Europe and North America. The group collected information on all patients diagnosed with DLBCL of nodal origin in the geographical area without using any pre-selection.

In our cohort the median age of 1863 evaluable patients was 66 years with a slight female majority at 51.2%. The median age of patients with DLBCL reported in the study by Krol et al. was comparable at 67 years (Krol et al., 2003) and in the study of Hasselblom et al. was higher at 73 years (Hasselblom et al., 2007). Smith et al in his evaluation of the british cohort reports the median age of 70 years. Kroll et al reported equal numbers of male and female patients (Krol et al., 2003) and in the study of Hasselblom et al. and in the study of Smith et al there was a slight dominance of male patients with 52% in both studies (Hasselblom et al., 2007) {Smith et al., 2015, #30493}. There is no explanation for these small differences.

In our cohort of patients a performance status with ECOG >1was reported in 26.1% of patients, a comparable number to the study of Hasselblom et al. (29%) (Hasselblom et al., 2007). By contrast in the study of Krol et al. (Krol et al., 2003) this number was significantly higher at 39%. There is no known explanation for this difference. Patients in our study presented almost equally with early and advanced stage disease with only a minimal predominance of advanced stage at 52.2%. By contrast in the study of Krol et al. more patients presented with early stage disease: 56% (Krol et al., 2003) and in the study of Hasselblom et al. there was the same number of patients with early and advanced disease (Hasselblom et al., 2007). B symptoms were present in 41.7% and were comparable with data from Hasselblom et al. (45%) (Hasselblom et al., 2007). Krol et al. (Krol et al., 2003) did not assess the presence of B-symptoms. Comparison of patients with bulky disease is difficult because of different definitions used in the studies and thus it will not be further discussed. Extranodal involvement in our cohort was seen in 48.1% of patients versus 59% in the study of Hasselblom et al. (Hasselblom et al., 2007). Krol et al. did not report on extranodal involvement, except for BM involvement, which was 20% followed by 16.4% in our cohort and 13% in the study of Hasselblom (Hasselblom et al., 2007) (Krol et al., 2003). Importantly BM

assessment and serum LDH levels were missing in significant numbers of patients in all studies. Elevated LDH serum levels were observed in 59.5%, 49.5% and 53.5% of tested patients in our study, Hasselblom et al. and Kroll et al.; respectively (Hasselblom et al., 2007) (Krol et al., 2003). The laboratory values were reported only in our study.

Regarding therapy, individual studies used different definitions for their treatment modalities thus direct comparisons are difficult. However, it was possible to observe some common trends. Approximately 75% of our patients were treated with anthracycline-based systemic chemotherapy and 4.9% of patients received anthracycline-based chemotherapy with ASCT in first remission. Only 5.7% received systemic chemotherapy without anthracyclines, 7.1% were treated with radiotherapy only and 7.6% received no chemotherapy or radiotherapy. During the time period of patient recruitment curative standard of care for patients with localised disease was radiotherapy alone. In fact the vast majority of patients in this group are patients with stage I disease. This is not common practice anymore, and has caused problems in classification of patients. In order to stay uniform with other studies we could not classify radiotherapy alone as curative treatment. Thus curative treatment was given to 79.6% of patients and palliative treatment to 20.4%. In the study of Hasselblom et al., 70% of patients received curative treatment and the remaining 30% palliative treatment (Hasselblom et al., 2007). By contrast in the study of Krol et al. only 40.5% of patients received an anthracycline-based systemic chemotherapy, 31.5% a localised treatment (mostly radiotherapy), 21.5% received systemic chemotherapy without anthracyclines and 6.8% no treatment at all (Krol et al., 2003). Mitoxantrone containing regimens were considered as a palliative treatment in contrast to our and other studies, which defined this treatment as curative. The standard treatment for patient with localised lymphoma in CS I was radiotherapy only. And indeed, the majority of patients treated with localised treatment had CS I. According to these numbers 40.5% of patients only were treated with curative treatment, increasing for patients in CS II – IV to 55%. The number of patients treated with palliative treatment is actually the lowest in our study, which was recruiting patients during the most recent period of time between 1990 and 2003, and it was highest in the study of Krol et al which recruited between 1985-1989 (Krol et al., 2003). Looking closer at the chemotherapy regimens CHOP, CNOP and VACOP-B were the three most often used in our group and by Hasselblom et al (Hasselblom et al., 2007).

We could observe differences in patient characteristics between the different treatment groups in our cohort. Patients treated with consolidation with ASCT in first remission were significantly different from the group of patients treated with standard anthracycline-based chemotherapy with more advanced/aggressive disease; statistically significant differences in CS, bulky disease, extranodal involvement and high LDH. This makes it obvious that the main reason for ASCT was more advanced disease at presentation in young, healthy patients. The group of patients treated with chemotherapy without anthracyclines as compared to patients treated with standard chemotherapy had significantly more elderly patients, female, patients with ECOG >1 and with higher IPI score, abnormal Hb, urea and AP. The patients treated without anthracyclines were older, with worse general condition and more or less the same incidence of advanced disease. This suggests that anthracyclines were usually omitted due to advanced age and comorbidities. The higher number of female patients may be associated with the higher average age of patients treated without anthracyclines and the number of females is higher among an elderly population. The differences were even more significant in comparison to patients not receiving any treatment. Both groups differ in all aspects except CS, bulky disease and abnormal WBC.

Looking at the patients in the curative and palliative groups in our study they differ significantly in all aspects except for the number of patient with BM involvement, abnormal HB, WBC and AP. Generally patients in the palliative chemotherapy group had similar pictures of disease but were older and more fragile as compered with the patients from the curative group. In the evaluation of the patients given palliative versus curative treatment by age decade, the number of palliatively treated patients remained constant until the beginning of the 7th decade and then it started to increase reaching more than 50% by the 9th decade. In all other studies age remained the main reason for not administering curative treatment. In the study of Krol et al. patients in the palliative treatment group were older, there were more females in this group, patients with normal LDH, CS I/ II and low risk IPI (Krol et al., 2003). Hassleblom et al has not compared directly both groups but collected information on the reasons for not giving curative treatment. The most common were high age and concomitant disease, followed by high age (18%), concomitant disease (17%), poor performance status (17%), and other 9% or not specified 12% (Hasselblom et al., 2007). Taking into consideration all the above factors the reasons for the relatively high number of patients treated with palliative therapies could be either related to the evaluation method or to patient characteristics. In

the older studies the recommendations for using anthracycline-based treatment were less strong, and patients with localised disease were receiving radiotherapy alone. This could contribute to the increased number of "palliatively treated patients" particularly if localised disease was more common in the study; e.g. Krol et al as compared to our cohort (Krol et al., 2003). Additionally, in the past supportive therapies were not so successful as they are currently and this obviously contributed to a reduced number of patients treated with anthracyclines. On top of this some studies classified mitoxantrone containing protocols as not anthracycline-based and this increased the number of patients in palliative groups e.g. Krol et al and (Krol et al., 2003). In conclusion a significant group of patients with DLBCL did not receive appropriate curative treatment with anthracycline-based chemotherapy. However, this number may have been affected by several subjective reasons such as treatment recommendation, supportive therapies or the definition of cohorts studied. Age seems to be the most important patient related objective factor.

The ORR for all patients in our study was 49.4% and as expected patients with early stage disease had higher response rates than those with advanced stage. Direct comparison between the treatment groups can be difficult particularly if looking at patients treated with radiotherapy alone as this group includes mostly patients with CS I without B-symptoms. Excluding this group from the comparison the best results were achieved in the group treated with the anthracycline-based chemotherapy and patients with ASCT in first remission. The slightly inferior results in the latter group may be associated with patients with more advanced disease. The lowest ORR was observed as expected among patients treated with chemotherapy without anthracyclines and patients not receiving any treatment. The ORR in the curative treatment group was 54.5% and was comparable with the results described by Hasselblom et al. and Krol et al. (Krol et al., 2003) (Hasselblom et al., 2007). The response to treatment in the curative group from Hasselblom's study was CR/CRu/PR in 65% of patients (CR 44%, CRu 17% and PR 14%), 20% of patients failed to respond and 5% were not assessed because of death or were not assessed properly (Hasselblom et al., 2007). In Krol et al's report the ORR was calculated for all patient receiving treatment at 55% (Krol et al., 2003).

The 5-year PFS and OS for the whole group was 33% and 42%, respectively and was comparable with the results of Hasselblom et al who estimated 5 years OS and PFS for all patients of 37% and 36%; respectively (Hasselblom et al., 2007). In Krol et al. the 5-year OS was 34% and the median OS 20 months, both calculated for all patients

receiving treatment (Krol et al., 2003). In terms of survival results for individual treatment groups the best were as expected in the groups treated with anthracycline-based chemotherapy, ASCT in first remission or radiotherapy only and worst among patients treated with no anthracycline-based chemotherapy, and patients treated with no chemo and no radiotherapy. The 5 year PFS and OS for the curative group was 37% and 46%, respectively and in the palliative group 16% and 21%, respectively: directly comparable to the results of Hasselblom et al. In the curative group he estimated 5-year PFS and OS of 48% and 46%; respectively and in the palliative group 5-years OS was 9% only (Hasselblom et al., 2007).

Role of age in the choice of therapy and outcome in DLBCL

Next to disease stage age is the most important predictive factor, which stratifies studies for DLBCL. Therefore we had a closer look at the population of younger and elderly patients in our cohort. This was done in two different ways; firstly by comparing the group of patients aged ≤60 years and >60 years using the age limit used by the IPI and most clinical trials, and secondly using age decades, in order to achieve a more detailed picture.

The issue of age in patients presenting with DLBCL was assessed in the two bigger population-based studies of Maartense et al. (Maartense et al., 1998) (Maartense et al., 2000). Both studies used the capacity of the Dutch Comprehensive Cancer Centre West population-based registry previously described in detail by Krol et al (Krol et al., 2003). One of the studies focused on comparing younger and elderly patients (Maartense et al., 1998) and the other on a detailed analysis of influence of the age as continuous variable for outcome of aggressive and indolent NHL (Maartense et al., 2000). Unfortunately both studies based their evaluation on the WF classification and the first one analyzes patients with all subtypes of NHL together. On the positive side they include a representative population-based cohort of patients. In addition, two other single centre studies focus on the aging population of patients with DLBCL or NHL, in terms of presentation, possible treatment and outcome. The first one by Varga et al. comes from the Jewish General Hospital in Montreal, Canada (Varga et al., 2014) and the other from Thieblemont et al. in Lyon, France (Thieblemont et al., 2008). Unfortunately the results of both evaluations are influenced and biased by the small populations they cover. In addition the latter evaluated cumulatively all patients with Non-Hodgkin lymphoma. Hasselblom et al. also included age as an independent

prognostic factor in their study, which delivered some interesting results (Hasselblom et al., 2007).

In our cohort, in terms of patient characteristics, the younger and older groups differed significantly in the number of female patients, patients with ECOG >1, BM and other extranodal involvement, abnormal Hb, albumin and urea – all more frequently present in the elderly group. Varga et all reported that elderly patients aged more than 80 were more likely to have ECOG >2 and higher IPI scores (Varga et al., 2014). In the study of Maartense, a majority of patients aged 70 or more were female and there were a higher number of patients with ECOG >2; these findings were significantly different from patients aged 70 years or less (Maartense et al., 1998). The significantly higher number of female patients among elderly patients can be explained by the larger proportion of female in aging population due to longer life expentancies of female as compare with male. The higher number of patients with higher ECOG scores in elderly groups can be associated with increased incidence of co-morbidities in an aging population. The higher number of patients with high IPI can be explained by age and ECOG score that is used in calculation of the IPI. Low albumin status and abnormal Hb is also associated with the aging population.

Anthracycline-based chemotherapy with or without consolidation with ASCT was by far the most commonly used regimen in younger patients. By contrast in elderly patients there were almost none treated with ASCT, but significant numbers of patients treated with less powerful regimens like no anthracycline-based chemotherapy, radiotherapy alone, or no treatment at all. All three options were equally distributed. Interestingly, among these less intense modalities radiotherapy alone was the most common one used in early stage disease in elderly and in younger patients. The treatment was efficient and without generalised side effects. As expected in the evaluation of curative and palliative treatment groups, a higher number of patients treated with palliative regimens were observed in elderly patients and in patients with early stages. In the latter group it was most likely due to frequent application of radiotherapy only for stage I disease. It is likely that clinicians treating patients with limited disease opted for less intense treatments. In advanced stage disease the choice of a less powerful treatment was most probably associated with the increasing age of patients and performance status. By contrast a young age encourages clinicians to choose more intensive regimens. In Varga et al standard anthracycline-based chemotherapy was successfully administered to 32.5% of patients aged 80 or more as

compared with 86.6% of the younger group (Varga et al., 2014). Suboptimal therapy was given to 25% vs. 6% and no therapy to 40% versus 5.2%. Patients, who did not receive standard chemotherapy were characterized by significantly higher ECOG scores, more advanced stage disease and lower albumin levels as compared with those who received standard treatment. Importantly, there were no differences in the Charlson comorbidity index (Varga et al., 2014). The data of Maartense et al. shows that the cohort of elderly patients was characterised by significantly lower numbers treated with anthracycline-based chemotherapy and higher numbers of patients treated with chemotherapy not including anthracyclines. Also significantly more elderly patients received no treatment (Maartense et al., 1998). The number of patients treated with surgery only or radiotherapy only was comparable in both groups. Obviously it needs to be noted that these numbers refer to all types of NHL. Thieblemont et al, studied very elderly patients aged 80 or more. The majority of those with an aggressive lymphoma were treated with anthracycline-based chemotherapy (47.1%), followed by 22.5% administered a single agent, 21.6% administered multidrug chemotherapy not including anthracyclines and 8.8% who received no treatment (Thieblemont et al., 2008). This data indicates that 47.1% of this cohort was treated with curative intent and 52.9% palliatively. This approach is in keeping with our results. The study by Boslooper et al confirmed these results in the rituximab era (Boslooper et al., 2014). A regional cancer registry was employed to analyse patients aged 75 and over in terms of treatment and outcome (Boslooper et al., 2014). The majority of patients - 80 (78%) received immunochemotherapy, 9 (9%) radiotherapy only, and 12 patients (11%) supportive treatment only. This means that approximately 22% of patients received palliative treatment (Boslooper et al., 2014).

Within anthracycline-based regimens we observed some variation. CNOP differs from standard CHOP by using mitoxantrone instead of doxorubicin and was used mostly in elderly patients. Mitoxantrone is an antineoplastic agent belonging to the anthracenediones group. It is thought to be less cardiotoxic than anthracyclines e.g. doxorubicin and thus is commonly used in patients vulnerable to cardiac side effects e.g. elderly. Unfortunately it is not as efficient as anthracyclines. VACOP-B, a third generation regimen, in our cohort was used more often in younger patients, particularly in those with early stage disease. This regimen is more time intense and thus probably more often used in younger individuals.

Combined modality treatment was more often used in younger patients, and in patients with early stage disease in both age groups. Although the data on radiotherapy in aggressive lymphomas is very poor, the treatment is associated with relatively small numbers of side effects and patients may still benefit, particularly those with limited or bulky disease. Clinicians were possibly motivated by the latter to employ this modality in patients with high-risk tumours.

Although this study was not designed as a direct comparison of the outcome of different treatment modalities we will briefly discuss the results. The ORR, PFS and OS as expected were statistically significantly higher in younger patients, and in patients with early stage disease in both age groups. This was also true for all treatment modalities and groups and the evaluation of curative and palliative treatment groups. An ORR above 50% was observed in younger and elderly patients with early stage disease. In patients with advanced disease it was 46.8% in younger patients and 32% in the elderly. The results of different modalities suggest that patients with DLBCL have poor response rates unless they receive anthracycline-based chemotherapy. The ORR for chemotherapy without anthracyclines or groups receiving no chemotherapy and no radiotherapy was as low as 20% in both groups. The radiotherapy only group deserves special mention. As the only treatment group, which did not use anthracyclines, it was characterized by an ORR comparable with that from anthracycline-based chemotherapy group. This was however the case for early-stage disease only, the results for advanced stage were poor. Because the radiotherapy only group was classified as palliative treatment for the purpose of this evaluation it contributes to the relatively high ORR in this group in younger patients. Varga et al. also reports significantly lower CR rates in elderly patients, aged 80 years or over (Varga et al., 2014). Hasselblom et al divided patients into two age groups ≤68 years and >68 years; the CR and CRu rates did not significantly differ between the two groups (61% and 60%; p=0.86) (Hasselblom et al., 2007). However in the group aged >80 years the CR / CRu rate was significantly lower at 44%. Maartense et al. reports a significantly higher CR rate in patients aged 70 or less in an evaluation of all patients and for all assessed treatment modalities in patients with NHL (Maartense et al., 1998). The differences between both age groups were significant for intermediate grade lymphoma and DLBCL. In Thieblemont et al the CR rate in very elderly patients with aggressive lymphoma aged > 80 years was 36% only (Thieblemont et al., 2008).

The results of PFS and OS follow those of the ORR. Despite all the limitations of this study, we see that the PFS and OS in patients treated with anthracycline-based chemotherapy were significantly lower than those reported in clinical trials, only being over 50% in early stage disease in younger patients. By contrast PFS and OS for palliative groups in elderly patients were only 10-20% or lower depending on the PS of patients. It should be emphasized that a direct comparison between treatment groups is not appropriate for this evaluation, as the design of the study did not allow for this. This is particularly important for patients with or without ASCT as consolidation, as those undergoing ASCT were preselected for this treatment at diagnosis due to high-risk disease. Interestingly, the relatively high ORR achieved in patients with early stage disease treated with radiotherapy alone were not reflected by the results of PFS and OS when compared with patients treated with standard anthracycline-based chemotherapy. In Varga et al, OS at 12 months calculated for all patients was significantly higher in younger patients. In the elderly group, patients who completed standard chemotherapy achieved the best survival rates. The worst survival data came from the group given no treatment. Patients with sub-optimal treatment were between these two groups. Elderly patients who completed standard treatment and obtained a CR had comparable OS to the younger patients. The data on event-free survival confirms these observations (Varga et al., 2014). The group of patients with NHL aged 70 or more in Maartense et al. was characterised by a significantly lower 5-year OS when compared with the younger patients (Maartense et al., 1998). This was confirmed in patients with intermediated grade lymphoma by WF and in DLBCL. By contrast there were no differences in disease free survival for all the above named groups. Thieblemont et al reports a median OS of 1.2 years only in very elderly patients aged > 80 years (Thieblemont et al., 2008). Hasselblom et al reports that age ≤60 years and >60 years was a statistically significant factor in univariate and multivariate analysis for OS in curatively treated patients and in all patients (Hasselblom et al., 2007). In the evaluation of PFS it was a significant factor in multivariate analysis for all patients but not for curatively treated patients. In conclusion the outcome of elderly patients was worse in all these publications.

Evaluation of impact of age as continuous variable on progression free survival and overall survival

The analysis of patients according to age decades delivered interesting results. In the evaluation of all patients, three separate groups were defined on the basis of differences in the OS rates between patients in different age decades: patients aged 60 years or less, patients between 60 – 70 years and those above 70. By contrast, based on PFS we could separate only two groups; 70 years or less and more than 70. In early stage disease in patients analysed by OS the same three groups were defined and according to the PFS we could still separate two groups. However the age limit was 60 years. In advanced stage disease we could distinguish between patients aged 70 years or less and more than 70 by both OS and PFS. This data corresponds with the observation that patients with advanced disease in their 60s should be treated with regimens designed for younger patients rather than with those for elderly patients. In patients with early stage disease the situation is not so obvious with the age limit of 60 years. This should be taken into consideration when new trials for patients with DLBCL are designed. The Dutch group of Maartense et al also studied the significance of age limits for patients with DLBCL (Maartense et al., 2000). They found that patients aged 65 or more had rapidly decreasing rates of CR and OS. This data is the opposite of ours, confirming the use of an age limit of 60 – 65 years for distinguishing younger and older patients. Importantly, the study of Maartense et al. included patients treated with different modalities whereas ours included only those receiving anthracycline-based chemotherapy. It is likely that patients receiving other treatment modalities were less fit and thus their response to treatment were worse confounding the age limits.

Evaluation of International Prognostic Index

The IPI is the most commonly used prognostic index for patients with DLBCL. It was developed in the late 1980's and published in the early 1990's as a response to increasing demand to supplement the Ann Arbor staging system in patients with DLBCL. The latter was primarily developed for HL and emphasises the distribution of nodal disease, because HL commonly spreads through continuous groups of lymph nodes (Carbone et al., 1971). Since the pattern of disease spread is different in NHL it is obvious that the Ann Arbor system is less accurate in DLBCL (Rosenberg, 1977). The IPI was developed based on a cohort of 3273 patients coming from different centres in Northern America and Western Europe. Complete data was available for 1872 patients

and 1385 were randomly selected for the dataset. Patients had a de novo diagnosis of diffused mixed, diffuse large-cell, or large-cell immunoblastic lymphoma (WF categories F, G, and H); diffuse centroblastic-centrocytic, centroblastic, immunoblastic or unclassified high-grade lymphoma (Kiel classification); or diffuse mixed lymphocytic-hystiocytic or diffuse histiocytic lymphoma (Rappaport classification). Unfortunately, at this time several different classification systems for lymphomas were in use in participating countries, which could lead to significant heterogeneity of the cohort. All patients were treated with a combination-chemotherapy regimen containing doxorubicin. The IPI and aaIPI for patients younger than 60 years were developed.

Our cohort includes only patients with a diagnosis of DLBCL and only patients from Northern England and Scotland. Like the original paper all our patients received anthracycline-based chemotherapy. The IPI was previously evaluated in other cohorts of patients however ours is the most representative as it has the highest number of patients and is population-based. Comparing both cohorts the distribution of patients in each risk group was very similar; for low and intermediate low risk groups with 35% vs. 38% and 27% vs. 26%; original vs. SNLG cohort respectively. For high intermediate and high risk groups the values were 16% vs. 22% and 22% vs. 14%; original vs. SNLG cohort respectively. For the aaIPI in the cohort of patients aged <60 years the comparisons were very similar with 22% vs. 29% in low risk group, 32% vs. 32% in low intermediate risk group, 32% vs. 31% in high intermediate risk group and 14% vs. 8% in high risk group; original vs. SNLG.

The 5-years OS in IPI groups was comparable for low, low intermediate and high-risk groups with 73% vs. 67%, 51% vs. 46% and 26% vs. 27%. For the high intermediate group the 5-year OS of our cohort was surprisingly low 43% vs. 25%, original vs. SNLG cohort. We cannot find any explanation for this phenomenon. For the aaIPI the 5-years OS was comparable for all risk groups: 83% vs. 88% (low); 69% vs. 62.5% (low intermediate); 46% vs. 41% (high intermediate) and 32% vs. 33% (high); original vs. SNLG cohort respectively. In the original IPI cohort and also in our cohort group the differences in OS between the individual risk groups were statistically significant in over all evaluation. The pair-wise evaluation was not presented in the original paper, however in our cohort we found significant differences except for low risk group and low intermediate risk group. Additionally, an evaluation of PFS was performed in our cohort and statistically significant differences were recorded in over all evaluation. In pairwise evaluation there were no statistically significant differences

between low risk group and low intermediate risk group and between low intermediate risk group and high intermediate risk group. For aaIPI, in an overall evaluation, the differences in OS were statistically significant for all risk group in the original evaluation and in our cohort, also for PFS. In pairwise evaluation in our cohort, we were not able to show significant differences between the high intermediate risk group and high risk group.

Armitage et al presented similar results on IPI in a study, which collected newly diagnosed NHL cases from 8 centres. The first 200 eligible cases from each centre were included (Armitage and Weisenburger, 1998). However both intermediate groups low and high were merged for the purposes of the evaluation. The IPI was also re-valuated in population-based studies on DLBCL by Hasselblom and Krol (Hasselblom et al., 2007) (Krol et al., 2003). In the study of Hasselblom the IPI and aaIPI strongly predicted OS but the differences between the low intermediate and high intermediate groups were rather small though still significant in the aaIPI model but not in the IPI (Hasselblom et al., 2007). The IPI and aaIPI were strongly predictive factors in the multivariate analysis of the curative group and all patients in this study. In Krol et al population-based registry study the IPI was available in 376 of 470 patients and 143 patients were in the low risk group, 96 in the low intermediate risk group, 66 in the high intermediate risk group and 71 in the high risk group (Krol et al., 2003). Median survival for the four groups was 80, 20, 7 and 5 months respectively ($p<0.001$).

In conclusion, the IPI is a robust and stable tool for prediction of survival in patients with DLBCL. However it has its limitations in certain groups of patients. Unfortunately the IPI is not universally used for patients with DLBCL. Thus comparison of results between individual studies still remains difficult.

Prognostic value of duration of first remission on overall survival

Some recent publications emphasize the influence of duration of first remission on the OS of patients with DLBCL. The GELA assessed the role of duration of first remission in DLBCL patients included in its trials. They found significantly shorter OS in patients who did not achieve remission, and those with shorter remission duration compared with those with PR, longer remission duration and patients with CR (Coiffier et al., 2008).

One of our aims was to assess the influence of duration of first remission in a population-based setting in patients with DLBCL treated with standard anthracycline-

based chemotherapy, and to further assess the possible differences in clinical features, (including IPI), between patients with different durations of first remission. Unfortunately it was not possible to obtain a precise remission status (CR vs. PR) for the majority of our patients. Thus the duration of remission was used as the main factor to select patients for the purposes of the evaluation. Patients were assigned to one of the following groups: 1) "refractory group" - patients who did not respond to first line treatment or relapsed within nine months from diagnosis (566 patients – 41%), 2) "early relapse group" - patients who relapsed within 9 – 18 months from diagnosis (92 patients – 7%), 3) "late relapse group" - patients who relapsed later than 18 months from diagnosis (120 patients – 9%) and 4) "no relapse group" - patients who did not relapse (597 patients – 43%). In younger patients, aged ≤60 years, the proportions were 33%, 7%, 8% and 52% respectively, and in elderly patients aged >60 years 46%, 7%, 9% and 38%. The increased number of patients in the "refractory group" in elderly patients could be explained by the generally worse prognosis of the disease in this group of patients.

Our results confirm the significant role of duration of first remission on OS of patients with DLBCL. The statistically significant highest 5-years OS was in the group with the longest remission time; the "no relapse group" (90%), followed by the group with the second longest, remission time; the "late relapse group" (50%), the "early relapse group" (16%) and the group with the shortest remission, the "refractory group" (8%). These results were confirmed both in younger patients aged ≤60 years – where 5 year. OS was 94%, 66%, 31% and 13% respectively ($p<0.001$) and in elderly patients aged >60 years, 85%, 40%, 65 and 4% respectively, $p<0.001$.

The GELA study assigned 7198 participating patients into five groups depending on quality and duration of remission (Coiffier et al., 2008). The 7 year OS was as follows: 1) patients with CR – above 90%, 2) patients with PR and 3) patients with early relapse (within first year) – both 38%, 4) patients with late relapse and 5) patients non-responding / refractory – both 12%. The data for patients treated with immunochemotherapy were similar to these except that OS for all groups tended to be higher. If we compare the GELA and SNLG results: we can conclude that the results are similar indicating that obtaining a remission and sustaining it for the longest possible period is crucial for long-term survival. Unfortunately the GELA PR group has no counterpart in our evaluation as our "no relapse group" does not distinguish between CR and PR.

To assess potential differences in clinical features of patients at presentation the individual groups were compared pair-wise: “refractory group” vs. “early relapse group”, “early relapse group” vs. “late relapse group” and “late relapse group” vs. “no relapse group”. The groups were compared for the following 13 clinical factors: age, sex, CS, B-symptoms, ECOG, extranodal and BM involvement, bulky disease, Hb, leukocytes, LDH, albumin, urea, and serum AP level. As suspected, the groups with the largest differences in OS were characterised by the largest differences in clinical characteristics at diagnosis – “the refractory group” vs. “early relapse group” and “late relapse group” and “no relapse group” followed by the “early relapse group” vs. “no relapse group”. By contrast, the groups with the smallest differences in OS, varied only by a single factor, e.g. “early relapse group” vs. “late relapse group” and “late relapse group” vs. “no relapse group”. CS, B-symptoms and ECOG were the most frequently variable factors. All are included in the IPI. CS and BM involvement were the only factors, which statistically distinguished between groups with small differences in OS, for example the “early relapse” and “late relapse group” and more importantly the “late relapse ” vs. “no relapse group”. The IPI, has the power to distinguish between the groups with the largest differences, but was not enough strong to distinguish between the groups with similar survival e.g. “early relapse group” vs “late relapse group” and “no relapse group” vs “late relapse group”. Additionally it lost power in individual evaluations between younger and elderly patients, probably due to decreasing patient numbers in the groups. In the GELA study the IPI also could not distinguish between individual groups (Coiffier et al., 2008). It is difficult to discuss these results further, as the IPI was not developed for this purpose.

Interestingly, the data on remission quality in patients with FL treated in GELA studies also confirms statistically significant longer OS for patients with CR as compared to those with PR (Bachy et al., 2010).

In conclusion, the data from both clinical trials and population-based studies on the duration and depth of remission in patients with DLBCL shows that better quality and longer duration of the achieved remission are associated with longer OS. This possibly indicates that regimens that result in achievement of the best possible, and longest remissions should be favoured at diagnosis. Additionally, it is impossible to entirely distinguish between the individual remission groups by clinical features or IPI at diagnosis. We conclude that the current prognostic indices based on clinical factors need to be enhanced using biological features of the tumour.

Evaluation of addition of rituximab to anthracycline-based chemotherapy

The advent of rituximab in the treatment of DLBCL was a break through event. It has contributed to the creation of new terms like pre-rituximab and post-rituximab era. The first results of the addition of rituximab come from randomized trials (Coiffier et al., 2002) (Feugier et al., 2005) (Habermann et al., 2006) (Pfreundschuh et al., 2006) (Pfreundschuh et al., 2008), and represent generally better results than expected in the patient population, mostly due to patient pre-selection. More recently, several population-based analyses of patients with DLBCL in the post-rituximab era were published (Sehn et al., 2005) (Krause et al., 2012) (Keegan et al., 2013) (Lee et al., 2012). The first population-based study to assess the impact of rituximab on outcomes of patients with DLBCL comes from the province of British Columbia in Canada. This retrospective study was carried out between September 1999 and August 2002, a 3-years period representing 18 months pre-rituximab (140 patients) and 18 months post the introduction of rituximab (152 patients) (Sehn et al., 2005). Lee et al performed a population-based analysis of all patients in the province of Ontario between Jan 1997 and Dec 2007 (Lee et al., 2012). All patients had a new diagnosis of DLBCL and received at least one cycle of CHOP-based chemotherapy, with or without the addition of rituximab (Lee et al., 2012). Patients from the CHOP group were then matched according to the clinical data at presentation, and the OS was calculated for both groups, and for individual age groups. Keegan et al evaluated the impact of adding rituximab to chemotherapy in DLBCL patients based on the National Cancer Institute's Patterns of Care studies (Keegan et al., 2013). Krause et al performed a population-based study based on the data of a single tumour centre cancer registry comparing the outcome of patients with de novo aggressive lymphomas (Krause et al., 2012).

In our study we assessed for the first time in a population-based setting the influence of the addition of rituximab to anthracycline-based chemotherapy for patients with DLBCL, treated in the NHS. The new data was compared to an historical cohort of patients treated with CHOP in the same service. Both cohorts were comparable with the exception for a smaller number of patients with BM involvement in the immunochemotherapy group and more elderly patients. In the study of Lee et al. all patients in the CHOP-R group were significantly older, and had higher comorbidity scores (Lee et al., 2012). By contrast in Krause et al there were no differences between the treatment groups in terms of age and sex (Krause et al., 2012). In the British Columbia study by Sehn et al no differences were described between the patients from

the pre-rituximab and post-rituximab eras according to sex, age, performance status, LDH, extranodal site numbers, CS, bulky disease and IPI.

In our cohort of patients doxorubicin was changed to mitoxantrone in the same number of patients treated with chemotherapy only and immunochemotherapy. However, we observed an increase in the use of additional radiotherapy in the patients treated with immunochemotherapy, particularly in the patients aged ≤60 years. This may be due either to improvement of results with the new regimen or new data supporting the use of combined modality treatment, plus general motivation to sustain remissions in younger patients. Krause et al. reported the same number of patients receiving additional radiotherapy in the chemotherapy only and chemotherapy with addition of rituximab groups (Krause et al., 2012). By contrast Sehn et al described more patients receiving post-chemotherapy radiation in the post-rituximab era compared with patients from the pre-rituximab era (24% vs. 14%, respectively; p=0.04) (Sehn et al., 2005).

Statistically significant differences in ORR were observed in the evaluations of all patients, younger and elderly patients, and in all stages with the exception of early stage in younger patients. Anthracycline-based chemotherapy boosted by radiotherapy, (combined modality treatment), which was commonly used in the region in the pre-rituximab era was probably sufficient for this group of patients.

As suspected in the evaluation of all patients there was a statistically significant increase in the duration of PFS and OS. However the differences were significant only in patients with advanced stage disease and not early stage disease. This was also observed in the evaluation of younger patient. By contrast in the evaluation of elderly patients the difference remained for PFS but not for OS. Importantly, there were no statistically significant differences in OS for any of the stage groups in elderly patients. The study of Sehn et al, which was characterised by short follow-up described differences in OS at 2 years between patients treated with and without rituximab. In the evaluation of all patients there were significant differences in PFS and OS between both groups (PFS: 51% vs. 69%, p=0.002 and OS: 52% vs. 78%, p<0.0001), and in elderly patients (PFS: 44% vs. 68%, p=0.007 and OS: 42% vs. 73%, p=0.0003). However, in younger patients there were significant differences in OS but not in PFS; PFS: 60% vs. 70%, p=0.13 and OS: 67% vs. 85%, p=0.02). Lee et al reported a similar 5-year OS of 57% in the group of all patients treated with R-CHOP. By contrast, in an evaluation of a matched group of patients the difference in 5-year OS was significant for all patients,

patients aged 60 or more, and patients aged 60 – 80, but not for very elderly patients aged 80 or over. In Keegan et al. patients who received chemotherapy with the addition of rituximab had better OS, but patients with impaired performance status, elevated LDH and advanced stage disease had worse OS (Keegan et al., 2013). In Krause et al. 5-year survival in the chemotherapy plus rituximab group was significantly better than the chemotherapy only group, 69.6% vs. 56.8% (Krause et al., 2012).

In conclusion data from clinical trials and population-based studies confirms the positive influence of the addition of rituximab on the outcome of patients with DLBCL. However this influence varies in different patient groups.

2.5 References

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Chapter 3. Studies on new prognostic molecular markers for DLBCL

3.1 Introduction

Better risk stratification and prediction of response to standard therapy and selection of the high-risk groups suitable for more intensive and novel therapy approaches are essential in order to target the treatment of DLBCL more effectively. All previously described prognostic markers including: individual biomarkers, GEP studies or IHC models are currently not used in routine practice due to different reasons including costs, requirement of fresh material or reliability. The limited amount of diagnostic tissue in the era of small biopsies is another currently arising issue (Rimsza et al., 2008).

In recent years the qPCR became a very reliable and efficient tool in investigating gene expression. Originally the method required use of fresh or snap-frozen material in order to achieve true and reproducible results. Thus, it was mostly used in the diagnosis of leukaemias and other conditions with easy access to fresh material. The measurement of BCR-ABL fusion gene in diagnostic and treatment monitoring of chronic myeloid leukaemia (CML) is one of the best known examples of employment of qPCR in routine diagnostics. The introduction of modern highly efficient RNA extraction methods and enzymes for reverse transcription (RT) and qPCR changed the situation. Now qPCR can be successfully applied in the genetic material extracted from FFPE tissue. This opened entirely new opportunities for qPCR application in routine diagnosis of solid tumours. However, the degradation of RNA still remains one of the main unsolved issues. The selection of primer sets for possibly short amplicon and housekeeper genes for control are crucial conditions of success.

In the following studies, we aim to investigate the application of qPCR in assessment of gene expression for survival prognosis in patients with DLBCL. We selected two genes: *c-MYC* and *HLA-DRβ*. Both were previously described as prognostic in single biomarker studies and GEP studies (Rosenwald et al., 2002) (Rimsza et al., 2004) (Rimsza et al., 2007) (Veelken et al., 2007) (Chang et al., 2000). The overexpression of c-MYC protein is associated with increased tumour proliferation and the loss of expression of HLA-DRβ protein with absence or reduction in immunosurveillance. Importantly, Rimsza et al. re-analyzed the expression of 36 genes with significant impact on patient survival in previous GEP studies in RNA extracted

from FFPE tissue samples of DLBCL patients treated with R-CHOP (Rimsza et al., 2008). The applied quantitative S1 nuclease protection assay is suitable for analysis of gene expression in the material extracted form FFPE samples. The expression of the only two genes: *c-MYC* and *HLA-DRβ* was significantly associated with patient survival, and they together with IPI were included in a new prognostic model. The model was based on high expression of one of the major proto-oncogenes responsible for cell proliferation and low expression of an MHC gene playing a key role in immunosurveillance. The abundance in expression of these two genes is also a defining attribute of BL, suggesting that lack of immunosurveillance and high proliferation are the key features defining the most aggressive lymphomas (Rosenwald et al., 2002) (Dave et al., 2006) (Hummel et al., 2006) (Rimsza et al., 2007). Bearing in mind significant improvement of outcome in patients with BL with introduction of more intensive chemotherapy regimens (R-CODOX-M/IVAC or GMAIL), there could be a hope that the patients selected with this predictive model would benefit from these regimens despite toxicity.

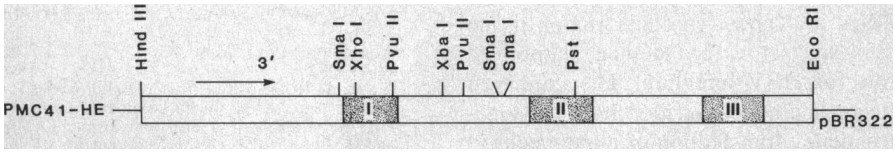
We also studied the prognostic role of a non-coding transcript: version 2 of the gene *chromosome 13 open reading frame 25 (C13orf25)*, called also *v2-transcript*. The *v2-transcript* is encoded within the 13q31-32 chromosomal region and a high degree of amplification of this region was identified in haematological malignancies including DLBCL (Rao et al., 1998) (Ota et al., 2004). *v2-transcript* plays potential role in oncogenesis of haematological malignancies (Ota et al., 2004). It encodes a miRNA cluster (miR-17-92), consisting of seven mature miRNAs: miR-17-5p, miR-17-3p, miR-18, miR-19a, miR-19b, miR-20, and miR-92 (Ota et al., 2004) (He et al., 2005), which have been shown to act in an oncogenic capacity (He et al., 2005). Thus, additionally we aim to address the question of application of qPCR in assessment of *v2-transcript* and miRNAs encoded in *v2-transcript* as new prognostic biomarkers in DLBCL. The techniques used for miRNA assessment are novel and required completely new approach including new extraction methods of RNA and qPCR.

3.1.1 *c-MYC*

c-MYC oncogene was originally identified in the avian myelocytomatosis virus and subsequently its oncogenic potential was described in several cancer types (Adams et al., 1985). The normal cellular proto-oncogene, *c-MYC*, encodes the protein c-MYC,

a transcription factor. The *c-MYC* is located in chromosome 8 and consists of 3 exons separated by 2 introns. The first exon codes for an untranslated leader; see figure 3.1.a.

(A)



(B)

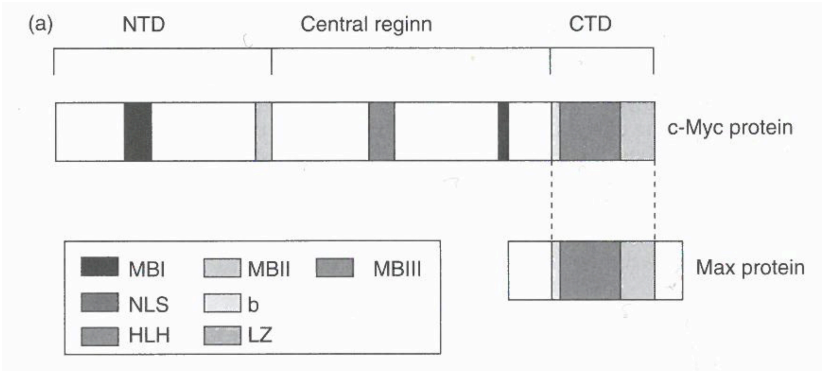


Figure 3.1. c-MYC structure. **(A)** A simplified map of then human *c-MYC* gene (ar-Rushdi et al., 1983) **(B)** Functional domains of human c-MYC protein (Pelengaris and Khan, 2006).

The physiological role of c-MYC protein is very versatile in healthy cell; it promotes cell cycle progression and is involved in cell differentiation, cell growth, apoptosis and angiogenesis (Coller et al., 2000). In order to become transcriptionally active c-MYC requires dimerization with another protein called c-myc-associated factor X (MAX). c-MYC possesses three functional domains: 1) the C-terminal domain (CTD) including helix-loop-helix leucine zipper motif for dimerization with MAX, 2) the N-terminal domain (NTD) containing conserved c-MYC boxes I and II (MB I and II) domain essential for transactivation of c-MYC target genes and 3) the central region including MB III which negatively influences apoptosis (see figure 3.1.b) (Pelengaris and Khan, 2006) (Nilsson and Cleveland, 2004) (Herbst et al., 2005). In health, c-MYC is not expressed in all cells. The cells possessing high proliferative capacity (e.g. epidermis) are characterized by high expression whereas it is undetected in cells which have exited the cell cycle (Pelengaris and Khan, 2006).

c-MYC has a very important role in cell growth and proliferation. *c-MYC* genes belongs to the early-response genes induced within 15 minutes from the binding of growth factor to the receptor and allowing cells to exit G_0 and enter the cell cycle. By

contrast, in non-proliferating cells, *c-MYC* is inactive. During oncogenic activation of *c-MYC* the cell is a subject of constant stimulation. The regulation of cell proliferation happens via induction of cyclin dependent kinase E-CKD2 (major factor in G_1 – S progression) by c-MYC protein. Additionally c-MYC protein influences the cell-cycle by repression of genes encoding cyclin-dependent kinase inhibitor (CKIs) $p15^{INK4b}$ and $p21^{CIP1}$, factors involved in cell cycle arrest (Pelengaris and Khan, 2006). Finally, c-MYC potein has an ability to remodel chromatin by methylation (Brenner et al., 2005). The mechanism of the influence of c-MYC protein on cell growth is not entirely understood but it happens via the activation of RNA polymerase III, however the recent studies indicate that all three nuclear RNA polymerases are activated by c-MYC protein (Gomez-Roman et al., 2003).

The terminal differentiation of proliferating cells requires their exit from the cell cycle and as the c-MYC protein keeps cells in the cell cycle it prevents their differentiation. Thus, unregulated levels of c-MYC protein can lead to excessive proliferation of cells and maturation arrest. By contrast, the differentiation of cells requires down regulation of c-MYC protein. The important molecule playing a role in c-MYC suppression is Max-interacting transcriptional repressor protein (MAD) MXII, which polymerizes with the MAX protein preventing the synthesis of active c-MYC-MAX dimers (Ayer et al., 1995). Some recent studies indicate that the onset of differentiation does not necessary involve cell cycle arrest (e.g. haematopoietic differentiation). Other recent studies emphasise the role of c-MYC in maintaining stem cell pluripotency and self-renewal (Pelengaris and Khan, 2006).

The pro-apoptotic features of c-MYC were discovered in the 1990's and were breakthrough news (Evan et al., 1992). There is not a single mechanism of c-MYC induced apoptosis activation (Evan et al., 1992). One described pathway runs through indirect activation of tumour suppressor gene tumor protein p53 (p53) via activation of other tumour suppressor $p19^{ARF}$ (Zindy et al., 1998). c-MYC can also promote apoptosis by interactions with BCL2 family (repression of anti-apoptotic molecules BCL2 and $BCLx_L$ and induction of pro-apoptotic molecule BIM) (Pelengaris and Khan, 2006). Lately, it has been postulated that highly conserved c-MYC box III region is involved in the pro-apoptotic activity of c-MYC (Herbst et al., 2005).

Bearing in mind the enormous importance of c-MYC in cell life and homeostasis, cells have evolved several control mechanism of c-MYC activity and accumulation (Pelengaris and Khan, 2006) (Amati, 2004). The activity of c-MYC is

controlled by a repertoire of promoting factors: growth factors, mitogens and β-catenin and inhibiting factors such as TGF-β (Amati, 2004). Mature c-MYC is also a very unstable protein, being a subject of ubiquitin-dependent degradation (Amati, 2004) and protein acetylation (Patel et al., 2004). Interestingly, not all c-MYC molecules undergo degradation at the same time leaving preserved pools of molecules.

Regarding the expression of c-MYC protein in human cancer, the most characteristic example is BL with *c-MYC* translocation (Swerdlow et al., 2008). In addition the elevated or deregulated expression of c-MYC has been detected in numerous other cancer types e.g. breast, colon, cervical, small-cell lung carcinomas, osteosarcomas, glioblastomas, melanoma, and myeloid leukaemia (Pelengaris et al., 2002). Usually, the overexpression of c-MYC is an adverse prognostic factor and is associated with aggressive, poorly differentiated tumours. The mechanism of overexpression of c-MYC varies from translocation, through gene amplification, stabilization of *c-MYC* mRNA, enhanced initialization of translation due to mutations or activation of upstream signalling pathways to affected stability of the c-MYC molecule (Pelengaris and Khan, 2006).

c-MYC plays an important role in lymphomagenesis. The *c-MYC*, human sequence related to the transforming gene (*v-MYC*) of avian myelocytomatosis virus (MC29), was found for the first time in the translocated region of chromosome 8q24 in BL cell lines (Dalla-Favera et al., 1982). The reciprocal translocation involves portions of chromosome 8 and chromosome 14 t(8;14)(q24;q32), or less frequently chromosomes 2 t(2;8)(p11;q24) and 22 t(8;22)(q11;q24). In the chromosomes 14, 2 and 22, the breakpoints occur within the regions carrying Ig genes: on chromosome 14 (heavy chains) or chromosome 2 and 22 (kappa and lambda light chains respectively). Regarding the breakpoints in chromosome 8, three different classes of translocation were described: class I with the breakpoint within the first exon or intron, class II with the breakpoints immediately upstream of the gene and class III with distant breakpoints. Class I and II translocation are predominant in sporadic BL and class III with the breakpoints up to 300 kb upstream of the gene are more frequent in endemic BL (Joos et al., 1992) (Siebert et al., 1998).

The cytogenetic changes in DLBCL are complex, and the rearrangements of c-*MYC*, *BCL2* and *BCL6* are the most common and the best characterized (Johnson et al., 2009). According to the data of population-based NHL Registry of the Comprehensive Cancer Centre West (CCCW), the *c-MYC* rearrangements were found in 7% of samples

(10/151) among this group two patients had additional *BCL6* rearrangements. Importantly, the *c-MYC* translocation was found predominantly in patients with primary extranodal disease, mostly involving the gastrointestinal tract and surprisingly was associated with favourable DFS (Kramer et al., 1998). In the data of Niitsu et al, the frequency of *c-MYC* rearrangements was estimated at 5.7% (28/489 and eight patients had concurrent t(14;18) (q32;q21) translocation. The patients with *c-MYC* rearrangements were characterised by more advanced disease, worse response and reduced survival. This was particularly evident in patients with concurrent *BCL2* rearrangements (Niitsu et al., 2009). The negative predictive value of *c-MYC* translocation was also confirmed in patients treated with CHOP-R (Barrans et al., 2010) (Savage et al., 2009). Moreover, it was confirmed that the *c-MYC* translocation usually occurs with concurrent *BCL2* and *BCL6* translocations and that patients carrying these complex karyotypes have very poor outcome. In addition, Barrans et al were able to create a new prognostic index based on age, IPI and *c-MYC* translocation status. Interestingly, the GELA group could not confirm the predictive value of *c-MYC* rearrangement status in their cohort of patients treated with CHOP-R. By contrast, they were able to develop an immune-fluorescence index predicting survival in DLBCL patients treated with CHOP-R. The index is based on expression of MUM1/IRF4 and FOXP1 and *BCL6* rearrangement status (Copie-Bergman et al., 2009). The issue of concurrent translocation of *c-MYC*, *BCL2* and *BCL6* in B-cell lymphomas, so called "double or triple-hit" was addressed by several groups confirming their poor prognosis (Johnson et al., 2009) (Snuderl et al., 2010). This lymphoma has not benefitted from addition of rituximab to the anthracycline-based chemotherapy and furthermore they are usually resistant to standard salvage chemotherapy with R-ICE and R-DHAP (Cuccuini et al., 2012).

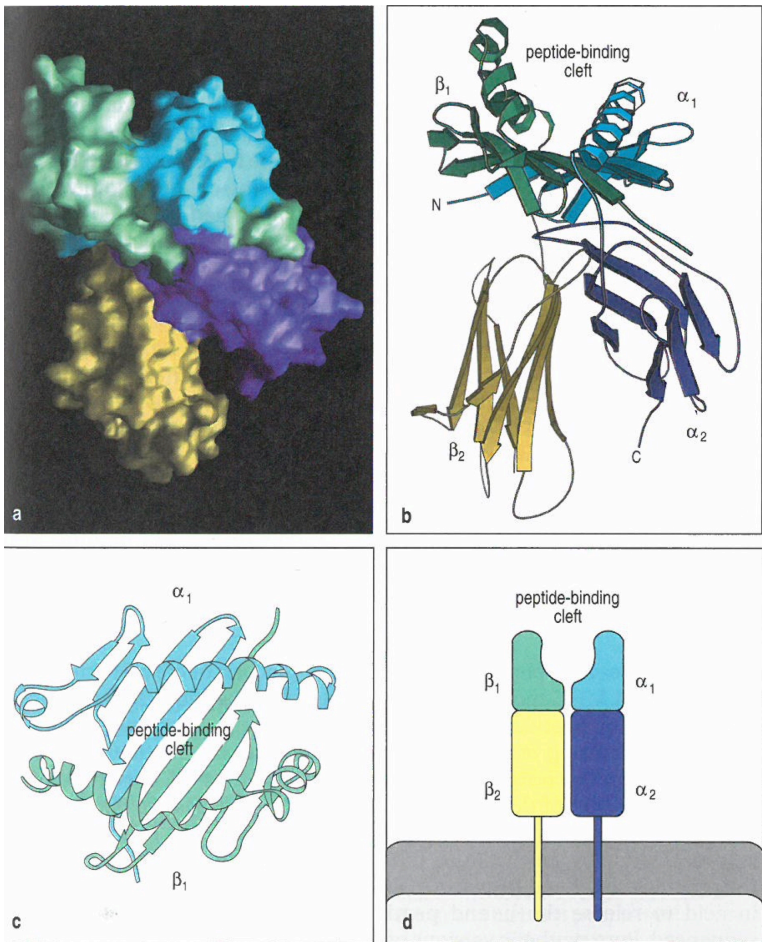
Despite the interesting data on prognostic value of *c-MYC* rearrangements there are only few data available on the predictive value of c-MYC protein expression in DLBCL. This is possibly due to difficulties in IHC staining for c-MYC. However, since the introduction of new antibodies the situation has changed (Gurel et al., 2008). The pilot study of Ruzinova et al. confirmed the association between the presence of primarily nuclear or mixed nuclear / cytoplasmic staining pattern and *c-MYC* translocation in aggressive lymphoma (Ruzinova et al., 2010). Green et al in their study on 219 DLBCLs and BLs could confirm positive correlation between presence of *c-MYC* translocation and expression of c-MYC protein (Green et al., 2012a). The recent

study from DSHNHL assessed the predictive impact of *c-MYC*, *BCL2* and *BCL6* rearrangement status and c-MYC, BCL2 and BCL6 protein expression in patients aged >60 years treated with CHOP-R (Horn et al., 2013). The expression status c-MYC (high), BCL2 (high) and BCL6 (low) and *c-MYC* rearrangement status were predictive independently from IPI for inferior outcome. However, it is still not clear if the *c-MYC* rearrangements itself or overexpression of c-MYC protein has a conclusive impact on patient survival. Additionally, there is still little known about the regulation of expression of c-MYC in DLBCL. There is very limited information available on data on predictive value of increased expression of *c-MYC* mRNA. Some results come from a GEP study of Rosenwald et al and this was confirmed by the study of Rimsza et al (Rosenwald et al., 2002) (Rimsza et al., 2008). Hummel et al in his GEP study on BL proved that the presence of *c-MYC* rearrangements is not necessarily associated with an increased in *c-MYC* mRNA expression (Hummel et al., 2006).

3.1.2 *HLA-DRβ*

HLA-DRβ is one of the genes encoding the β chain of the MHC class II molecule. MHC class II molecules are dimers of one α and one β transmembrane chain. Each chain consists of two domains α1 and α2 and β1 and β2 respectively (figure 3.2.a) (Janeway et al., 1999). The MHC II class molecules are present on macrophages and B-lymphocytes and are responsible for presentation of intra-vesicular antigens to the CD4+ cells (Th1 and Th2) cells (Janeway et al., 1999). There are three pairs of MHC class II α and β chain genes: *HLA-DP*, *HLA-DR* and *HLA-DQ*; see figure 3.2.b. In some haplotypes the *HLA-DR* cluster contains an additional β–chain gene, which can pair with the *HLA-DR* α chain gene. Thus these three sets of genes can build four types of MHC class II molecules. The MHC class II genes are highly polymorphic (e.g. there are >200 alleles of *HLA-DRβ* chain) and they are characterized by co-dominant expression. Together with polygeny, this contributes to the diversity of MHC molecules expressed by an individual (Janeway et al., 1999).

(A)



(B)

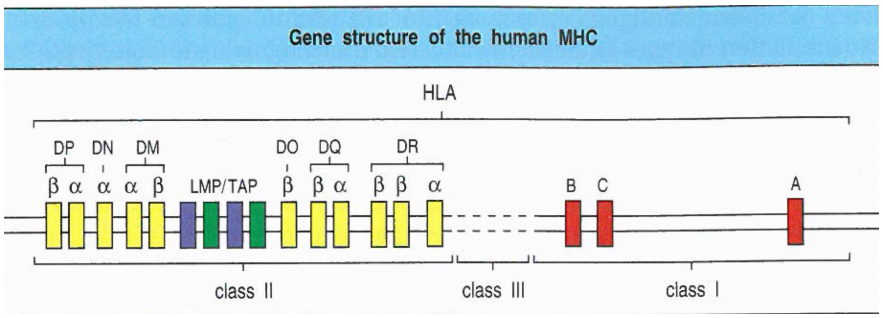


Figure 3.2 HLA-DRβ structure. **(A)** The structure of an MHC class II molecule and **(B)** the genetic organization of the MHC in humans (Janeway et al., 1999).

The expression of MHC class I and II molecules, or more precisely the lack of expression, in DLBCL was described as a negative prognostic factor for patients with DLBCL (Rimsza et al., 2004) (Rimsza et al., 2007) (Veelken et al., 2007). The predictive value of HLA-DRβ increases with its low expression, significantly higher with cut-off of 20% as compared to median cut off (Rimsza et al., 2008). The importance of lost MHC class II appears to be related to a loss of tumour

immunosurveillance as evidenced by decreased numbers of tumour-infiltrating T-cells (Rimsza et al., 2004) (Chang et al., 2007) (Lippman et al., 1990) (List et al., 1993). Bernd et al described the loss of expression of HLA-DRβ molecule together with immunoblastic tumour phenotype to predict adverse outcome in patients with DLBCL (Bernd et al., 2009). This was also confirmed by others (Rimsza et al., 2004) (Veelken et al., 2007). Additionally, the loss expression of MHC class II molecule genes was reported as related with poor patient survival. Rosenwald et al in his GEP study described lack of expression of *HLA-DRβ*, *HLA-DRα*, *HLA-DPα* and *HLA-DQα* as a prognostic factor (Rosenwald et al., 2002). Rimsza et al confirmed this results in the study on the FFEP tissue samples of DLBCL patients treated with R-CHOP, where *HLA-DRβ* and *c-MYC* were the only significant prognostic factors (Rimsza et al., 2008).

3.1.3 *v2-transcript and microRNAs (miRNAs) of miR-17-92 cluster*

The miRNAs are small (~ 22 nucleotides [nt]) endogenous RNAs that are diverse in sequence and expression pattern and are evolutionarily widespread (Lagos-Quintana et al., 2001) (Lau et al., 2001). MiRNAs are involved in sequence-specific, posttranscriptional regulatory mechanisms of gene expression. Together with short interfering RNAs (siRNAs), the miRNAs form a part of the RNA interference process. RNA interference is an RNA-dependent gene-silencing process that is mediated by the RNA-induced silencing complex (RISC) and is initiated by short double-stranded RNA molecules in the cytoplasm. The short RNA fragments are known as siRNA when they derive from exogenous sources such as viruses and miRNA when they are produced from RNA-coding genes in the cell's own genome.

The history of miRNAs begun in the early 1990's, when two groups, one led by Amros and the other by Ruvkun, found independently that larval development of the nematode *Caenorhabditis elegans* required a tiny RNA (mir lin-4) to inhibit the expression of the *lin-14* gene (Ruvkun et al., 2004) (Lee et al., 2004). The further studies on miRNAs expanded the work on RNA interference and posttranscriptional gene silencing. At the moment it is known that miRNA genes are encoded in the genome of most eukaryotic organisms, and thousands of miRNAs have been identified to date (Griffiths-Jones, 2004).

The miRNA are coded by miRNA genes, which are transcribed by RNA polymerase II into primary miRNAs (pri-miRNA). The longer pri-miRNAs are then processed into 60 to 70 nt long stem loop miRNA precursors miRNAs (pre-miRNA) by

the complex of nuclear RNase III Drosha and the DiGeorge syndrome critical region 8 (DGRC8) protein (Denli et al., 2004) (Gregory et al., 2004) (Landthaler et al., 2004). The pre-miRNAs are transported from the nucleus to the cytoplasm by Exportin-5, a guanosine triphosphate (GTP) dependent transporter (Bohnsack et al., 2004) (Brownawell and Macara, 2002) (Lund et al., 2004) (Yi et al., 2003). In the cytoplasm, the pre-miRNAs are cleaved at the base of the loop by a second RNase II enzyme, Dicer, to generate an imperfect miRNA : miRNA duplex of about 21 to 24 nt (Ouellet et al., 2006) (Bernstein and Whitington, 1988). Dicer has been recently reported to act in association with the trans-activating response RNA-binding protein (TRBP) within a pre-miRNA processing complex (Gregory et al., 2005). After the strand selection/separation, mature miRNAs of about 22 nts in length in combination with Argonaute 2 (Ago2), Dicer and TRBP form the RISC. RISC is then guided within the cytoplasm towards specific mRNA. RISC can then mediate the cleavage or repression of target mRNA, depending whether the miRNA : mRNA pairing is perfect or not (Bartel, 2004). The miRNAs pathway is shown in figure 3.3.

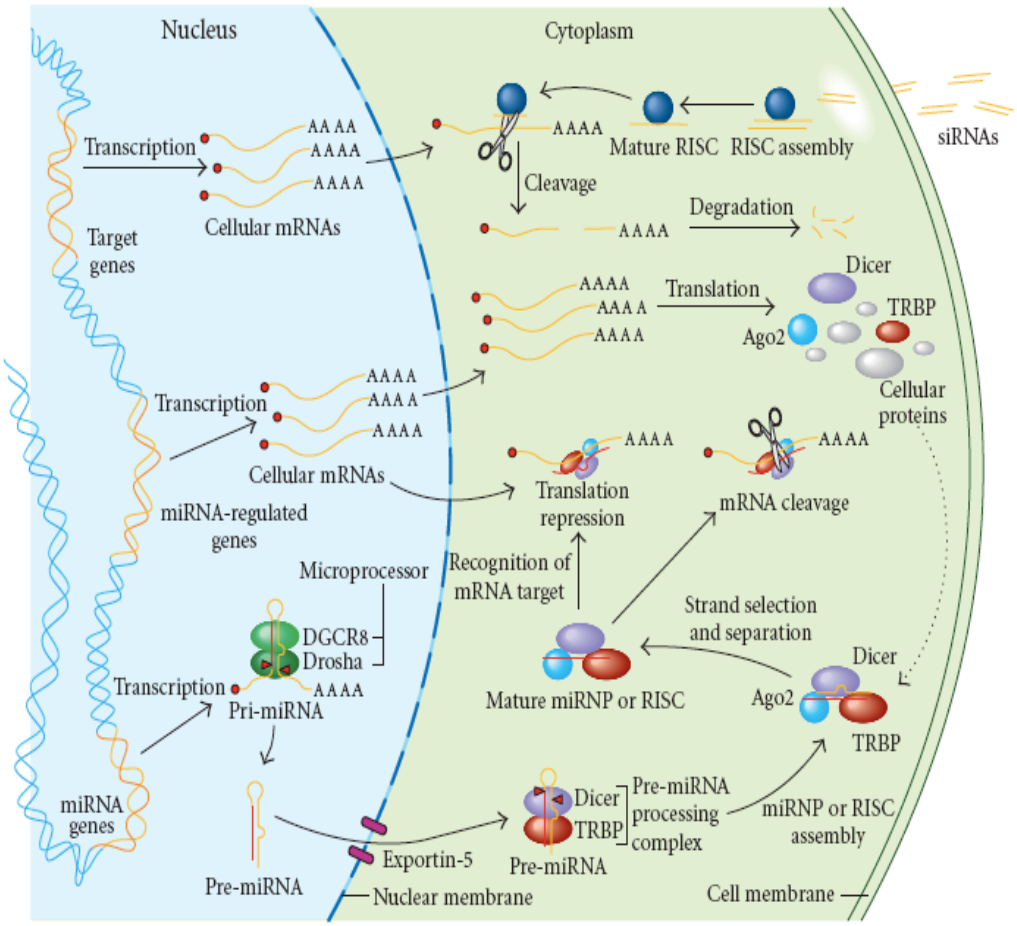


Figure 3.3 miRNA pathways (Ouellet et al., 2006).

miRNAs are involved in different biological processes including the regulation of developmental processes (Wightman et al., 1991) (Lee et al., 1993) (Kloosterman et al., 2004), genome rearrangement (Mochizuki et al., 2002), gene inhibition (Volpe et al., 2002) (Provost et al., 2002) (Savage et al., 2006) and chromosome segregation (Provost et al., 2002). MiRNAs are also known to regulate normal haematopoiesis (Kluiver et al., 2006), the immune response (Rodriguez et al., 2007) and are involved in macrophage inflammatory responses (Taganov et al., 2007). It has also been reported that miRNAs play a vital role in cell growth and apoptosis (Brennecke et al., 2003) (Cheng et al., 2005) (Chan et al., 2005) (Cimmino et al., 2005) (Calin et al., 2002). However the greatest interest is focused on the role of miRNAs in oncogenesis.

The role of miRNA in disease pathogenesis was primarily assessed in leukaemia, because of the easy access to genetic material in this condition. The original data arrives from studies on involvement of miRNAs in the pathogenesis of CLL. They reported loss of expression of miR-15 and miR-16 in CLL cells (Calin et al., 2002) (Calin et al., 2004) or presence of different miRNA signatures in CLL cells and normal lymphocytes (Calin et al., 2004) (Calin et al., 2005). Venturini et al. assessed the role of miRNAs in pathogenesis of CML indicating increased expression of miRNAs from the miR-17-92 cluster (Venturini et al., 2007). Interesting data come also from studies on the oncogenic role of miRNAs in solid tumours. The studies of Volinia et al indicate overexpression of miRNAs in tumour tissue of lung, breast, stomach, prostate, colon and pancreas (Volinia et al., 2006). Takamizawa confirmed this in studies on lung and colon cancers (Takamizawa et al., 2004). By contrast the reduced expression of miR-143 and miR-145 was associated with oncogenesis of colon cancer (Michael et al., 2003). The work of Kulshreshtha focused on the role of miRNAs in mechanisms of hypoxia in oncogenesis indicating several miRNAs being involved (Kulshreshtha et al., 2007).

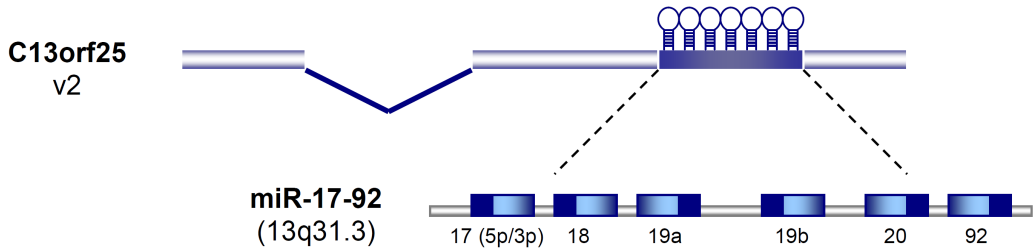
Research on the involvement of miRNAs in the pathogenesis of lymphoma focused on the expression of miR-155 and its pri-miRNA called B-cell integration cluster (BIC) as well on the role of the miRNAs encoded by the miR-17-92 polycistron of the *c13orf25 transcript variant 2*. BIC was originally identified as a target for pro-viral insertions in the avian leucosis virus (AVL) – induced lymphomas (Clurman and Hayward, 1989). Tuschl et al. demonstrated that BIC is the pri-miRNA of the mir-155 (Tuschl and Borkhardt, 2002). The expression of mir155 and BIC was analysed in HL- and NHL-derived cell lines and patient tissue samples by both Northern blotting and in

situ hybridisation (ISH) (Kluiver et al., 2005). BIC was expressed in 90% of the HL cases, in 67% of DLBCL and in 100% of PMLBL cases. However, other cases of NHL revealed no expression of BIC. The expression of BIC was higher in ABC-like DLBCL cases compared with that observed in GC-like DLBCL, implying involvement of BIC in aggressiveness of the disease. These results were confirmed by qPCR in both DLBCL and PMLBL cases. In addition Northern blot analysis showed the expression of miR155 in all HL, DLBCL and PMLBL cell lines and in some patient samples (Tuschl and Borkhardt, 2002). Recently, using semi-quantitative RT-PCR and Northern-blotting, Eis et al also investigated the expression of BIC and miR155 in a number of lymphoma cell lines (Eis et al., 2005). The expression of BIC was in concordance with the expression of mir155 and, as reported before by Kluiver et al (Kluiver et al., 2005). The highest expression was observed in ABC-like DLBCL-cells followed by HL-cells and GC-like DLBCL-cells. In contrast the cell lines of CML, myeloma and acute promyelocytic lymphoma were negative. Transfection of the HEK-293T cell line, which is negative for BIC and miR-155 expression, with miR-155-coding sequence (BIC) or with miR-155 negative sequence (control) resulted in the overexpression of mature miR-155 in the BIC transfected cells. These findings confirmed the association between BIC and miR-155. Quantification of BIC and miR-155 expression using the Invader RNA assay revealed higher expression of BIC and miR-155 in the ABC-like DLCBL samples than in GC-like DLBCL samples. As the ABC-like DLCBL phenotype is a more aggressive disease, the above findings may imply that miR-155 expression could be used for assessing prognosis in patients with DLBCL. These results were in concordance with those obtained using semi-quantitative RT-PCR and Northern blotting. Examination of the correlation between BIC and miR-155 revealed a weak correlation implying that the amount of BIC-RNA should not be taken as an accurate measure of the amount of miR-155. Eis et al evaluated as well the expression of other miRNAs such miR-15a and miR-16 and let70 in the DLBCL cells (Eis et al., 2005). Reduced expression of miR-15a and miR-16 was observed in 30-70% and in 25% of samples respectively, while a heterogeneous level of expression was reported with let 70.

Amplification of the 13q31 locus is frequently observed in various subtypes of NHL including DLBCL, FL, MCL and primary cutaneous lymphoma (Neat et al., 2001) (Mao et al., 2002) (Ota et al., 2004). The candidate target gene at 13q31 is *c13orf25*. There are two major transcripts in the *C13orf25* – variant 1 and 2 (Ota et al., 2004). The *C13orf25* transcript variant 1 (*V1-trancript*) has been reported as bA121J7.2 (Ota et al.,

2004). The *c13orf25* transcript variant 2 (*V2-trancript*) encodes a miRNA cluster (miR-17-92) consisting of seven mature miRNAs: miR-17-5p, miR-17-3p, miR-18, miR-19a, miR-19b, miR-20 and miR-92, see figure 3.4 (O'Donnell et al., 2005).

(A)



(B)

miR-17-5p	c**aaagug**cuuacagugcagguag
miR-17-3p	a**cugcag**ugaaggcacuuguag
miR-18	u**aaggug**caucuagugcagauag
miR-19a	u**gugcaa**aucuaugcaaaacuga
miR-19b	u**gugcaa**auccaugcaaaacuga
miR-20	u**aaagug**cuuauagugcagguag
miR-92	u**auugca**cuugucccggccugu

Figure 3.4 Structure of *v2-transcript* and miR-17-92 cluster. **(A)** The *C13orf25* (*v2-transcript*) gene is located on chromosome 13q31.3 and encodes a polycistronic cluster of 7 mature miRNAs (Ota et al., 2004). **(B)** The sequences of the mature miRNAs are shown and the 'seed sequences' indicated in bold and red (Griffiths-Jones et al., 2006).

The miR-17-92 mature miRNAs have been shown to act in an oncogenic capacity via targeting genes with roles in apoptosis (Olive et al., 2009) (Mu et al., 2009) (Xiao et al., 2008) (Ventura et al., 2008) and cell proliferation (Inomata et al., 2009) (Cloonan et al., 2008). He et al reported that the mir-17-92 cluster co-operates with *c-MYC* to induce lymphomagenesis, thus identifying the first oncogenic miRNA, termed oncomir-1 (He et al., 2005). Tagawa et al. investigated the expression *v2-transcript* and of its 7 miRNAs in 12 malignant lymphoma cell lines and 21 cases of DLBCL (Tagawa and Seto, 2005). The expression of *v2-transcript* measured with RT-PCR was significantly higher in the cell lines and tumour samples with a 13q31 gain. Northern blot analysis of the seven mature miRNAs revealed that these were significantly more highly-expressed in the cell lines with a 13q31 gain/amplification when compared with those without the

gain. The abundance of 191 mature miRNAs was assessed using microarray technology in four lymphoma cell lines that expressed the *v2-transcript* and such expression was compared to six cell lines lacking the amplicon (Ota et al., 2004). Six miRNAs were identified for which high-level expression correlated with the expression level of *v2-transcript*. Five of them belong to the mir-17-92 polycistron. Further, the expression of the *v2-transcript* measured with RT-PCR was performed on series of human lymphoma and colorectal carcinomas. A significant overexpression of *v2-transcript* was observed in 65% of lymphoma cases and only in 15% of colorectal carcinoma samples. Ota et al assessed the oncogenic potential of the mir-17-92 cluster of *c13orf25* locus using a mouse model, which confirmed its oncogenic role in the development of lymphoma (Ota et al., 2004). Transplantation of hematopoietic stem cells (HSC) from Eμ-MYC/+ mice to lethally irradiated recipient mice resulted in the development of B-cell lymphoma after 4-6 months post transplantation. Co-expression of the mir-17-92 cluster in HSC from Eμ-MYC/+ mice and transplantation of these cells to lethally irradiated recipients resulted in the development of lymphoma at a significant shorter time interval. Additionally, in the work of Rinaldi et al. the expression of the c-MYC gene and of the mir-17-92 cluster was measured by FISH in a human MCL cell line and the concomitant expression of *v2-transcript* was defined (Rinaldi et al., 2007). The overexpression of 9 mature miRNAs (4 of which were encoded within the polycistronic mir-17-92 cluster) was found by RNA microarray and RT-PCR in the 3 BCR-ABL-positive CML cell-lines (Venturini et al., 2007). The expression could be down regulated by both imatinib and anti-BCR-ABL-RNA interference. The expression of mir-17-92 was also reduced by anti-cMYC-RNA interference and enhanced by *c-MYC* transfection, indicating that the expression of mir-17-92 c-Myc is possibly regulated by *c-MYC*. Additionally, the expression of *v2-transcript*, *c-MYC* and mature miRNAs was investigated in patients with chronic phase and in those with blast-crisis of CML. *v2-transcript* was overexpressed in 86% of patients in chronic phase and in a lower level in all blast crisis patients. *c-MYC* was overexpressed in 79% of patients in chronic phase and had a heterogeneous expression pattern in patients with blast crisis. By contrast, the mature miRNAs were overexpressed in patients in chronic phase but not in blast crisis patients. The overexpression of mature miRNAs in patients with chronic phase could be reduced by treatment with imatinib. Interesting data on miR-17-92 cluster from *C13orf25* gene come from the study of Hayashita et al on lung cancer (Hayashita et al., 2005). The miR-17-92 cluster was overexpressed in lung cancer cell lines, especially of

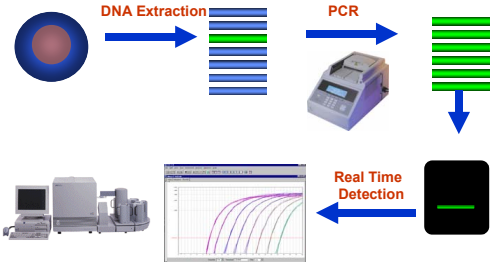
small-cell lung cancer origin and was predominately localized in the cell nucleus. The authors reported that the growth of lung cancer cell was enhanced by the introduction of the expression construct of the miR-17-92 cluster, but not by introduction of the putative open reading frame of *C13orf25*; implying the involvement of the miR-17-92 in oncogenesis.

The above studies confirm that miRNAs play an important role in oncogenesis. Although, the expression of miRNAs was assessed not only in cell lines but also in clinical samples, investigation into the relationship with disease outcome is less well documented. Because of their small size miRNA are supposed to be resistant to fixation and storage-induced degradation in FFPE blocks. Thus they have potential to represent an important new potential source of biomarkers. Numerous studies showed that the miRNAs from the miR-17-92 cluster and their pri-miRNA *v2-transcript* are involved in the oncogenesis of different tumours, including lymphomas and DLBCL. Particularly, the expression of miRNAs and *v2-transcript* was associated with the aggressive course of the disease in studies on clinical samples and also successfully proven experimentally in an in vitro mice model. Thus, the *v2-transcript* and miRNAs of miR-17-92 cluster were selected for further assessment and will be assessed as potential biomarkers.

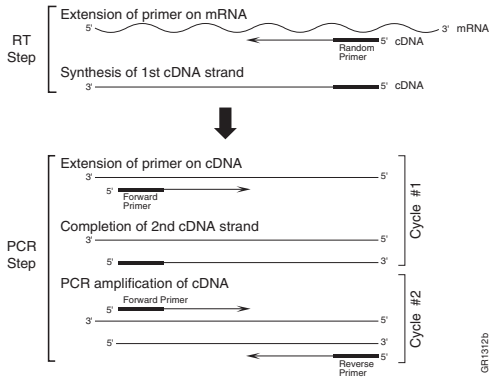
3.1.4 *Quantitative polymerase chain reaction (qPCR)*

Discovery of the polymerase chain reaction (PCR) entirely revolutionized studies on nucleic acids. The introduction of quantitative PCR (qPCR) was the next development step in molecular biology (figure 3.5.A). Briefly, the conventional PCR is a qualitative method allowing the confirmation of presence or absence of the assessed gene or its mRNA. By contrast the qPCR allows additionally the quantitative analysis of expression of the studied amplicon.

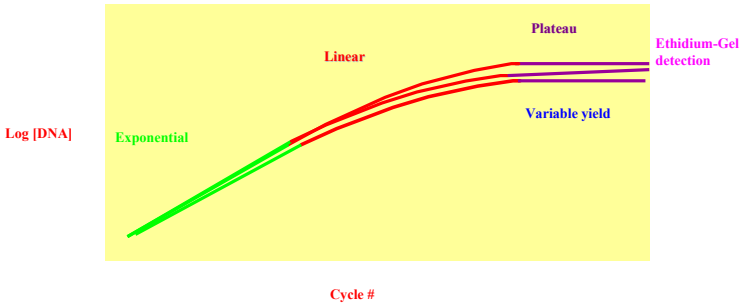
(A)



(B)



(C)



(D)

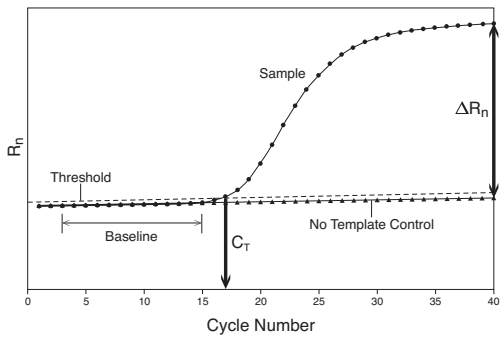


Figure 3.5 Introduction to PCR and qPCR. **(A)** PCR vs. qPCR **(B)** PCR principal reactions **(C)** PCR phazes and **(D)** amplification plot and qPCR terms (Biosystems, 2006b).

Principals of PCR and qPCR

The PCR synthesises of a copy of the target amplicon via a DNA polymerase, usually a Taq DNA polymerase. The selectivity of the reaction is guaranteed by specific primers, which are also necessary for initiation of the reaction. The basic required

components of each PCR reaction are: a nucleic acid sample, a DNA polymerase, a set of primers and nucleotides. For the assays detecting the DNA sequence, the DNA sample is simply used in the reaction. By contrast, the assays detecting mRNA require RT of the mRNA into cDNA see figure 3.5.B. Usually the whole mRNA is transcribed into a cDNA library using the random hexamer primers set or oligo-dT or sequence specific primers. The PCR reaction runs in a particular pattern accompanied by changes in the reaction temperature. In the first phase of the single reaction cycle, the temperature is increased up to above 90°C and the double strands of nucleic acids separate. In the next phase the temperature decreases, the primers bind to specific fragments of single strand nucleic acid and DNA polymerase starts to build a copy of the desired fragment of nucleic acid using nucleotides. After the competition of synthesis the temperature of reaction is increased again and the cycle starts from beginning. The whole PCR run is composed of about 20 – 40 such cycles.

In order to understand the principals and differences of conventional PCR and qPCR, the knowledge of the kinetics of a PCR run is required. Below, the short introduction to the method based on the materials of ABI is summarized (Biosystems, 2006b) (Biosystems, 2006a) (Biosystems, 2004). The basic single PCR run can be divided into three phases (see figure 3.5.C): 1) exponential phase: exact doubling is accumulating at every cycle (assuming 100% reaction efficiency). At this phase the reaction is very specific and precise. 2) linear phase: as the reaction components are being consumed, the reaction is slowing and products are starting to degrade. This phase of the reaction is characterized by high variability. 3) Plateau (end-point) phase: the reaction has stopped, no more products are being made and if left long enough, the PCR products will begin to degrade. Assuming the replicates have the same starting quantity of the amplicon (shown in figure 3.5.C), with the progress of the reaction, the samples begin to amplify in a very precise manner. A doubling of product occurs every cycle during the exponential phase. During the course of the reaction some of the reagents are being consumed as a result of amplification. This will occur at different rates for each reaction. The reaction starts to slow down and the PCR product is no longer being doubled at each cycle. The linear amplification can be seen on the product diagram and the reaction enters the linear phase. The three example samples in figure 3.5.C begin to diverge in their course during the linear phase as the reaction efficacy will be different in each sample and depends on multiple factors. In further course, the reaction slows down more and stops altogether reaching the plateau. Each reaction will plateau at a

different point due to the different kinetics for each sample. The plateau phase is where conventional PCR takes its measurement and thus it can say nothing on the initial quantity of the product. Usually the PCR product is separated in gel electrophoresis and consequently marked by an intercalating agent binding to double strand nucleic acid. By contrast the qPCR takes measurements of the amount of the reaction product, by measuring the fluorescence level, during the whole reaction as it occurs and particularly during exponential phase, where the replicate samples are amplifying exponentially. The data is therefore collected through the PCR process, rather than at the end of the PCR like in conventional PCR. qPCR is characterized by the point in time during cycling, when amplification of a target is first detected by measured fluorescence. In other words, the fluorescence level reached the set up limit (threshold) in the certain qPCR cycle so called cycle threshold (Ct), see figure 3.5.D. The higher the starting copy number of the target amplicon, the sooner detection occurs in an earlier cycle. Thus amplicons with high expression levels are associated with lower Ct value and those with low expression levels with higher Ct value.

qPCR chemistry

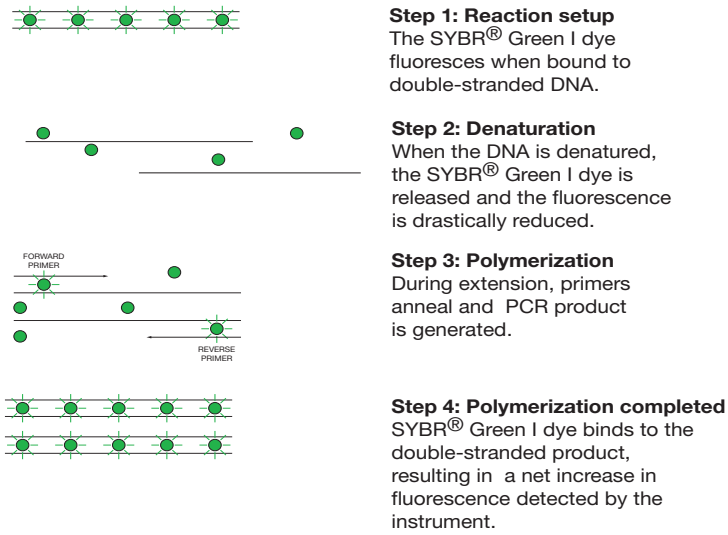
Currently there are two different technologies to detect PCR products during qPCR: i) SYBR Green dye chemistry and ii) TaqMan® chemistry, both using fluorescence (figure 3.6).

i) The SYBR Green chemistry uses in addition to the primer set the SYBR Green I dye to detect PCR products by binding to the minor groove of double-strand DNA synthesized during PCR reaction. The bound SYBR Green dyes have higher fluorescence emission as compared with unbound ones. The increase in fluorescence is detected during the PCR reaction, see figure 3.6.A.

ii) The TaqMan® chemistry uses in addition to the set of primers a specific probe. The probe is a nucleic acid strand that has incorporated a reporter dye at the 5' end and a quencher dye at the 3'end. When the probe is intact and the reporter and quencher dye lie close to each other, the fluorescence energy of the reporter dye is absorbed by the quencher dye. During the reaction, if the target sequence is present, the probe binds to the specific target nucleic acid fragment downstream one of the primers. The Taq DNA polymerase works from 3'to 5'direction on the leading DNA strand, synthesising the copy from its 3' to its 5' end and cleaving the probe from the leading DNA strand by its 5' nuclease activity. First the reporter dye and subsequently the

quencher dye are released. As the distance between the both dyes increases, the energy of the reporter dye is no longer absorbed and can be sent free and detected. The cleaved probes do not interfere with the Taq DNA polymerase function which continues the synthesis of the target amplicon. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced see figure 3.6.B.

(A)



(B)

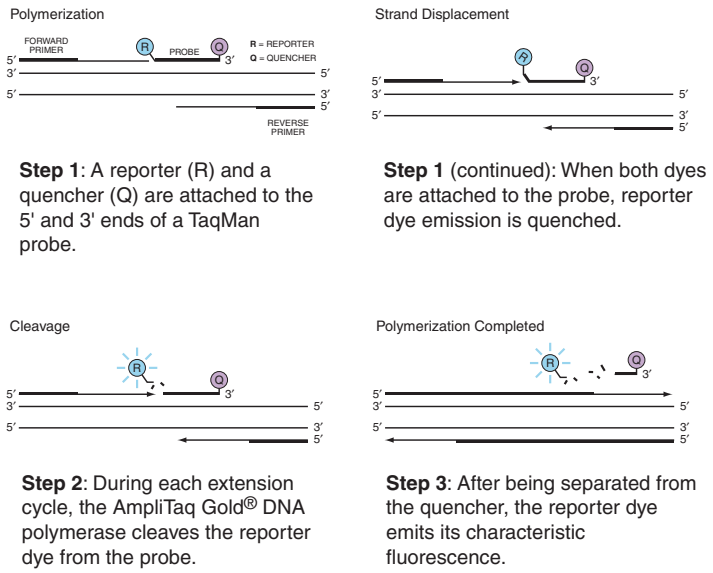


Figure 3.6 qPCR chemistry. **(A)** SYBR® Green and **(B)** Taqman (Biosystems, 2006a).

The most important difference between the TaqMan® and SYBR green chemistry is that the SYBR Green I dye will detect all double stranded DNA, including non specific reaction products. A well-optimized reaction is therefore essential for accurate results. By contrast the TaqMan® chemistry will detect only one specific PCR product. The primary disadvantage of the TaqMan® chemistry is that the synthesis of different probes is required for different sequences.

Absolute and relative qPCR

There are two different ways to calculate the results of qPCR assays: absolute and relative quantitation. The absolute quantitation assay is used to quantitate unknown samples by interpolating their quantity from a standard curve with known concentration of the target (in mass unit or copies number). This method might be used to correlate exact viral copy number with a disease state when it is of interest to the researcher to know the exact copy number of the target in a given biological sample in order to monitor the progress of disease.

A relative quantitation assay does not give the result of expression as a definite number but as the ratio of the expression of target gene in a given sample (sample obtained from treated patient or sick patient) to the expression in reference sample (such as untreated or healthy control sample). The relative quantitation maybe used to measure gene expression in response to a drug or in a tumour sample. The level of gene expression of a particular gene of interest in a treated sample or cancer sample would be compared relative to the level of gene expression in an untreated sample or healthy sample, so called calibrator. There are two different methods used for relative quantitation: i) standard curve method and ii) comparative Ct method ($2^{-\Delta\Delta Ct}$ method). In both methods, it is recommended to obtain the ratio of the target gene to the endogenous control (reference) in a tested sample and in a calibrator for control and normalization purposes (for differences in amount of cDNA added to each reaction, imprecise RNA measurement after extraction, RNA integrity or inaccurate pipetting). The endogenous control is usually constitutively expressed gene or so called 'housekeeper gene'.

i) The standard curve method is based on interpolating quantity of target and endogenous control in tested sample and in calibrator sample from a relevant standard curve. As the tested sample quantity is divided by target quantity, the unit of the standard is cancelled out. Thus, only the relative dilution of samples in standard curve is

required. For relative standard curve method any stock of cDNA containing the appropriate target can be used.

ii) The comparative Ct method is similar to the standard curve method, except it uses the arithmetic formula to achieve the same result for relative quantitation. It is possible to eliminate the use of standard curves and to use comparative Ct method $2^{-\Delta\Delta Ct}$ method as long the PCR efficiencies of target and endogenous control(s) are relatively equivalent and close to one. The following formula can be used:

$$\mathbf{2^{-\Delta\Delta Ct}}$$

The ΔCt in tested sample and in calibrator sample can be calculated by:

$$\mathbf{\Delta Ct = Ct\ (target) - Ct\ (reference)}$$

The ΔΔCt is calculated by:

$$\mathbf{\Delta\Delta Ct = \Delta Ct\ (tested\ sample) - \Delta Ct\ (calibrator\ sample)}$$

Conditions for successful and precise qPCR

In order to achieve precise and correct results in all different types of qPCR several conditions need to be met. As comparative Ct method $2^{-\Delta\Delta Ct}$ will be used in this study, we focus on conditions relevant for this method.

i) The input of high quality RNA is required for successful quantitation of gene expression. Protein contamination, including RNAses, can inhibit the PCR reaction or cause degradation of RNA. Carry-over chemicals e.g. phenol can cause PCR inhibition. Degraded RNA sample (samples with low RNA integrity) can contribute to loss of detection of rare transcripts. Co-extracted genomic DNA can serve as a PCR template and can cofound RNA detection results. The latter can be avoided by use of exon-exon spanning primers or use of DNAse before the RT. It is recommended to assess the RNA quality by spectrophotometr: $A_{260/280} \geq 2.0$ is the recommended quality indicating a protein free RNA isolate.

ii) The efficacy of qPCR needs to be close to one for target genes and reference gene(s). This particularly important if the comparative Ct method $2^{-\Delta\Delta Ct}$ is used and the custom designed primers and probes are used. The efficacy of reaction for all assessed targets needs to be checked in a validation experiment. When standard curves for the targets are produced and the efficacy of the reaction calculated, it is expected to achieve the correlation coefficient (R^2 value >0.99). All TaqMan® Gene Expression Assays are tested for efficacy of reaction equal one and do not require a validation experiment.

iii) All reactions should be performed in triplicates and the mean Ct value used with SD <0.3. This is particularly important for samples with high Ct values (34-40) when due to statistical distribution there is always a high level of Ct variation. In the case that one Ct value from a triplicate is outstanding, the duplicate can be used if SD <0.3.

iv) Regarding the reference genes, it is recommended to use at least two genes, with different range of Ct. Geometric mean of average Ct of both genes is used for calculation purposes. The Ct of reference gene should be ≤34 and equal distribution over all samples is required; fold change in distribution should not excide 1.3.

Summary

There are several advantages of using qPCR over the conventional PCR. The most important is the quantitative character of the method. The qPCR requires no-post PCR processing and detection is capable down to a 2-fold change. Beyond the competition with conventional PCR, the qPCR using the TaqMan® chemistry is currently the most sensitive and specific method in analysis of gene expression. Its capabilities surpass those of e.g. Northern blotting, microarray and other assays. Particularly, the real-time PCR is method of choice for confirmation of results of studies using microarrays. Thus, in our studies we have chosen qPCR with TaqMan® chemistry for prognostic assessment of gene and mature miRNA expression.

3.2 Materials

3.2.1 *Selection and culture of lymphoma cell lines*

Sixteen different cell lines of lymphoid origin: 10 DLBCL, 2 FL, 2 MCL and 2 lymphoblastic lymphomas, were selected for this study. All cell lines were obtained from The German Collection of Microorganisms and Cell Cultures (DSMZ). Table 3.1 provides a list and characteristics of origin of the cell lines.

Cell line	Origin
NU-DHL-1	DLBCL (lymph node)
SU-DHL-4	DLBCL (peritoneal effusion)
SU-DHL-5	DLBCL (lymph node)
SU-DHL-6	DLBCL (peritoneal effusion)
SU-DHL-8	DLBCL (pleural effusion)
SU-DHL-10	DLBCL (pleural effusion)
HT	DLBCL (peritoneal effusion)
DB	DLBCL (peritoneal effusion)
KARPAS-422	DLBCL (pleural effusion)
OCI-LY-19	DLBCL (bone marrow)
SC-1	Follicular lymphoma (peritoneal effusion)
WSU-FSCLL	Follicular lymphoma (peripheral blood)
GRANTA-519	Mantle cell lymphoma (peripheral blood)
REC-1	Mantle cell lymphoma (lymph node & peripheral blood)
CI-1	Lymphoblastic lymphoma (peritoneal effusion)
U-698-M	Lymphoblastic lymphoma (tonsil)

Table 3.1 Cell lines and cell line origin.

Cell lines were cultured according to the supplier's instruction: WSU-FSCLL, REC-1, U-698-M, NU-DHL-1, SU-DHL-4, SU-DHL-5, SU-DHL-6, SU-DHL-10, HT and KARPAS-422 in Roswell Park Memorial Institute Medium (RPMI) 1640 [Gibco UK] supplemented with 10% v/v heat inactivated foetal calf serum [Sera Lab LTD, UK]; CI-1, SU-DHL-8 and DB in RPMI 1640 supplemented with 20% v/v heat inactivated foetal calf serum; GRANTA-519 in Dulbecco's minimal essential medium (DMEM) (4.5g/L glucose) [Gibco UK] supplemented with 10% v/v heat inactivated foetal calf serum and OCI-LY-1 in 80% alpha minimal essential medium (MEM) [Gibco UK] supplemented with 20% v/v heat inactivated foetal calf serum. All media contained 100IU/ml penicillin [Gibco UK], 100µg/ml streptomycin [Gibco UK] and 2mM L-glutamine [Gibco UK]. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air incubator Galaxy B [Scientific Laboratory Supplies LTD, UK].

3.2.2 *Selection of formalin fixed paraffin embedded (FFPE) with corresponding frozen tissue*

A cohort of 11 patients with diagnosis of DLBCL lymphoma with available FFPE tissue blocks with corresponding snap frozen tissue blocks was randomly selected from the Tissue Bio-bank, Department of Cellular Pathology, Newcastle upon Tyne Hospital NHS Foundation Trust for the study.

3.2.3 *Selection of RNA samples isolated from FFPE of patients with DLBCL for confirmation studies*

A series of RNA samples isolated from the FFPE of patients with DLBCL were kindly provided by Dr R Crossland for confirmation work. Those samples were isolated from FFFP blocks using the High Pure FFPET RNA Isolation Kit [Roche®, UK]. All RNA samples were treated with the DNAse TURBO DNA-free™ [Ambion®, UK].

3.2.4 *Selection of FFPE tissue of population-based cohort of DLBCL patients*

A cohort of 92 patients with DLBCL and available FFPE tissue blocks was selected from the Lymphoma Bio-bank, Northern Institute of Cancer Research, Newcastle University. The patients had to fulfil all following inclusion criteria and to fulfil none of the following exclusion criteria:

Inclusion criteria:

- First diagnosis of DLBCL NOS
- No previous history of chemotherapy, immunotherapy and radiotherapy
- Age at diagnosis ≥18 years
- Started treatment with CHOP or CNOP polychemotherapy in combination with rituximab (at least one cycle)
- Available actual information at least on patients survival

Exclusion criteria:

- Previous or concurrent diagnosis of other malignant disorder including leukaemia and lymphoma
- Origin of disease in CNS, testicle or stomach
- HIV infection

The clinical information available for included patients incorporates the detailed demographic and clinical data at presentation (gender, age at diagnosis, performance status by ECOG, CS, B-symptoms, BM and other extranodal involvement, bulky

disease, laboratory data and IPI), data on treatment (kind of chemotherapy, number of administered cycles, eventual reason for discontinuation of chemotherapy and information on radiotherapy if given) and finally outcome data (response, date of eventual progression if occurred and date of last seen or date of death). All samples were additionally reviewed by expert haematopathologists and the detailed immunohistochemical staining allowing assignment of the patients to GC or ABC phenotype by Hans classification were also performed.

The patient characteristics of the cohort are provided in table 3.2. The median age of the group was 66 years, majority of patients were male and had ECOG <1.

Clinical parameter	Patients n (%)	Univariate analysis for PFS	Univariate analysis for OS
Median age, range	66, 27 – 91	0.001	0.002
Female gender	44/92 (47.8)	0.032	0.104
ECOG >1	12/83 (14.5)	0.004	0.001
CS I / II	34/68 (50.0)	0.001	0.003
B symptoms	24/77 (31.2)	0.025	0.023
Bulk disease	33/77 (42.8)	0.669	0.563
BM involvement	3/65 (4.6)	<0.001	<0.001
Extra nodal involvement	28/76 (36.8)	0.002	0.01
IPI Group			
Low	19/42 (45.2)	<0.001	<0.001
Low intermediate	8/42 (19.0)		
High intermediate	9/42 (21.4)		
High	6/42 (14.3)		
Abnormal Hb	27/84 (32.1)	0.566	0.478
Abnormal WBC	18/84 (21.4)	0.654	0.887
Abnormal albumin	18/79 (22.8)	0.011	0.001
Abnormal urea	23/81 (28.4)	<0.001	<0.001
Abnormal AP	17/80 (21.3)	0.671	0.816
Abnormal LDH	41/58 (70.7)	0.063	0.044
Hans phenotype			
Activated B-cell	24/80 (30.0)	0.059	0.082
Germinal centre	56/80 (70.0)		

Table 3.2 Patient characteristics of the cohort with *v2-transcript*, *c-MYC* and *HLA-DRβ* evaluation and significance of clinical factors on PFS and OS in univariate analysis Cox regression (n=92).

Among the patients with established CS exactly the half of patients were in advance stage disease and one third had B-symptoms at the diagnosis. The IPI could be calculated for 42 only. The major reason so few patients had an IPI was lack of BM biopsy and LDH level. The majority of patients had low and intermediate low IPI index (64.3%). The Hans phenotype was established for 80 patients and 30% had ABC phenotype and 70% GC. The remaining clinical data are provided in table 3.2.

The vast majority of patients received the combined immunochemotherapy of CHOP with rituximab and approximately 10.9% of patients received CNOP in combination with rituximab; see table 3.3.

Clinical parameter	**Patients n (%)**
Chemotherapy regimen	
CHOP	74 / 92 (80.4)
CNOP	10 / 92 (10.9)
NK	8 / 92 (8.7)
Combined modality	
Yes	31 / 92 (33.9)
No	52 / 92 (56.5)
NK	9 / 92 (9.8)
Scheduled chemotherapy discontinued	
No	70 / 92 (76.1)
Yes	14 / 92 (15.2)
NK / ND	8 / 92 (8.7)
Reason for discontinuation	
Progression	3 / 14 (21.4)
Infection	2 / 14 (14.3)
Organ failure	2 / 14 (14.3)
Other	7 / 14 (50%)
Overall responce	
CR	74 / 92 (68.5)
PR	10 / 92 (7.6)
Failure	14 / 92 (15.2)
NK / ND	8 /92 (8.7)
3 years PFS	62.9%
3 years OS	68.3%

Table 3.3 Treatment and treatment outcome of the cohort with *v2-transcript*, *c-MYC* and *HLA-DRβ* evaluation (n=92).

Approximately one third of patients received additional radiotherapy. The vast majority of patients completed the scheduled chemotherapy and the most common reason for the discontinuation were: progression, infection and organ failure. The ORR was 76% with 68.5% CR and 7.6% PR. The 3-year PFS and OS were 62.9% and 68.3% respectively; see table 3.3.

All clinical factors were included in univariate analysis of risk factors for PFS and OS. For PFS age, gender, CS, presence of B-symptoms, BM involvement, abnormal urea and albumin were statistically significant and for OS additionally the LDH serum level was statistically significant but not patient gender. For the results with corresponding p-values see table 3.2.

3.2.5 *Selection of control tissue*

The pooled RNA of normal CD19+ B-cells isolated from peripheral blood of three healthy individuals was used as control tissue for the studies on cell lines, on corresponding frozen and FFPE tissue and confirmation studies. The isolation of CD19+ B-cells was performed using positive selection of CD19+ cells directly from anti-coagulated whole blood using Whole Blood CD19 MicroBeads [Miltenyi Biotec, UK] in an autoMACS^{TM} Separator [Miltenyi Biotec, UK].

For the patient cohort study pooled RNA extracted from 2 FFPE tissue blocks of reactive lymph nodes and no evidence of any malignancy was used as control tissue. The two independent patients were randomly selected from the collection of the Tissue Bio-bank, Department of Cellular Pathology, Newcastle upon Tyne Hospital NHS Foundation Trust for the study.

3.3 Methods

3.3.1 *Extraction of RNA*

For studies on gene expression, the RNA was isolated from cell lines and CD19+ lymphocytes using *mir*Vana^{TM} miRNA Isolation Kit [Ambion®, UK] with the protocol for a total RNA isolation. This protocol was also employed for isolation of RNA from frozen DLBCL tissues. Extraction of RNA from FFPE tissues was performed with the RecoverAll™ Total Nucleic Acid Isolation Kit [Ambion®, UK].

For the studies on expression of miRNAs, the *mir*Vana^{TM} miRNA Isolation Kit provides a possibility of extracting RNA by two different protocols: i) the protocol for a

total RNA isolation (called *mir*Vana™ total) and ii) the protocol for an enrichment isolation of small RNAs (called *mir*Vana™ micro). Both methods of the *mir*Vana™ miRNA Isolation Kit were employed in the preliminary study on expression of miRNAs in cell lines. Subsequently for all future studies on miRNAs, the protocol for isolation of total RNA only was employed on cell lines and frozen samples and the RecoverAll™ Total Nucleic Acid Isolation Kit [Ambion®, UK] was used for extraction of the RNA from FFPE.

The residual DNA contamination in samples provided for assessment of gene expression was removed using the TURBO DNA-*free*™ [Ambion®, UK] DNAse treatment and removal reagents.

The concentration and purity of all isolated RNA samples was determined spectrophotometrically using ND-1000 spectrophotometer [NanoDrop Technologies. Inc].

3.3.2 *Assessment of gene expression*

Expression of *V2-transcript*, *c-MYC* and *HLD-DRβ* was determined with qPCR with TaqMan® chemistry by [Applied Biosystem®, UK] and was normalized using the $2^{-\Delta\Delta CT}$ method relative to selection of housekeeper genes (*GAPDH*, *PGK1* and *TBP*). The expression of target genes in lymphoma cell lines, frozen tissue blocs and corresponding FFPE tissue blocks and confirmation samples was normalized to their expression in pooled RNA of normal CD19+ B-cells isolated from peripheral blood of 3 healthy individuals. By contrast in cohort study, the expression of the target genes was normalized to their expression in pooled RNA extracted from 2 FFPE tissue blocks of lymph nodes with no evidence of any malignancy.

The RT reaction was performed using the High Capacity RNA-to-cDNA Kit [Applied Biosystem®, UK] in 2720 Thermal Cycler [Applied Biosystem®, UK]. The reaction conditions are summarized in table 3.4.

Step	Step Type	Time	Temperature
1.	Hold	60 min	37°C
2.	Hold	5 min	95°C
3.	Hold	∞	4°C

Table 3.4 Parameter values for RT of total cDNA library.

The real time PCR step was performed using TaqMan® Universal Maser Mix I and II in ABI Prism® 7900HT Sequence Detection System [Applied Biosystem®, UK]. The thermal cycling parameters are provided in table 3.5.

Step	Step Type	PCR Phase	Time	Temperature
Activation	Hold		10 min	95°C
PCR	Cycle	Denaturation	15 sec	95°C
		Annealing / extension	60 sec	60°C

Table 3.5 Parameter values for qPCR for assessment of expression of the genes and miRNAs.

All qPCR reactions were performed in triplicate and the reported Ct value is an average from those three, further it is defined as Ct only. All used primers and probes were supplied by Applied Biosystem®, UK:

1) For *c-MYC* expression the ready to use probe and primers set was used:
   MYC - v-myc avian myelocytomatosis viral oncogene homolog
   Amplicon size: 107
   Assay ID: Hs00153408_m1
   NM: NM_002467.4
   Cat No: 4453320
2) For *HLA-DRβ* expression the ready to use probe and primers set was used:
   HLA-DRB1 - major histocompatibility complex, class II, DR beta 1
   Amplicon size: 75
   Assay ID: Hs99999917_m1
   NM: NM_001243965.1
   Cat. No: 4453320
3) For *V2-transcript* of *c13orf25* the primers and probe described by Venturini et al. were used (Venturini et al., 2007). These primers and probe bind to the latter sequence of the *V2-transcript* beyond the region encoding mature miRNAs and the length of the amplicon is approximately 170 nb. The screening of the *V2-transcript* for alternative primers and probe binding sites, preferably within the region encoding the miRNA and shorter amplicon remained unsuccessful.

V2-transcript forward primer:

CAGTAAAGGTAAGGAGAGCTCAATCTG

V2-transcript reverse primer:

CATACAACCACTAAGCTAAAGAATAATCTGA

V2-transcript FAM-TAMRA probe:

TGGAAATAAGATCATCATGCCCACTTGAGAC

4) For *GAPDH* expression a ready to use endogenous control assay including primers and probe were used:

   GAPDH - glyceraldehyde-3-phosphate dehydrogenase

   Amplicon size: 122

   Assay name: Human GAPD (GAPDH)

   Endogenous Control (FAM™/MGB probe, non-primer limited)

   NM: NM_002046.3

   Cat No: 4333764

5) For *PGK1* expression a ready to use endogenous control assay including primers and probe were used:

   PGK1 - Human PGK1 - Phosphoglycerate Kinase 1

   Amplicon size: 75

   Assay name: Human PGK1 (Phosphoglycerate Kinase 1) Endogenous Control (FAM™/MGB Probe, non-primer limited)

   NM: NM_000291.2

   Cat No: 4333765

6) For *TBP* expression a ready to use endogenous control assay including primers and probe were used:

   TBP – Human TBP (Tata-box binding Protein)

   Amplicon size: NK

   Assay name: Human TBP (TATA-box binding Protein) Endogenous Control (FAM™/MGB probe, non-primer limited)

   NM: NK

   Cat No: 4333769

3.3.3 *Assessment of expression of mature miRNAs*

TaqMan® MicroRNA Assay [Applied Biosystem®, UK] is a novel tool in assessment of expression of miRNAs. It is based on the principles of TaqMan® gene expression assays with several differences such as transcript specific RT. It specifically detects the miRNA and does not react with genomic DNA.

Expression of mature miRNAs (miR155, miR17-5p, miR20 and miR92) was determined using the TaqMan® MicroRNA Assay [Applied Biosystem®, UK] and was normalized using the $2^{-\Delta\Delta CT}$ method relative to expression of miR24, "house keeping miRNA". The expression of target miRNAs in lymphoma cell lines, frozen tissue blocks and corresponding FFPE tissue blocks and in the confirmation FFPE tissue cohort was normalized to their expression in pooled RNA of normal CD19+ B-cells isolated as described in the materials section.

The RT step with specific primers from TaqMan® MicroRNA Assay was performed in a 2720 Thermal Cycler [Applied Biosystem®, UK]. The reaction conditions are summarized in table 3.6.

Step	Step Type	Time	Temperature
1.	Hold	30 min	16°C
2.	Hold	30 min	42°C
3.	Hold	5 min	85°C
4.	Hold	∞	4°C

Table 3.6 Parameter values for RT of miRNAs.

The qPCR step was performed using TaqMan® Universal Maser Mix, UDG Free in ABI Prism® 7900HT Sequence Detection System [Applied Biosystem®, UK]. The thermal cycling parameters are provided in table 5. Likewise in the assessment of gene expression all qPCR reactions for miRNA were performed in triplicate and the reported Ct value is an average from those three, further it is defined as Ct only.

Following TaqMan® MicroRNA Assay [Applied Biosystem®, UK] were employed in this study for assessment of individual miRNAs:

1) For miR-155 TaqMan® MicroRNA Assay:
   sequence: UUAAUGCUAAUCGUGAUAGGGGU
   Assay Name: hsa-miR-155
   Assay ID: 002623
   Cat No: 4427975

2) For miR-17-5p TaqMan® MicroRNA Assay:
   sequence: CAAAGUGCUUACAGUGCAGGUAGU
   Assay Name: hsa-miR-17-5p
   Assay ID: 000393
   Cat No: 4427975
3) For miR-20 TaqMan® MicroRNA Assay:
   sequence: UAAAGUGCUUAUAGUGCAGGUAG
   Assay name: hsa-miR-20a
   Assay ID: 000580
   Cat No: 4427975
4) For miR-92 TaqMan® MicroRNA Assay:
   sequence: UAUUGCACUUGUCCCGGCCUGU
   Assay name: hsa-miR-92a
   Assay ID: 000431
   Cat No: 4427975
5) For miR-24 TaqMan® MicroRNA Assay:
   sequence: UGGCUCAGUUCAGCAGGAACAG
   Assay Name: hsa-miR-24
   Assay ID: 000402
   Cat No: 4427975

3.3.4 *Statistics*

Demographics and disease characteristics were summarized using descriptive statistics. The Mann-Whitney-U-test was used to investigate differences between means. Spearman's R correlation was used to assess the correlation between distributions of factors. Bland-Altman plots were used to assess the level of agreement. In brief, for Bland-Altman analysis the two methods agreed if the bias between them was equal to or less than one as this equated to less than one qRT-PCR cycle difference. For correlation, significance was set at $p<0.05$ and trend to significant set at $p<0.10$. The PFS and OS times were calculated from date of diagnosis to the first documentation of progression during therapy, failure at the end of therapy, or further relapse or death from any cause during or after the end of treatment. OS was calculated from date of diagnosis to the date of death, or if no death occurred, to the last documented follow up for the patient. The rates of PFS and OS were estimated according to the method of

Kaplan and Meier and comparison between the groups was with the log-rank test. Cox-regression analysis was used for assessment of PFS and OS and gene expression as a continuous variable. The limits for dichotomizing the gene expression were defined using receiver operating characteristic (ROC) curve analysis and the assessment of PFS and OS and gene expression as dichotomous variables. All tests were performed with a confidence interval of 95 % and statistical significance was defined as $p \leq 0.05$. Statistical analysis was performed with SPSS Version 13.0 for MAC OS X (SPSS Incorporated, Chicago, IL), Prism Version 6 for MAC OS X and SIGMA Plot Version.

3.4 *Results - studies on expression of v2-transcript, c-MYC and HLA-DRβ*

3.4.1 *Validation of qPCR for v2-transcript*

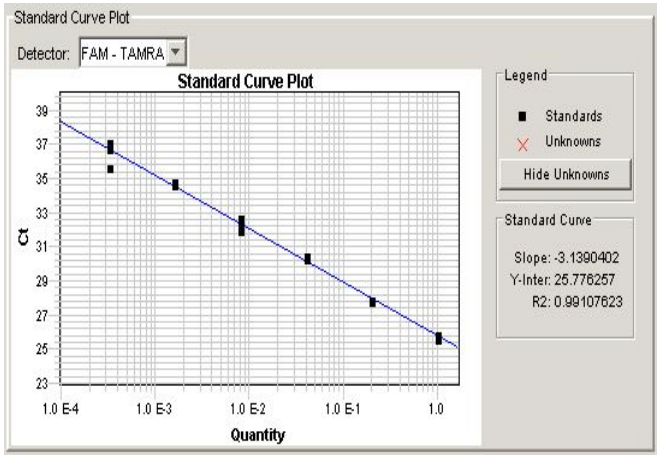
As the primers for *v2-transcript* of *c13orf25* were custom designed they had to be checked to ensure they are specific to mRNA and do not react with genomic DNA during qPCR. The "standard" and "NO RT" (without reverse transcriptase) RT reactions were performed on RNA samples treated and not treated with DNAse. The cDNA from each reaction has been then used in qPCR for *v2-transcript* and, for control purposes, for *GAPDH*.

In samples using RNA not treated with DNAse, the qPCR for *v2-transcript* was strongly positive in cDNA samples from both "standard" and "NO RT" RT reactions. By contrast the qPCR for *GAPDH* was significantly positive in "standard" RT reactions with no signal in "NO RT" RT reactions. In samples using RNA treated with DNAse, the qPCR for both *v2-transcript* and *GAPDH* was positive in "standard" RT reactions and negative in "NO RT" RT reactions. These results indicate that primers for *v2-transcript* react with both mRNA and genomic DNA. Thus RNA treatment with DNAse is a necessary step before RT and qPCR.

Additionally, in order to prove the efficiency and dynamic range of qPCR for *v2-transcript* and *GAPDH,* a validation experiment was performed. The cDNA library was created from RNA extracted from GRANTA19 cell line. Subsequently the qPCR was perfrmed in the reactions in samples with six consequent dilutions of cDNA: 1:1, 1:5, 1:25, 1:125, 1:625 and 1:3125. The RNA input in RT reaction was 10 ng/µl of RT reaction giving the final input of cDNA in qPCR reaction as follows: 1.0ng/µl of qPCR reaction, 0.2ng/µl, 0.04ng/µl, 0.008ng/µl, 0.0016ng/µl, 0.00032ng/µl and 0.000064ng/µl (making assumption that the evfficiency of the RT was 1.0). The standard curves have

been established. For *v2-transcript* the slope was -3.1390402 and R2 0.99107 and for GAPDH -3.2888095 and 0.99829805. These results allow using $2^{-\Delta\Delta Ct}$ method for normalization of expression of *v2-transcript* (figure 3.7).

(A)



(B)

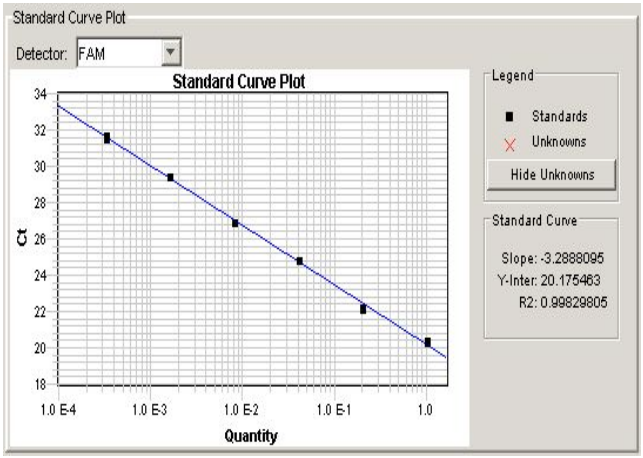


Figure 3.7 qPCR standard curve for *v2–transcript* and *GAPDH*, total RNA extracted from GRANTA 519 cell line. **(A)** *v2–transcript* and **(B)** *GAPDH*.

3.4.2 *Expression of v2-transcript, c-MYC and HLA-DRB in lymphoma cell lines*

Validation of endogenous control (GAPDH) for qPCR

In preliminary experiments for genes expression using qPCR in lymphoma cell lines, *GAPDH* was proposed as housekeeping gene for normalization purposes of $2^{-\Delta\Delta Ct}$ method. In order to validate the expression of *GAPDH* as a reference gene the qPCR for *GAPDH* was performed in RNA extracted from all studied cell lines. The RT and qPCR were performed according to the standard conditions on DNAse treated, total RNA extracted from each cell line. The concentration of RNA in the RT reaction was 10ng/µl

of RT reaction and cDNA in the qPCR reaction 1ng/µl of qPCR reaction. These conditions were maintained through all experiments on gene expression in cell lines. In order to avoid changes in condition of reaction, RT and qPCR were performed simultaneously in all samples. *GAPDH* was expressed nearly equally in all tested cell lines with fold change of 1.23 in all cell lines. This met the conditions for a housekeeper gene. The mean Ct value for all cell lines was 17.05, range from 14.98 to 18.42 (figure 3.8). According to these results *GAPDH* fulfilled the criteria for a housekeeping gene in $2^{-\Delta\Delta Ct}$ method.

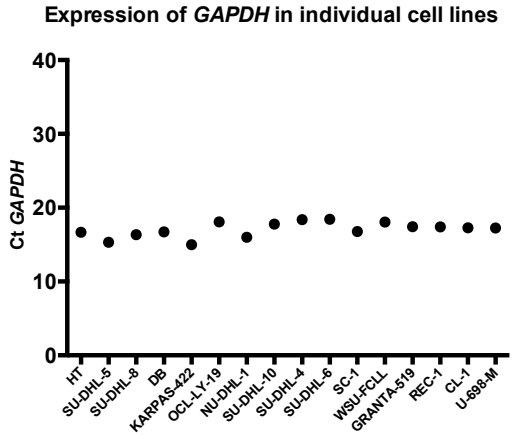


Figure 3.8 Expression of *GAPDH* (Ct value) in individual cell lines.

Expression of v2-transcript, c-MYC and HLA-DRB in lymphoma cell lines

The RT and qPCR of all assessed genes in the lymphoma cell lines were performed to the standard conditions described above. The expression was evaluated according to $2^{-\Delta\Delta Ct}$ using *GAPDH* as endogenous control and RNA extracted from normal CD19+ B-cell from 3 healthy donors as calibrator.

v2-transcript was expressed in all analyzed cell lines (see figure 3.9.A). As compared with the normal calibrator the *v2-transcript* was under-expressed in all cell lines except from SC1 (FL cell line) and REC1 (MCL cell line). In comparison between DLBCL cell lines and other lymphoma cell lines, *v2-transcript* had significantly lower expression in DLBCL cell lines ($\log^2$ RQ range from -1.72 to -6.35; average -3.56) than in other lymphoma cell lines ($\log^2$ RQ range from -4.18 to 0.83; average -1.46); ($p=0.042$, Mann-Whitney *U* Test), figure 3.9.B.

c-MYC and *HLA-DRβ* were also under-expressed in all cell lines as compared to the normal calibrator (figure 3.9.C and 3.9.E). There were no significant difference between the DLBCL cell lines and other cell lines in expression of *c-MYC*: $\log^2$ RQ

range from -0.44 to -7.41; average -2.88 in DLBCL cell lines vs. $\log^2$ RQ range from -1.01 to -5.57; average -2.58 in other cell lines (p=0.713) and *HLA-DRβ*: $\log^2$ RQ range from -1.77 to -29.90; average -14.64 in DLBCL cell lines vs. $\log^2$ RQ range from -3.36 to -29.90; average -12.49 in other cell lines (p=0.792); (figure 3.9.D and 3.9.F).

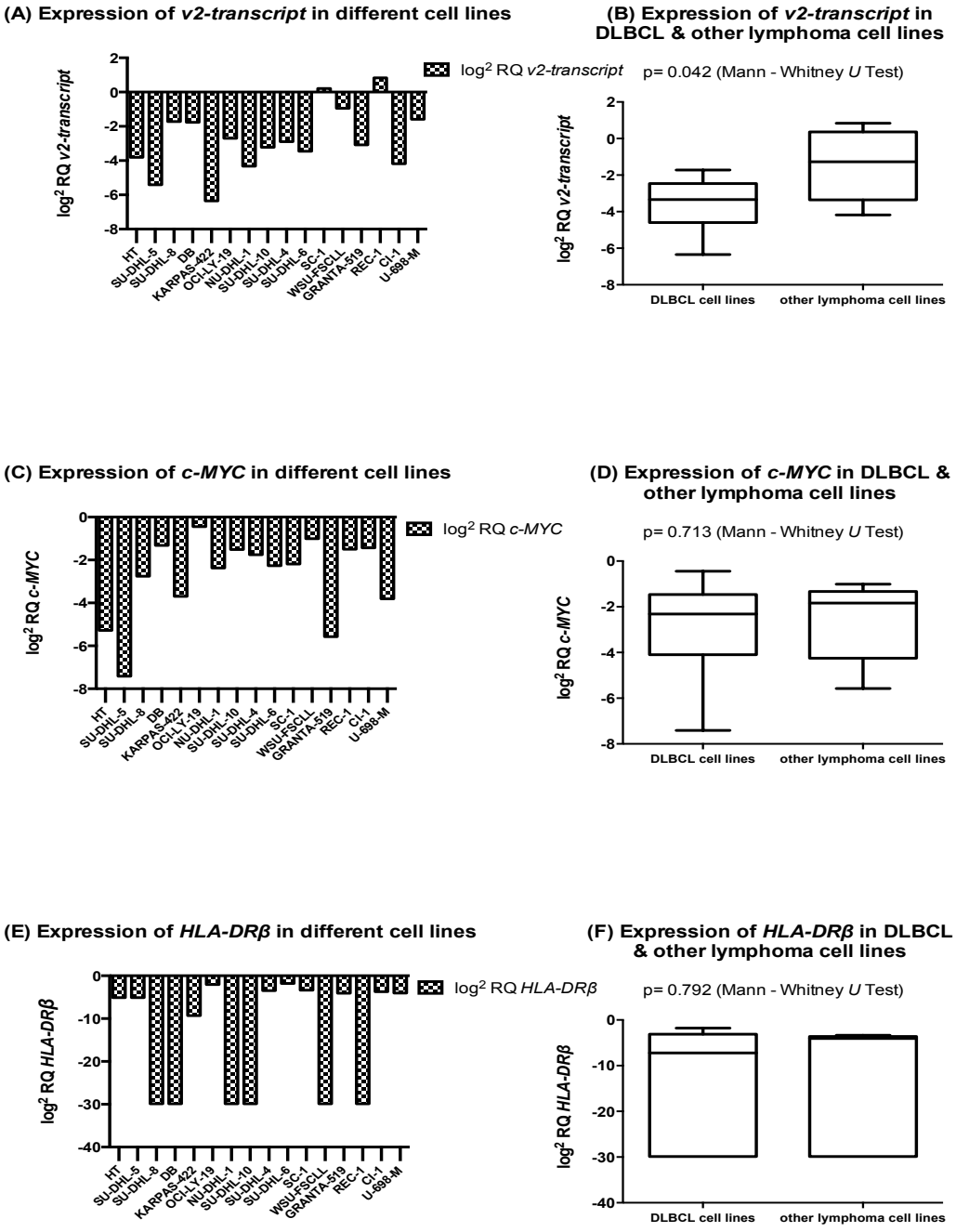


Figure 3.9 Expression of *v2-transcript*, *c-MYC* and *HLA-DRβ* in lymphoma cell lines. Expression ($\log^2$ RQ) in individual lymphoma cell lines **(A)** *v2-transcript*, **(C)** *c-MYC,* **(E)** *HLA-DRβ* and a comparison of expression ($\log^2$ RQ) in DLBCL vs. other lymphoma cell lines (Mann-Whitney U test). **(B)** *v2-transcript*, **(D)** *c-MYC,* **(F)** *HLA-DRβ*

3.4.3 *Expression of v2-transcript, c-MYC and HLA-DRB in FFPE and frozen lymphoma tissue samples*

As the proposed study material should be FFPE tissue and qPCR on nucleic acid extracted from FFPE usually encountered significant difficulties due to loss of their integrity the qPCR was performed on the RNA extracted from both FFPE and corresponding frozen tissue from the series eleven patients with lymphoma. The RNA input in RT was 10ng/µl of RT reaction and cDNA input in qPCR was 0.5ng/µl of qPCR reaction throughout this study. The calibrator was RNA extracted from normal B-cells.

Validation of endogenous control (GAPDH) for qPCR in FFPE and frozen lymphoma tissue samples

The expression of *GAPDH* was assessed in RNA extracted from eleven FFPE tissue blocks and from corresponding eleven frozen tissue blocks. *GAPDH* was expressed in all assessed samples; see figure 3.10.A. The expression in frozen tissue was characterized by lower Ct values with min Ct of 19.87 and max Ct of 24.46 (mean Ct 21.64) as compared to FFPE tissue (min Ct of 26.36 and max Ct of 32.14; mean Ct 29.29). This difference was statistically significant (p=0.000), figure 13.10.B. The fold of difference among both frozen tissue samples and FFPE tissue samples was lower <1.3 with respectively: 1.23 and 1.22. According to this data the *GAPDH* can be used as endogenous control for normalization purposes of $2^{-\Delta\Delta Ct}$ method.

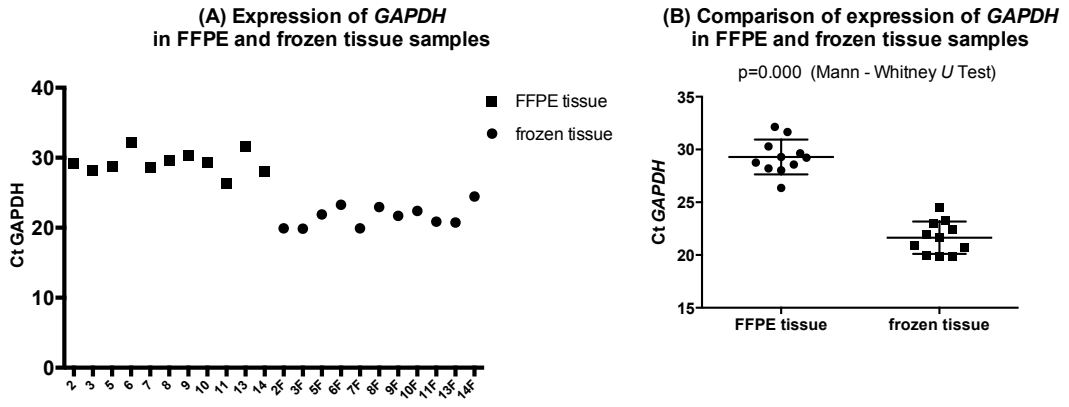


Figure 3.10 Expression of *GAPDH* in FFPE tissue and corresponding frozen tissue. **(A)** expression of *GAPDH* (Ct value) in all samples **(B)** comparison of *GAPDH* expression (Ct value) in FFPE tissue and frozen tissue samples (Mann-Whitney U test).

Expression of v2-transcript, c-MYC and HLA-DRB in FFPE and frozen lymphoma tissue samples

All assessed genes were expressed in the frozen tissue and corresponding FFPE blocks. There were significant differences in Ct values between both types of material, see figure 3.11. The mean Ct of *v2-transcript* in frozen tissue was 30.25 (range from 26.59 to 32.90) and in FFPE was 35.00 (range from 30.23 to 36.54); $p<0.001$ (see figure 3.11.A). Regarding the expression of *c-MYC*, the mean Ct value in frozen tissue was 26.14 (range from 23.33 to 28.77) and in FFPE 33.31 (range from 30.61 to 36.38); $p<0.001$ (see 3.11.C). The same pattern of expression was observed in *HLA-DRβ*, the mean Ct value in frozen tissue was 31.12 (range from 21.79 to 40.00) and in FFPE was 34.60 (range from 27.65 to 40.00), however the difference was not significant; $p=0.243$ (figure 3.11.E).

Additionally, correlation between gene expression measured in FFPE tissue blocks and in corresponding frozen tissue was evaluated using Spearman's correlation. The expression of *c-MYC* and *HLA-DRβ* showed significant correlation; for *c-MYC* $Rs=0.7727$ and $p=0.0074$ (figure 3.11.B) and for *HLA-DRβ* $Rs=1.00$ and $p<0.0001$ (figure 3.11.D). But there was no significant correlation for expression of *v2-transcript* $Rs=0.2273$ and $p=0.5034$ (figure 3.11.F).

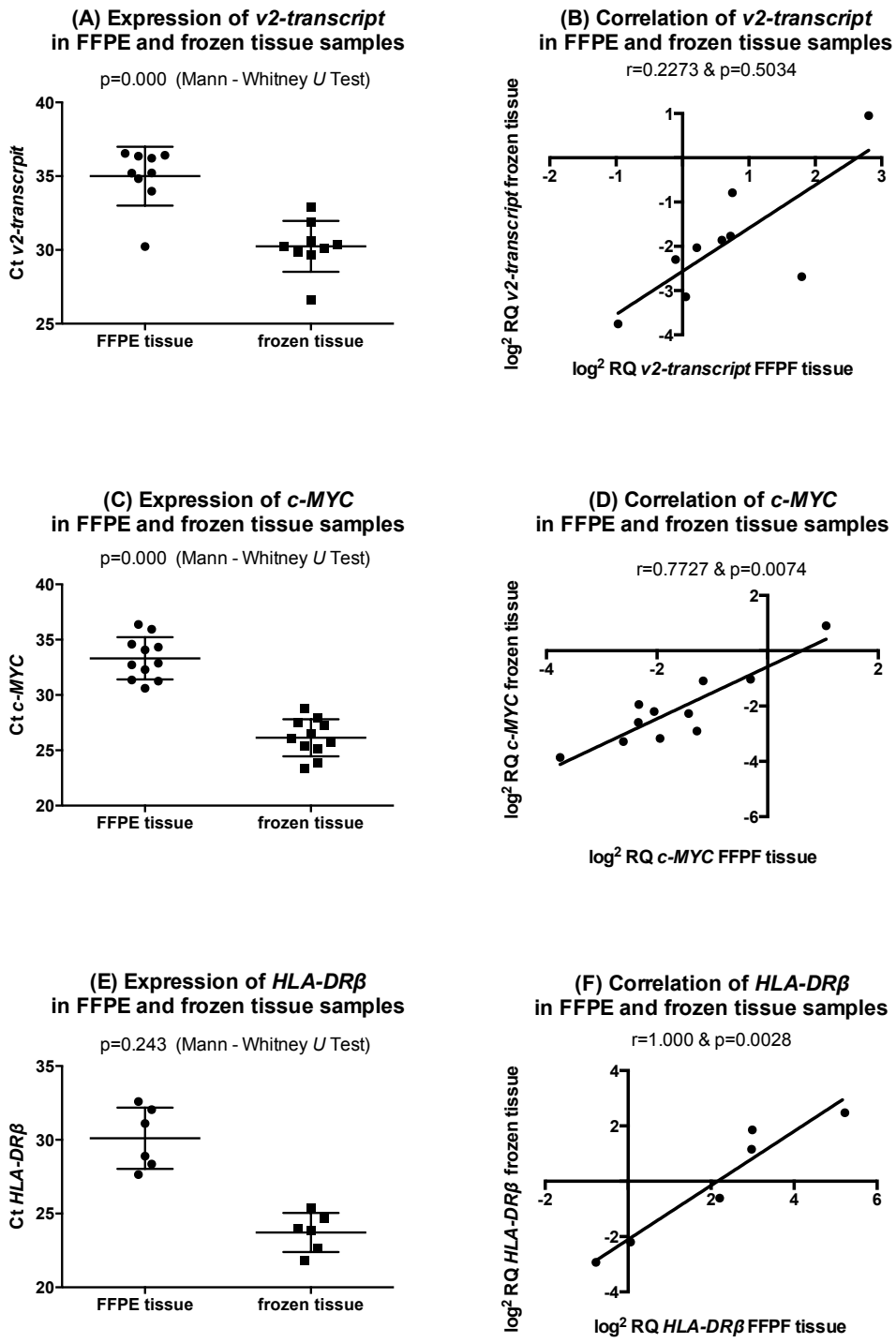


Figure 3.11 Expression of *v2-transcript*, *c-MYC* and *HLA-DRβ* in FFPE tissue and corresponding frozen tissue. Comparison of expression (Ct-values) in FFPE tissue and corresponding frozen tissue samples (Mann-Whitney *U* test). **(A)** *v2-transcript*, **(C)** *c-MYC* and **(E)** *HLA-DRβ*. Correlation of expression (log^2 RQ) in FFPE tissue and corresponding frozen tissue samples (r Spearman). **(B)** *v2-transcript,* **(D)** *c-MYC* and **(F)** *HLA-DRβ*.

3.4.4 *Validation of repeated qPCR for V2-transcript, c-MYC and HLA-DRB in series of DLBCL FFPE clinical tissue samples*

In order to assess the precision and repeatability of the applied assays, the expression of the studied genes was assessed twice using two different concentrations of the mRNA extracted from the FFPE of a cohort patients with DLBCL for the RT and subsequently qPCR. In the first series of experiments the concentration of RNA used for RT was 10ng/µl of RT reaction and in subsequent series this was reduced to 5ng/µl of RT reaction. The concentration of cDNA in qPCR was 0.5ng/µl of qPCR reaction in the first series of experiments and 0.25ng/µl of qPCR reaction in the next series.

Concordance between the expressions of the genes in both series of experiments was evaluated using Bland-Altman method to measure agreement and Spearman's correlation to assess correlation. Ad described above for Bland-Altman analysis the two methods agreed if the bias between them was equal to or less than one as this equated to less than one qPCR cycle difference. For correlation, significance was set at $p<0.05$. The evaluation showed that the expression of all assessed genes: *v2-transcript*, *c-MYC* and *HLA-DRβ* agreed between the two series of experiments with bias of -0.2559, -0.1046 and -0.2038 respectively (figure 3.12A, C and E). There was a significant correlation between the expression of all genes from two series of experiments with Rs=0.791 and $p<0.001$ for *v2-transcript*, 0.761 and $p<0.001$ for *c-MYC* and Rs=0.998 and $p<0.001$ for *HLA-DRβ* (figure 3.12.B, D and F).

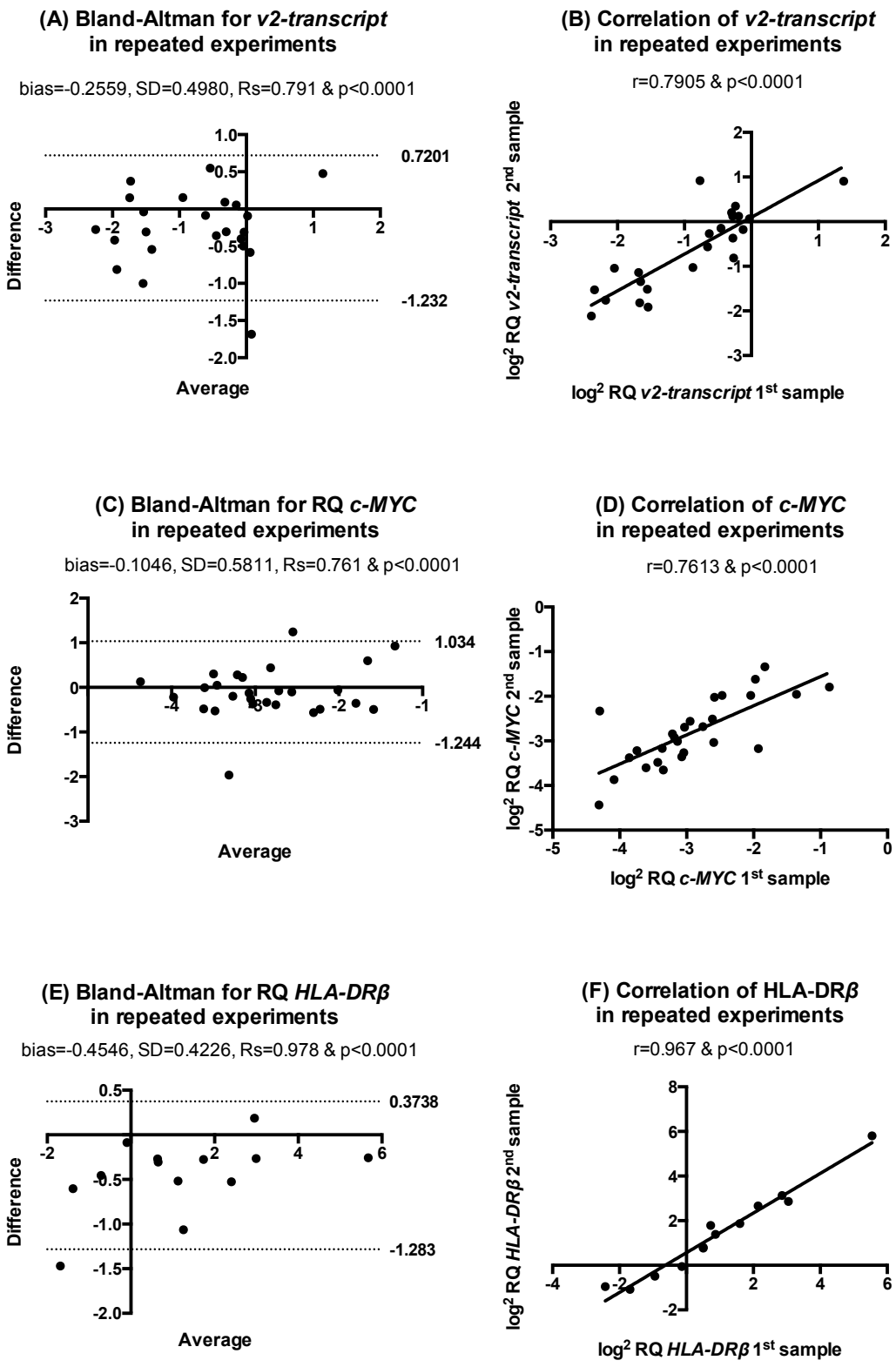


Figure 3.12 Expression of *v2-transcript*, *c-MYC* and *HLA-DRβ* in two repeated series of FFPE tissue samples. Bland-Altman plots for agreement of expression (log^2 RQ). **(A)** *v2-transcript,* **(C)** *c-MYC* and **(E)** HLA-DRβ. Correlation of expression (log^2 RQ), r Spearman. **(B)** *v2-transcript,* **(D)** *c-MYC* and **(F)** *HLA-DRβ.*

3.4.5 *Expression of v2-transcript, c-MYC and HLA-DRβ in a population-based cohort of DLBCL patients*

Validation of endogenous controls for qPCR in a cohort of patients with DLBCL

For the purposes of the $2^{-\Delta\Delta Ct}$ three individual housekeeper genes with estimated different Ct values were evaluated: *GAPDH*, *PGK1* and *TBP*. In order to fulfil the criteria of a valid housekeeper the Ct value should be <34 and SD of triplicate expression ≤0.3. The *GAPDH* was evaluable in 80 samples. In remaining 12 samples, it was not detected in one sample, Ct was ≥34 in 8 samples, SD was >0.3 in one sample and Ct was ≥34 and SD >0.3 in two samples. The mean Ct value of *GAPDH* was 29.29 (range from 24.71 to 33.61) and the ratio between the lowest and highest Ct value was 1.36, being just above the conventional limit of 1.3 (figure 3.13.A). The *PGK1* was evaluable in 87 samples. Among 5 non-evaluable samples the Ct was ≥34 in four samples and SD >0.3 in one sample. The mean Ct value of *PGK1* was 28.05 (range from 24.64 to 32.69) and the ratio between the lowest and highest Ct value was 1.32, being just above the conventional limit of 1.3 (figure 3.13.B). By contrast the *TBP* was evaluable in two samples only. In remaining 90 samples the gene was not expressed in 37 samples, in 32 samples the Ct was ≥34 and in 21 Ct was ≥34 and the SD was >0.3. Following these results the *GAPDH* and *PGK1* were used as housekeeper genes for the purposes of the $2^{-\Delta\Delta Ct}$ evaluation.

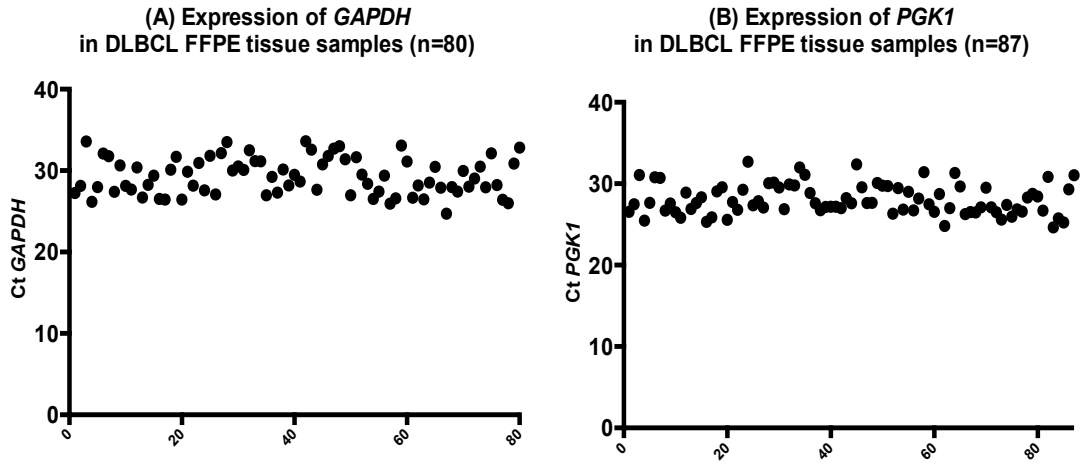


Figure 3.13 Expression of *GAPDH* and *PGK1* (Ct value) in FFPE tissue samples of patient cohort with DLBCL. **(A)** *GAPDH* and **(B)** *PGK1*.

Expression of v2-transcript, c-MYC and HLA-DRβ in a cohort of patients with DLBCL

The expression of all assessed target genes was performed in 92 samples. The expression of target genes was evaluable if the gene was expressed with SD ≤0.3. For the evaluation purposes both housekeeper genes: *GAPDH* and *PGK1* had to be measurable in the tested samples.

The *v2-transcript* was expressed with SD ≤0.3 in 74 samples. In 9 samples there was no expression of the gene and in remaining 9 samples the SD was >0.3. Among 74 patients with evaluable expression, the housekeeper genes were not evaluable in 3 samples, thus the *v2-transcript* was assessed with a $2^{-\Delta\Delta Ct}$ method in 71 samples. The total efficacy of the method was 77%. The mean $\log^2$ RQ was -0.44 (range from -3.89 to 1.94, SD 1.16); (Figure 3.14.A).

c-MYC was expressed with SD ≤0.3 in 71 samples. In 6 samples there was no measured expression and in further 15 samples the SD was >0.3. Among 71 samples with evaluable *c-MYC* expression, the housekeeper genes were not evaluable in 3 samples, thus the expression of *c-MYC* assessed with a $2^{-\Delta\Delta Ct}$ method was performed in 68 samples, giving the total efficacy of method of 74%. The mean $\log^2$ RQ was -0.82 (range from -3.03 to 1.81, SD 0.92); (Figure 3.14.B).

HLA-DRβ was expressed in 89 samples with SD ≤0.3. In the remaining 3 samples, the gene was expressed, however the SD was >0.3. Among 89 samples with evaluable *HLA-DRβ* expression, the housekeeper genes were not evaluable in 9, thus the expression of *HLA-DRβ* by $2^{-\Delta\Delta Ct}$ method was performed in 80 samples with a total efficacy of the method of 87%. The mean $\log^2$ RQ was -17.81 (range from -29.90 to 1.85, SD 14.19); (Figure 3.14.C).

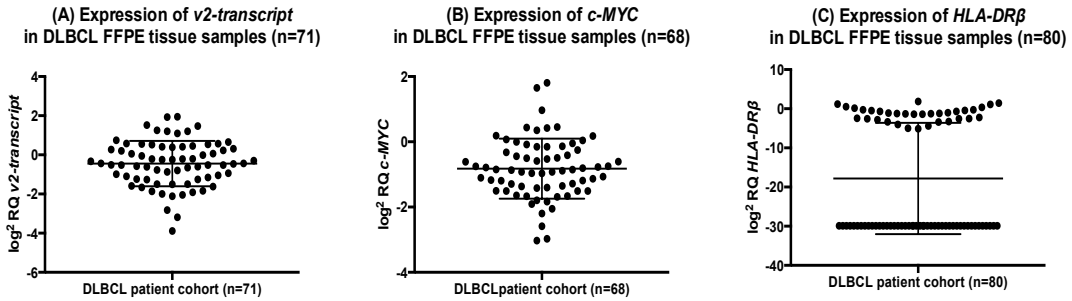


Figure 3.14 Expression of *v2-transcript, c-MYC and HLA-DRβ* ($\log^2$ RQ) in samples of patient cohort with DLBCL. **(A)** *v2-transcript*, **(B)** *c-MYC* and **(C)** *HLA-DRβ*.

Association of expression of v2-transcript, c-MYC and HLA-DRβ with clinical characteristics in a cohort of patients with DLBCL

The expression of all measured genes was correlated with the patient characteristic parameters in all evaluable patients, see table 3.7. The *v2-transcript* was significantly more highly-expressed in patients with BM involvement and with higher ECOG (p=0.015 and p=0.049; respectively). By contrast *c-MYC* was significantly more expressed in patients with bulky disease (p=0.027), higher serum LDH levels (p=0.030) and lower albumin levels (p=0.046). The expression of *HLA-DRβ* was not associated with any parameter.

Factor	*v2-transcript*	*c-MYC*	HLA-Drβ
Sex	n.s.	n.s.	n.s.
Age	n.s.	n.s.	n.s.
CS	n.s.	n.s.	n.s.
ECOG	0.049	n.s.	n.s.
B-symptoms	n.s.	n.s.	n.s.
Extranodal involvement	n.s.	n.s.	n.s.
Bulk	n.s.	0.027	n.s.
BM involvement	0.015	n.s.	n.s.
LDH	n.s	0.030	n.s.
B2-microglobulin	n.s	n.s	n.s
Hb	n.s.	n.s.	n.s.
WBC	n.s.	n.s.	n.s.
Albumin	n.s.	0.046	n.s.
Urea	n.s.	n.s.	n.s.
AP	n.s.	n.s.	n.s.
IPI	n.s.	n.s.	n.s.
Hans phenotype	n.s.	n.s.	n.s.

Table 3.7 Expression of *v2-transcript*, *c-MYC* and *HLA-DRβ* and their relation to patient characteristics by Mann-Whitney-Test.

Association of expression of v2-transcript, c-MYC and HLA-DRβ with response to treatment and survival in a cohort of patients with DLBCL

The expression of *v2-transcript*, *c-MYC* and *HLA-DRβ* was tested in patients with different response to treatment rates and different survival status (alive or death at the end of the treatment). There were no statistical differences.

The predictive value of expression of *v2-transcript*, *c-MYC* and *HLA-DRβ* on PFS and OS was assessed as a continuous variable using the Cox-regression model. The expression of none of assessed genes (*v2-transcript*, *c-MYC* and *HLA-DRβ*) was predictive as a continuous variable for PFS or OS (see table 3.8).

PFS	
Target	**H.R.; (C.I.); p-value**
v2-transcript	0.864; (0.618 – 1.207); 0.391
c-MYC	1.030; (0.662 – 1.604); 0.895
HLA-DRβ	0.988; (0.964 – 1.013); 0.348
OS	
Target	**H.R.; (C.I.); p-value**
v2-transcript	0.879; (0.621 – 1.244); 0.467
cMYC	0.911; (0.564 – 1.470); 0.702
HLA-DRB	0.986; (0.960 – 1.013); 0.292

Table 3.8 Survival analysis (PFS and OS) and expression of *v2-transcript*, *c-MYC* and *HLA-DRβ* as continuous variables by Cox regression.

In order to assess the predictive value of *v2-transcript*, *c-MYC* and *HLA-DRβ* expression as a dichotomous variable and the limits for dichotomizing expression values were set up using the ROC curves methods. The mean PFS and OS should be compared for both values (high and low expression) using Log-rank test.

The ROC curves analysis could calculate the limits for dichotomized evaluation of v*2-transcript* and *c-MYC* for PFS and OS and no valid limits for *HLA-DRB* for both PFS and OS, see table 3.9. However, the calculated limits for v*2-transcript* and *c-MYC* had restricted predictive value and could not be used for appropriate evaluation.

PFS						
	Direction	**Cutoff**	**Sensitivity**	**Specificity**	**ROC**	**p-value**
v2-transcript	Low	-0.8350	0.4815	0.7619	0.5573	0.4242
c-MYC	high	-3.000	1.0000	0.0000	0.5188	0.8001
OS						
	Direction	**Cutoff**	**Sensitivity**	**Specificity**	**ROC**	**p-value**
v2-transcript	low	1.9340	1.0000	0.0222	0.5308	0.6674
c-MYC	low	0.3877	0.9545	0.1087	0.5326	0.6653

Table 3.9 ROC curve analysis for limits of expression of *v2-transcript* and *c-MYC*; PFS (pre-test probability = 0.58) and OS (pre-test probability = 0.65).

3.5 *Results - studies on expression of miRNAs*

3.5.1 *Influence of different RNA extraction methods on qPCR of mature miRNAs*

As the practical knowledge of molecular techniques in assessment of miRNAs is very limited, there is a need for novel laboratory methods including the extraction, processing and storage of nucleic acids and expression assays. In order to choose the best method for extraction of RNA from cell lines for expression studies of miRNAs two different protocols of the *mir*Vana^{TM} miRNA Isolation Kit were compared: the protocol for a total RNA isolation (called *mir*Vana^{TM} total) and the protocol for an enrichment isolation of small RNAs (called *mir*Vana^{TM} micro). The amplification of two miRNAs was performed: miR-24, which is used as a housekeeping miRNA in the studies on lymphoma and miR-155 a miRNA, which is known to be expressed in lymphoid tissue. Three lymphoma cell lines were used: NU-DHL-1 a DLBCL cell line, U-698-M a lymphoblastic lymphoma cell line and SC-1 a FL cell line. The amplification was performed in two separate series of RNA; one with storage time of 2 weeks or shorter and the second series with preservation time of approximately 12 months. Both sets of samples were stored in -70°C.

Both miRNAs could be amplified successfully in all samples. The differences in the mean Ct value were statistically significant between the samples with RNA extracted with two different protocols in amplification of miR24 (p=0.004) and also present but not significant in amplification of miR155 (p=0.548), figure 3.15.

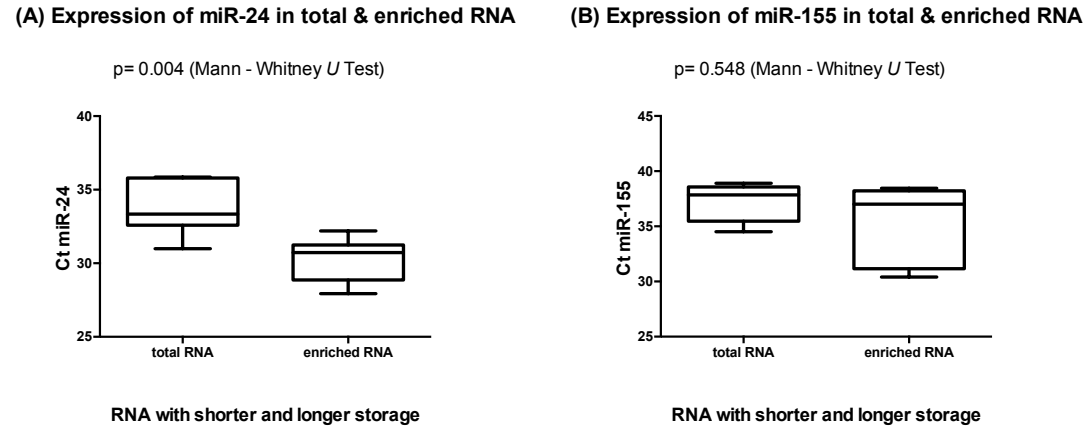


Figure 3.15 Expression of miRNAs in total and enriched RNA extracted from GRANTA519. Comparison of expression (Ct values) in total and enriched RNA (Mann-Whitney U test). **(A)** miR-24 and **(B)** miR-155.

Hence the amplification of miRNAs was possible in RNA samples extracted both protocols but the *mir*Vana™ micro can bias the results of amplification of the regular amplicons and it would be of importance to amplify both miRNAs and regular genes in one sample it was decide to use the *mir*Vana™ total in future analysis. This will be additionally assessed in the samples with RNA with shorter and longer storage.

3.5.2 *Influence of the storage time of RNA on qPCR of mature miRNAs*

Just as two different extraction methods were compared in the preliminary studies, a similar experiment was designed in order to assesse the potential degradation of miRNAs during the storage of RNA samples. The amplification of two mature miRNAs miR-24 and miR-155 was performed on the RNA samples with shorter storage (less than 2 weeks) and longer storage time (approximately 12 months). All samples were stored at temperature of approximately -70°C. The RNA was extracted from 3 different lymphoma cell lines: NU-DHL-1, U-698-M and SC-1 with *mir*Vana™ micro and total.

Both of the miRNA could be amplified in all samples and there were no significant differences in the sample pairs with different storage times. This pattern was seen in samples extracted with both extraction methods: *mir*Vana™ micro and total. Figure 3.16.A and B show the average Ct values for miR24 and miR155 respectively in all RNA samples with shorter and longer storage time. The differences were not statistically significant (p>0.999 for both).

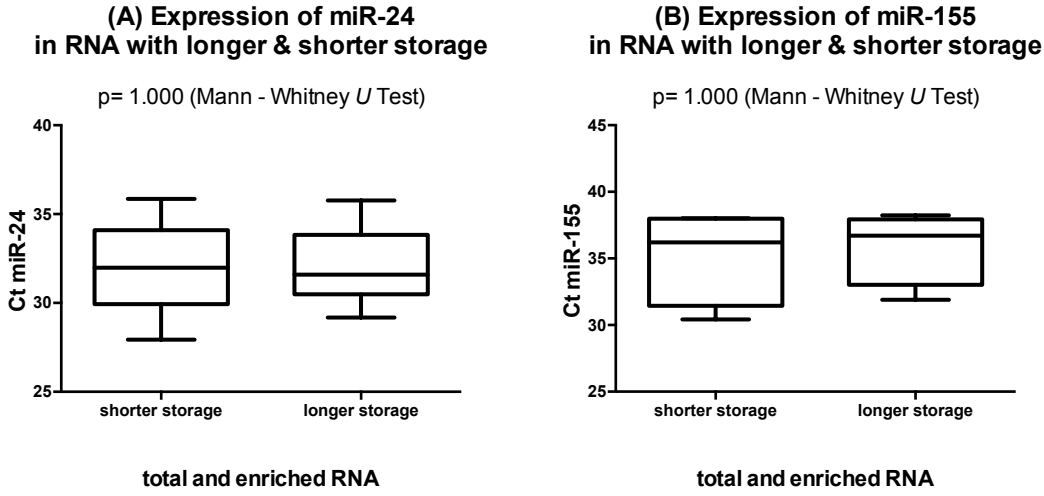


Figure 3.16 Expression of miRNAs in RNA with shorter and longer storage time, extracted from GRANTA519. Comparison of expression (Ct values) in samples with shorter and longer storage time (Mann-Whitney U test). **(A)** miR-24 and **(B)** miR-155.

The results of this study confirmed that the RNA for studies on miRNA can be stored at least up to a year and there were no advantages in using the enrichment extraction method.

3.5.3 *Validation of qPCR for mature miRNAs*

In order to prove the efficiency of RT and qPCR of mature miRNAs and to select the optimal amount of RNA the reactions for two different miRNAs: miR-24 and miR-155 were performed with three different total amounts of RNA used in each RT reaction: 25ng, 125ng and 250ng in each RT reaction. These amounts correspond with the following concentrations of RNA in RT reactions: 1.66ng/µl, 8.33ng/µl and 16.66ng/µl of RT reaction and qPCR reactions: 0.074ng/µl, 037ng/µl and 0.74ng/µl of qPCR reaction. The RNA was extracted with *mir*Vana™ total from a DLBCL cell line (NU-DHL-1). For both miR-24 and miR-155 the lowest Ct values were measured in samples with the highest amount of RNA used, see figure 3.17.

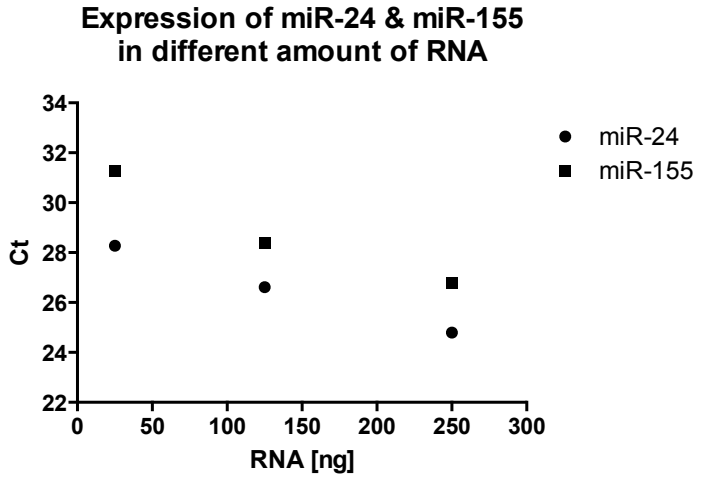


Figure 3.17 Expression of miR-24 and miR-155 (Ct value) in samples with different RNA amount (25ng, 125ng, 250ng of total RNA extracted from GRANTA519 per RT reaction).

To assess the correlation between the Ct values and amount of RNA used the Pearson Correlation test has been performed. There was a linear correlation between the Ct values and amount of RNA used for both miRNAs (for miR-24 $R=0.999$, $p<0.001$ and for miR-155 $R=0.973$, $p<0.0001$). These results allow the usage of $2^{-\Delta\Delta CT}$ method for normalization of expression of mature miRNAs. It was decided that in the future

experiments the total amount of RNA will be 200ng per one miRNA RT (13.33ng/µl of RT reaction).

3.5.4 *Expression of mature miRNAs in lymphoma cell lines*

Validation of miR-24 as a reference miRNA for qPCR for mature miRNAs

miR-24 was reported in several studies as an appropriate candidate for the studies on lymphomas. To validate the expression of miR-24 as a reference miRNA its expression was assessed in the samples of RNA extracted from all cell lines. The RNA was extracted with *mir*Vana^{TM} total from each cell line and was used in a concentration of 13.33ng/µl of RT reaction and concentration of RT-product in qPCR was 0.059ng/µl of qPCR reaction. The RT and qPCR were performed as per standard conditions. In order to stay with homogenous conditions the RT and qPCR of all samples were performed simultaneously in all samples. miR-24 was expressed nearly equally in all samples (i.e. <1.3-fold of change in all cell lines). The median Ct value for all cell lines was 28.72, range from 24.11 in KARPAS-422 to 29.86 in DB (figure 3.18). According to these results the expression of mature miRNAs in tested cell lines can be normalized to expression of miR-24 using $2^{-\Delta\Delta CT}$ method.

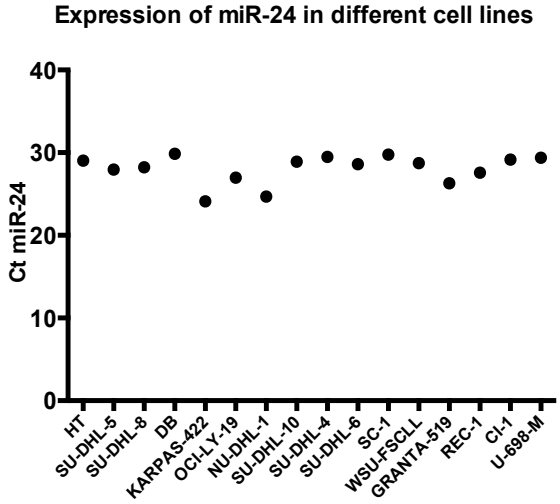


Figure 3.18 Expression of miR-24 (Ct value) in different cell lines.

Expression of mature miRNAs in lymphoma cell lines

The RT and qPCR was performed according to the standard conditions on RNA isolated with *mir*Vana^{TM} total from each cell line used in RT in concentration of

13.33ng/µl of RT reaction and concentration of RT-product in qPCR was 0.059ng/µl of qPCR reaction. The expression of each miRNA was compared to endogenous control miR-24. The ratio of target miRNA and endogenous control in each cell line was compared to the ratio of target miRNA and endogenous control in calibrator (RNA from normal CD19+ B-cells from 3 healthy donors). Fold change was calculated using $2^{-\Delta\Delta CT}$ method.

Four miRNAs were chosen for these experiment: miR-155 as an internal control as this miRNA is known to be expressed in lymphoid malignancies and 3 miRNAs from miR-17-92 cluster: miR-17-5p, miR-20 and miR-92. miR-17-5p is encoded in the beginning of the cluster, miR-20 in the middle and miR-92 at its end. All analysed miRNAs were expressed in the tested cell lines. The expression pattern of miR-155 over different cell lines was the most heterogeneous. It was mostly under-expressed in the DLBCL cell line, highly under-expressed in FL cell lines SC-1 and WSU-FSCLL, slightly over-expressed in both MCL (GRANTA-519 and REC-1) and had mixed pattern in lymphoblastic lymphoma cell lines (CI-1 and U-698-M); figure 3.19.A. miR-155 was slightly more highly-expressed among lymphoma cell lines as compared with DLBCL cell lines (figure 3.19.B), $p>0.999$. By contrast, the expression of miRNAs from miR-17-92 cluster had a more homogenous pattern. miR-17-5p was overexpressed in all cell lines (figure 3.19.C), like miR20 which was slightly under expressed only in two cell lines – one DLBCL cell line (NU-DHL-1) and one MCL line (GRANTA-519); figure 3.20.E. miR-92 was also overexpressed in all cell lines with an exception of NU-DHL-1 and GRANTA-519 and additionally KARPAS-422 (figure 3.19.G). All three miRNA were significantly more highly-expressed among other lymphoma cell lines as compared with DLBCL cell lines (figure 3.19 D, F and H).

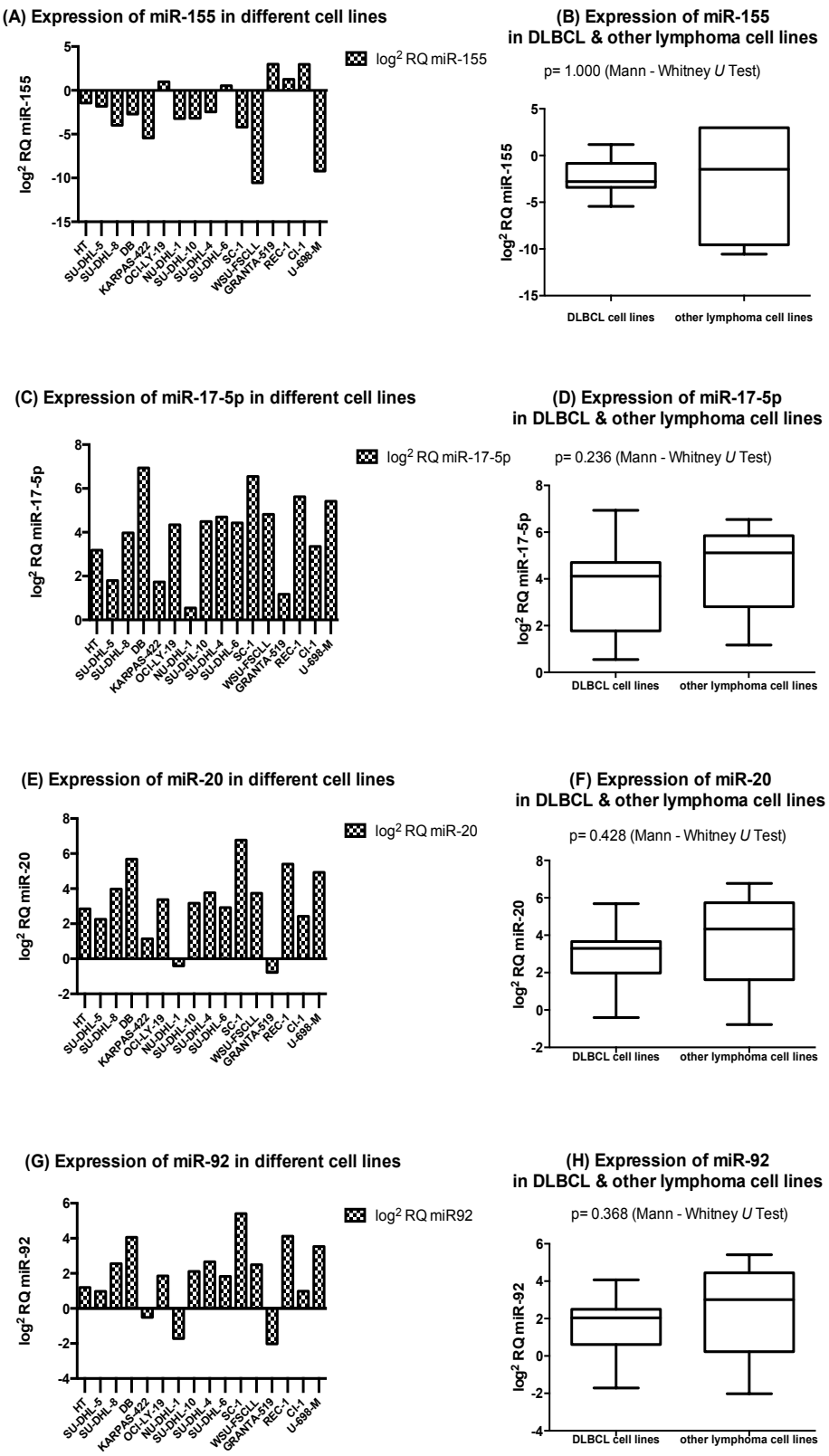


Figure 3.19 Expression of miR-155, miR-17-5p, miR-20 and miR-92 in lymphoma cell lines. Expression (log^2 RQ) in individual lymphoma cell lines **(A)** miR-155, **(C)** miR-17-5p, **(E)** miR-20 and **(G)** miR-92 and a comparison of expression (log^2 RQ) in DLBCL vs. other lymphoma cell lines (Mann-Whitney U test). **(B)** miR-155, **(D)** miR-17-5p, **(F)** miR-20 and **(H)** miR-92.

3.5.5 *Expression of mature miRNAs in FFPE and frozen lymphoma tissue samples*

As previously mentioned in the section on studies on gene expression, the proposed study material should be FFPE tissue. One of the common limitations of qPCR is its efficacy in nucleic acid extracted from FFPE tissue, particularly for amplicons >150nt. It usually encounters significant difficulties due to loss of their integrity. The small size of miRNAs could suggest they could be better targets for qPCR. In order to prove this hypothesis the qPCR for mature miRNAs was performed on the RNA extracted from both FFPE and corresponding frozen tissue from the series of eleven patients with lymphoma. The concentration of RNA in RT reaction was 13.3ng/µl of RT reaction and the concentration of RT product in qPCR reaction was 0.059ng/µl of qPCR reaction through out this study.

Validation of endogenous control (miR-24) for qPCR in FFPE and frozen lymphoma tissue samples

The expression of miR-24 was assessed in RNA extracted from eleven FFPE tissue blocks and from corresponding eleven frozen tissue blocks. miR-24 was expressed in all assessed samples; see figure 3.20.A. Importantly, by contrast to the studies on *GAPDH*, there was no statistical difference in measured Ct values of miR-24 between the FFPE tissue and frozen samples, p=0.898. Ct values in frozen tissue samples ranged from 22.79 to 25.64 (mean 24.30) and in FFPE tissue samples from 22.58 to 26.20 (mean 24.51); figure 3.20.B. The fold of difference among both frozen tissue samples and FFPE tissue samples was lower <1.3 with respectively: 1.13 and 1.16. According to this data miR-24 can be used as endogenous control for normalization purposes of $2^{-\Delta\Delta Ct}$ method.

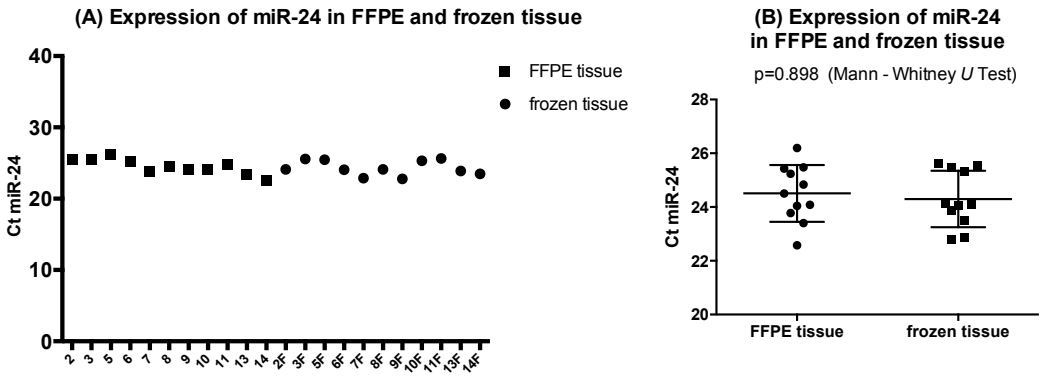


Figure 3.20 Expression of miR-24 in FFPE tissue and corresponding frozen tissue. **(A)** expression (Ct value) of miR-24 in all samples and **(B)** comparison of miR-24 expression (Ct value) in FFPE tissue and frozen tissue samples (Mann-Whitney U test).

Expression of mature micro RNAs in FFPE and frozen lymphoma tissue samples

All assessed miRNAs were expressed in the frozen tissue and corresponding FFPE blocks. By contrast to the results of the similar experiments in expression of the genes, there were no significant differences in Ct values between both types of material. The mean Ct of miR-155 in frozen tissue was 24.69 (range from 20.91 to 27.63) and in FFPE was 24.46 (range from 21.69 to 28.37); p=0.699; figure 3.21.A. The same pattern of expression was observed in expression of miRNAs from the miR-17-92 cluster: the mean Ct value of miR-17-5p in frozen tissue was 25.82 (range from 24.23 to 27.48) and in FFPE 25.91 (range from 23.50 to 27.73); p=0.699, figure 3.21.C. The mean Ct of miR-20 in frozen tissue was 22.59 (range from 21.25 to 23.83) and in FFPE 22.63 (range from 21.06 to 24.55); p=0.949 (figure 3.21.E) and regarding miR-92, the mean Ct value in frozen tissue was 22.03 (range from 20.23 to 22.89) and in FFPE was 22.35 (range from 20.90 to 23.44), p=0.332; figure 3.21.G.

Additionally, correlation between miRNA expression measured in FFPE tissue blocks and in corresponding frozen tissue was evaluated using Spearman's correlation. All assessed miRNAs showed significant correlation; for miR-155 Rs=0.6818 and p=0.0251 (figure 3.21.B), for miR-17-5p Rs=0.6636 and p=0.030 (figure 3.21.D), for miR-20 Rs=0.5545 and p=0.0818 (figure 3.21.F) and for miR-92 Rs=0.6091 and p=0.0519; see figure 3.21.H.

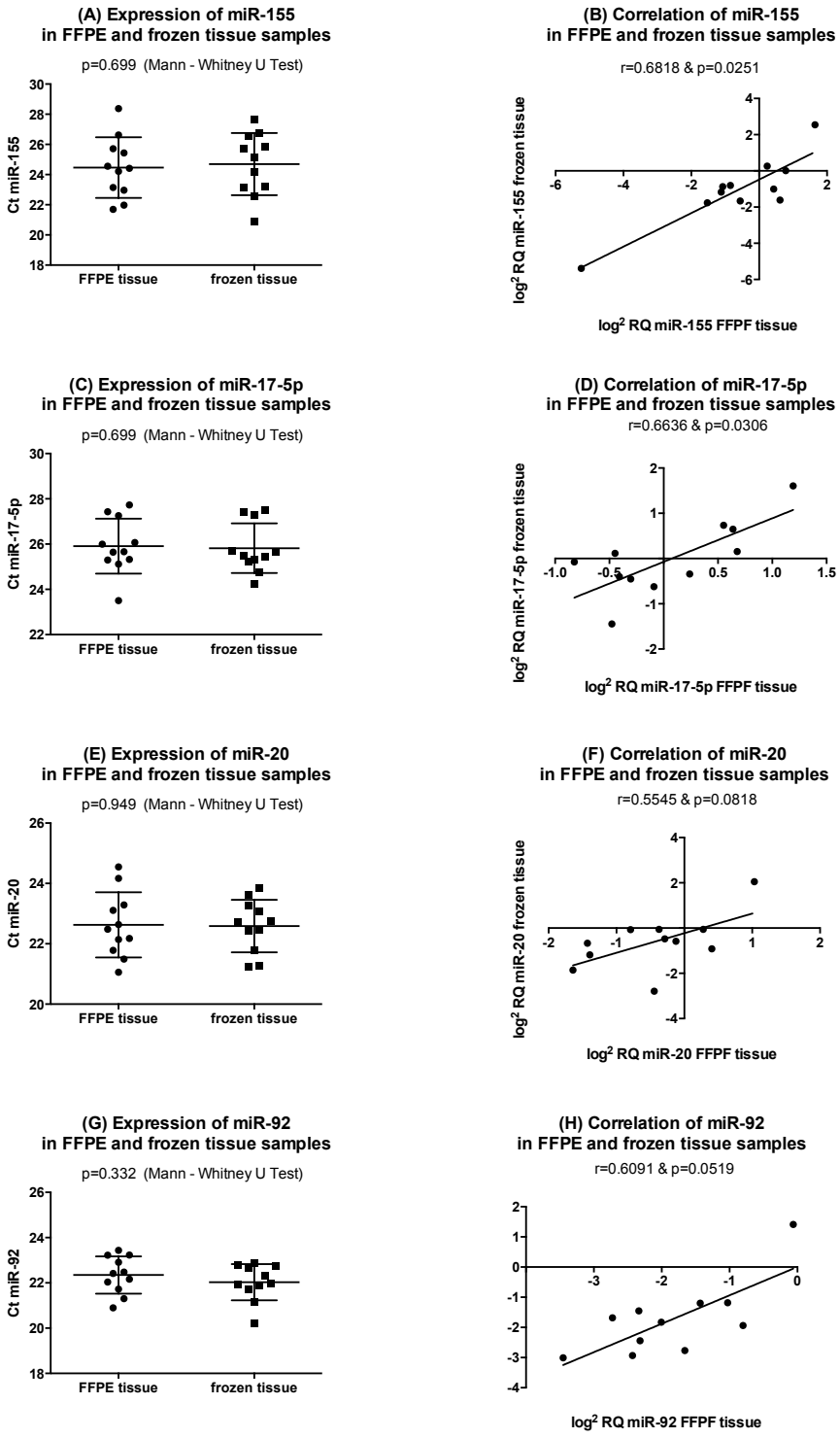


Figure 3.21 Expression of mature miRNAs in FFPE tissue and corresponding frozen tissue. Comparison of expression (Ct-values) in FFPE tissue and corresponding frozen tissue samples (Mann-Whitney U test): **(A)** miR-155, **(C)** miR-17-5p, **(E)** miR-20 and **(G)** miR-92. Correlation of expression (log^2 RQ) in FFPE tissue and corresponding frozen tissue samples (r Spearman): **(B)** miR-155, **(D)** miR-17-5p, **(F)** miR-20 and **(H)** miR-92.

3.5.6 Validation of repeated qPCR for mature miRNA in series of DLBCL FFPE clinical tissue samples

In order to assess the precision and repeatability of the applied assays, the expression of the studied miRNAs was assessed in two series of experiments using two different concentrations of the RNA extracted from the FFPE tissue of a cohort of patients with DLBCL. In the first series the concentration of RNA used for RT reaction was 1.38ng/µl of RT reaction and subsequently in the second series of experiments it was reduced to 0.66ng/µl of RT reaction. The concentration of the miRNA RT product in qPCR reaction was 0.006147ng/µl of qPCR reaction in the first series and 0.00325ng/µl of qPCR reaction in the second series.

The evaluation showed that the expression of all assessed miRNAs: miR-155 and miR-17-5p agreed between the two series of experiments with bias of 0.2905 and 0.6856, retrospectively (figure 3.22.A and C). By contrast there was no agreement in expression of miR-20 with bias of 1.1194 (figure 3.22.E). There was a significant correlation between the expression of all miRNAs from two series of experiments with Rs of 0.601, p=0.043 for miR-155 (figure 3.22B), Rs of 0.717 and p=0.037 for miR-17-5 (figure 3.22.D) and Rs of 0.839 and p=0.001 for miR-20 (figure 3.22.D).

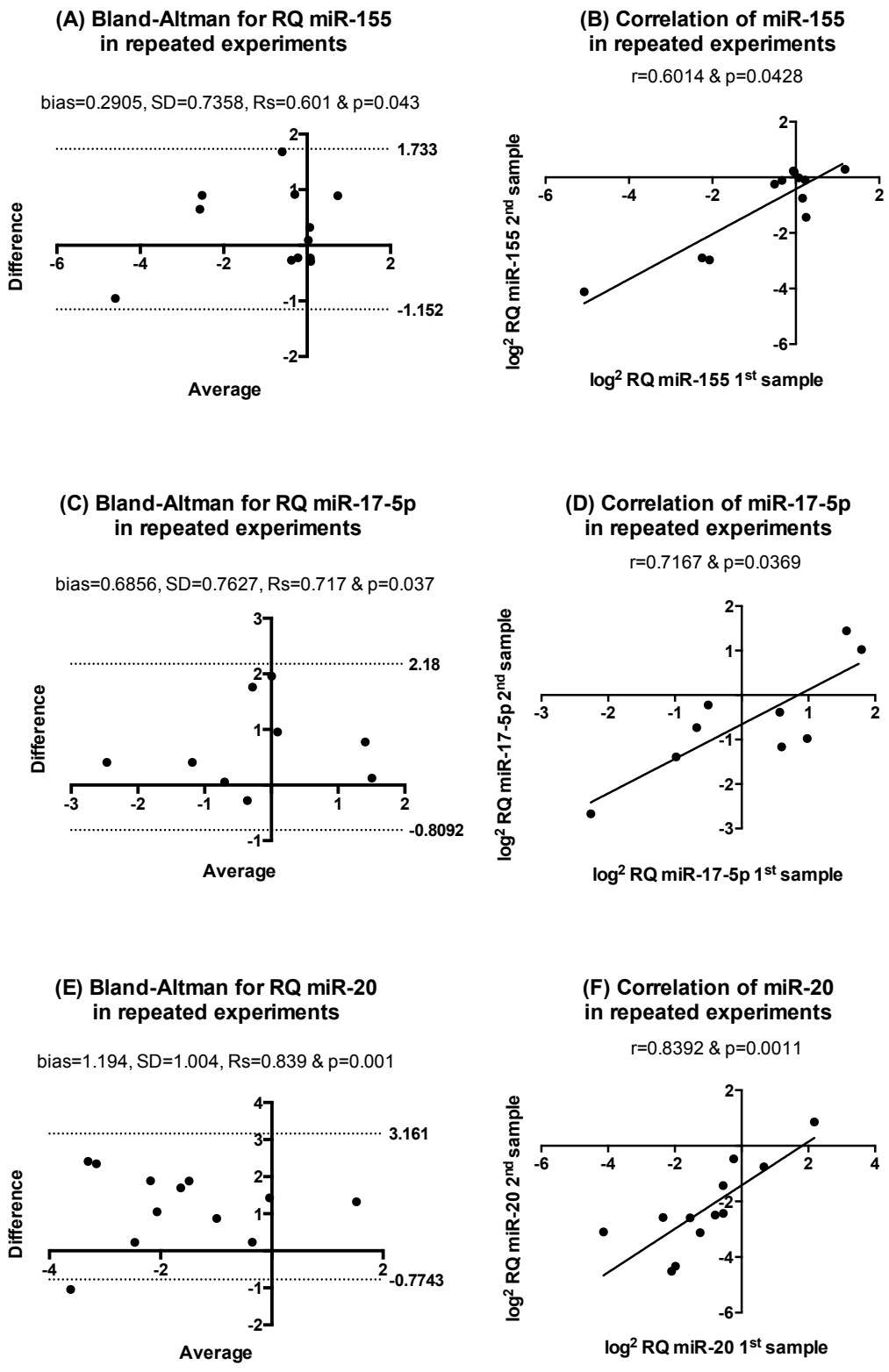


Figure 3.22 Expression of mature miR-155, miR-17-5p and miR-20 in two repeated series of FFPE tissue samples. Bland-Altman plots for agreement of expression (log^2 RQ) **(A)** miR-155, **(C)** miR-17-5p and **(E)** miR-20. Correlation of expression (log^2 RQ), r Spearman: **(B)** miR-155, **(D)** miR-17-5p and **(F)** miR-20.

3.6 Discussion

The research in this chapter focused on assessment of expression of *c-MYC*, *HLA-DRβ* and *v2-transcript* at the RNA level using qPCR on the material extracted from FFPE tissue samples as predictive factors for DLBCL. Additionally, we assessed the potential role of mature miRNAs encoded in the *v2-transcript* as predictive biomarkers in DLBCL. All these genes have been reported to play important roles in oncogenesis and also have prognostic value in DLBCL. *c-MYC* is a known oncogene and chromosomal aberrations involving its locus are well defined in lymphomagenesis, as are alterations its expression at the protein level. *HLA-DRβ* is one of the MHC class II genes and its under-expression plays important role in limiting immune-surveillance. Finally, *v2-transcript* is a new candidate for a new class of proto-oncogenes encoding miRNAs involved in RNA interference processes.

The expression of *c-MYC* and *HLA-DRβ* was assessed using ready-to-use commercial TaqMan® Gene Expression Assays including sets of primers and probes and they do not require any additional testing. By contrast, there were no commercially available ready-to-use assays for assessment of *v2-transcript*. We used the set of primers and probes previously described by Venturini et al (Venturini et al., 2007). Hence, these primers and probe are characterized by some limitations e.g. they bind to the amplicon beyond the region encoding mature microRNAs and the transcribed amplicon has significant length of 172nt. The amplicon was re-scanned using Applied Biosystem software in order to find a more optimal set of primers and probes. Unfortunately, no further candidates were found. The chosen *v2-transcript* primers and probes passed successfully the standard curve experiment. It was also proven that primers and probes can react with genomic DNA thus treatment with DNAse is necessary.

In preliminary experiments the expression of *c-MYC*, *HLA-DRβ* and *v2-transcript* was successfully measured in RNA extracted from a collection of lymphoma cell lines. Generally, the mean Ct values fluctuated around 15-25. The *c-MYC* and *HLA-DRβ* were under-expressed in all cell lines as compared with the expression in reference healthy B-cells. There were no differences in the expression of both genes between the DLBCL cell lines and other lymphoma cell lines. The *v2-transcript* was under-expressed in all cell lines with an exception of two cell lines with a random origin. It showed lower expression in DLBCL cell lines as compared with other lymphoma cell

lines. The results of expression in cell line studies should be taken with big reserve, as all cell lines are in vitro models, which do not necessary show any association with natural disease picture. Most of the cell lines were developed from cells isolated from pleural effusions or ascites from patients with advanced, usually chemotherapy-resistant tumours. It also difficult to compare the results of our experiment with other cell line studies because the cell lines tent to change their characteristics the culture. In our studies they were used for the purposes of establishing a reaction condition only. Additionally, in case of *c-MYC* the expression of the gene in control sample (B-cell collection) was higher than in cell line samples and thus could bias the results.

Hence the aim of the study was to assess expression of the genes in RNA extracted form FFPE tissue samples. As quantity and quality (particularly integrity) of nucleic acid is often affected by the process of formalin fixation, paraffin embedding and sample storage, we moved experiments to materials extracted from FFPE and corresponding frozen tissue. A selection of eleven pairs of FFPE tissue and corresponding frozen samples with diagnosis of lymphoma was established. Unfortunately, there was no guarantee that both samples came from same region of the tumours and the individual samples can obviously differ in the amount of tumour cells and connective tissue. This obviously can affect the results of the studies and limited the predication of number of performed statistical tests. Generally, all assessed genes were expressed in both materials; however there was a significant difference in measured Ct values between them. Importantly, the Ct values of a reference gene *GAPDH* in FFPE tissue samples ranged between 26.36 and 32.14 with mean Ct of 29.29 and the fold of difference between the lowest and the highest value was 1.22. In the material extracted from frozen tissue samples the Ct values for *GAPDH* ranged from 19.87 to 24.46 (mean Ct 21.64) with fold change of 1.23. This confirmed the suitability of *GAPDH* as a reference gene. The mean Ct value of *v2-transcript* in frozen tissue and FFPE tissue samples was 30.25 and 35.00, respectively, for *c-MYC* 26.14 and 33.31 and for *HLA-DRB* 23.33 and 34.60. With the mean Ct value measured in RNA extracted from FFPE tissue varied from 33.31 for *c-MYC*, through 34.60 for *HLA-DRB* to 35.00 for *v2-transcript*, the qPCR is reaching its limits of dynamic range and requires very careful controls e.g. only samples with SD <0.3 in triplicates could be used. Despite applying theses special conditions, significant variation of results is possible, as the expression of genes is measured at the single copy level and can be easily affected by number of factors including RNA quality, technical problems or inhibition of reaction.

Additionally, we performed the correlation studies on the gene expression in frozen and FFPE tissue samples. We found the significant correlation for *c-MYC* and *HLA-DRβ* (r=0.7727 & p=0.0074 and r=1.0 & p=0.0028; respectively) but not for *v2-transcript* (r=0.2273 & 0.5034).

Subsequently, in order to check the precision and reproducibility of assays the expression of studied genes was assessed in two series of RNA samples with different concentrations extracted from the FFPE tissue samples in a cohort of patients with DLBCL. The Bland-Altman assessment confirmed the agreement in expression in both experiments with bias of -0.2559 (SD=0.4980) for *v2-transcript*, -0.1046 (SD=0.5811) for *c-Myc* and -0.4546 (SD=0.4226) for *HLA-DRβ*. Spearman's correlation tests also showed significant correlation for all assessed genes with r=0.7905 & p<0.001 for *v2-transcript*, r=0.7613 & p<0.001 for *c-Myc* and r=0.967 & p<0.001 for *HLA-DRβ*.

Although reaching the technical limits of qPCR we were able to perform the assessment of the genes in FFPE samples collected from a population-based cohort of patients with DLBCL. In order to deliver the most precise result we applied strict criteria for assessment of the genes with low abundance, as recommended by Applied Biosystem. Additionally, following the recent recommendation, two control genes were used for evaluation purposes: *PGK1* and *GAPDH*. The *GAPDH* was evaluable in 80 samples (87%), the mean Ct value was 29.29 (range from 24.71 to 33.61) with the fold of difference between the lowest and the highest value of 1.36 and *PGK1* was evaluable in 87 samples (95%) with a mean Ct value of 28.05 (range from 24.64 to 32.69) with the fold of difference between the lowest and the highest value of 1.33. Both genes passed successfully the criteria for control genes in the material extracted from FFPE tissue.

The efficacy of qPCR for individual genes varied from 74% for *c-MYC*, through to 77% for *v2-transcript* to 87% for *HLA-DRβ*. All tested genes had a mean RQ lower than in control tissue with mean RQ for *v2-transcript* of -0.44 (range -3.89 – 1.94, SD 1.16), for *c-MYC* -0.82 (range -3.03 – 1.81, SD 0.92) and for *HLA-DRβ* -17.81 (range -29.90 – 1.85, SD 14.19). The expression of *v2-transcript* was significantly higher in patients with lower ECOG status and BM involvement. This could be explained by higher aggressiveness of tumours with higher expression of *v2-transcript*. The c-*MYC* showed higher expression in patients with bulky disease, increased LDH serum levels and lower albumin levels. All these factors can be also associated with more aggressive tumours with higher expression of *c-MYC*. In our evaluation there was no association of

the expression of *v2-transcript*, *c-MYC* or *HLA-DRβ* measured as continuous or dichotomized variables and patient survival.

The role of *c-MYC* in lymphomagenesis of DLBCL was firstly associated with the presence of the chromosomal translocation involving its locus (Johnson et al., 2009) (Kramer et al., 1998). It was usually described together with the presence of chromosomal translocations involving *BCL2* and *BCL6*. The tumours harbouring both translocations are called "double hit lymphomas" and those with all translocation "triple hit lymphomas". The outcome of patients with *c-MYC* translocation, particularly those with additional translocations of *BCL2* or *BCL6*, was very dismal when treated with standard anthracycline-based chemotherapy with or without addition of rituximab (Niitsu et al., 2009) (Barrans et al., 2010) (Savage et al., 2009) (Johnson et al., 2012). They often are classified as B-cell lymphoma unclassifiable, with features between DLBCL and BL (Snuderl et al., 2010) (Swerdlow, 2014). The "double and triple hit lymphomas" are also usually resistant to salvage chemotherapy with r-DHAP or R-ICE (Cuccuini et al., 2012). Due to lack of reliable monoclonal antibodies against c-MYC, immunohistochemical studies on c-MYC expression were scarce until the advent of new type of antibodies in 2008 (Gurel et al., 2008) (Ruzinova et al., 2010). The recent studies described the adverse predictive value of c-MYC expression alone or in association with expression of BCL2 and BCL6, so called "double and triple expressors" usually with the rearrangement status of assessed genes (Horn et al., 2013) (Green et al., 2012b) (Valera et al., 2013) (Hu et al., 2013). However there are also some reports, which could not support a predictive role of *c-MYC* rearrangement status (Kramer et al., 1998) (Visco et al., 2013). Despite these encouraging results on *c-MYC* rearrangements and protein expression, there is still little known about the role of expression of *c-MYC* at mRNA level, although it would be logical to focus the research in this direction. The data from GEP studies of Rosenwald and confirming studies of Rimsza were suggesting negative predictive value of *c-MYC* RNA expression in DLBCL (Rosenwald et al., 2002) (Rimsza et al., 2008). In our studies we could not confirmed these results. There are several possible explanations for this. With increasing knowledge of c-MYC it can be confirmed that its role in oncogenesis is multidirectional. Particularly the complex regulation of c-MYC expression on all levels (DNA, mRNA and protein) and c-MYC pro-proliferative and pro-apoptotic properties contribute towards it (Pelengaris and Khan, 2006). Recently it was postulated that in order to assess the c-MYC equilibrium in the cell it is necessary to measure the

expression of c-MYC on all expression levels. The *c-MYC* rearrangements seem to have more predictive value as compared with *c-MYC* mRNA expression or c-MYC protein expression. The *c-MYC* rearrangements usually lead to constant overflow of a cell with the amount of c-MYC protein overcoming the regulation mechanism. Other mechanisms leading to increased *c-MYC* overexpression preserve the complex regulatory machinery. Additionally, the factors associated with selection of the patients and technical limitation of qPCR could interfere with our results, as discussed below.

Regarding the expression of MHC class I and II protein, the low or absent expression of these molecules was associated with worse survival of patients with DLBCL, most likely due to loss of immunosurveillance (Rimsza et al., 2004) (Rimsza et al., 2007) (Veelken et al., 2007). The loss of expression of HLA-DRβ molecule together with immunoblastic tumour phenotype was predictive for adverse outcome of patients with DLBCL in the study of Bernd et al (Bernd et al., 2009). The loss of expression of MHC class II genes at the mRNA level was a negative prognostic factor in the GEP studies of Rosenwald. Subsequently this was confirmed by the study of Rimsza et al (Rosenwald et al., 2002) (Rimsza et al., 2008). Unfortunately, we could not confirm these results. As the association between reduced expressions of *HLA-DRβ* seems to be established, the possible explanation of our results should be more likely found in the applied technology or patient selection.

The *v2-transcript* is a new candidate for an oncogene acting through miRNAs encoded within it. Its increased amplification was observed in various subtypes of lymphoma including DLBCL (Neat et al., 2001) (Ota et al., 2004) (Mao et al., 2002). Its oncogenic capacity has been described in several pre-clinical studies. It was also assessed in a cohort of patients with CML via qPCR with TaqMan technology, showing not only a predictive value but also positive answer to the treatment with tyrosine kinase inhibitor (Venturini et al., 2007). Data on expression of *v2-transcript* in patients with DLBCL were lacking. We aimed to address this in the population-based cohort of patients with DLBCL. We could not find any predictive value of the *v2-transcript* expression in our cohort. However it is worth mentioning that the set of primers and probes was suboptimal for assessment in the material extracted from FFPE tissue with the length of amplicon of 170nt and high G-C content. Further limitations of the method and cohort described below also apply.

In terms of the selection of patients, our cohort was based on an unselected group of patients with characteristics that did not differ from those presented by others.

Solely the general health condition of our patients could be different as we evaluated samples from unselected cohort of patients. By contrast, in the majority of other studies, the samples of patients included in clinical trials were evaluated. There is known bias in patient selection in clinical trials that usually favours more healthy patients. This can obviously differ depending on used inclusion criteria. In this case the response and survival data are usually better.

Also, looking from the perspective of applied methodology, there can be a different explanation of our results. Regarding the technical limitation of qPCR, the expression of all genes was characterized by high Ct values reaching the dynamic range of the method. In this situation, the expression of single copies of the genes is detected and it can be easily affected by several factors including quality of RNA, inhibition of reaction or technical failures. Despite promising preliminary results, the use of qPCR in material extracted from FFPE tissue appears to be complex and not reliable. Probably the use of a non-enzymatic method would have more advantages, particularly that several new technologies have been recently introduced e.g. NanoString.

Additionally, in all studies the RNA was extracted from curls obtained from whole tumour blocks. It is obvious that in some cases not a whole block was infiltrated by tumour cells. Thus, the extracted RNA could be easily contaminated by RNA coming from non- malignant cells e.g. connective tissue cells. In the situation when the Ct values are very high and we measure single copies of the amplicon, this can affect easily the results. The optimal solution to this problem would be to work on RNA extracted from tissue microarrays. However, the amount of extracted RNA can be an issue when using TMA.

Regarding the pilot studies on the expression of miRNA as potential biomarkers, firstly we assessed the most appropriate way of extracting RNA. At that time, experiments on miRNAs were novel and little was known about the optimal techniques. As we aimed to work on the same material for gene and miRNA expression analysis we opted for the normal protocol of RNA extraction rather than the one which involved enriching extracted miRNAs. The both methods delivered comparable Ct values of assessed miRNAs. In order to assess the potential influence of storage time of extracted RNA, we compare the expression of miRNA in the RNA extracted from the same cell lines but with different storage time. We could not find any significant difference in expression rates, confirming the stability of miRNA over a one year in appropriate conditions.

One of the main conditions for successful relative qPCR is finding an appropriate candidate for reference gene (amplicon). In our studies we aimed for miR-24, which was previously successfully used in the studies on DLBCL. The expression of miR-24 was equally distributed over the selected cell lines, with mean Ct value of 28.72 (range 24.11 – 29.86) and the fold difference in the expression between the lowest and highest Ct value of 1.24. For our studies we choose 4 different miRNAs: miR-155m (it has already an established role in the lymphomagenesis) and three miRNAs encoded in different loci of *v2-transcript*: miR-17-5p (at the beginning), miR-20 (in the middle) and miR-92 (at the end). We were able to detect and measure the expression signal for all assessed miRNAs in the RNA extracted from the selection of lymphoma cell lines. MiR-155 showed the most heterogeneous expression pattern, being under-expressed in DLBCL cell lines, highly under-expressed in FL cell lines, slightly over-expressed in both MCL cell lines and variably expressed in lymphoblastic lymphoma cell lines. MiR-155 showed higher expression in non-DLBCL cell lines as compared with DLBCL cell lines. The miRNAs from the *v2-transcript* had a more homogenous expression pattern, being overexpressed in almost all assessed lymphoma cell lines. Expression was slightly higher in non-DLBCL cell lines than DLBCL cell lines. As the aim of this study was not to focus on expression in the cell line model we used these results as a preliminary model.

Similar to the studies on gene expression, we moved to the studies on the material extracted from FFPE tissue. The first studies compared the expression of mature miRNAs in the material extracted from FFPE tissue samples and corresponding frozen tissue samples. The expression of miR-24 met the criteria for a reference gene with mean Ct value in FFPE tissue and corresponding frozen tissue samples of 24.51 (range 22.58 – 26.20) with the fold of difference between the lowest and the highest value of 1.16 and 24.30 (range 22.79 – 25.64) with the fold of difference between the lowest and the highest value of 1.13, respectively. By contrast to the studies on gene expression, the difference between Ct levels in frozen tissue and FFPE tissue was not significant. This indicates better resistance of miRNAs for material fixation and embedding and storage associated degradation, as it was assumed. This was also confirmed for the all assessed miRNAs. The mean Ct value of miR-155 in frozen tissue and FFPE tissue was 24.69 and 24.46, respectively, for miR-17-5p 25.82 and 25.91, for miR-20 22.59 and 22.63 and for miR-92 22.03 and 22.35. There were no statistically significant differences between the expression in both materials, confirming the

hypothesis of better preservation of miRNAs in FFPE tissue. By contrast to the gene expression the qPCR method was within its dynamic range for expression of miRNA in FFPE tissue. There was a significant correlation between the expression of miR-155 and miR-17-5p in the frozen tissue and FFPE tissue (r=0.6818 & p=0.0251 and 0.6636 & 0.0306) but no correlation was found in expression of miR-20 and miR-92 (r=0.5545 & 0.082 and 0.6091 & p=0.0519). Notably, the R-values were smaller as compared with the gene expression studies, indicating worse correlation. Taking into consideration the tumour homogeneity in the corresponding FFPE and frozen tissue samples this could reflect potentially greater sensitivity of qPCR for miRNAs as compared with genes assessments.

Subsequently, in order to check the precision and repeatability of the assays the expression of studied miRNAs was assessed in two different concentrations of RNA extracted from the FFPE tissue samples from a cohort of patients with DLBCL. The Bland-Altman assessment confirmed the agreement in expression in both experiments for miR-155 (bias 0.2905 & SD=0.7358) and miR-17-5p (0.6856 & 0.7627) but not for miR-20 (bias 1.194 & SD=1.004). Spearman's correlation tests showed significant correlation for all assessed miRNAs with r=0.014 & p=0.043 for miR-155, r=0.7167 & p=0.0369 for miR-17-5p and r=0.8392 & p=0.0011 for miR-20. However, the statistical values were worse than those obtained in the gene expression studies. Additionally, looking closer at the results, surprisingly the measured Ct values were higher than those measured in FFPE tissue samples from studies on comparison of expression in FFPE tissue samples and corresponding frozen tissue samples. The differences were statistically significant: for miR-24 24.05 vs. 31.38 (p=0.000), for miR-155 24.64 vs. 31.73 (p=0.000), for miR-17-5p 25.91 vs. 33.06 (p=0.000) and for miR-20 22.63 vs. 31.31 (p=0.000). Importantly, the differences for the same comparison performed on gene expression were not significant: for *GAPDH* 29.81 vs. 28.27 (p=0.196), for *v2-transcript* 35.00 vs. 34.80 (p=0.869), for *c-MYC* 33.31 vs. 33.32 (p=0.924) and for *HLA-DRβ* 30.11 vs. 30.50 (p=1.000). Importantly, the Ct values for miRNAs from these studies lie again at the limits of the dynamic range of the method and can be easily interfered with by several factors, as discussed above. One of the potential explanations could be differences in processing the extracted RNA in both studies. In these studies, the RNA samples were uniformly treated with DNAse, for both miRNAs and gene assessment. By contrast, in studies on comparison of expression in FFPE tissue samples and corresponding frozen tissue samples, the RNA samples for gene assessment were

treated with DNAse but those for miRNAs assessment were not. The treatment with DNAse could potentially reduce amount of the miRNAs in the samples. Importantly, the DNAse protocol incorporates a step of inhibition and deactivation of DNAse using absorbing micro particles. Most likely the micro particles could absorb the microRNAs. This observation shows how sensitive techniques used in qPCR can be and how fragile the material used for assessment of miRNAs is. Despite this, we could confirm that assessment of miRNA expression in FFPE tissue samples on an unselected cohort of patients with DLBCL is possible and can be evaluated further. The technical limitation of used materials and methods discussed above apply here as well. During this work, several studies have successfully assessed miRNAs as biomarkers in DLBCL in material extracted from FFPE tissue (Lawrie et al., 2007) (Alencar et al., 2011) (Iqbal et al., 2015) (Fassina et al., 2012).

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Chapter 4. Epidemiology, clinical presentation, traditional and novel treatment modalities in EATL

4.1 Introduction

Primary gastrointestinal lymphomas comprise 4% - 12% of all NHL and 1 - 4% of all gastrointestinal tumours (Gale et al., 2000). Intestinal lymphomas account for 20 – 40% of all lymphomas of the gastrointestinal tract (Domizio et al., 1993). Primary T-cell gastrointestinal lymphomas are rare. The only defined clinicopathological entity is EATL (Isaacson and Du, 2005). The current 2008 WHO Classification of Tumours of Hematopoietic and Lymphoid Tissues defines two types of EATL: type I and type II EATL. The detailed histopathological picture of the tumour has been presented in the Chapter 1.

EATL is an uncommon type of aggressive lymphoma in most parts of the world, with an estimated annual incidence rate of 0.5 -1 per million people in Western countries (Catassi et al., 2002). Type I EATL is seen with greater frequency in areas with a high prevalence of coeliac disease (particularly Northern Europe), by contrast, type II EATL has a broader geographic distribution and is also seen in regions where coeliac disease is rare (Swerdlow et al., 2008).

Typically EATL occurs primarily in patients in the sixth or seventh decade of life. Usually, patients present with abdominal pain frequently associated with intestinal perforation. The published data on the clinical features of the disease are restricted to small retrospective series of patients, single centre studies (Gale et al., 2000) or subanalysis (Daum et al., 2003). In type I EATL, a small proportion of patients have a history of childhood onset of coeliac disease but most have a history of adult onset coeliac disease with or without a disease free period. In some patients type I EATL is diagnosed simultaneously with coeliac disease. Others do not have a diagnosis of coeliac disease at all. The clinical presentation of type II EATL is similar, with the exception that most patients do not demonstrate evidence of previous or co-existing coeliac disease (Swerdlow et al., 2008).

The relationship between type I EATL and coeliac disease is well established and characterised by positive serological tests, HLA-DQ2 or HLA-DQ8 expression and associated clinical findings such as dermatitis herpetiformis and hyposplenism (Diamanti et al., 2006) (Green and Cellier, 2007) (Cheung et al., 1998) (Isaacson, 1985)

(Howdle et al., 2003) (Salmi et al., 2006). By contrast, type II EATL, may be diagnosed in patients without a history of coeliac disease and thus an association with coeliac disease and other risk factors is not proven (Deleeuw et al., 2007).

Coeliac disease is the commonest food intolerance disorder in Western populations with a prevalence estimated at 0.5% – 1.0% (Dube et al., 2005) (Fasano et al., 2003) (Verbeek et al., 2008). In patients with coeliac disease the ingestion of gluten leads to chronic inflammation of the small intestinal mucosa, resulting in villous atrophy, crypt hyperplasia, plasmacytosis of the lamina propria and increased intraepithelial lymphocytes (Isaacson and Du, 2005). Previously, coeliac disease was considered a gastrointestinal disorder of childhood with classical symptoms (Green and Cellier, 2007). Currently, it is regarded as a chronic systemic autoimmune disease with a complex clinical picture (Green and Cellier, 2007), more commonly diagnosed in adults (Verbeek et al., 2008). The clinical picture includes such symptoms as: diarrhoea with abdominal pain or discomfort, gastroesophageal reflux, constipation or a more subtle presentation with iron-deficiency anaemia, osteoporosis or neurological symptoms (Green and Cellier, 2007). The only accepted treatment for coeliac disease is lifelong elimination of gluten from the diet (Green and Cellier, 2007). A small number of patients (2% – 5%) fail to improve on a gluten-free diet and are considered to have refractory coeliac disease (Daum et al., 2005). Refractory coeliac disease is defined as persisting villous atrophy with crypt hyperplasia and increased intraepithelial T lymphocytes in spite of a strict gluten-free diet for more than 12 months, or severe symptoms necessitating intervention independent of the duration of the diet (Cheung et al., 1998) (Al-Toma et al., 2007a). Refractory coeliac disease is classified as type 1, with intraepithelial lymphocytes of normal phenotype, or type 2 with clonal expansion of intraepithelial lymphocytes with aberrant phenotype (Green and Cellier, 2007). Patients with type 2 refractory coeliac disease have a limited life expectancy with a 5-year OS rate of 50% - 58% (Cheung et al., 1998) (Al-Toma et al., 2007a). The main cause of death in these patients is EATL (88.4%) (Al-Toma et al., 2007a).

The treatment options have historically included surgical resection with or without anthracycline-based chemotherapy (Daum et al., 2003) (Gale et al., 2000) or less commonly HDCT with ASCT (Al-Toma et al., 2007b) (Okuda et al., 2002) (Rongey et al., 2006). The results of treatment with surgery or conventional chemotherapy are poor and the reported prognosis of EATL is dismal with 5-year PFS and OS of approximately 3.2% and 19.7%, respectively (Gale et al., 2000). The results

of HDCT with ASCT are available only as case reports (Okuda et al., 2002) (Rongey et al., 2006) or small series of patients (Al-Toma et al., 2007b).

In the following two studies, we approached the problem of the treatment of EATL. In the first one, data on clinical presentation, treatment and outcome of patients with EATL were collected prospectively in a population-based setting in order to obtain a more accurate picture of the disease. In the second study, a novel treatment regimen, HDCT with IVE/MTX and ASCT was evaluated prospectively in a cohort of patients with EATL. As most research on EATL was performed before the introduction of the new WHO classification for purposes of clarity where the generic term EATL is used in this article it will refer to both forms of the disease.

4.2 Population-based study on epidemiology, clinical presentation and treatments modalities in EATL

4.2.1 *Materials and methods*

4.2.1.a *Patient selection*

The SNLG prospectively collected data on all patients with a diagnosis of lymphoproliferative disease in Scotland and the Northern region of England (population, circa 7.6 million) beginning in 1979. Total registration of all new cases was achieved during 1994 to 1998. The data collection was performed with a help of a specially designed questionnaire, see Appendix III. Records of patients with a new diagnosis of EATL made between 1994 and 1998 were selected for further evaluation and case records were reviewed. Original histopathological material was re-valuated by haematopathology experts.

4.2.1.b *Data collection and response assessment*

Data obtained included patient demographics, details of baseline evaluations, treatment and patient outcomes.

At baseline evaluation the collected data included: the results of clinical examination, laboratory tests, BM biopsy, endoscopy and imaging, including X-ray, ultrasound and computed tomography. Where appropriate, detailed operation notes were available to further inform definitive staging of disease. The extent of disease was recorded using published staging systems for primary gastrointestinal lymphomas:

Lugano and Manchester scores (table 4.1).

Stage	Definition
Lugano score for gastrointestinal lymphomas	
I	Confined to GI-tract (single primary or multiple non-contiguous sites of disease)
II1*	Local abdominal nodal involvement
II2*	Distant abdominal nodal involvement
IIE	Serosal penetration to involve adjacent organ or tissues
IV	Disseminated extranodal involvement or supradiaphragmatic nodal involvement
Manchester score for gastrointestinal lymphomas	
Ia	Tumour confined to one area of the gastrointestinal tract without penetration of the serosa
Ib	Multiple tumours confined to the gastrointestinal tract without penetration of the serosa
IIa	Tumour with local nodes histologically involved (gastric or mesenteric)
IIb	Tumour with perforation and / or adherence to adjacent structures
IIc	Tumour with perforation and peritonitis
III	Tumour with widespread nodal involvement (paraaortic or more distant nodes)
IV	Disseminated disease involving extra-lymphatic tissues not adjacent to the tumour (eg. liver, bone marrow, bone, lung etc.)

Table 4.1 Lugano and Manchester scores for gastrointestinal lymphomas. *) combinations like II1 E and II2 E are also possible

The available data on treatment included the treatment modality (surgery or chemotherapy), type of surgical procedure and chemotherapy type and number of cycles given.

Evaluation of the final response was performed three months after the end of treatment (day of operation or last day of chemotherapy, whichever was appropriate). Further follow-up was carried once a year when the patient records were re-valuated for possible relapse, treatment given and survival status. CR, PR and PD were defined according to the criteria reported by Cheson et al (Cheson et al., 2007).

4.2.1.c *Statistics*

Demographics and disease characteristics were summarized using descriptive statistics. The χ^2-test, Fisher's exact test, and t-test were used as appropriate to

investigate differences on proportions and means, respectively. PFS and OS rates were estimated according to the method of Kaplan and Meier and comparison between the groups was with log-rank test. PFS was calculated from date of diagnosis to the first documentation of progression during therapy, failure at the end of therapy, or further relapse or death from any cause during or after the end of treatment. OS was calculated from date of diagnosis to the date of death, or if no death occurred, to the last documented follow up for the patient. All tests were performed with a confidence interval of 95 % and statistical significance was defined as $p \leq 0.05$. Statistical analysis was performed with SPSS Version 13.0 for MAC OS X (SPSS Incorporated, Chicago, IL).

4.2.2 *Results*

4.2.2.a *Epidemiology of EATL in SNLG*

Between 1994 and 1998, in the population of approximately 7.6 million people in Scotland and the Northern Region of England, 4542 patients were diagnosed with NHL. Fifty-four had features of EATL, giving an overall incidence of 0.14/100 000 per year. The distribution of the registered EATL cases remained constant over the study period.

4.2.2.b *Clinical presentation of the patients*

The detailed patient characteristics are summarised in table 4.2. Median age at diagnosis was 57 years (range 28 – 82 years) and 21/54 (39%) patients were female. The majority of patients 37/54 (70%) had extensive disease (defined as Lugano stages II2, IIE and IV and Manchester stages IIb, IIc, III and IV). Tumour was arising from the jejunum and ileum in 52/54 (96%) patients; in the duodenum in one patient and the iliocaecal region in the remaining patient. BM was assessed in 33 patients at diagnosis with two patients having BM involvement 2/33 (6%). The majority of patients 46/52 (88%) presented with a reduced performance status (ECOG >1).

Clinical parameter	N (%)
Median age (range)	57 (28 – 82)
Female sex	21/54 (39)
Lugano stage	
I	8/53 (15)
II 1	8/53 (15)
II 2	6/53 (11)
II E	0/53 (0)
II 1E	0/53 (0)
II 2E	21/53 (40)
IV	10/53 (19)
Manchester stage	
Ia	5/53 (9)
Ib	3/53 (6)
IIa	8/53 (15)
IIb	4/53 (8)
IIc	17/53 (32)
III	6/53 (11)
IV	10/53 (19)
Site of disease in GT	
Jejunum / ileum	52/54 (96)
Stomach / duodenum	0/54 (0)
Duodeno-jejunal flexure	1/54 (2)
Ileocaecal	1/54 (2)
Bone marrow involvement	2/33 (6)
ECOG > 1	46/52 (88)
Presenting features	
Pain	43/53 (81)
Lump	3/53 (6)
Nausea / vomiting	2/53 (38)
Weight loss	30/53(57)
Bowel upset	17/53 (32)
Perforation	21/53 (40)
Subacute obstruction	16/53 (30)

Table 4.2.A Population-based evaluation of EATL patient characteristics (n=54).

Clinical parameter	N (%)
Duration of symptoms pre-diagnosis	
0 – 1 months	17/51 (33)
1 – 3 months	13/51 (26)
3 – 6 months	15/51 (29)
> 6 months	6/51 (12)
Presence of coeliac disease	48/52 (92)
Prior to diagnosis	18/52 (34)
At diagnosis	30/52 (58)
Diagnosis at	
Surgery	49/54 (91)
Endoscopy	4/54 (7)
Post mortem	1/54 (2)
Abnormal chest X ray at diagnosis	3/45 (7)
Abnormal abdomen ultrasound at diagnosis	8/24 (33)
Abnormal chest CT at diagnosis	2/28 (7)
Abnormal abdomen CT at diagnosis	25/35 (71)
Abnormal Hb	27/50 (54)
Abnormal WBC	18/50 (36)
Abnormal kidney function	16/50 (32)
Abnormal liver function	39/51 (76)
Abnormal LDH	11/26 (42)

Table 4.2.B Population-based evaluation of EATL patient characteristics (n=54).

Presenting symptoms were assessed in 53 patients. Pain was the most commonly observed symptom in 43/53 (81%) of patients, followed by weight loss 30/53 (57%), visceral perforation 21/53 (40%), nausea/vomiting 20/53 (38%), change in bowel habit 17/53 (32%), subacute obstruction 16/53 (30%) and palpable mass 3/53 (6%). Details of the duration of symptoms before the diagnosis of EATL were assessed in 51 patients. In 30/51 patients (59%) symptoms were present for less than 3 months before diagnosis and in 21/51 patients (41%) for more than 3 months. The coeliac disease status was available in 52 patients. The majority of patients 48/52 (92%) had co-existing coeliac disease; 18/52 (35%) were known coeliac patients and 30/52 (58%) had coeliac disease diagnosed co-incidentally with their lymphoma. Diagnosis of EATL was made at laparotomy in 49/54 patients (91%), in four patients at endoscopy and in one patient at post mortem.

Various imaging techniques were used for diagnostic and staging purposes including computed tomography of the abdomen in 35/54 (65%) patients. Seventy-one per cent of studies (25/35) were abnormal. Abdominal ultrasound was performed in 25/54 (46%) patients with abnormal results in 8/25 (32%). Additional staging procedures included chest X-ray in 45/54 (83%) patients with only 3 patients having abnormal results (7%) and computed tomography of the chest in 28/54 (52%) patients, with pathological findings in only 2/28 (7%) patients.

At diagnosis the majority of patients 39/51 (76%) had abnormal liver function tests mainly due to low albumin levels. Hb was abnormal in 27/50 (54%) and LDH level in 11/26 (42%) patients. WBC and kidney function tests were abnormal in approximately one third of patients.

4.2.2.c *Treatment modalities*

Forty-nine patients (91%) presented with acute abdominal symptoms, necessitating emergency surgical procedures performed with diagnostic and therapeutic intent. Thirty of those patients (56%) were treated with additional systemic chemotherapy. Five patients (9%) received systemic chemotherapy only and no surgery. Thus, 35 patients were treated with chemotherapy with or without surgery, and 19 patients were treated with surgery alone.

The majority of patients treated with chemotherapy 31/35 (89%) received anthracycline-based chemotherapy: 17 patients received CHOP, 7 patients CNOP, 7 vincristine, doxorubicin, etoposide, prednisolone, cyclophosphamide, bleomycin (VAPEC-B) and one patient cyclophosphamide, vincristine and prednisolone (CVP). Three patients were treated up-front with the HD regimen IVE/MTX, one went on to receive BEAM and ASCT. Twenty-three patients (66%) completed their scheduled chemotherapy regimen. The remaining 12 patients (34%) discontinued the scheduled treatment due to PD or side effects. Importantly, there were no statistically significant differences between patients treated with systemic chemotherapy with or without surgery and those with surgery alone, with the exception of a higher number of elderly patients in the latter group.

4.2.2.d *Responses and outcome*

Seventeen patients (32%) achieved CR at the end of their treatment: 2/19 (11%) patients treated with surgery alone and 15/35 (43%) patients treated with chemotherapy

with or without surgery. The difference between groups was statistically significant, p=0.02; see table 4.3. Forty-four patients (81%) died; more in the surgery alone group - 18/19 (95%) than in the chemotherapy with or without surgery group - 26/35 (74%); though the difference was not statistically significant, p=0.08. The majority of patients died due to disease progression or complications of treatment.

	all patients No (%)	surgery alone No (%)	chemotherapy +/- surgery No (%)	p-value*
Response				
CR/PR	17/54 (32)	2/19 (11)	15/35 (43)	0.02
Failure	37/54 (69)	17/19 (89)	20/35 (57)	
Death	44/54 (81)	18/19 (95)	26/35 (74)	0.08

Table 4.3 Patient outcome in population-based evaluation, all patients (n=54), patients treated with surgery alone (n=19) vs patients treated with chemotherapy +/- surgery (n=35). *) patients treated with surgery alone vs patients treated with chemotherapy +/- surgery.

For all patients median PFS was 3.4 months and median OS 7.1 months (figure 4.1). 5-year PFS and OS for all patients were 18% and 20%, respectively (figure 4.1).

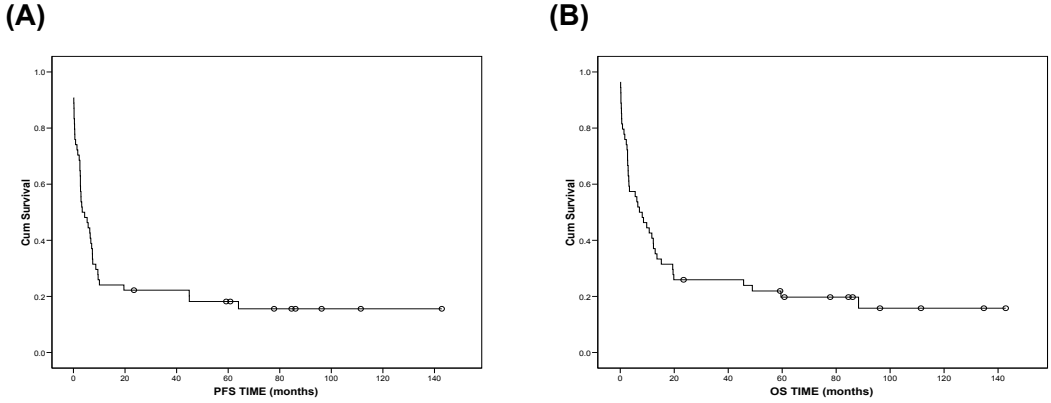

Figure 4.1 Kaplan-Meier plots for PFS and OS in population-based evaluation of patients with EATL, all patients (n=54). **(A)** PFS and **(B)** OS.

There were statistically significant differences for both PFS and OS between patients treated with chemotherapy with or without surgery and those treated with surgery alone (p<0.001). Five-year PFS and OS for patients from the chemotherapy with or without surgery group were 26% and 28%, respectively whereas none of patients treated with surgery alone survived at this time.

4.2.3 *Discussion*

The epidemiology, clinical features, treatment and outcome of EATL have not been studied extensively particularly in a population-based setting. Most currently available information comes from sub-analyses of bigger trials (Daum et al., 2003), retrospective evaluations of single centre experiences (Gale et al., 2000) or evaluation of national registries (Verbeek et al., 2008). Verbeek et al, conducted a nation-wide study of incidence of EATL in the Netherlands (Verbeek et al., 2008). This study utilised the resources of the nation-wide network and registry of histo- and cytopathology reports available in the Netherlands (PALGA). The study covered the entire population of the country, however the data evaluated were rather sparse and restricted to age, gender, site of disease and coeliac disease status. Daum et al, performed a non-randomized prospective trial of CHOP in intestinal lymphoma, however EATL was evaluated together with other subtypes of T-cell lymphoma (Daum et al., 2003). Al-Toma, assessed the survival in refractory coeliac disease and EATL (Al-Toma et al., 2007a). This was a single-centre retrospective evaluation and delivered some very interesting information on EATL that developed from refractory coeliac disease type II. The most comprehensive data was presented in a retrospective evaluation of a lymphoma database from the Wessex Regional Medical Oncology Unit in Southampton by Gale et al (Gale et al., 2000). The cases were carefully evaluated for presenting features, treatment and outcome.

The incidence of EATL in our population study was 0.14/100.000/year and EATL accounted for only 1.4% of all registered NHL cases. The median age of patients was 57 years and the majority of patients were male (61%). The results of another population-based study by Verbeek et al are comparable with ours, showing an incidence of EATL of 0.10/100.000/year, a median age of 64 years and 64% of patients were male (Verbeek et al., 2008). The predominance of male gender with 74% male patients was also reported by Gale et al (Gale et al., 2000), but by contrast in the study of Daum et al the majority of patients were female (Daum et al., 2003). Al-Toma reported predominance of male patients in de-novo EATLs but of females in EATLs that developed on a background of refractory coeliac disease type 2 (Al-Toma et al., 2007a). The median age of patients was lower than in our group in the other British study by Gale et al (50 years) (Gale et al., 2000) whereas in the remaining studies the median age of patients was greater than 60 years (Daum et al., 2003) (Verbeek et al., 2008).

The majority of our patients presented with a reduced performance status, with an ECOG score of >1 in 88% of patients, mainly due to high numbers of patients with acute abdominal symptoms and poor nutritional status. This observation was confirmed in the studies of Gale et al and Daum et al (Gale et al., 2000) (Daum et al., 2003). In our study, as in the studies published by Gale et al, Daum et al and Verbeek et al the diagnosis was predominantly made at laparotomy (Gale et al., 2000) (Daum et al., 2003) (Verbeek et al., 2008). Daum et al additionally distinguish between an elective and emergency procedure, both being equal in the T-cell lymphoma group. In our cohort of patients the majority underwent an emergency procedure as they presented with an acute abdomen. The pattern of presenting symptoms in our evaluation and those presented by Gale et al and Daum et al was comparable with pain, nausea/vomiting and weight loss being the most common (Gale et al., 2000) (Daum et al., 2003).

The majority of patients (70%) in our study presented with advanced stage disease. This was confirmed by Daum et al and Gale et al, however both these studies used the Ann Arbor staging system, which presents significant limitations for the assessment of intestinal lymphomas (Gale et al., 2000) (Daum et al., 2003). Although, both the surgical and pathological reports were reviewed, it was very difficult to determine the tumour localisation in the small intestine. Thus all the tumours of jejunum and ileum were assigned to one common group. However, other studies have confirmed the jejunum as the most common site (Gale et al., 2000) (Daum et al., 2003). As in other studies, infiltration of the colon was continuous with the iliocaecal region. We did not observe any patients with secondary involvement of the colon.

Coeliac disease was diagnosed in 92% of patients, 34% had previous records of coeliac disease and in 58% of patients the diagnosis of coeliac disease and of EATL were made simultaneously. In the Dutch population-base registry study, coeliac disease was reported in 47% of patients, however this was not a mandatory field in the register questionnaire (Verbeek et al., 2008). In the study of Gale et al, a third of patients had a previous history of coeliac disease but there is no information on the presence or absence of coeliac disease in the patient's histopathological samples (Gale et al., 2000). The recent WHO 2008 classification of EATL distinguishes between EATL with and without a diagnosis of coeliac disease. However, we should bear in mind that due to changes in diagnostic criteria for coeliac disease and the current availability of more advanced laboratory tests any more valid conclusions from previous studies are difficult.

Sixty-five per cent of our population study patients were treated with systemic chemotherapy, mostly anthracycline-based, with or without a surgical procedure and 35% were treated with surgery alone. These results are similar to those previously published by Gale et al and Daum et al (Gale et al., 2000) (Daum et al., 2003). The study of Al-Toma, which includes more recent patients, additionally registered several patients treated with ASCT (Al-Toma et al., 2007a). One reason for withholding chemotherapy was the poor condition of patients after surgery. In addition, the median age of patients treated with surgery alone was statistically significantly higher than patients treated with systemic chemotherapy with or without surgery. As in other published studies, only around half of our population study patients completed their scheduled chemotherapy; the main reasons for discontinuation being refractory disease and toxicity (Gale et al., 2000) (Daum et al., 2003).

The outcome of the whole population study group was very poor; 81% died and the median PFS and OS were 3.4 months and 7 months, respectively. 5-year PFS and OS for the whole group were 20% and 18%, respectively. Gale et al in their study reported 5-year PFS and OS of 3.2% and 19.7%, (Gale et al., 2000) respectively and Daum et al 2 year OS of 28% (Daum et al., 2003). Patients treated with chemotherapy with or without surgery had a significantly better outcome than those treated with surgery alone. Because of the lack of published data on survival of patients treated with surgery alone, further comparison is impossible. However, the outcome in our study for patients treated with chemotherapy with and without surgery was comparable with that previously reported (Gale et al., 2000) (Daum et al., 2003).

In conclusion, EATL is a rare lymphoma, which mostly presents in male patients aged more than 50 years. Approximately one third of patients have a previous history of coeliac disease and other 50% are diagnosed with coeliac disease at the same time as their diagnosis of EATL. Most patients undergo emergency surgical procedures at presentation due to acute abdominal symptoms and are treated with conservative treatment procedures including surgery and chemotherapy with anthracycline-based regimens. The outcome of patients treated with surgery alone is dismal but results can be improved by the addition of systemic chemotherapy.

4.3 Study on novel treatment approach: ifosfamide, etoposide, epirubicin / methotrexate (IVE/MTX) followed by autologous stem cell transplant (ASCT) in EATL

4.3.1 *Materials and methods*

4.3.1.a *Patient selection*

In view of the dismal outcomes obtained with conventional anthracycline-based chemotherapy, from 1998 onwards the SNLG introduced a novel treatment regimen for patients with a diagnosis of EATL. The treatment decision was made by a panel of specialist haematologists. The eligibility criteria for the IVE/MTX regimen were: de novo EATL, age ≥18 years and ability to tolerate HD treatment. Patients were discussed at multidisciplinary unit meetings, and all patients who were assigned to receive the new treatment were evaluated. The data collection was performed with a help of a specially designed questionnaire, see Appendix III.

4.3.1.b *Study design and treatment*

The IVE/MTX regimen begins with one course of CHOP chemotherapy (cyclopospham ide 750mg/m^2 - day 1, doxorubicin 50mg/m^2 - day 1, vincristine 2mg - day1 and prednisone 100mg - days 1-5); followed by three courses IVE (ifosfamide 3000mg/m^2 on days 1 – 3, epirubicine 50mg/m^2 on day 1 and etoposide 200mg/m^2 on days 1 – 3) alternating with intermediate dose methotrexate 1500mg/m^2 in a 24 hr infusion (table 4.4).

Drug	Dose	Route	Day of cycle
IVE			
Epirubicin	50mg/m^2	IV	1
Etoposide	200mg/m^2	IV	1 - 3
Ifosfamide	3000mg/m^2	IV	1 - 3
Intermediate MTX			
Methotrexate	1500mg/m^2	IV	1

Table 4.4 Chemotherapy dosage for a novel IVE/MTX regimen.

ASCT was performed 4 – 6 weeks after the last course of IVE/MTX if the patient was in remission and sufficient numbers of PBSC had been collected. Stem cell harvesting

was performed after the second or third course of IVE. The treatment schedule is shown in figure 4.2.

Conditioning for ASCT was either HD melphalan/total body irradiation (TBI): melphalan, 140mg/m^2 on day 1; TBI, 1200 cGy in 6 fractions over 3 days or BEAM: carmustine, 300mg/m^2 on day 1, etoposide: 200mg/m^2, cytarabine: 200mg/m^2 bd on days 2–5, and melphalan: 140 mg/m^2 on day 6.

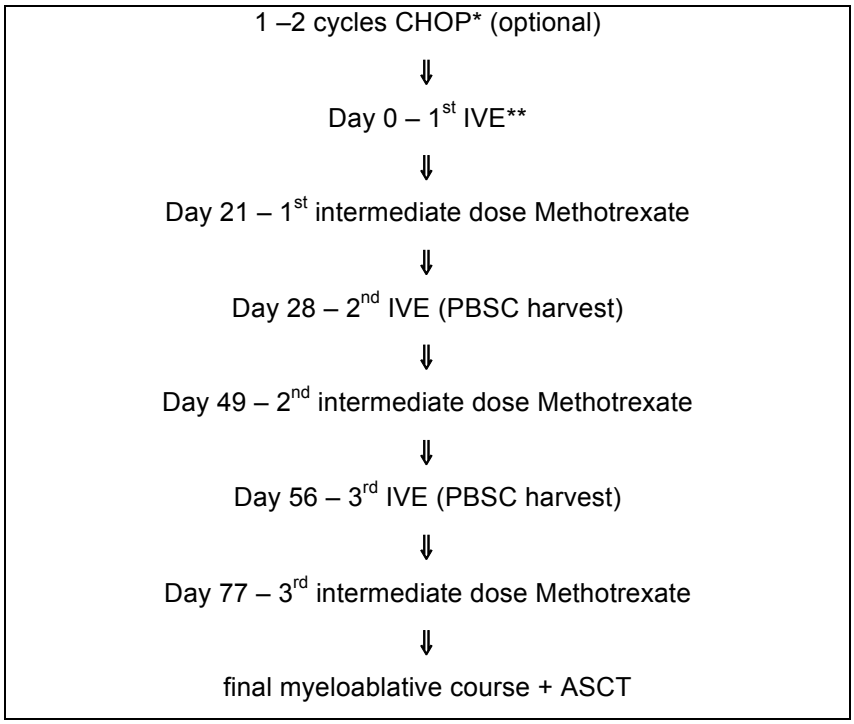

Figure 4.2 Treatment flowchart for IVE/MTX. *) CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone **) IVE: ifosfamide, epirubicin, etoposide

4.3.1.c *Response assessment*

At baseline evaluation the extent of disease was assessed by clinical examination, laboratory tests, BM biopsy and imaging, including X-ray, ultrasound and computed tomography. Where appropriate endoscopy and emergency surgery were performed. Detailed operation notes were available to further inform definitive staging of disease. The extent of disease was recorded using Lugano and Manchester scores (table 4.1).

Evaluation of the final response was performed 3 months after the end of treatment. Further follow-up was carried out at 3 monthly intervals during the first year and thereafter at 6 monthly intervals. All sites of initial disease were reassessed using appropriate techniques.

CR, PR and PD were defined according to the criteria reported by Cheson et al

(Cheson et al., 2007). Safety was assessed continuously during the study on the basis of reports, laboratory tests and vital sign measurements. Adverse events were categorized according to the Common Toxicity Criteria of WHO.

4.2.1.d *Statistics*

Demographics and disease characteristics were summarized using descriptive statistics. The χ^2-test, Fisher's exact test, and t-test were used as appropriate to investigate differences on proportions and means, respectively. PFS and OS rates were estimated according to the method of Kaplan and Meier and comparison between the groups was with log-rank test. PFS was calculated from date of diagnosis to the first documentation of progression during therapy, failure at the end of therapy, or further relapse or death from any cause during or after the end of treatment. OS was calculated from date of diagnosis to the date of death, or if no death occurred, to the last documented follow up for the patient. All tests were performed with a confidence interval of 95 % and statistical significance was defined as $p \leq 0.05$. Statistical analysis was performed with SPSS Version 13.0 for MAC OS X (SPSS Incorporated, Chicago, IL).

4.3.2 *Results*

4.3.2.a *Patient characteristics*

From 1998 onwards 26 patients with de novo EATL were treated with IVE/MTX in 7 centres around the UK (Newcastle upon Tyne, Stoke on Trent, Edinburgh, Inverness, Poole and Stafford). In our experience, the novel treatment can be tolerated by most patients presenting with EATL. During the study time 17 patients presented with a diagnosis of EATL to the Newcastle Hospitals Lymphoma Multidisciplinary Team, the primary site where the protocol was initiated, and 12 patients (71%) were enrolled. The remaining 5 patients were excluded from the new protocol because of advanced age in 3 cases and poor general condition in 2 (failure to recover from primary surgery).

Generally, patient characteristics at baseline were similar to those of patients treated with anthracycline-based chemotherapy from our population-based evaluation, see table 4.5. There was no statistically significant difference between both groups according to age, sex, and features at presentation except for a greater number of

patients presenting with nausea/vomiting (p=0.029) and a lower number of patients with diagnosed coeliac disease (p=0.02).

Clinical parameter	IVE/MTX No (%)	Anthracycline chemotherapy No (%)	p-value
Median age (range)	56 (36 – 69)	56 (30 – 82)	0.284
Female sex	9/26 (35)	10/31 (32)	0.851
Lugano stage			
I	4/26 (15)	6/30 (20)	0.055
II 1	5/26 (19)	4/30 (13)	
II 2	2/26 (8)	5/30 (17)	
II E	3/26 (12)	10/30 (33)	
II 1E	6/26 (23)	0/30 (0)	
II 2E	1/26 (4)	0/30 (0)	
IV	5/26 (19)	5/30 (17)	
Manchester stage			
Ia	1/26 (4)	3/30 (10)	0.875
Ib	3/26 (12)	3/30 (10)	
IIa	5/26 (19)	4/30 (13)	
IIb	5/26 (19)	3/30 (10)	
IIc	4/26 (15)	7/30 (23)	
III	3/26 (12)	5/30 (17)	
IV	5/26 (19)	5/30 (17)	
Site of disease in GT			
Jejunum / ileum	22/26 (84)	30/31 (97)	0.231
Stomach / duodenum	2/26 (8)	0/31 (0)	
Duodeno-jejunal flexure	2/26 (8)	1/31 (3)	
Ileocaecal	0/26 (0)	0/31 (0)	
Bone marrow involvement	1/22 (5)	1/23 (4)	0.999
Presenting features			
Pain	25/26 (96)	23/30 (77)	0.056
Lump	1/26 (4)	1/30 (3)	0.999
Nausea / vomiting	18/26 (69)	12/30 (40)	0.029
Weight loss	19/26 (73)	16/30 (53)	0.128
Bowel upset	15/26 (58)	10/30 (33)	0.067
Perforation	8/26 (31)	12/30 (40)	0.472
Subacute obstruction	10/26 (39)	10/30 (33)	0.690

Table 4.5.A Patient characteristics – patients treated with IVE/MTX (n=26) vs patients treated with anthracycline-based chemotherapy (n=31).

Clinical parameter	IVE/MTX No (%)	Anthracycline chemotherapy No (%)	p-value
ECOG > 1	20/26 (77)	25/29 (86)	0.490
Pre-diagnosis symptom duration			
0 – 1 months	6/25 (24)	11/28 (39%)	0.567
1 – 3 months	7/25 (28)	7/28 (25%)	
3 – 6 months	5/25 (20)	6/28 (21%)	
> 6 months	7/25 (28)	4/28 (14%)	
Presence of coeliac disease	19/26 (73)	28/29 (97)	0.020
Prior to diagnosis	11/26 (42)	11/29 (38)	
At diagnosis	8/26 (31)	17/29 (59)	
Diagnosis at			
Surgery	23/26 (89)	26/31 (84)	0.999
Endoscopy	3/26 (11)	4/31 (13)	
Post mortem	0/26 (0)	1/31 (3)	
Abnormal Hb	11/26 (42)	14/28 (50)	0.571
Abnormal WBC	9/26 (35)	9/28 (32)	0.847
Abnormal kidney function	11/25 (44)	8/29 (28)	0.208
Abnormal liver function	16/25 (64)	22/29 (76)	0.341
Abnormal LDH	9/19 (47)	6/19 (32)	0.319

Table 4.5.B Patient characteristics – patients treated with IVE/MTX (n=26) vs patients treated with anthracycline-based chemotherapy (n=31).

The median age was 56 years (range 36 – 69 years) and 9/26 (35%) patients were female. The majority of patients 17/26 (66%) presented with advanced disease (Lugano stage II2, II E, II1 E, II2 E and IV or Manchester stage IIb, IIc, III and IV). In the majority of patients 22/26 (84%), the primary site of disease in the gastrointestinal tract was jejunum/ileum. Of the remaining four patients, two had involvement of stomach and duodenum and the other two the duodeno-jejunal flexure. Among 22 patients with BM assessment at diagnosis, marrow involvement was confirmed in one. The performance status as measured by ECOG was greater than 1 in 20/26 (77%) patients. The most frequently presenting symptoms at diagnosis were: pain in 25/26 (95%) patients, weight loss in 19/26 (73%) patients and nausea/vomiting in 18/26 (80%) patients. In half of the patients the symptoms had been present for less than 6 months. The majority of patients (19/26 (73%)) were diagnosed with coeliac disease; 11/26 (42%) were known to have coeliac disease before the diagnosis of EATL and 8/26

(31%) were diagnosed co-incidentally with their lymphoma. In the majority of patients, 23/26 (89%) the diagnosis of EATL was made at laparotomy. The laboratory tests most frequently abnormal were: liver function in 16/25 (64%) patients and serum LDH level in 9/19 (47%).

4.3.2.b *Treatment feasibility and toxicity*

All 26 patients who were assigned to the treatment went on to start it and were available for evaluation (figure 4.3). In 3 patients methotrexate was omitted from the scheduled chemotherapy because of kidney insufficiency or poor general condition. Five patients (19%) discontinued treatment prematurely; 4 because of toxicities (1 severe sepsis, 1 severe encephalopathy, 1 BM failure and 1 gastrointestinal bleeding), and the other patient progressed after the initial cycle of CHOP. Of the remaining 21 patients, 14 received ASCT. In the remaining 7 patients ASCT was omitted because of poor general condition in 3 patients, refractory disease in 2 patients, insufficient stem cell mobilisation in one, and one patient declined further treatment. Importantly, there was no statistically significant difference in the number of patients who discontinued treatment between the group of patients treated with IVE/MTX and the historical group of 31 patients treated with anthracycline-based regimens (54% vs. 65%; p=0.43).

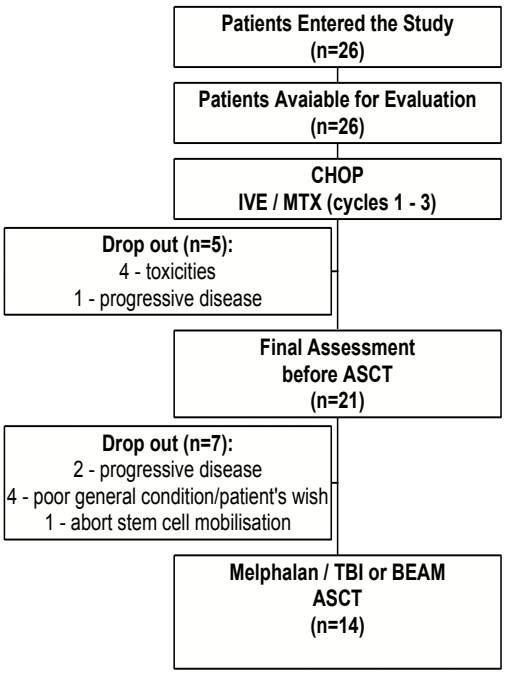

Figure 4.3 Flow diagram of patients with EATL treated with IVE/MTX.

Details of the conditioning regimen for the ASCT procedure were available in 13 patients; 7 of them received BEAM and 6 HD melphalan/TBI. The source of stem cells was peripheral blood in the majority of patients, 11/13 (85%) Data on the number of $CD34^+$ stem cells re-infused was available in 11 patients, and the median number was 6.0 $x10^6$/kg (range 1.0 $x10^6$/kg to 14.8 $x10^6$/kg). Information on growth factor use after ASCT was available in 10 patients, 5 received granulocyte colony-stimulating factor. Table 4.6 provides detailed description of the transplant procedures.

	No (%)
Stem cell source	
Peripheral blood	11/13 (85)
Bone marrow	2/13 (15)
Median number of CD34+ re-infused	6.0 $x10^6$/kg
	(range 1.0 – 14.8 $x10^6$/kg)
Growth factor used after ASCT	5 / 10 (50)
Conditioning regimen	
HD melphalan/TBI	7 / 13 (54)
BEAM	6 / 13 (46)

Table 4.6 Characteristic of autologous stem cell transplant (n=13).

The toxicity profile was acceptable for an intensive regimen. The most common toxicities were infection/sepsis in 14/26 (54%) patients. Other severe toxicities affecting 1 patient each were encephalopathy, BM failure, gastrointestinal bleeding and chronic renal failure. Six patients had disease-related complications during the treatment; 4 intestinal obstruction and 2 visceral perforation.

4.3.2.c *Responses and outcome*

Treatment results were compared with an historical control group treated with conventional anthracycline-based chemotherapy from our population-based series. There were no statistically significant differences between both groups according to age, sex and features at presentation, except for a greater number of patients presenting with nausea / vomiting (p=0.029) and a lower number of patients with diagnosed coeliac disease (p=0.02). Patients given IVE/MTX had an improved remission rate compared with patients treated with anthracycline-based chemotherapy (69% vs. 42%; p=0.06); table 4.7.

Death rates were significantly lower in patients treated with IVE/MTX, than in patients treated with anthracycline-based chemotherapy (39% vs. 81%; p=0.001). In both groups most deaths were due to underlying disease. Among the 14 patients who received a transplant, 2 died as a result of non-disease-related reasons: 1 because of *Pneumocistis jiroveci* pneumonia 4 months after ASCT and 1 because of sepsis and recurrent chest infections 12 months after ASCT.

	IVE/MTX No (%)	**Anthracycline chemotherapy No (%)**	**p-value***
Response to treatment			
CR	17/26 (65)	13/31 (42)	0.06
PR	1/26 (4)	0/31 (0)	
failure	8/26 (31)	18/31 (58)	
Death	10/26 (39)	25/31 (81)	0.001
Death lymphoma	8/26 (31)	19/25 (61)	0.005

Table 4.7 Patient outcome – patients treated with IVE/MTX (n=26) vs patients treated with anthracycline-based chemotherapy (n=31).*) p-value: patients treated with IVE/MTX vs patients treated with anthracycline-based chemotherapy from population-based evaluation

Five-year PFS and OS were significantly higher in patients receiving IVE/MTX than in those treated with anthracycline-based chemotherapy, (PFS: 52% vs. 22%; p=0.01 and OS: 60% vs. 22%; p=0.003).

Evaluation of patients who entered the protocol and completed the scheduled treatment versus those who failed to complete it confirmed that the number of patients with remissions was statistically significantly higher in the former group than in the latter: 13 patients (93%) vs. 5 (41%), respectively; p=0.004. There were fewer deaths and EATL – related deaths in the group that completed the protocol compared with the group that did not; however the difference was not statistically significant: 4 (29%) and 2 (14%) vs. 6 (50%) and 6 (50%); p=0.0422 and p=0.09, respectively. 5-years PFS and OS were both 68% for patients who completed the whole treatment and 33% and 50% for patients who did not; the difference is statistically significant for PFS (p=0.028) but not for OS (p=0.0251).

4.3.3 *Discussion*

Our study describes the role of HD chemotherapy followed by ASCT for the primary treatment of EATL in a cohort of representative patients. The outcome of patients treated with HDCT and ASCT reported previously varies from no response to remissions lasting more than 32 months (Al-Toma et al., 2007b) (Okuda et al., 2002) (Rongey et al., 2006) (Jantunen et al., 2004) (Blystad et al., 2001). In some cases the ASCT was performed in first remission (Al-Toma et al., 2007b), and in some second and subsequent remissions (Okuda et al., 2002) (Rongey et al., 2006), or remission status was not specified (Blystad et al., 2001). Therefore it is difficult to make any valid conclusions. Generally, the patients were treated with several cycles of anthracycline-based chemotherapy followed by myeloablative conditioning and ASCT.

The high number of patients failing conventional anthracycline-based chemotherapy mainly due to primary refractory disease/ disease progression and toxicity in our observation study and those published by others (Daum et al., 2003) (Gale et al., 2000), prompted the SNLG to reconsider our approach to this difficult group of patients. The promising results using IVE in patients with refractory / relapsed lymphomas (Zinzani et al., 2002) (McQuaker et al., 1997) (Proctor et al., 2001), encouraged us to introduce this regimen as the first-line treatment of choice for patients with EATL. We added intermediate dose methotrexate to the protocol as prophylaxis against previously described relapse / progression of disease in the central nervous system (Berman et al., 1998) (Tutt et al., 1997). Treatment starts with one cycle of CHOP to establish initial disease control to minimize the risk of side effects such as perforation, anastomotic breakdown, or wound dehiscence (in the post surgery patient), and to allow a limited recovery period before the more intensive regimen begins.

The new treatment was generally well tolerated compared with reports of anthracycline-based therapies from our group (64%) and other studies (Daum et al., 2003) (Gale et al., 2000). Most patients benefit from parenteral nutrition at least initially. The toxicity profile was similar to that described in the literature for HDCT regimens with ASCT (Okuda et al., 2002) (Rongey et al., 2006) (Al-Toma et al., 2007b). However, as expected, there was a higher incidence of neutropenic fever and sepsis when compared with anthracycline-based chemotherapy (Gale et al., 2000) (Daum et al., 2003). Significantly, stem cell harvest after IVE was successful in all

except one patient and engraftment was successful in all patients who received a transplant.

Patients treated with the novel regimen had statistically higher remission rates and lower mortality rates compared with patients receiving anthracycline-based chemotherapy from our population-based study (p=0.06 and p=0.001) and with series of patients from other studies (Gale et al., 2000) (Daum et al., 2003). Observed remissions, lasting up to 143 months, are longer than other published data on EATL treated with ASCT (Okuda et al., 2002) (Rongey et al., 2006) (Al-Toma et al., 2007b). The 5-years PFS and OS were also significantly higher compared with the group of patients treated with anthracycline-based chemotherapy from our population-based evaluation (p=0.01and p=0.003; respectively) and from other studies (Gale et al., 2000) (Daum et al., 2003).

Patients developing de novo EATL without a previous history of coeliac disease present no treatment dilemmas, and we have described improved results with our intensive approach. By contrast, the time to initiate treatment in patients with type II refractory coeliac disease is controversial. Some investigators suggest the early introduction of therapy before a diagnosis of EATL (Al-Toma et al., 2007c) as large numbers of patients with type II refractory coeliac disease may sooner or later develop EATL (Al-Toma et al., 2007a). Additionally, treatment of established EATL is associated with increased complications (Daum et al., 2003). In our opinion, the IVE/MTX is a very powerful regimen with potential severe toxicities and should be reserved for patients with a confirmed diagnosis of EATL. Future studies should evaluate techniques for early diagnosis of EATL in patients with type II refractory coeliac disease for example capsule enteroscopy, CT (Mallant et al., 2007), or PET-CT. No simple effective treatment has yet been shown to prevent development of EATL in such patients, but promising results are reported (Hadithi et al., 2006).

In conclusion, a novel treatment with IVE/MTX and ASCT was feasible with acceptable toxicities and could be applied to a majority of patients with de-novo EATL. The outcome of patients treated with the new regimen was significantly improved when compared with conventional treatment with anthracycline-based chemotherapy. Patients with EATL should be treated with systemic chemotherapy and where feasible with an aggressive treatment like IVE/MTX.

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Chapter 5. IVE/MTX followed by ASCT for the treatment of PTCL other than EATL

5.1 Introduction

PTCL are characterised by an unsatisfactory outcome. The reported OS rates at 5 years for PTCL treated with standard CHOP chemotherapy are 25-35%, (d'Amore, 2010), with the exception of ALK positive PTCL which has a more encouraging 5 year OS of 70% (Vose et al., 2008).

The prognostic impact of HDCT-ASCT in the treatment of PTCL was evaluated for the first time by the GELA group (Mounier et al., 2004). A matched pair analysis was performed for patients treated with HDCT-ASCT and patients receiving sequential consolidation for high-risk lymphomas, including PTCL, treated within studies LNH 87 and LNH 93. There was a trend towards a better outcome in HDCT-ASCT for B-cell lymphomas and particularly high-risk B-cell lymphomas, but not in patients with non-anaplastic T-cell lymphomas. There are several possible explanations for this finding. This study was the first to assess the role of HDCT–ASCT in the treatment of PTCL in a randomised fashion.

With increasing experience and improving supportive care, ASCT has become a more popular treatment modality for all types of lymphomas including PTCL and several analyses have been performed and published. The British Society of Bone Marrow Transplantation and Australasian Bone Marrow Transplant Recipient Registry conducted a study on PTCL patients treated with ASCT (Feyler et al., 2007). Among 82 evaluated patients, 31 received ASCT as consolidation therapy in first CR. The 2 year PFS and OS were 59% and 62%, respectively. The results are difficult to interpret as the study included many subtypes of PTCL namely ALCL, ATLL and cutaneous T-cell lymphoma. Additionally some of the patients received an allo-transplant. The Finnish survey on patients with PTCL treated with ASCT between 1990 and 2001 identified 37 patients. Histologies included PTCL-NOS, ALCL and other non-characterised cases (Jantunen et al., 2004). The 5-year PFS and OS were 64% and 63% with ALCL patients having a significantly better outcome. A further Scandinavian series of 40 patients with PTCL treated at two centres also reported better outcomes for ASCT patients in comparison to patients treated with standard anthracycline-based regimens (Blystad et al., 2001). The 3-year PFS and OS were 48% and 58%, respectively, but again the study

included patients with different subtypes and of different remission status. The Spanish Lymphoma and Autologous Transplantation Group presented data on a more homogenous cohort of 74 patients with ASCT in first CR after an anthracycline-based induction (Rodriguez et al., 2007b). With a median follow-up time of 67 months from diagnosis, the 5-year PFS and OS were 63% and 68%, respectively and transplant related mortality (TRM) was 4%. Several other analyses have been published on specific subtypes of PTCL like angioimmunoblastic lymphoma (AIL) (Schetelig et al., 2003) (Rodriguez et al., 2007a) with the biggest analysis by EBMT evaluating 146 patients (Kyriakou et al., 2008), ALCL (Deconinck et al., 2000) and NK/T-cell extranodal lymphoma, nasal type (Au et al., 2003). All these studies reported better outcomes compared with the results previously published for PTCL (The Non-Hodgkin's Lymphoma Classification Project, 1997) and its subtypes (Vose et al., 2008). All these studies were restricted only to evaluation of the conditioning regimen and the ASCT procedure itself. As most of the transplants were performed on patients in remission, they omitted the patients who never responded to induction chemotherapy. Additionally, most of the studies included patients of different remission status (first or subsequent remission, sensitive or non-sensitive disease). Thus, their value in evaluation of the role of HDCT and ASCT in improvement of 1st line treatment of PTCL is limited.

Currently there are only four published studies and one conference abstract which assess the role of upfront ASCT in PTCL, evaluating all patients assigned to the procedure from the beginning of the protocol (Reimer et al., 2009) (Mercadal et al., 2008) (Corradini et al., 2006) (Rodriguez et al., 2007c) (d'Amore et al., 2012). Even though none of the studies are randomized, they are presently our most reliable source of information.

After achieving very good results with a new regimen IVE/MTX followed by ASCT in EATL, the subgroup of PTCL with one of the worst prognosis, we elected to assess the regimen in other subtypes of PTCL. This approach aimed to evaluate both the effect of replacing the standard anthracycline-based induction with the new HD regimen and the impact of an up-front ASCT.

5.2 Materials and methods

5.2.1 *Patients selection*

From 1997 onwards, patients with a de-novo diagnosis of peripheral T-cell lymphoma including: ALCL, ALK negative, ALCL, ALK positive, PTCL, NOS, extranodal NK/T-cell lymphoma nasal type and hepatosplenic T-cell lymphoma were treated with the new protocol IVE/MTX-ASCT. The patients needed to be able to tolerate HD treatment. Patients were discussed at multidisciplinary unit meetings, and all patients who were assigned to receive the new treatment were evaluated. The data collection was performed with a help of a specially designed questionnaire, see Appendix IV.

5.2.2 *Study design and treatment*

The study protocol and regimen doses were the same as described in Chapter 4, paragraph 4.3.1.b *Study design and treatment*.

5.2.3 *Response assessment*

Documentation of disease extent at diagnosis, treatment and toxicities as well as evaluation of response remission criteria were the same as described in Chapter 4, paragraph 4.3.1.c *Response assessment*. However the Ann Arbor classification was used instead of the Lugano and Manchester scores, as these are designed particularly for gastro-intestinal disease and would have been inappropriate for nodal lymphomas. For used questionnaire see Appendix IV.

5.2.4 *Statistics*

The statistical methods used in the evaluation were the same as described in the Chapter 4, paragraph 4.2.1.d *Statistics*.

5.3 Results

5.3.1 *Patient characteristics*

Between 1998 and 2009, 31 patients with a diagnosis of PTCL were treated with the novel regimen in 3 centres (Newcastle upon Tyne, Middlesbrough and Stockton on Tees) around the Northern Region of England. The histopathology of patients was

heterogeneous with PTCL NOS being the most representative group with 17/31 (55%) cases; followed by 6 cases of ALCL, ALK positive; 4 cases of extranodal NK/T-cell lymphoma nasal type; 3 cases of ALCL, ALK negative and one case of hepatosplenic T-cell lymphoma (table 5.1).

Histopathological identity	No (%)
Peripheral T–cell Lymphoma not other specified	17 /31 (55)
Anaplastic large cell lymphoma (ALK positive)	6 / 31 (19)
Extranodal NK/T-cell lymphoma, nasal type	4 / 31 (13)
Anaplastic large cell lymphoma (ALK negative)	3 / 31 (10)
Hepatosplenic T-cell lymphoma	1 / 31 (3)

Table 5.1 Histopathological diagnosis of patients with PTCL treated with IVE/MTX, all patients (n=31).

The median age of the patients was 42 years (range 22 – 64) and approximately a third of patients were female. Most patients presented with a good performance status, with only 8/30 (27%) of patients having an ECOG >1. Early and advanced stages of disease were distributed equally within the group with 13/31 (42%) defined as early and 18/31 (58%) defined as advanced stage. Half of all patients presented with B-symptoms. One fifth of patients had bulky disease and two thirds had extranodal organs involved; nasopharynx in 8/30 (27%) patients, lung and sinuses each in 5/30 (15%) patients and BM in 4/30 (13%) patients. aaIPI could be calculated in 26 patients; 4/26 (15%) were in the low risk group, 13/26 (50%) in the low intermediate risk group, 6/26 (23%) in the high intermediate risk group and 3/26 (12%) in the high-risk group. Seven of twenty-nine (24%) patients had abnormal Hb and WBC levels at diagnosis and 14/27 (52%) had elevated LDH levels. Table 5.2 gives a summary of patient characteristics.

Clinical parameter	No (%)
Median age	42 (range 22 – 64)
Female sex	12 / 31 (39)
ECOG >1	8 / 30 (27)
B – symptoms	14 / 27 (52)
Clinical stage	
Early (I / II)	13 / 31 (42)
Advanced (III / IV)	18 / 31 (58)
aaIPI (for nodal disease origin only)	
Low	4 / 26 (15)
Low intermediate	13 / 26 (50)
High intermediate	6 / 26 (23)
High	3 / 26 (12)
Bulky disease	6 / 30 (20)
Extranodal disease	20 / 30 (66)
Extranodal organ involved	
Nasopharynx	8 / 30 (27)
Lung	5 / 30 (17)
Sinuses	5 / 30 (17)
Marrow involvement	4 / 30 (13)
Gastrointestinal tract	3 / 30 (10)
Lever and biliary tract	2 / 30 (7)
Spleen	2 / 30 (7)
Tongue	1 / 30 (3)
Breast	1 / 30 (3)
Heart	1 / 30 (3)
Pancreas	1 / 30 (3)
Abnormal Hb	7 / 29 (24)
Abnormal WBC	7 / 29 (24)
Abnormal LDH	15 / 27 (52)

Table 5.2 Characteristics of patients with PTCL treated with IVE/MTX, all patients (n=31).

5.3.2 *Treatment feasibility and toxicity*

All 31 patients who were allocated the new regimen started it and were available for evaluation. In four patients the methotrexate treatment had to be adjusted. In one patient because of poor general condition at diagnosis it was omitted completely and in another it was omitted after the first cycle. In the third patient methotrexate was stopped

because of liver failure after cycle 1 and in the fourth patient methotrexate was discontinued because of the suspicion of disease progression after 1st cycle. All patients continued treatment with IVE. Four patients (13%) discontinued treatment prematurely, three because of rapid disease progression and one because of poor general condition. Of the remaining 27 patients, 20 (65%) received ASCT. The ASCT was omitted because of inadequate stem cell collection in 4 patients, progressive disease in 2 patients and poor general condition in one patient (figure 5.1). Twelve patients (39%) received radiotherapy.

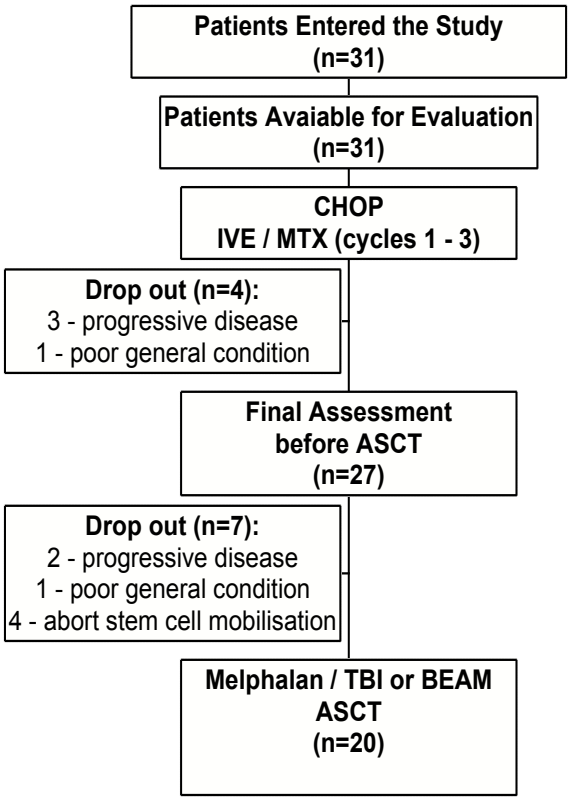

Figure 5.1 Flow diagram of patients with PTCL treated IVE/MTX.

Detailed information on ASCT was available in all transplanted patients (Table 5.3). The majority of patients, 13 (63%) received HD melphalan based conditioning regimens including melphalan/TBI in 6 patients (30%), melphalan/etoposide in 5 patients (25%) or melphalan alone in 2 patients (11%). The remaining 7 (35%) patients received BEAM conditioning. In all but one patient peripheral blood was the source of the CD34+ cells, the median number of transplanted cells was 5.2 $x10^6$/kg (range 1.7 – 26.3 $x10^6$).

	No (%)
Stem cell source	
Peripheral blood	19 / 20 (95)
Bone marrow	1 / 20 (5)
Median number of CD34+ re-infused	6.0 x10^6/kg
	(range 1.7 – 26.3 x10^6/kg)
Conditioning regimen	
BEAM	7 / 20 (35)
HD melphalan / total body irradiation	6 / 20 (30)
HD melphalan / etoposide	5 / 20 (25)
HD melphalan alone	2 / 20 (10)

Table 5.3 ASCT characteristic in patients with PTCL treated with IVE/MTX, all patients with ASCT (n=20).

The toxicity profile was acceptable for an intensive regimen. The most common acute toxicities were neutropenic sepsis / infection in 14/31 (45%) patients. Two patients suffered from severe nausea and vomiting requiring hospital admission. Other recorded severe toxicities occurring in one patient each were: deep fungal infection, haematuria, diarrhoea, visual hallucinations, pulmonary oedema, methotrexate induced liver failure and severe mucositis. Three patients suffered from late toxicities; two mood disorders, (in one patient accompanied by psoriasis arthritis) and one paraneoplastic pemphigus in the irradiated area. One patient developed a secondary malignancy: acute myeloid leukaemia.

5.3.3 *Response and outcome*

At evaluation after completion of induction and before ASCT 26/31 (84%) patients were in remission; 24/31 (77%) in CR and 2/31 (7%) in PR; 5/31 (16%) had PD. At evaluation after ASCT all transplanted patients were in remission: 19/20 (95%) in CR and 1/20 (5%) in PR. Using an intention to treat analysis: at the end of treatment 26/31 (84%) patients were in remission: 24/31 (77%) in CR and 2/31 (7%) in PR and 5/31 (16%) patients had PD (Table 5.4).

	all patients No (%)	PTCL other than ALCL, ALK positive No (%)	p-value
Response to treatment			
CR	24 / 31 (77)	19 / 25 (76)	>0.999
PR	2 / 31 (7)	2 / 25 (8)	
Failure	5 / 31 (16)	4 / 25 (16)	
Death	11 / 31 (35)	10 / 25 (40)	0.786
Death lymphoma	10 / 31 (32)	9 / 25 (36)	>0.999

Table 5.4 Response to treatment and outcome of patients with PTCL treated with IVE/MTX, all patients (n=31) and PTCL other than ALCL, ALK positive (n=25).

The remission rates remained the same when six patients with ALCL, ALK positive were excluded from the evaluation, with ORR of 21/25 (84%) with 19/25 (76%) CR and 2/25 (8%) PR and 4/25 (16%) with PD; (p>0.999). At the median follow-up time of 33 months, 11/31 (35%) patients had died, ten of them from lymphoma (32%) and one patient from infection complicating severe paraneoplastic pemphigus in the irradiated field. There were no differences in death rates after exclusion of ALCL, ALK positive cases with 10/25 (40%) patients dying (p=0.786); 9/25 (36%) from lymphoma (p>0.999).

The 3-year PFS for the whole group was 57% and OS 71% (figure 5.2). There were no differences in 3-year PFS (51%) and OS (68%) in an evaluation of all patients with PTCL and after exclusion of ALCL, ALK positive cases; p=0.709 and p=0.828 (figure 5.3). The 3-year PFS and OS of histological subgroups were as follows: ALCL, ALK positive: both 83%, PTCL NOS: 45% and 64%, ALCL, ALK negative: 33% and 67%, extranodal NK/T-cell lymphoma, nasal type: both 100% and for hepatosplenic T-cell lymphoma: both 0% (figure 5.4). Although the ALCL, ALK positive cases seem to have better outcomes than other PTCL subtypes with 3-year PFS and OS of 83%, the differences were not statistically significant when compared with the whole group of PTCL patients (p=0.244 and p=0.469).

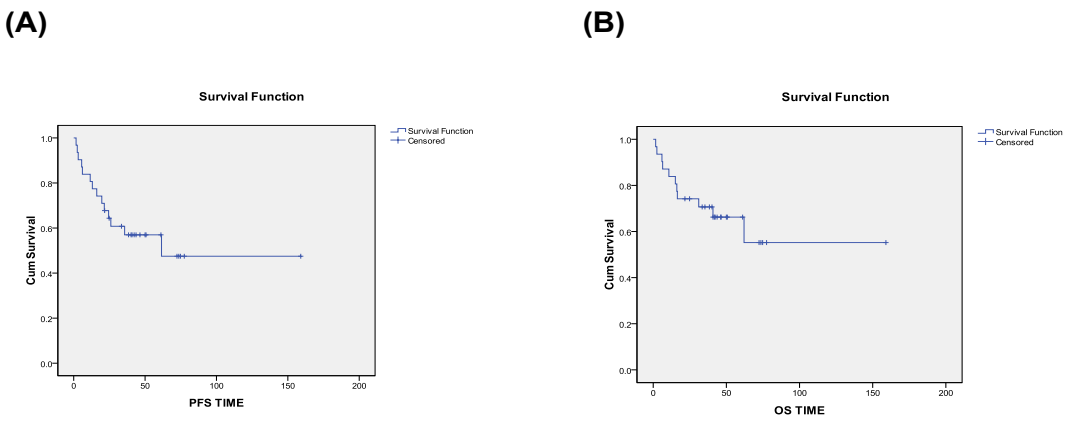

Figure 5.2 Kaplan-Meier plots for PFS and OS in patients with PTCL treated with IVE/MTX (n=31). **(A)** PFS and **(B)** OS.

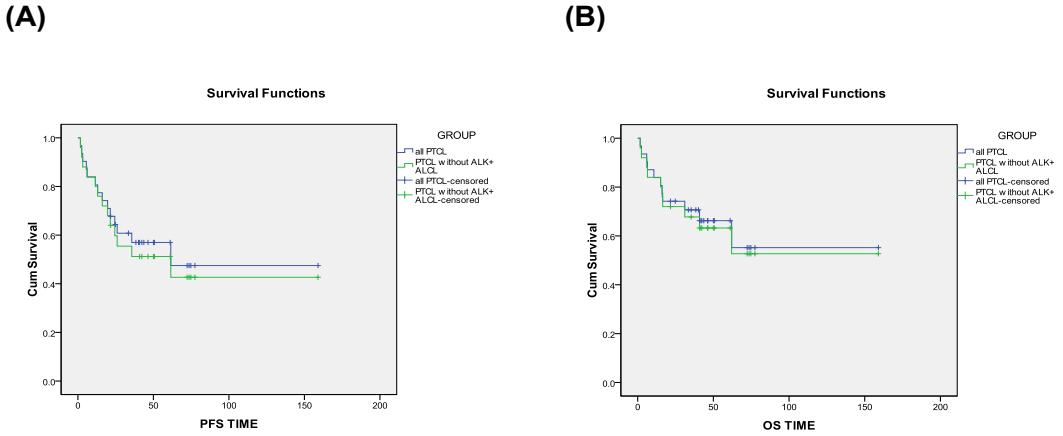

Figure 5.3 Kaplan-Meier plots for PFS and OS in patients with PTCL treated with IVE/MTX, all patients (n=31) vs PTCL other than ALCL, ALK positive (n=26); p=0.709 and p=0.828, respectively. **(A)** PFS and **(B)** OS.

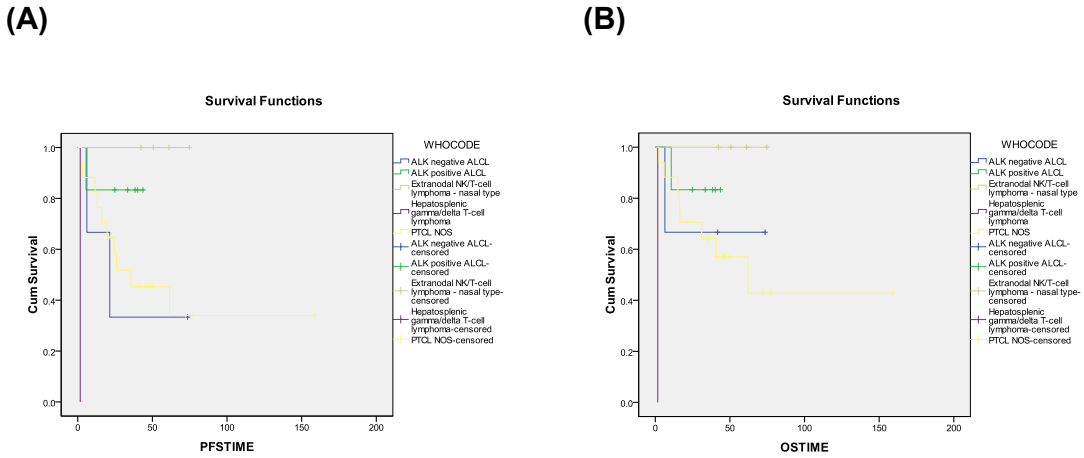

Figure 5.4 Kaplan-Meier plots for PFS and OS in patients with PTCL treated with IVE/MTX, different histological subtypes, all patients (n=31); p<0.001 for both. **(A)** PFS and **(B)** OS.

We observed a difference in PFS and OS between groups with low and low intermediate aaIPI and those with high and high intermediate aaIPI with 3-year PFS of 70% vs. 28% (p=0.068) and 76% vs. 67% (p=0.247), (figure 5.5), respectively.

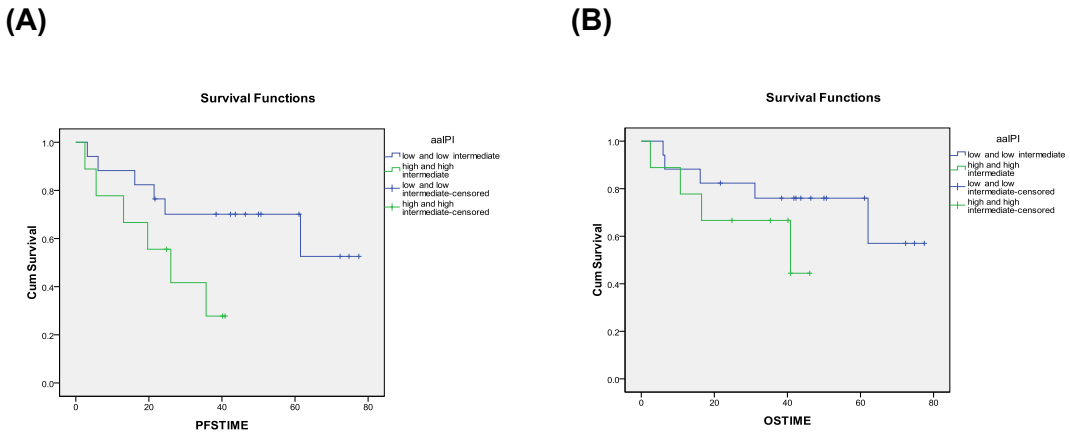

Figure 5.5 Kaplan-Meier plots for PFS and OS in patients with PTCL treated with IVE/MTX, patients with low and low intermediate aaIPI (n=17) vs high and high intermediate aaIPI (n=9); p=0.068 and p=0.247, respectively. **(A)** PFS and **(B)** OS.

After exclusion of ALCL, ALK positive the figures were 66% vs. 17% (p=0.057) and 73% vs. 67% (p=0.333), respectively (figure 5.6). The differences were however not statistically significant, probably because of small patients numbers.

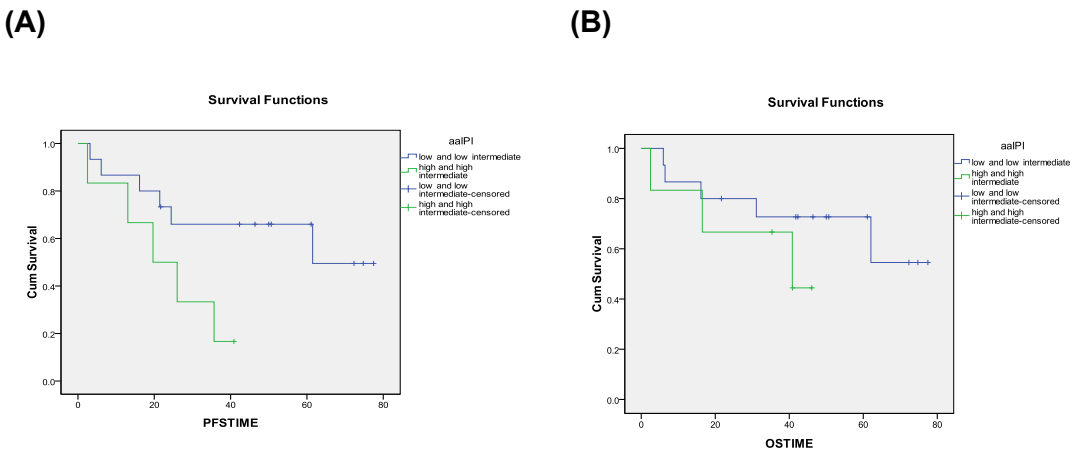

Figure 5.6 Kaplan-Meier plots for PFS and OS in patients with PTCL without ALCL, ALK positive treated with IVE/MTX, patients with low and low intermediate aaIPI (n=15) vs. high and high intermediate aaIPI (n=6); p=0.057 and p=0.333, respectively. **(A)** PFS and **(B)** OS

In an evaluation of response and failure patterns in all patients we observed that 16% (5 patients) progressed during the treatment, 10% (3 patients) had early post

treatment relapses (within 1 year from the end of treatment), 16% (5 patients) had late post treatment relapses (later than 1 year after the end of treatment) and 55% (17 patients) remained in long term remission.

5.3.4 *Role of ASCT*

In the evaluation of patients who completed the whole scheduled treatment including the ASCT and those who failed to complete it the ORR was statistically significantly higher in the former group than in the latter (table 5.5).

	Patients with ASCT No (%)	**Patients without ASCT No (%)**	**p-value**
Response to treatment			
CR	19 / 20 (95)	5 / 11 (45.5)	<0.001
PR	1 / 20 (5)	1 / 11 (9)	
Failure	0 / 20 (0)	5 / 11 (45.5)	
Death	5 / 20 (25)	6 / 11 (54.5)	0.132
Death lymphoma	4 / 20 (20)	6 / 11 (54.5)	0.455

Table 5.5 Response to treatment and outcome of patients with PTCL treated with IVE/MTX, patients with ASCT (n=20) vs. patients without ASCT (n=11).

In the group who completed treatment all 20 patients achieved remission; 19/20 (95%) CR and 1/20 (5%) PR. In the group of patients who failed to complete the regimen only 6/11 (55%) of patients achieved remission; 5/11 (45.5%) CR and 1/11 (9%) PR ($p<0.001$). There were also fewer deaths in the group of patients who completed the protocol - 5/20 (25%) compared with the group of patients who did not complete the regimen 6/11 (55%). However, the difference was not significant ($p=0.455$). All four patients who relapsed during the IVE/MTX treatment or before the ASCT died shortly after of progressive disease. Of two patients who stopped the regimen due to poor condition (one after 2nd methotrexate), one is alive and in ongoing remission at 6 years and the other who stopped the treatment just before ASCT died at 3 years. Three from four patients who stopped the regimen just before ASCT because of failed stem cell mobilisation are alive in remission at 3 years, 5 years and 6 years respectively; one patient relapsed after 2 years but is alive at 3 years.

The 3-year PFS and OS were 69% and 84%, respectively for patients who completed the whole treatment and 36% and 54% for patients who did not, the differences are not significant; p=0.072 and p=0.069, respectively (figure 5.7).

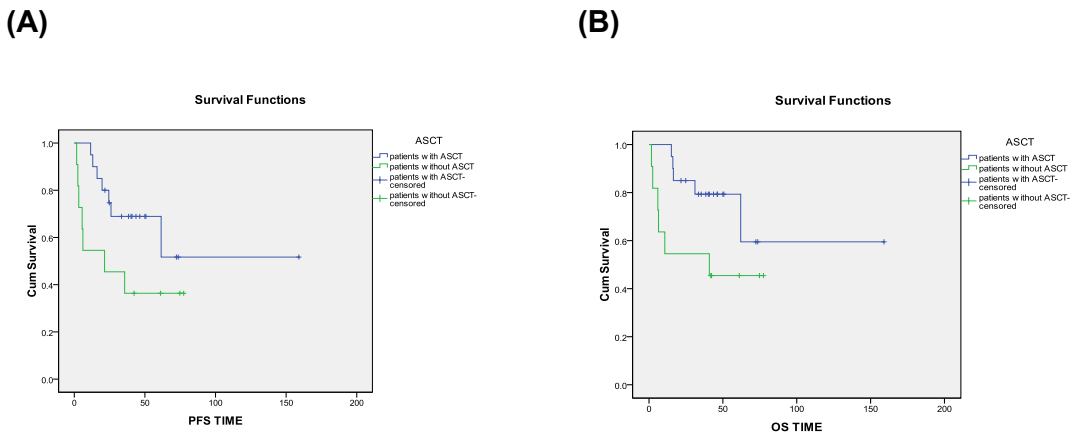

Figure 5.7 Kaplan-Meier plots for PFS and OS in patients with PTCL treated with IVE/MTX, patients with ASCT (n=20) and patients without ASCT (n=11), all patients (n=31); p=0.072 and p=0.069, respectively. **(A)** PFS and **(B)** OS.

The difference remained the same after exclusion of patients with ALCL, ALK positive: PFS: 59% and 40% (p=0.36), OS: 73% vs. 60% (p=0.294); (figure 5.8).

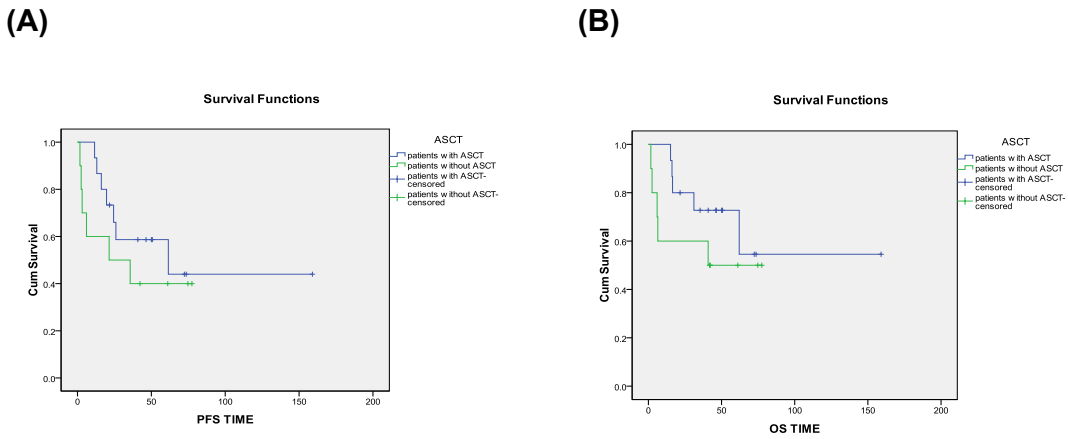

Figure 5.8 Kaplan-Meier plots for PFS and OS in patients with PTCL without ALCL, ALK positive treated with IVE/MTX, patients with ASCT (n=15) and patients without ASCT (n=10), p=0.36 and p=0.294, respectively. **(A)** PFS and **(B)** OS.

Comparison of patients who achieved CR/PR after completion of 3 cycles of IVE/MTX but did not receive ASCT due to insufficient stem cell harvest (4 patients) or poor general condition (1 patient), with those who received the whole protocol including

ASCT, revealed no differences in PFS and OS. The 3 year PFS and OS were 60% vs. 69% (p=0.998) and 100% vs. 79% (p=0.692); respectively.

5.4 Discussion

In view of disappointing results with conventional CHOP based treatment and after achieving very promising results with the new intensive regimen IVE/MTX followed by a myeloablative ASCT in EATL, we prospectively assessed the value of this regimen in other PTCL. The new regimen was feasible with acceptable toxicities. The overall response rate was 84% with a 3-year PFS of 57% and OS of 71%. There was no difference in ORR (84%), 3 year PFS (51%) and OS (68%) after exclusion of ALCL, ALK positive.

As previously mentioned, the study of Mounier et al was the first to assess HDCT-ASCT in patients with PTCL. A matched pair analysis was performed to assess the impact of phenotype on the role of HDCT-ASCT when compared with sequential consolidation for high-risk lymphomas treated within studies LNH 87 and LNH 93 (Mounier et al., 2004). Patients received a standard induction of four cycles of a CHOP-like regimen given every 2 weeks: doxorubicin 75mg/m^2 (day 1), cyclophosphamide 1200mg/m^2 (day 1), vindesine 2mg/m^2 (days 1 – 5), bleomycin 10mg/m^2 (days 1 – 5) and prednisone 60mg/m^2 with intrathecal methotrexate 15mg (day 2) or alternatively the same regimen with mitoxantrone 12mg/m^2 (day 1) instead of doxorubicin (NCVBP). Patients achieving CR/CRu in the HDCT-ASCT group received two cycles of HD methotrexate 3000mg/m^2 with leucovorin rescue, followed by myeloablative conditioning with CBV (cyclophosphamide 6000mg/m^2, BCNU 300mg/m^2 and etoposide 1000mg/m^2) followed by ASCT. Patients in the sequential consolidation group received two cycles of HD methotrexate 3000mg/m^2, with two cycles of ifosfamide 1500mg/m^2 and etoposide 300mg/m^2, one cycle of L-asparaginase 50000 mg/m^2 and a final two cycles of cytarabine 100 mg/m^2 (days 1 - 4). A description of the regimens and doses is given in table 5.6.

	Mounier	Mounier	Corradini	Corradini	Sieniawski
	4 ACVB ⇓ 2 HD MTX ⇓ 2 ifosfamide/etoposide ⇓ L-asparginase ⇓ 2 cytarabine	4 ACVB ⇓ 2 HD MTX	2 APO ⇓ 2 DHAP ⇓ HD cyclophosphamide ⇓ HD cytarabine ⇓ HD cisplatin/etoposide	MACOP-B ⇓ mitoxantrone / HD cytarabine	3 IVE/MTX
Cyclophosphamide	4800	4800		1400	
Doxorubicine	300	300	300	200	
Vincristine			5.6	5.6	
Prednisone	1200	1200	2100	5250	
Vindesine	16	16			
Bleomycin	80	80		20	
Methotrexare				800	
Dexamethasone			320		
Cytarabine			8000		
Cisplatin			200		
Epirubicine					150
L - asparginase	50000				
HD mitoxantrone				24	
HD cyclophosphamide			7000		
HD araC	1600		24000	12000	
HD cisplatin			100		
HD etoposide	600		2400		1800
HD methotrexate	6000	6000			4500
HD ifosfamide	3000				27000

Table 5.6.A Cumulative doses of different induction regimen for PTCL.

	Nickelsen	Reimer	Reimer	D'amore	Mercadal	Rodriguez	Rodriguez
	4 – 6 megaCHOEP	4 - 6 CHOP ⇓ ESHAP	4 - 6 CHOP ⇓ DexaBEAM	6 CHOEP	3 megaCHOP ⇓ 3 ESHAP	3 megaCHOP ⇓ 2 IFE	4 megaCHOP
Cyclophosphamide	16500 – 19500	3000 - 4500	3000 - 4500	4500	6000	6000	8000
Doxorubicine	280 – 420	200 - 300	200 - 300	300	270	270	360
Vincristine	8 – 12	8 - 12	8 - 12	12	6	2.8	2.8
Prednisone	2000 - 3000	2000 - 3000	2000 - 3000	3000	900	900	1200
Etoposide	3130 - 5200	160	300	1800	480	1800	
Dexamethasone			168				
Carmustine			60				
Melphalan			20				
Cytarabine		2000	800		6000		
Prednisone		1000			3000		
Ifosfamide						30000	
Cisplatin		100			300		

Table 5.6.B Cumulative doses of different induction regimen for PTCL.

There was an overall trend for better outcomes in the HDCT-ASCT group for B-cell lymphomas (5-year DFS: 72% vs. 67%; p=0.13 and OS: 79% vs. 77%; p=0.64). This was particularly marked in B-cell lymphomas with 2 - 3 aaIPI risk factors. This group of patients benefitted from HDCT-ASCT with statistically significance differences in 5-year DFS: 75% vs. 63%, p=0.001 and OS: 78% vs. 72%, p=0.04. By contrast, among 28 evaluable patients with non-anaplastic T-cell lymphoma there was no difference between HDCT-ASCT and sequential consolidation arms: with 5-year DFS of 49% and 44%, p=0.87 and OS of 45% vs. 38%, p=0.89 (Table 5.7). There are several possible reasons why HDCT-ASCT might not have shown an advantage in this group of patients. Firstly the overall results were unexpectedly very good in both treatment groups compared with other studies. Secondly the standard arm included not only established anthracycline-based chemotherapy but also additional sequential chemotherapy with relatively high-doses of very effective drugs including methotrexate, ifosfamide, etoposide, L-asparaginase and cytosine arabinoside. Thirdly the total number of patients with T-cell lymphomas was rather small, making comparison difficult. Fourthly there were no available details on histology, therefore the number of potential cases with a better prognosis (for example ALCL, ALK positive) remains unknown. However, this was the first study assessing the role of HDCT–ASCT in the treatment of PTCL in a prospective randomised trial.

Currently there are only four published studies and one conference abstract, which assess the role of upfront ASCT in PTCL and evaluate all patients assigned to the procedure from the beginning of the treatment (Reimer et al., 2009) (Mercadal et al., 2008) (Corradini et al., 2006) (Rodriguez et al., 2007c) (d'Amore et al., 2012). These studies will be briefly described below. Tables 5.6 and 5.7 provide an overview of drugs doses and responses.

Study	Patients	ORR after induction	ORR after ASCT	ORR intent to treat	PFS / OS
Mounier	28	NA	NA	NA	5yr PFS – 38% 5yr OS – 44%
Mounier	29	NA	NA	NA	5yr PFS – 45% 5yr OS – 49%
Nickelsen	33	NA	NA	ORR 18/33 (55%) CR 16/33 (49%) PR 2/33 (6%)	3yr PFS – 26% 3yr OS – 46%
Corradini	62	ORR 45/62 (72%) CR 35/62 (56%) PR 10/62 (16%)	ORR 46/46 (100%) CR 41/46 (89%) PR 5/46 (11%)	ORR 46/62 (74%) CR 41/62 (66%) PR 5/62 (8%)	3yr PFS - 49% 3yr OS - 62%
Rodriguez	26	ORR 19/26 (77%) CR 17/26 (65%) PR 3/26 (12%)	ORR 18/19 (94%) CR 17/19 (89%) PR 1/19 (5%)	ORR 19/26 (77%) CR 18/26 (69%) PR 1/26 (8%)	3yr PFS - 53% 3yr OS - 73%

Table 5.7.A Patient outcome in different high-dose regimen for PTCL.

Study	Patients	ORR after induction	ORR after ASCT	ORR intent to treat	PFS / OS
Mercadal	41	ORR 24/41 (59%) CR 20/41 (39%) PR 4/41 (10%)	ORR 19/19 (100%) NK	ORR 24/41 (59%) CR 21/41 (51%) PR 3/41 (8%)	3yr PFS - 34% 3yr OS - 43%
Reimer	83	ORR 59/83 (71%) CR 39/83 (47%) PR 20/83 (24%)	ORR 55/55 (100%) CR 48/55 (87%) PR 7/55 (13%)	ORR 55/83 (66%) CR 48/83 (58%) PR 7/83 (8%)	3yr PFS – 36% 3yr OS – 48%
D'Amore	160	ORR 132/155 (85%) CR 81/155 (52%) PR 51/155 (33%)	ORR 99/112 (88%) CR 81/112 (72%) PR 51/112 (16%)	NK	3yr PFS – 48% 3 yr OS – 57%
Sieniawski	31	ORR 26/31 (84%) CR 24/31 (77%) PR 2/31 (7%)	ORR 20/20 (100%) CR 19/20 (95%) PR 1/20 (5%)	ORR 26/31 (84%) CR 24/31 (77%) PR 2/31 (7%)	3yr PFS – 57% 3yr OS – 71%

Table 5.7.B Patient outcome in different high-dose regimen for PTCL.

Reimer et al performed a prospective study evaluating up-front ASCT in patients with PTCL younger than 65 years; ALCL, ALK positive and primary cutaneous lymphomas were excluded (Reimer et al., 2009). The patients were treated with 4 – 6 cycles of CHOP: cyclophosphamide 750mg/m^2 (day 1), doxorubicin 50mg/m^2 (day 1), vincristine 2mg (day1) and prednisone 100mg (days 1-5). This was followed by stem cell mobilizing therapy with either dexaBEAM: dexamethasone 8mg tds (days 1 – 7), melphalan 30mg/m^2 (day 3), BCNU 60mg/m^2 (day 3), etoposide 75mg/m^2 (days 4 - 7), Ara-C 100mg/m^2 twice daily (days 4 - 7) or ESHAP: etoposide 40mg/m^2 (days 1 - 4), methylprednisolone 500mg/m^2 (days 1 – 5), cytarabine 2000mg/m^2 (day 5) and cisplatin 25mg/m^2 (days 1 – 4) and stem-cell collection. Patients in CR or PR underwent myeloablative chemotherapy with TBI and HD cyclophosphamide. Eighty-three patients were included in the study, 32 patients with PTCL NOS; 27 with AITL; 13 with ALCL, ALK negative; 5 with intestinal lymphoma; 4 with extranodal NK/T-cell lymphoma nasal type and 2 with hepatosplenic T-cell lymphoma. Fifty-five patients (66%) received the planned ASCT. ORR was 66% (56% CR and 8% PR). With a median follow-up of 33 months 48% of patients had died. The 3-year PFS and OS were 36% and 48%, respectively.

The biggest prospective phase II multicentre study of ASCT in PTCL has been completed by the Nordic Lymphoma Group. They assessed the impact of a dose-intensified induction with 6 courses of two-weekly CHOEP: cyclophosphamide 750mg/m^2 (day 1), doxorubicin 50mg/m^2 (day 1), vincristine 2mg (day1), etoposide 100mg/m^2 (days 1-3) and prednisone 100mg (days 1-5) consolidated in CR/PR with BEAM-ASCT (Reimer et al., 2009). The patients were previously untreated and aged 18 – 67 years. ALCL, ALK positive; primary cutaneous; leukaemic and lymphoblastic subtypes were excluded. 160 patients with a confirmed histological diagnosis of PTCL were included, 62 PTCL NOS; 32 ALCL, ALK negative; 30 AITL; 21 EATL; 6 panniculitis-like; 5 extranodal NK/T-cell nasal type and 5 hepatosplenic T-cell lymphomas. From the original cohort 155 patients were evaluable after induction and 112 patients (72%) underwent ASCT. With a median follow-up time of 45 months, 46% of patients have died. The 3 / 5 year PFS and OS were: 48% / 43% and 57% / 50%; respectively.

A multicentre phase II study of Mercadal et al included patients with PTCL in CS II - IV under the age of 65 (Mercadal et al., 2008). ALCL, ALK positive and

mycosis fungoides patients were excluded. Patients received 3 cycles of megaCHOP: cyclophosphamide 2000mg/m^2 on day 1 (2.7 x standard CHOP), doxorubicin 90mg/m^2 on day 1 (1.8 x standard CHOP), vincristine 2mg on day 1 and prednisone 60mg on days 1 – 5, followed by three courses of standard ESHAP: etoposide 40mg/m^2 (days 1 - 4), methyprednisolone 500mg/m^2 (days 1 – 5), cytarabine 2000mg/m^2 (day 5) and cisplatin 25mg/m^2 (days 1 – 4) and stem-cell collection. Patients with CR or PR were given myeloablative chemotherapy with BEAM or carmustine, etoposide, cytarabine and cyclophosphamide (BEAC) followed by ASCT. 41 patients were included in the trial: 20 PTCL NOS; 12 AITL 2 hepatosplenic T-cell lymphoma; 2 extranodal NK/T-cell nasal type; 2 panniculitis like and one each of Sezary syndrome; ALCL, ALK positive and other γ/δ lymphoma. Seventeen (41%) patients received ASCT. ORR was 58% (51% CR/CRu and 7% PR). With a median follow-up time of 3.2 years, 54% of patients have died. 3-year PFS and OS were 34% and 43%, respectively.

In the study of Rodriguez et al 26 gallium-scan-positive patients with high-risk PTCL were treated with three courses of megaCHOP: cyclophosphamide 2000mg/m^2 on day 1 (2.7 x standard CHOP), doxorubicin 90mg/m^2 on day 1 (1.8 x standard CHOP), vincristine 2mg on day 1 and prednisone 60mg on days 1 - 5 before they were re-evaluated (Rodriguez et al., 2007c). Patients with negative gallium scans received an additional cycle of megaCHOP and went on to ASCT, and patients, who remained gallium positive received two courses of salvage IFE: ifosfamide 10000mg/m^2 continuous infusion on days 1 - 3 and etoposide 150mg/m^2 twice a day on days 1 – 3. All patients with at least PR received BEAM and ASCT. Twenty-six patients were included in this study: 11 PTCL NOS; 8 ALCL, ALK negative and 7 AITL. ASCT was performed in 19 (73%) patients. The ORR was 77% (19 CR – 69% and 1 PR – 8%), with a median follow-up time of 35 months. Thirty-one per cent of patients died and 3-year PFS and OS were 53% and 73%.

Corradini et al reported on sixty-two patients with PTCL: 28 PTCL NOS; 19 ALCL, ALK positive; 10 AITL; 4 ALK negative PTCL and one EATL (Corradini et al., 2006). Thirty-two patients received an intensified version of the HD sequential chemotherapy regimen, which consisted of:

(i) A debulking phase with two cycles of APO: doxorubicin 75mg/m^2 (days 1 and 21), vincristine 1.4mg/m^2 (days 1 and 21) prednisone 50mg/m^2 (days 1 – 21) and two courses of DHAP: cisplatin 100mg/m^2 (day 1), cytarabine 2000mg/m^2 twice a day (day 2) and dexamethasone 40mg/m^2 (days 1 – 4)

(ii) A HD phase, which involved the sequential administration of cyclophosphamide 7000mg/m^2, cytarabine 2000mg/m^2 twice daily for six days, cisplatin 100mg/m^2 and etoposide 2400mg/m^2. The conditioning regimen for ASCT was HD mitoxantrone and melphalan.

The remaining 30 patients were treated for 8 weeks with MACOP-B: methotrexate 400mg/m^2 (days 8 and 36), doxorubicin 50mg/m^2 (days 1, 15, 29 and 43), cyclophosphamide 350mg/m^2 (days 1, 15, 29 and 43), vincristine 1.4mg/m^2 (days 8, 22, 36, 50), bleomycin 10units/m^2 (days 22 and 50) and prednisone 75mg (days 1 - 56), followed by intensification with a course of mitoxantrone 8 mg/m^2 (days 1 – 3) with HD cytarabine 2000mg/m^2 twice daily (days 1 – 3). The conditioning regimen for ASCT was BEAM. Thirty-five patients (56%) received ASCT. The ORR after ASCT was 74% (66% CR and 8% PR). The 3-year PFS and OS were: 49% and 62%.

The other study worthy of closer scrutiny because of the treatment approach is the DSHNHL trial assessing maximum intensification of the CHOP regimen with additional etoposide in the treatment of aggressive lymphomas (Nickelsen et al., 2009). Separate evaluations were performed to compare the feasibility and efficacy of the regimen in lymphomas with T-cell and B-cell phenotype. Patients were randomised to four different dose levels: the total regimen doses and increase / decrease factor compared with the standard of 6 cycles of CHOP are given in brackets: cyclophosphamide (16500mg/m^2 - 19500mg/m^2; 3.7x – 4.3x), doxorubicin (280mg/m^2 – 420mg/m^2; 0.93x – 1.4x), vincristine (8mg/m^2 - 12mg/m^2; 0.7x – 1x), etoposide (3130mg/m^2 - 5200mg/m^2; 1.7x – 2.9x) and prednisone (2000mg/m^2 - 3000mg/m^2; 0.7x – 1x). Patients received 4 or 6 cycles of the regimen, with stem cell support beginning from the second cycle. Thirty-three patients in this prospective study had T-cell phenotype lymphomas; 13 ALCL, ALK positive; 11 PTCL NOS; 4 AITL; 3 NK/T-cell lymphoma; 1 EATL and 1 lymphoblastic lymphoma. The outcome of T-cell lymphoma patients was significantly worse than B-cell lymphoma patients in all evaluated aspects, including, number of patients who completed the planned treatment (66.7% vs. 84.4%), ORR (55% vs. 76%), PFS and OS at 3 years (25.9% and 44.5% vs. 60.1% and 63.4%).

Our novel regimen include a solitary initial cycle of standard CHOP followed by three cycles of high dose therapy with IVE/MTX (the cumulative regimen doses are given in brackets): ifosfamide 3000mg/m^2 on days 1 – 3 (27000mg/m^2), epirubicin 50mg/m^2 on day 1 (150mg/m^2) and etoposide 200mg/m^2 on days 1 -3 (1800mg/m^2) alternating with intermediate dose methotrexate 1500mg/m^2 by 24 hr infusion (4500

mg/m^2). When compared with other studies our patients receive from the beginning of their treatment high doses of effective drugs in a unique combination. Ifosfamide was previously used in the treatment of PTCL in the sequential chemotherapy of the GELA group, but in a lower dose of 3000mg/m^2 (Gisselbrecht et al., 1998) (Coiffier et al., 1990), and at the higher dose of 30000mg/m^2 in salvage therapy in combination with etoposide in the study of Rodriguez (Rodriguez et al., 2007c) compared with 27000mg/m^2 in our regime. Etoposide is a part of several other treatments mostly as an addition to CHOP or as part of a mobilisation regimen (ESHAP or DexaBEAM) (Mercadal et al., 2008) (Reimer et al., 2009), or salvage therapy (Rodriguez et al., 2007c). Epirubicin was used in our study for the first time whilst other protocols use mostly doxorubicin as an anthracycline (Reimer et al., 2009) (d'Amore et al., 2012) (Mercadal et al., 2008). The final drug of our regimen methotrexate was used in an intermediate dose of 1500mg/m^2 and has not been used in other regimens for PTCL except in the GELA sequential chemotherapy and HDCT-ASCT regimen (Mounier et al., 2004).

All patients in our study who were assigned to receive the treatment were prospectively evaluated on intent to treat basis. This approach allowed us to assess this entirely new regimen in the first line treatment of other histological subtypes of PTCL after achieving promissing results in EATL patients (a group of patients with a dismal prognosis when treated with conventional therapies).

In order to perform a better and more structure comparison of outcome of patients treated in the above-described prospective studies of PTCL, the studies can be divided into three categories according to the type of induction.

(i) induction based on conventional CHOP-like regimens followed by a mobilising cycle with more intensive chemotherapy and a final myeloablative regimen with ASCT “conventional CHOP – myeloablative – ASCT”. The two largest studies of Reimer et al and D’Amore et al belong (Reimer et al., 2009) (d'Amore et al., 2012) to this category, and the second part of Corradini’s study, which includes treatment with MACOP-B followed by one cycle of mitoxantrone with HD cytarabine (Corradini et al., 2006).

(ii) induction with high dose CHOP (e.g. megaCHOP) either alone as in the standard arm of Rodriguez et al, or with additional HD cycles e.g. the intensified arm of Rodriguez et al (Rodriguez et al., 2007c) or the study of Mercadal - “HD CHOP – myeloablative - ASCT” (Mercadal et al., 2008).

(iii) The third category includes studies, which are not based on conventional CHOP but use different drugs in high doses "HD - myeloablative – ASCT", for example, the present study and Corradini's first study (Corradini et al., 2006).

Comparison between the different studies is difficult, as several studies included different treatment arms, which were evaluated together (Corradini et al., 2006) (Rodriguez et al., 2007c). The most straightforward comparison is possible with studies, which included only one treatment regimen (d'Amore et al., 2012) (Reimer et al., 2009) (Mercadal et al., 2008).

The general characteristics of our patients were comparable with those of patients in other prospective trials in PTCL. The median age was 42 years (range from 43 years to 57 years in other trials), and the majority of patients were male with a male : female ratio of 1.6, in other studies this ranged from 1.6 to 2.72 (d'Amore et al., 2012) (Corradini et al., 2006) (Mercadal et al., 2008) (Rodriguez et al., 2007c). Performance status as measured by ECOG was less than 1 in 73% of patients and was comparable with those from the biggest studies of Reimer et al and d'Amore et al with 71% in both (d'Amore et al., 2012) (Reimer et al., 2009). However, the percentage of patients with a good performance status was higher than that reported in the studies of Rodriguez and Mercadal; 50% and 46%, respectively (Rodriguez et al., 2007c) (Mercadal et al., 2008). Over half of our patients (52%) presented with B-symptoms. In other studies this ranged between 54% and 63%. LDH was increased in 52% of patients and in other studies the range was between 54% and 62%. However, the percentage of our patients with advanced stage disease was at 58% lower when compared with other studies, where it ranged from 75% and 96% (Corradini et al., 2006) (Mercadal et al., 2008) (Reimer et al., 2009) (d'Amore et al., 2012). One possible explanation is the fact that some of the studies were specifically designed for high-risk patients and included patients with advanced stage disease only.

The response rates to induction treatment before ASCT were highest in our study, with an 84% overall response rate and 77% CR. The two biggest studies belonging to the category "conventional CHOP – myeloablative – ASCT", reported comparable response rates with respect to overall response rate but the CR rate was significantly lower when compared with the present study; d'Amore at al 85% with 52% CR (d'Amore et al., 2012) and Reimer et al 71% with 47% CR (Reimer et al., 2009). In Mercadal's study using "HD CHOP – myeloablative - ASCT" methodology with HD CHOP followed by 3 cycles of ESHAP the ORR was lowest with 59% (39%

CR) (Mercadal et al., 2008). Nickelsen et al applied the highest possible doses of drugs in their CHOP regimen (cyclophosphamide 3.7 – 4.3x dose increase, doxorubicin 0.93 – 1.4x and etoposide 1.7 – 2.9x), but the results of the study were disappointing and did not support any further investigation of this approach (Nickelsen et al., 2009). In the third category of studies where different treatment modalities at high doses were employed the response rates were comparable with our study, 72% (56% CR) in the study of Rodriguez (Rodriguez et al., 2007c) and 77% (65% CR) in the study of Corradini et al (Corradini et al., 2006). Importantly, Corradini reported no differences between the HD part of his study (representing "HDCT – myeloablative - ASCT") and the anthracycline-based followed by HDCT part, (representing "conventional CHOP – myeloablative – ASCT") (Corradini et al., 2006).

The comparison of responses to ASCT is more straightforward as all studies used ASCT only for patients in CR or PR (Corradini et al., 2006) (Mercadal et al., 2008) (Rodriguez et al., 2007c) (d'Amore et al., 2012). Despite the different conditioning regimens used all transplanted patients achieved CR or PR except in the studies of Rodriguez and d'Amore (d'Amore et al., 2012) (Rodriguez et al., 2007c). The latter study reported 13 (12%) patients with failure, and in the former one patient failed. One possible explanation for the failures is that both studies used almost the same induction (with minimal intensification in Reimer's study). D'Amore et al reported an ORR after induction 14% higher than Reimer (Reimer et al., 2009) (d'Amore et al., 2012). Some of these patients responded to the modified induction for a very short time only and relapsed quickly after ASCT.

With respect to ORR and rates of CR after the end of treatment the best response rates were achieved in the present study with 84% ORR and 77% CR, followed by the studies of Rodriguez, (77% ORR and 69% CR) (Rodriguez et al., 2007c) and Corradini (74% and 66%) (Corradini et al., 2006). Studies using induction with anthracycline-based chemotherapy were characterised by the worst results; 66% ORR and 58% CR in Reimer et al (Reimer et al., 2009) and 59% ORR and 51% CR in Mercadal et al where an intensified CHOP regimen was applied (Mercadal et al., 2008).

The 3-year PFS and OS were highest in the study of Rodriguez (53% and 73%) (Rodriguez et al., 2007c) and the present study (57% and 71%). Both studies were characterized by early intensification with ifosfamide and etoposide – in our approach in all patients and in the study of Rodriguez in patients selected by positive Gallium scans. The two biggest studies applying a "conventional CHOP – myeloablative - ASCT"

design reported significantly lower 3 year PFS and OS of 36% and 48% (Reimer et al., 2009) and 48% and 57% (d'Amore et al., 2012). Results were worst in the study of Mercadal et al. using a HD CHOP-like regimen followed by HDCT with 3 year PFS of 34% and OS of 43% (Mercadal et al., 2008), This study and that of Nickelsen et al (26% and 46%) confirm that there is no advantage to increasing the dose of CHOP-like regimens (Nickelsen et al., 2009). Corradini et al reported 3 year PFS and OS of 49% and 62%, however this study included a number of ALK positive T-cell lymphomas (Corradini et al., 2006).

A comparison between outcomes of histological subgroups was possible for our study and the patients included in the ITLP (Vose et al., 2008). In our study the 3-year OS was improved for all histological groups except for one patient with hepatosplenic T-cell lymphoma; ALCL, ALK positive: 83% vs. 73%; PTCL NOS: 64% vs. 41%; ALCL, ALK negative: 67% vs. 54%; extranodal NK/T-cell lymphoma, nasal type: 100% vs. 13%. In addition it is possible to compare our patients with PTCL NOS and ALCL, ALK negative with those treated in d'Amore's study (d'Amore et al., 2012). The 3-year PFS and OS was better for patients treated with our protocol for PTCL NOS: 45% and 64% vs. 42% and 51% and but not for ALCL, ALK negative: 33% and 67% vs. 64% and 77%. This might suggest that patients with PTCL NOS benefit more from an intensified induction than patients with ALCL. However because of the small numbers of patients these results need to be viewed with some caution.

In our study we observed a trend towards a longer PFS and OS according to aaIPI (high / intermediate high vs low / intermediate low), however these differences were not significant. Reimer et al also found a trend towards better survival in patients with a low and intermediate low aaIPI score (Reimer et al., 2009) and in the study of Corradini et al aaIPI predicted for significantly longer OS and a trend for PFS (Corradini et al., 2006). By contrast, Rodriguez et al did not find any predictive value for aaIPI score (Rodriguez et al., 2007c). However once again the numbers of patients were too small to make any valid conclusions on the significance of the aaIPI.

In an evaluation of patients who completed the whole treatment including ASCT and those who did not we observed differences in PFS and OS for all patients, and after exclusion of ALCL, ALK positive cases, the differences were not statistically significant. By contrast, Reimer et al reported statistically significant differences between patients who received a transplant and those who did not (Reimer et al., 2009). We did not observe any such difference in our patients with and without a transplant in

an evaluation of patients who achieved CR / PR after induction. This might suggest that our intensive HD induction is powerful enough and does not necessarily require an ASCT as compared with conventional induction with CHOP. Mercadal et al did not notice a difference in the same evaluation in his study (Mercadal et al., 2008). Both studies used a more intensified induction than standard CHOP. This data may be important when we consider the treatment of elderly patients who may be suitable for IVE/MTX but not necessarily for a myeloablative ASCT.

When evaluating the response and failure patterns we observed a 16% progression during treatment, 10% early post treatment relapses, 16% late post treatment relapses and 55% long-term remissions. A similar evaluation was performed by d'Amore et al (d'Amore, 2010) (d'Amore et al., 2012) and Reimer et al (Reimer et al., 2009). Progression during treatment was found in 30 – 35% of patients, early post treatment relapses in 15 – 20%, late post treatment relapses in 5% and long-term remissions in 35 – 50%. According to these results with the new regimen we are able to reduce the number of failures appearing during treatment, early post treatment relapses and to increase the number of long term remissions. However, the number of late relapses increased.

In conclusion, the novel intensified regimen was evaluated prospectively by an intention to treat method in all suitable patients presenting with PTCL in our region. The therapy was based on early intensive induction with a unique combination of very effective anti-neoplastic drugs, and moved away from conventional anthracycline-based induction therapies. The treatment was feasible with acceptable toxicities for a HD therapy. The achieved ORR, PFS and OS were better than those previously published. Particularly, the incidence of primary progressive patients and early relapses could be reduced. Importantly, the patients also benefitted from the treatment when they responded with CR/PR to the 3 cycles of induction therapy but did not proceed to the final myeloablative ASCT. This option may potentially be applied and further evaluated in more fragile and elderly patients. Furthermore, the newer folate analogues with significant powerful efficacy in PTCL (O'Connor et al., 2009) could be incorporated into the regimen and replace methotrexate in future when they become more widely available.

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Chapter 6. Conclusive discussion

6.1 Discussion

The aim of this thesis was to perform several independent studies in order to improve treatment strategies for patients with aggressive lymphomas. Almost 45 years have past since the Working Formulation for NHL was presented in 1982 and the term 'aggressive lymphomas' was introduced (National Cancer Institute, 1982). Despite significant improvement in the understanding of biology and pathology, including a new classification, there has been limited improvement in treatment strategies. The current standard treatment is based on a multidrug, anthracycline-based regimen CHOP which has been used since 1976 (McKelvey et al., 1976). Tumours of B-cell origin are treated with the addition of the anti-CD20 agent rituximab (Coiffier et al., 2002). Because of the limits of this thesis, the research was focused on several aspects only and it was not possible to cover all important issues.

According to the recent WHO Lymphoma Classification the former group of aggressive lymphoma was subdivided into approximately 15-20 identities with different biology, prognosis and individual requirements for improvement of their treatment strategies (Swerdlow et al., 2008). The research of this thesis was focused on the DLBCL-NOS and PTCL with EATL particularly.

Diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma is the most common and the most heterogeneous lymphoma in terms of pathobiology and outcome (Swerdlow et al., 2008). The current treatment for intermediate and advanced stage disease is 6 – 8 cycles of anthracycline-based chemotherapy with CHOP in combination with rituximab and for early stage two cycles of CHOP with rituximab and adjuvant radiotherapy (Coiffier et al., 2002) (Coiffier et al., 2010) (Habermann et al., 2006) (Pfreundschuh et al., 2008b) (Pfreundschuh et al., 2008a) (Sehn et al., 2005). The outcome, particularly for patients with intermittent / advanced stage disease, is limited. The data justifying this approach in intermittent and advanced stage disease comes from clinical trials, which included mostly patients with intermittent stages only, leaving the advanced stage / high IPI cases. The data on patients with early stage disease is even less supported by clinical trials, all of which come from the pre-rituximab era (Bonnet et al., 2007) (Horning et

al., 2004) (Miller et al., 1998) (Reyes et al., 2005). Additionally, the majority of data on DLBCL comes from clinical trials usually performed on highly selected patients. Thus, the results may be biased and not always representative for patients presenting in clinical practice.

Performing the population-based studies on DLBCL of nodal origin we could describe the epidemiological background of the disease, which should be successively used in the design of future research. We confirmed that DLBCL is a disease of the elderly population and is diagnosed in advanced stage. This group of patients has a worse outcome and is often treated inadequately or cannot tolerate the standard treatment and is switched to more palliative options. This group of patients was usually not included in clinical trials. Interestingly, a significant number of patients in the whole population have not received standard treatment. The other important finding was the fact that patients who achieved a better quality remission with first line treatment had a better outcome. This emphasizes the importance of the curative role of the first line treatment, particularly in view of very limited results for currently available salvage treatments. Our evaluation of patients treated with a combination of immunochemotherapy, although performed in selected NHS trusts, could only confirm the limited outcome of the treatment in patients with advanced stage disease, particularly in the elderly population.

In conclusion, new treatment strategies should focus on advanced stage disease, in the fragile elderly population who often cannot tolerate standard anthracycline-based treatment. Recent progress in the understanding of the biology of DLBCL, resulting from the employment of next generation sequencing on whole genome / exome, provides some help (Pasqualucci and Dalla-Favera, 2015). New discovered oncogenic pathways can be targeted with novel anticancer drugs (Pasqualucci and Dalla-Favera, 2015) (Schneider et al., 2011). The most advanced research focuses on the following targets: NF-κβ with Bortezomib or Lenalidomide or inhibitors of BCR cascade (e.g. SYK, BTK and PKCβ) with fostamatinib, ibrutinib and enzastaurin, all of them used in ABC-DLBCL (Sehn and Gascoyne, 2015). The new drugs proposed for GCB-DLBCL are PI3K inhibitor idelalisib, BCL2 inhibitor ABT-199 or EZH2 and BCL6 inhibitors (Sehn and Gascoyne, 2015). Finally, the new anti-CD20 and anti-CD79b antibodies can be used in all types of DLBCL (Sehn and Gascoyne, 2015). Most likely these new drugs can be added to the backbone of standard chemotherapy with CHOP-R or perhaps more rationally in studies of elderly or fragile patients to less intensive treatments like

bendamustine or DECC, which was successfully evaluated in palliative setting for patients with DLBCL in our group and showed very good results with an excellent toxicological profile (Proctor et al., 2010).

The remaining question is if there is any place for intensification of the chemotherapy in the treatment strategies of patients with DLBCL? Studies of the role of high-dose chemotherapy with autologous stem cell transplant as first line treatment for patients with DLBCL deliver conflicting results (Greb et al., 2007) (Schmitz et al., 2012) (Schmitz et al., 2012). However, their design was not optimal to answer the above question, as they usually recruited a very heterogeneous group of patients. Even very simple studies of intensified chemotherapy / autologous stem cell transplant in high risk IPI DLBCL are still missing. According to current knowledge the intensive approach can be reserved for the patients with advanced stage disease / high IPI with aggressive tumours in the group of patients suitable for high-dose treatment. The unresolved issues remain the selection of patients and choice of induction regimen / consolidation regimen. The use of IPI for selection of high-risk patients has limited success, as proven in our studies on a larger cohort of patients treated with anthracycline-based chemotherapy only and with addition of immunotherapy as well. Currently the intensification of treatment is postulated in patients with double translocations involving *c-MYC* and *BCL2*, so called "double hit" lymphoma (Friedberg, 2012). The double translocation occurs in approximately 5% of DLBCL cases, these cases are characterized by very poor response to standard treatment with CHOP-R with a median survival of approximately eight months only. Recently, since the introduction of improved antibodies against c-MYC, so called dual expressers of c-MYC / BCL2 can also be considered for intensive treatment options (Johnson et al., 2012). Depending on the evaluation/technical method approximately 25% of DLBCL patients belong to this group and their 5-years PFS is 25%.

In our studies we attempted to establish a prognostic model based on the expression at the RNA level of *c-MYC* and *HLA-DRβ*. The choice of the prognostic genes was based on the results of the studies on the role of *c-MYC* in lymphomagenesis of DLBCL and Burkitt lymphoma (Joos et al., 1992) (Johnson et al., 2009), particularly we noticed the fact that since the intensification of chemotherapy with the introduction of specific regimens like CODOX-M/IVAC or GMAIL protocol in Burkitt lymphoma there has been a significant improvement in outcome. The role of the microenvironment and immunosurveillance justify the use of *HLA-DRβ* as a second prognostic gene

(Rimsza et al., 2004). In our model we also included the *V2-transcript* encoding the 17-92 microRNA cluster, which has a previously described role in lymphomagenesis (Tagawa and Seto, 2005). We could not confirm the prognostic value of expression of the assessed genes in our cohort of patients. As discussed above, this could be due to methodological reasons like selection of the assessment method, extraction of RNA from samples, characteristic of the amplicon and of the primers and probe for *V2-transcript*. These technical obstacles can be improved by using the improved technic of obtaining representative tumour samples (micro-sections from several separate sides of tumour) and applying other non–enzyme based gene expression assessment methods like NanoString (Scott et al., 2015). Particularly, given that recent data indicate that this technology can be successfully used in FFPE tissue samples in clinical practice (Scott et al., 2015). By contrast the whole genome / exome next generation sequencing methods, although very important in basic research, have very limited value in routine practice because of costs and requirement of fresh tissue. Additionally, the complex character of control of gene expression as in the case of *c-MYC* can contribute to our results.

The choice of type of intensive treatment in DLBCL is also not yet determined. One of the options is based on a conventional anthracycline-based regimen with some modification such as the addition of etoposide (CHOEP or DA-EPOCH-R) (Petrich et al., 2014). Alternatively the regimen used in the treatment of Burkitt lymphoma could be applied. A final option is the use of a completely novel strategy such as the NEALL VI protocol, used primarily by SNLG for the treatment of ALL, but subsequently successfully used in patients with primary refractory DLBCL and bone marrow involvement (Sieniawski et al., 2009). From the view of tumour biology it seems that using the two latter options is more justified but this needs further assessment in randomized clinical trials. The role of consolidation with stem cell remains open, and if so, whether this should be autologous or allogeneic.

Peripheral T-cell lymphoma

The outcome of patients with peripheral T-cell lymphoma was described as generally worse than the outcome of DLBCL. This conclusion is based on the subanalysis of the peripheral T-cell lymphomas treated within the trials for aggressive lymphomas before the rituximab era and evaluation of international cohort trials (Vose et al., 2008) (Mounier et al., 2004). Prospective population-based cohort trials would be a very helpful source of information about patients with peripheral T-cell lymphomas in

terms of patient characteristics, presentation and outcome of the standard treatment, particularly for those cases with rare subtypes. In our prospective population-based trial on EATL we collected the most accurate information on epidemiology and presentation of this entity. This also allowed the performance of an exact evaluation of the outcome of routinely applied treatments including surgery and chemotherapy and the very poor prognosis of patients when treated with conservative treatment was confirmed. This data subsequently led to implementation of new high dose regimen with IVE/MTX, which significantly improved the outcome of patients with EATL.

The IVE/MTX regimen was then used in the treatment of other subtypes of PTCL including: PTCL NOS, ALCL ALK negative, ALCL ALK positive, extranodal NK/T-cell lymphoma, nasal type or hepatosplenic T-cell lymphoma. The outcome of treated patients was significantly better as compared with historical data for patients with these entities treated with standard anthracycline-based treatment with CHOP. In addition, in comparison to the largest study on modified anthracycline-based chemotherapy with CHOEP and up-front autologous stem cell transplant our results seem to be slightly better (d'Amore et al., 2012). Particularly, the number of cases with primary resistant disease and early relapses was reduced. Our cohort of patients is currently undergoing late follow-up and we are expecting very interesting data on the late events.

The question remains if upfront high dose chemotherapy is needed for the treatment of PTCL. According to currently available data only ALCL ALK positive have more or less satisfactory outcome when treated with standard anthracycline-based treatment with CHOP (Vose et al., 2008). Because of heterogeneity and rarity of PTCL, the available data on the pathobiology of PTCL is very sparse and described abnormalities in oncological pathways would support use of several new drugs only, e.g.: the patients with mutations in *TET2* and *DNMT3A* can be potentially treated with demethylating agents (Couronné et al., 2012) or the *ROHA* status can be helpful in directing targeted therapies (Palomero et al., 2014), finally the *FYN* mutations accounted in PTCL result in increased activation of the enzyme and could be potentially targeted with SRC kinase inhibitors (Palomero et al., 2014). Additionally, current data on new anticancer drugs used in PTCL would not support the idea of significant improvement on the standard anthracycline-based chemotherapy when given in combination. Obviously this combination can be used in fragile patients who would not tolerate the intensive treatment, see below. Thus there is still a need for the assessment

of more intensive treatment like CHOEP or IVE/MTX in consolidation with stem cell support. Both approaches have their positives and negatives. There is an option to add new drugs to induction with CHOEP but this would probably be more difficult with more intense IVE/MTX. In IVE/MTX the substitution of MTX by a novel antifolate could be an interesting possibility. Finally, the direct comparison of both regimens would be worthy of further consideration.

In conclusion this thesis focused on the chosen aspects of epidemiology, prognosis and treatment of aggressive lymphomas. This research can help prepare future studies on aggressive lymphomas. There is a significant discordance between the current knowledge of the biology of aggressive lymphomas and used therapies. Improvement in treatment strategies is greatly required, however it will require very close cooperation between haematooncology and particularly clinical pathology and other involved disciplines like radiotherapy or diagnostic imaging. However the accent should be put on the active role of pathological diagnostic.

6.2 References

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Appendix I – SNLG questionnaire

SCOTLAND AND NEWCASTLE LYMPHOMA GROUP

BASIC DATA RECORD SHEET

REF: 2002

Please DO NOT put patient's name on this sheet

GENERAL PRACTITIONER

(Initial and Surname) .....

DIAGNOSIS 1) ☐ Lymphoma
2) ☐ Not Lymphoma

NEWLY DIAGNOSED CASE?

a. 1) ☐ Yes 2) ☐ No

Date of Biopsy ☐☐/☐☐/☐☐☐☐

Diagnosed outwith SNLG?

b. 1) ☐ Yes 2) ☐ No

TYPE OF DISEASE (including origin)

a. 1) ☐ Hodgkins 2) ☐ Non-Hodgkins

b. 1) ☐ Nodal Origin 2) ☐ Extranodal Origin
9) ☐ NK

SYSTEMIC SYMPTOMS

1) Yes	2) No	9) NK	
☐	☐	☐	Night Sweats
☐	☐	☐	Weight Loss
☐	☐	☐	Fever
☐	☐	☐	Itch
☐	☐	☐	Alcohol pain

PREVIOUS OR CONCURRENT MALIGNANT DISEASE

a. 1) ☐ Yes 2) ☐ No 9) ☐ NK

If Yes, specify .....

b. Family history of lymphoma/leukaemia

1) ☐ Yes 2) ☐ No 9) ☐ NK

If Yes 1) ☐ HD 2) ☐ NHL 3) ☐ Leukaemia

c. Strong family history of malignant disease*

1) ☐ Yes 2) ☐ No 9) ☐ NK

* Two or more 1st degree relatives (excl skin Ca)

GENERAL CONDITION

Fitness Rating (Grade 0 - 5) [ECOG] .....

If comorbidity – reason .....

PALPATION OF SPLEEN

1) ☐ Not palpable 2) ☐ Palpable
3) ☐ Prev splenectomy 9) ☐ NK

LYMPHADENOPATHY

☐ None palpable

Right	Left	
☐		Waldeyer's ring (incl. tonsil)
☐	☐	Cervical (incl. supraclavic)
☐	☐	Infraclavicular
☐	☐	Axillary
☐		Coeliac
☐		Para-aortic
☐		Retroperitoneal
☐		Mesenteric
☐	☐	Pelvic (Iliac)
☐	☐	Inguinal
☐	☐	Popliteal
☐		Other (specify) .....

HAEMATOLOGY (enter numerical results in spaces below)

Plasma Viscosity ..... cp
E S R ..... mm 1st hour
Hb ..... g/l
W B C ..... x 10^9/l

DIFFERENTIAL COUNT

Neutrophils ..... x 10^9/l
Lymphocytes ..... x 10^9/l
Monocytes ..... x 10^9/l
Eosinophils ..... x 10^9/l
Basophils ..... x 10^9/l
Other (specify) ..... x 10^9/l
Platelets ..... x 10^9/l

MARROW ASPIRATE

1) ☐ No lymphoma 2) ☐ Lymphoma
3) ☐ Failed 4) ☐ Not done

MARROW TREPHINE

1) ☐ No lymphoma 2) ☐ Lymphoma
3) ☐ Failed 4) ☐ Not done

LIVER BIOPSY (Percutaneous or Laparoscopy)

1) ☐ No lymphoma 2) ☐ Lymphoma
3) ☐ Failed 4) ☐ Not done ☐

DATE FIRST STAGING CT SCAN ☐☐/☐☐/☐☐☐☐

CHEST X-RAY

1) ☐ Done 2) ☐ Not done
3) ☐ No lymphoma ☐

CT SCAN (Thoracic)

1) ☐ Done 2) ☐ Not done
3) ☐ No lymphoma ☐

RESULTS FROM CHEST X-RAY/CT SCAN

R	L		
☐	☐	Lungs - possible lymphoma	☐☐
☐	☐	Lungs - lymphoma	☐☐
☐		Mediastinum - possible lymphoma	☐
☐		Mediastinum - lymphoma	☐
☐	☐	Hilar - possible lymphoma	☐☐
☐	☐	Hilar - lymphoma	☐☐
☐	☐	pleural effusion–possible lymphoma	☐☐
☐	☐	pleural effusion–lymphoma	☐☐
☐		other – specify	☐

BONE SCAN

1) ☐ No lymphoma
2) ☐ Possible lymphoma
3) ☐ Lymphoma ☐
4) ☐ Not done

GALLIUM SCAN

1) ☐ No lymphoma
2) ☐ Possible lymphoma
3) ☐ Lymphoma ☐
4) ☐ Not done

CT SCAN (Abdominal+pelvis)

1) ☐ No lymphoma
2) ☐ Possible lymphoma
3) ☐ Lymphoma ☐
4) ☐ Not done

CT SCAN (Head and neck)

1) ☐ No lymphoma
2) ☐ Possible lymphoma
3) ☐ Lymphoma
4) ☐ Not done ☐

ULTRASOUND SCAN (any site)

1) ☐ No lymphoma
2) ☐ Possible lymphoma
3) ☐ Lymphoma
4) ☐ Not done ☐

MAGNETIC RESONANCE IMAGING

1) ☐ No lymphoma
2) ☐ Possible lymphoma
3) ☐ Lymphoma ☐
4) ☐ Not done

BONE RADIOGRAMS

1) ☐ No lymphoma
2) ☐ Possible lymphoma
3) ☐ Lymphoma
4) ☐ Not done ☐

LAPAROTOMY

a.1) ☐ Done 2) ☐ Not done ☐

⇩

Splenectomy

b.1) ☐ Done 2) ☐ Not done ☐

Laparotomy (histology of)

c.1) ☐ Negative 2) ☐ Positive ☐

⇩

Positive histology in:-

☐ Splenic hilum ☐
☐ Coeliac nodes ☐
☐ Para-aortic nodes ☐
☐ Mesenteric nodes ☐
☐ Iliac nodes ☐
☐ Other nodes ..... (specify) ☐
☐ Spleen ☐
☐ Liver ☐
☐ Other ..... (specify) ☐

INITIAL DIAGNOSTIC HISTOLOGY *Tick one box only

	Yes	No	
a.	☐	☐	* Lymph node
b.	☐	☐	* Other

If 'Other' specify .....

..... ☐☐

EVIDENCE OF DISEASE

(TICK if histologically proven)

- ☐ Bone marrow
- ☐ Bone
- ☐ C N S
- ☐ Genito-urinary
- ☐ Gut
- ☐ Liver
- ☐ Lung
- ☐ Orbit
- ☐ Skin
- ☐ Thymus
- ☐ Thyroid
- ☐ Other ..... (specify)
- ☐ Nodal (lymph nodes etc)
- ☐ Peripheral blood

STAGING (COTSWOLDS AND SNLG)

a. Clinical Stage (CS)

☐ I ☐ II ☐ III ☐ IV

CS based on clinical evidence found in:-

☐ L/N ☐ Spleen ☐ Lung

☐ Liver ☐ Bone ☐ CNS

☐ Other (specify) .....

b. Number of Involved Sites (Stage II ONLY)

State number .....

c. "B" Symptoms 1) ☐ "A" 2) ☐ "B"

d. Extranodal Disease 1) ☐ "E" (Site) .....

2) ☐ None 3) ☐ Stage IV

e. Bulk Disease

1) ☐ Yes 2) ☐ No 9) ☐ NK

If "Yes": 1) ☐ Abdo 2) ☐ Mediastinum

3) ☐ Other (Specify) .....

If Abdominal or Other

1) ☐ ≥5<10cm 2) ☐ ≥10cm

f. Pathological Stage

☐ M (Marrow) ☐ H (Liver) ☐ L (Lung)

☐ O (Bone) ☐ P (Pleura) ☐ D (Skin)

g. Extent of staging

1) ☐ Complete 2) ☐ Incomplete

BLOOD UREA

1) ☐ Normal 2) ☐ Abnormal

3) ☐ Not known / Not done

If abnormal specify:

Level .....

Normal range) Lower .....

) Upper .....

SERUM LDH

1) ☐ Normal 2) ☐ Abnormal

3) ☐ Not known / Not done

If abnormal specify:

Level .....

Normal range) Lower .....

) Upper .....

SERUM AST

1) ☐ Normal 2) ☐ Abnormal

3) ☐ Not known / Not done

If abnormal specify:

Level .....

Normal range) Lower .....

) Upper .....

SERUM ALT

1) ☐ Normal 2) ☐ Abnormal

3) ☐ Not known / Not done

If abnormal specify:

Level .....

Normal range) Lower .....

) Upper .....

SERUM ALKALINE PHOSPHATASE

1) ☐ Normal 2) ☐ Abnormal

3) ☐ Not known / Not done

If abnormal specify:

Level .....

Normal range) Lower .....

) Upper .....

Also specify Liver or bone type

.....

B_2MICROGLOBULIN

1) ☐ Normal 2) ☐ Abnormal

3) ☐ Not known / Not done

If abnormal specify:

Level .....

Normal range) Lower .....

) Upper .....

SERUM ALBUMIN

1) ☐ Normal 2) ☐ Abnormal

3) ☐ Not known / Not done

If abnormal specify:

Level .....

Normal range) Lower .....

) Upper .....

CREATININE

1) ☐ Normal 2) ☐ Abnormal ☐
3) ☐ Not known / Not done

If abnormal specify:

Level .....
Normal range) Lower .....
) Upper .....

HIV-RELATED LYMPHOMA

1) ☐ + ve blood test ☐
2) ☐ – ve blood test
3) ☐ Not known / Not done
4) ☐ Suspected / Not proven
5) ☐ Refused blood test

PREVIOUS SIGNIFICANT ILLNESS/TRANSPLANT

Auto immune disease 1) ☐ Yes 2) ☐ No 3) ☐ NK ☐

If Yes specify .....

Coeliac disease 1) ☐ Yes 2) ☐ No 3) ☐ NK ☐

Thyroid dysfunction 1) ☐ Yes 2) ☐ No 3) ☐ NK ☐

Transplant 1) ☐ Yes 2) ☐ No 3) ☐ NK ☐

If Yes specify organ .....

Date of transplant ☐☐/☐☐/☐☐☐☐

REFERRAL (for suspicion of Lymphoma)

by 1. ☐ General Practitioner ☐
2. ☐ Hospital consultant

Date of Referral ☐☐/☐☐/☐☐☐☐

CLINICIANS SEEING PATIENT

(for Lymphoma)

1st Name ..... ☐☐☐☐
Speciality ..... ☐☐
Date of first seeing Clinician ☐☐/☐☐/☐☐☐☐

2nd Name ..... ☐☐☐☐
Speciality ..... ☐☐
Date of first seeing Clinician ☐☐/☐☐/☐☐☐☐

3rd Name ..... ☐☐☐☐
Speciality ..... ☐☐
Date of first seeing Clinician ☐☐/☐☐/☐☐☐☐

4th Name ..... ☐☐☐☐
Speciality ..... ☐☐
Date of first seeing Clinician ☐☐/☐☐/☐☐☐☐

Please ensure Pathological Classification is complete

HAS TREATMENT FOR LYMPHOMA STARTED?

☐ Yes ☐ No ☐

⇓

Date treatment started

Day/Month/Year ☐☐/☐☐/☐☐☐☐

COMMENTS: ☐

PATHOLOGY HISTOLOGY No: .....

Hospital .....

DATE REPORT ISSUED / /

PATHOLOGY REVIEW No: .....

Hospital .....

IMMUNOTYPING: at diagnosis

1) ☐ 'T' 2) ☐ 'B' 3) ☐ Null

4) ☐ Histiocytic 5) ☐ Other

6) ☐ ND 9) ☐ Not known

HTLVI 1) ☐ Yes 2) ☐ No 9) ☐ NK

EB Virus Status 1) ☐ +ve 2) ☐ –ve 9) ☐ NK

Other (specify) 1) ☐ Yes 2) ☐ No 9) ☐ NK

.....

PATHOLOGICAL CATEGORY – Attach Report if in doubt

If reviewed – specify review

THE REVISED EUROPEAN AMERICAN CLASSIFICATION OF LYMPHOID NEOPLASMS

B CELL

PRECURSOR

1) ☐ Lymphoblastic

PERIPHERAL

2) ☐ B-CLL/lymphocytic lymphoma

3) ☐ Lymphoplasmacytoid

4) ☐ Mantle cell lymphoma

5) ☐ Follicle centre lymphoma : follicular (Grade I, II, III)
: diffuse predominantly small cell

6) ☐ Marginal zone lymphoma : extra nodal low grade MALTOMA
: nodal,+/-monocytoid B cells

7) ☐ Splenic marginal zone lymphoma

8) ☐ Hairy cell leukaemia

9) ☐ Plasmacytoid/myeloma

10) ☐ Diffuse large B cell lymphoma

11) ☐ Burkitt's lymphoma

12) ☐ Burkitt-like lymphoma

13) ☐ Other (B cell) specify .....

T CELL

PRECURSOR

14) ☐ Lymphoblastic

PERIPHERAL

15) ☐ T CLL/prolymphocytic lymphoma

16) ☐ Large granular lymphocytic leukaemia

17) ☐ Mycosis fungoides

18) ☐ Peripheral T cell lymphomas unspecified

19) ☐ Angioimmunoblastic T cell lymphoma

20) ☐ Angiocentric lymphoma

21) ☐ Intestinal T cell lymphoma +/- enteropathy

22) ☐ Adult T cell lymphoma/leukaemia HTLV1+

23) ☐ Anaplastic large cell lymphoma (T and null)

24) ☐ Anaplastic large cell lymphoma Hodgkin's like

25) ☐ Other (T-cell) specify .....

26) ☐ unclassifiable as T or B specify .....

HODGKIN'S DISEASE

1) ☐ Lymphocyte predominance nodular (diffuse)

2) ☐ Nodular sclerosis

3) ☐ Mixed cellularity

4) ☐ Lymphocyte depletion

5) ☐ Lymphocyte rich classical HD

6) ☐ Other – specify .....

SCOTLAND AND NEWCASTLE LYMPHOMA GROUP

REF: 2002

'**NOTES ON COMPLETION**' are available – please use.

PLEASE DO NOT PUT PATIENT'S NAME ON THIS FORM

SECTION I Please write in current information:

CENTRE .....

TREATING HOSPITAL .....

CONSULTANT IN CLINICAL CHARGE .....

LYMPHOMA DIAGNOSIS DISCARDED:

Tick if no further entry required ☐

DATE LAST SEEN ☐☐/☐☐/☐☐☐☐

GENERAL CONDITION (Grade 0-5) ECOG .....

If comorbidity – reason .....

STATE AT DATE LAST SEEN

1) ☐ Alive 2) ☐ Dead 3) ☐ Untraceable

REMAINS IN COMPLETE REMISSION:

*** not applicable to first annual follow-up ***

TICK if remaining in CR ☐

Only Questions on 2nd Malignancy and Therapy Induced Menopause need be answered

SECTION II DEVELOPMENTS WITH DATES IN ABOVE 12 MONTHS

STATE OF LYMPHOMA

1) ☐ CR, no radiological abnormality
Day / Month / Year ☐☐/☐☐/☐☐☐☐

2) ☐ CR(u), unconfirmed /unproven uncertain
Day / Month / Year ☐☐/☐☐/☐☐☐☐

3) ☐ Part remission achieved
Day / Month / Year ☐☐/☐☐/☐☐☐☐

4) ☐ No change

5) ☐ Relapse/progression
Day / Month / Year ☐☐/☐☐/☐☐☐☐

APPEARANCE OR REAPPEARANCE OF SYSTEMIC SYMPTOMS

	Yes	No	If reappearance TICK	NK	
Night Sweats	1) ☐	2) ☐	3) ☐	9) ☐	☐
Weight Loss	1) ☐	2) ☐	3) ☐	9) ☐	☐
Fever	1) ☐	2) ☐	3) ☐	9) ☐	☐
Itch	1) ☐	2) ☐	3) ☐	9) ☐	☐
Herpes Zoster	1) ☐	2) ☐		9) ☐	☐

PRESENCE OF DISEASE

Was disease present during the above 12 months (excluding disease declared at diagnosis – UNLESS such disease is Persisting or Recurring after Primary Therapy)?

1) ☐ YES

If **NEW** sites: complete new disease
If **PERSISTING** sites: complete persisting disease
If **RECURRING/PROGRESSIVE** sites: complete recurring disease ☐

2) ☐ NO

3) ☐ UNSURE

SITES OF NEW DISEASE

1) ☐ Nodal 2) ☐ Extranodal 3) ☐ Both ☐

Nodal (Tick if histologically proven)

Right	Left		Right	Left	
☐		Waldeyer's ring (incl. tonsil)	☐		☐
☐	☐	Cervical (incl. supraclavic)	☐	☐	☐
☐	☐	Infraclavicular	☐	☐	☐
☐	☐	Axillary	☐	☐	☐
	☐	Mediastinal	☐		☐
☐	☐	Hilar	☐	☐	☐
	☐	Coeliac	☐		☐
	☐	Para-aortic	☐		☐
	☐	Retroperitoneal	☐		☐
	☐	Mesenteric	☐		☐
☐	☐	Pelvic (Iliac)	☐	☐	☐
☐	☐	Inguinal	☐	☐	☐
	☐	Other nodes (specify)	☐		☐
	☐	Spleen	☐		☐

Extranodal (Tick if histologically proven)

☐	Bone marrow	☐
☐	Bone	☐
☐	CNS	☐
☐	Genito-urinary	☐
☐	Gut	☐
☐	Liver	☐
☐	Lungs	☐
☐	Orbit	☐
☐	Skin	☐
☐	Thymus	☐
☐	Thyroid	☐
☐	Other (specify)	☐
☐	Peripheral Blood	☐

SITE OF PERSISTING DISEASE

1) ☐ Nodal 2) ☐ Extranodal 3) ☐ Both

Nodal (Tick if histologically proven)

Right	Left		Right	Left	
☐		Waldeyer's ring (incl. tonsil)	☐		
☐	☐	Cervical (incl. supraclavic)	☐	☐	
☐	☐	Infraclavicular	☐	☐	
☐	☐	Axillary	☐	☐	
	☐	Mediastinal		☐	
☐	☐	Hilar	☐	☐	
	☐	Coeliac		☐	
	☐	Para-aortic		☐	
	☐	Retroperitoneal		☐	
	☐	Mesenteric		☐	
☐	☐	Pelvic (Iliac)	☐	☐	
☐	☐	Inguinal	☐	☐	
	☐	Other nodes (specify)		☐	
	☐	Spleen		☐	

Extranodal (Tick if histologically proven)

☐	Bone marrow	☐
☐	Bone	☐
☐	CNS	☐
☐	Genito-urinary	☐
☐	Gut	☐
☐	Liver	☐
☐	Lungs	☐
☐	Orbit	☐
☐	Skin	☐
☐	Thymus	☐
☐	Thyroid	☐
☐	Other (specify)	☐
☐	Peripheral blood	☐

SITE OF RECURRING/PROGRESSIVE DISEASE

1) ☐ Nodal 2) ☐ Extranodal 3) ☐ Both

Nodal (Tick if histologically proven)

Right	Left		Right	Left	
☐		Waldeyer's ring (incl. tonsil)	☐		
☐	☐	Cervical (incl. supraclavic)	☐	☐	
☐	☐	Infraclavicular	☐	☐	
☐	☐	Axillary	☐	☐	
	☐	Mediastinal		☐	
☐	☐	Hilar	☐	☐	
	☐	Coeliac		☐	
	☐	Para-aortic		☐	
	☐	Retroperitoneal		☐	
	☐	Mesenteric		☐	
☐	☐	Pelvic (Iliac)	☐	☐	
☐	☐	Inguinal	☐	☐	
	☐	Other nodes (specify)		☐	
	☐	Spleen		☐	

Extranodal (Tick if histologically proven)

☐	Bone marrow	☐
☐	Bone	☐
☐	CNS	☐
☐	Genito-urinary	☐
☐	Gut	☐
☐	Liver	☐
☐	Lungs	☐
☐	Orbit	☐
☐	Skin	☐
☐	Thymus	☐
☐	Thyroid	☐
☐	Other (specify)	☐
☐	Peripheral blood	☐

TRANSFORMATION TO LEUKAEMIA

☐ Yes ☐ No

If Yes, specify: .....

TRANSFORMATION TO HIGH GRADE

☐ Yes ☐ No

If Pathology proven – tick box ☐
and complete Pathological Classification

TRANSFORMATION TO NHL

Please tick box ONLY if LPHD has transformed to TCR BCL ☐
and complete Pathological Classification

CHANGE OF DIAGNOSIS

☐ Yes ☐ No

a) from initial specimen ☐ Yes ☐ No
b) from second specimen ☐ Yes ☐ No

CHANGE OF HISTOLOGICAL SUB-TYPE

☐ Yes ☐ No

a) from initial specimen ☐ Yes ☐ No
b) from second specimen ☐ Yes ☐ No

DEVELOPMENT OF 2nd MALIGNANCY

☐ Yes ☐ No

if Yes a) ☐ non-haematalogical
specify .....

b) ☐ haematological
specify .....

**** Please refer to completion notes before completing this section***

SECTION III TREATMENT

TREATMENT FOR LYMPHOMA IN ABOVE 12 MONTHS

☐ YES ☐ NO⇒ If primary therapy has not been given reason .....

If YES –

1) ☐ Primary therapy only

2) ☐ Subsequent therapy only — complete appropriate questions

3) ☐ Both (ie Primary PLUS subsequent)

PRIMARY THERAPY

Was the primary therapy STARTED in the above 12 months?

☐ YES ☐ NO

If YES ⇓

DATE STARTED
Day / Month / Year

Complete details below

TYPE OF PRIMARY THERAPY

1) ☐ Radiotherapy

2) ☐ Chemotherapy

3) ☐ Surgery (specify) .....

If curative 1) ☐ YES 2) ☐ NO

4) ☐ Other (specify) .....

IF COMBINED MODALITY please indicate the sequence: eg 3 + 1 + 2

.....

RADIOTHERAPY

☐ Localised
☐ Regional
☐ Mantle
☐ Inverted Y
☐ TBI
☐ Other (specify) .....

.....

CHEMOTHERAPY

☐ Single agent (specify) .....

☐ Other (specify) .....

If growth factors used specify .....

ON TRIAL/STUDY 1) ☐ YES 2) ☐ NO

If yes specify .....

If no - why .....

ACUTE ADJUNCTIVE THERAPY (eg emergency radiotherapy)

☐ YES ☐ NO

If YES, specify .....

TREATMENT SUBSEQUENT TO PRIMARY THERAPY

Was the treatment subsequent to Primary Therapy STARTED in the above 12 months?

☐ YES ☐ NO ⇓

If YES ⇓ If NO, go to Section IV

DATE STARTED
Day / Month / Year

TYPE OF SUBSEQUENT THERAPY

1) ☐ Radiotherapy

2) ☐ Chemotherapy

3) ☐ Surgery (specify) .....

4) ☐ Other (specify) .....

5) ☐ BMT/PBSC

IF COMBINED MODALITY, please indicate the sequence: eg 3 + 1 + 2

.....

STEM CELL TRANSPLANT

1) ☐ PBSC 2) ☐ MARROW 3) ☐ BOTH

Day / Month / Year

1) ☐ Allogeneic 2) ☐ Autologous
3) ☐ Syngeneic 4) ☐ MUD

Done in:

1) ☐ 1st remission 2) ☐ 2nd remission
3) ☐ Partial remission
4) ☐ Other (specify) .....

RADIOTHERAPY

☐ Localised
☐ Regional
☐ Mantle
☐ Inverted Y
☐ TBI
☐ Other (specify) .....
☐ BMT conditioning (specify) .....

CHEMOTHERAPY

☐ Single agent (specify) .....

☐ BMT conditioning (specify) .....

.....

☐ Other (specify) .....

If growth factors used specify .....

THERAPY INDUCED PREMATURE MENOPAUSE?

1) ☐ YES 2) ☐ NO 9) ☐ N/K 8) ☐ N/A

If YES:

1) ☐ Chemotherapy 2) ☐ Radiotherapy
3) ☐ Both 4) ☐ Other (specify)

SECTION IV

CURRENT PATHOLOGICAL STATUS

THE REVISED EUROPEAN AMERICAN CLASSIFICATION OF LYMPHOID NEOPLASMS

B CELL

PRECURSOR

1) ☐ Lymphoblastic

PERIPHERAL

2) ☐ B-CLL/lymphocytic lymphoma
3) ☐ Lymphoplasmacytoid
4) ☐ Mantle cell lymphoma
5) ☐ Follicle centre lymphoma : follicular (Grade I, II, III)
: diffuse predominantly small cell
6) ☐ Marginal zone lymphoma : extra nodal low grade MALTOMA
: nodal,+/-monocytoid B cells
7) ☐ Splenic marginal zone lymphoma
8) ☐ Hairy cell leukaemia
9) ☐ Plasmacytoid/myeloma
10) ☐ Diffuse large B cell lymphoma
11) ☐ Burkitt's lymphoma
12) ☐ Burkitt-like lymphoma
13) ☐ Other (B cell) specify .....

T CELL

PRECURSOR

14) ☐ Lymphoblastic

PERIPHERAL

15) ☐ T CLL/prolymphocytic lymphoma
16) ☐ Large granular lymphocytic leukaemia
17) ☐ Mycosis fungoides
18) ☐ Peripheral T cell lymphomas unspecified
19) ☐ Angioimmunoblastic T cell lymphoma
20) ☐ Angiocentric lymphoma
21) ☐ Intestinal T cell lymphoma +/- enteropathy
22) ☐ Adult T cell lymphoma/leukaemia HTLV1+
23) ☐ Anaplastic large cell lymphoma (T and null)
24) ☐ Anaplastic large cell lymphoma Hodgkin's like
25) ☐ Other (T-cell) specify .....

26) ☐ unclassifiable as T or B - specify ..... ☐☐

HODGKIN'S DISEASE

1) ☐ Lymphocyte predominance nodular (diffuse)
2) ☐ Nodular sclerosis
3) ☐ Mixed cellularity
4) ☐ Lymphocyte depletion
5) ☐ Lymphocyte rich classical HD
6) ☐ Other – specify ..... ☐

SECTION V DEATH

DATE OF DEATH

Day / Month / Year ☐☐/☐☐/☐☐☐☐

POST MORTEM DONE ☐

☐ YES ☐ NO

If YES was there a change in the histological diagnosis? ☐

☐ YES ☐ NO

If YES, specify .....

PRINCIPAL CAUSE OF DEATH ☐

1) ☐ Lymphoma
2) ☐ Treatment of lymphoma
3) ☐ Other (specify) .....

DID TREATMENT CONTRIBUTE SIGNIFICANTLY TO DEATH? ☐

1) ☐ Yes 2) ☐ No 3) ☐ N/K

AT DEATH

a. Was lymphoma clinically in:- ☐

1) ☐ CR, no radiological abnormality
2) ☐ CR(u), unconfirmed/unproven
3) ☐ Partial remission
4) ☐ No change
5) ☐ Relapse/progression
6) ☐ Not known

b. At post mortem was lymphoma in:- ☐

1) ☐ CR, no pathological evidence of lymphoma
2) ☐ CR(u), pathological abnormalities – possibly lymphoma, but unproven
3) ☐ Partial remission
4) ☐ No change
5) ☐ Relapse/progression
6) ☐ Not known

NB Have you filled in the earlier sections which may pertain to the period before the patient's death

SECTION VI

COMMENTS: Please reference comments to appropriate question

.....

.....

.....

.....

Appendix II – Questionnaire for collection of immunochemotherapy data

R-CHOP – Diffuse Large B-cells Lymphoma Questionnaire

UPN (PI): _____ **Histopathology PR No:** _____ **Research:** ☐ yes ☐ no ☐ NK/ND

Hospital: ____ **Hospital No:** _____ **NHS No:** _____

Gender: ☐ male ☐ female

Date of birth: ___ /___ / _____ (DD / MM / YYYY)

Date of diagnosis: ___ /___ / _____ (DD / MM / YYYY)

Disease status and investigations at diagnosis

Clinical Stage: ☐ I ☐ II ☐ III ☐ IV ☐ NK/ND

ECOG: ☐ 0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ NK/ND

B-symptoms: ☐ yes ☐ no ☐ NK/ND

Marrow involvement: ☐ yes ☐ no ☐ NK/ND

Extranodal organs involvement excluding marrow: ☐ yes ☐ no ☐ NK/ND

Organs: _____ **No. of sites:** _____

Bulk (defined as: single node >5cm or group of nodes >10cm or mediastinal bulk >30% of chest diameter): ☐ yes ☐ no ☐ NK/ND

Hb: level: _____ ☐ normal ☐ abnormal ☐ NK/ND

WBC: level: _____ ☐ normal ☐ abnormal ☐ NK/ND

LDH: level: _____ ☐ normal ☐ abnormal ☐ NK/ND

LDH: lower limit _____ upper limit _____

$ß_2$ – microglobuline: ☐ normal ☐ abnormal ☐ NK/ND

Albumin: ☐ normal ☐ abnormal ☐ NK/ND

Urea: ☐ normal ☐ abnormal ☐ NK/ND

Alkaline phosphatase: ☐ normal ☐ abnormal ☐ NK/ND

Treatment

Planed chemotherapy completed: ☐ yes ☐ no ☐ NK/ND

No. CHOP-R cycles given: __ No CNOP-R cycles given: __ No. R cycles given: __

Reason for discontinuation of scheduled chemotherapy:

☐ Progression ☐ infection ☐ organ failure ☐ other ☐ NA ☐ NK/ND

Radiotherapy: ☐ yes ☐ no ☐ NK/ND

Outcome

Response after the end of first line treatment:

☐ complete remission ☐ partial remission ☐ failure

Progression: ☐ yes ☐ no

Date progression: ___ /___ / _____ (DD / MM / YYYY)

Death: ☐ yes ☐ no

Date last seen or date of death: ___ /___ / _____ (DD / MM / YYYY)

Appendix III – Questionnaire for collection of IVE/MTX followed by ASCT for the treatment of EATL data

EATL – QUESTIONNAIRE

1. **UPN (PI)**

   _____

2. **Date of presentation (DD/MMM/YYYY)**

   _____

3. **Age at presentation (years)**

   _____

4. **Gender**
   - male
   - female

5. **Presenting features at presentation**
   - pain
   - lump
   - nausea/vomiting
   - weight loss
   - bowel upset
   - perforation
   - subacute obstruction
   - other, please specify _____
   - unknown / not done

6. **Duration of symptoms pre-diagnosis**
   - 0-1 month
   - 1-3 months
   - 3-6 months
   - >6 months
   - unknown / not done

7. **Diagnosis at**
   - surgery
   - gastroscopy
   - other, please specify _____
   - unknown / not done

8. **Site of disease**
   - Stomach
   - Jejunum
   - Ileum
   - Ileocecal
   - other, please specify _____
   - unknown / not done

9. **General Fitness Rating (ECOG) at presentation**
    - ECOG 0
    - ECOG 1
    - ECOG 2
    - ECOG 3
    - ECOG 4
    - ECOG 5
    - unknown / not done
10. **B-symptoms at presentation**
    - yes
    - no
    - unknown / not done
11. **Hb level [g/dl] at presentation**

    _____
12. **Hb at presentation**
    - normal (≥ 12 g/dl)
    - abnormal (< 12 g/dl)
    - unknown / not done
13. **WBC level [x10^9/L] at presentation**

    _____
14. **WBC at presentation**
    - normal (4 x10^9/L – 11 x10^9/L)
    - abnormal (< 4 x10^9/L or > 11 x10^9/L)
    - unknown / not done
15. **Urea + electrolyte at presentation**
    - normal
    - abnormal
    - unknown / not done
16. **Liver function tests at presentation**
    - normal
    - abnormal
    - unknown / not done
17. **LDH at presentation**
    - normal
    - abnormal
    - unknown / not done
18. **Chest X ray at presentation**
    - normal
    - abnormal
    - unknown / not done
19. **CT abdomen at presentation**
    - normal
    - abnormal
    - unknown / not done
20. **CT chest at presentation**
    - normal
    - abnormal
    - unknown / not done

21. **Ultrasound abdomen at presentation**
    - normal
    - abnormal
    - unknown / not done
22. **Bone marrow at presentation**
    - normal
    - abnormal
    - unknown / not done
23. **Lugano stage at presentation**
    - Stage I
    - Stage II1
    - Stage II2
    - Stage II E
    - Stage II1 E
    - Stage II2 E
    - Stage IV
    - unknown / not done
24. **Manchester stage at presentation**
    - Stage Ia
    - Stage Ib
    - Stage IIa
    - Stage IIb
    - Stage IIc
    - Stage III
    - Stage IV
    - unknown / not done
25. **Primary treatment description [text]**

    _____
26. **Surgery**
    - yes
    - no
    - unknown / not done
27. **Type of surgery [text]**

    _____
28. **Chemotherapy**
    - yes
    - no
    - unknown / not done
29. **Type of chemotherapy [text]**

    _____
30. **Autologous stem cell transplant**
    - yes
    - no
    - unknown / not done

31. **Chemotherapy completed**
    - yes
    - no
    - not applicable
    - unknown / not done
32. **Outcome**
    - CR
    - PR
    - failure
    - unknown / not done
33. **Progression Free Survival Event**
    - relapse / death
    - no relapse / no death
34. **Progression Free Survival or Last Assessed Date (DD/MMM/YYYY)**

    _____
35. **Overall Survival Event**
    - death
    - no death
36. **Overall Survival or Last Seen Date (DD/MMM/YYYY)**

    _____
37. **Cause of Death**
    - lymphoma
    - treatment complications
    - other, please specify _____
    - not applicable
    - unknown / not done
38. **Presence of coeliac disease**
    - yes prior to lymphoma
    - yes at presentation
    - no
    - unknown / not done
39. **Complications [text]**

    _____
40. **Comments [text]**

    _____

Appendix IV – Questionnaire for collection of IVE/MTX followed by ASCT for the treatment of PTCL other than EATL data

T-cell – IVE / MTX – QUESTIONNAIRE

41. **UPN (PI)**

    _____

42. **Date of presentation (DD/MMM/YYYY)**

    _____

43. **Date of birth (DD/MMM/YYYY)**

    _____

44. **Gender**
    - male
    - female

45. **WHO classification**
    - copy of pathological report

    _____

46. **Site of disease**

    _____

47. **Clinical stage**
    - copy of CT / chest RTX / ultrasound report
        - I
        - II
        - III
        - IV

48. **B-symptoms at presentation**
    - yes
    - no
    - unknown / not done

49. **Bulky disease**
    - yes
    - no
    - unknown / not done

50. **Site of bulky disease**

    _____

    Extranodal disease
    - yes
    - no
    - unknown / not done

51. **Site of extranodal disease**

    _____

52. General Fitness Rating (ECOG) at presentation

- ECOG 0
- ECOG 1
- ECOG 2
- ECOG 3
- ECOG 4
- ECOG 5
- unknown / not done

53. Hb level [g/dl] at presentation

_____

54. Hb at presentation

- normal (≥ 12 g/dl)
- abnormal (< 12 g/dl)
- unknown / not done

55. WBC level [x10^9/L] at presentation

_____

56. WBC at presentation

- normal (4 x10^9/L – 11 x10^9/L)
- abnormal (< 4 x10^9/L or > 11 x10^9/L)
- unknown / not done

57. LDH at presentation

- normal
- abnormal
- unknown / not done

58. Bone marrow at presentation

- normal
- abnormal
- unknown / not done

59. Primary treatment description [text]

_____

60. CHOP – number of cycles

_____

61. IVE – number of cycles

_____

62. MTX – number of cycles

_____

63. Radiotherapy

- yes
- no
- unknown / not done

64. Autologous stem cell transplant

- yes
- no
- unknown / not done

65. Type of conditioninig

_____

66. Chemotherapy completed

- yes
- no
- not applicable
- unknown / not done

67. Outcome

- CR
- PR
- failure
- unknown / not done

68. Progression Free Survival Event

- relapse / death
- no relapse / no death

69. Progression Free Survival or Last Assessed Date (DD/MMM/YYYY)

_____

70. Overall Survival Event

- death
- no death

71. Overall Survival or Last Seen Date (DD/MMM/YYYY)

_____

72. Cause of Death

- lymphoma
- treatment complications
- other, please specify _____
- not applicable
- unknown / not done

73. Complications [text]

_____

_____

_____

74. Comments [text]

_____

_____

_____

75. UPN (PI)

_____

76. Type of salvage treatment

_____

77. Salvage treatment completed

- yes
- no
- unknown

78. Outcome of salvage treatment

- CR
- PR
- failure
- unknown / not done

79. Cause of Death

- lymphoma
- treatment complications
- other, please specify _____
- not applicable
- unknown / not done

80. Comments [text]

_____

_____

APPENDIX V - List of publications and presentations based on the work included in the thesis

Publications:

1. de Baaij LR, Berkhof J, van de Water JM, Sieniawski MK, Radersma M, Verbeek WH, Visser OJ, Oudejans JJ, Meijer CJ, Mulder CJ, Lennard AL, Cillessen SA. A new and validated clinical prognostic model (EPI) for enteropathy-associated T-cell lymphoma. Clin Cancer Res. 2015 Jul 1;21(13):3013-9.
2. Culpin RE, Sieniawski M, Angus B, Menon GK, Proctor SJ, Milne P, McCabe K, Mainou-Fowler T. Prognostic significance of immunohistochemistry-based markers and algorithms in immunochemotherapy-treated diffuse large B cell lymphoma patients. Histopathology. 2013 Dec;63(6):788-801.
3. Culpin RE, Sieniawski M, Proctor SJ, Menon G, Mainou-Fowler T. MicroRNAs are suitable for assessment as biomarkers from formalin-fixed paraffin-embedded tissue, and miR-24 represents an appropriate reference microRNA for diffuse large B-cell lymphoma studies. J Clin Pathol. 2013 Mar; 66 (3): 249-52.
4. Sieniawski MK, Lennard AL. Enteropathy-associated T-cell lymphoma: epidemiology, clinical features, and current treatment strategies. Curr Hematol Malig Rep. 2011 Dec; 6(4): 231-40 Review.
5. Sieniawski M, Angamuthu N, Boyd K, Chasty R, Davies J, Forsyth P, Jack F, Lyons S, Mounter P, Revell P, Proctor SJ, Lennard AL Evaluation of enteropathy associated T-cell lymphoma comparing standard therapies with a novel regimen including autologous stem cell transplant. Blood. 2010 May 6; 115(18): 3664-70.
6. Sieniawski M, Bhartia S, Wilkinson J, Proctor SJ Incidence and outcome of patients with diffuse large B-cell lymphoma with marrow involvement and preliminary experience of an adult acute lymphoblastic leukemia protocol (NEALL VI) in cyclophosphamide, doxorubicin, vincristin, and prednisolone – refractory patients. Leuk Lymphoma. 2009 Oct; 50(10): 1726-30.

Conference Presentations:

1. Sieniawski M, Lennard J, Lyons S, Maung Z, Mounter P, Proctor SJ, Lennard AL Aggressive primary chemotherapy with ifosfamide, etoposide, epirubicin / intermediate methotrexate and autologous stem cell transplantation in PTCL. International T-cell Lymphoma Phorum, Feb 2010, San Francisco
2. Sieniawski M, Angamuthu N, Boyd K, Chasty R, Davies J, Revell P, Proctor SJ, Lennard AL Sequential wvaluation of outcome in EATL comparing standard therapeutic approaches: surgery or CHOP-like chemotherapy with a new high-dose ifosfamide, etoposide, epirubicin / methotrexate and autologous stem cell transplant regimen: a prospective study of Scotland and Newcastle Lymphoma Group. 50th ASH Annual Meeting and Exposition, Dec 2008, San Francisco, USA
3. Sieniawski M, Lennard J, Millar C, Lyons S, Mounter P, Maung Z, Proctor SJ, Lennard AL Aggressive primary chemotherapy plus autologous stem cell transplantation improves outcome for PTCL compared with CHOP-like regimens. 51th ASH Annual Meeting and Exposition, Dec 2009, New Orleans, USA
4. Sieniawski M, Farrow M, Zhao X, Wilkinson J, Mainou–Fowler T, White J, MacIntyre J, Proctor SJ A novel Bayesian prognostic index for DLBCL: A new powerful tool for prediction of clinical outcome. 51th ASH Annual Meeting and Exposition, Dec 2009, New Orleans, USA
5. Sieniawski M, Wilkinson J, Culligan D, Davies J, Goodlad J, Jarrett R, Lennard AL, Lucraft H, Mainou-Fowler T, McKay P, Scott F, Tauro S, White J, Proctor SJ Duration of first remission in DLBCL defines groups of patients with different overall survival which cannot be entirely distinguished by clinical features or IPI at diagnosis: a prospective population-based study of the Scotland and Newcastle Lymphoma Group Blood. 50th ASH Annual Meeting and Exposition, Dec 2008, San Francisco, USA
6. Sieniawski M, Mainou-Fowler T, Wilkinson J, Culligan D, Davison J, Goodlad J, Jackson G, Lucraft H, Lewis J, Lennard A, McKay P, Miekeljohn D, Prescott R, White J, Proctor The effect of DLBCL treatment regimens on survival: a prospective-cohort study. 10th International Conference on Malignant Lymphoma, Jun 2008, Lugano, Switzerland