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Prognostic Markers in Diffuse Large B-cell Lymphoma

How Bad can it be

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Abstract

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Diffuse large B-cell lymphoma (DLBCL), which is the most common type of lymphoma, is characterised by its aggressiveness and poor outcome without adequate treatment and also for its biological and clinical heterogeneity. It is therefore highly desirable to gain a more profound understanding of the underlying biology of the disease, as well as predictive factors for the guidance of treatment. The studies presented here attempt to gain an overall grasp on DLBCL, from the epidemiological level down to the genomic level.

The tumour microenvironment consists of both tumour cells and normal infiltrating cells in a delicate interplay. By assessing the number of infiltrating mast cells (MCs) in the microenvironment, a correlation between low numbers of MCs and poorer prognosis of DLBCL was found.

However, malignant cells are not only affected by environmental conditions but also by intrinsic factors, such as small non-coding microRNAs. A low expression level of microRNA-129 was found to correlate with poor survival of DLBCL and the finding remained significant even for rituximab-treated patients.

An even smaller intracellular genomic unit is one single nucleotide. The single nucleotide polymorphism 309 (SNP309) is a T to G change in the promotor region of MDM2, a regulatory protein in the p53 pathway, which results in increased transcription of MDM2 and thus decreased levels of p53. It was found that homozygous T allele patients had longer overall survival, as well as disease-specific survival and disease-free survival. However, treatment with rituximab eliminated the predictive value of the SNP309 polymorphism.

In the last project presented in this thesis we used epidemiological methods to analyse all DLBCL cases diagnosed 2000-2013 in Sweden. Here it was possible to categorically show that higher age is an adverse prognostic factor, and most importantly, this starts from a young age.

In conclusion, within this thesis I have applied different laboratory and analysis techniques to examine DLBCL biology in relation to the clinic. I have identified potential new prognostic markers, contributed to an enhanced understanding of DLBCL biology and described epidemiological data from one of the largest DLBCL cohorts ever presented. All of these aspects provide important information for a deeper understanding of the disease DLBCL.

Keywords: DLBCL, Survival, Mast cell, Microenvironment, MicroRNA, MDM2, Polymorphism, Age, Epidemiology

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I may not have gone where I intended to go,
but I think I have ended up where I needed to be
D. Adams

To my family and friends

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Hedström, G., Berglund, M., Molin, D., Fisher, M.,Nilsson, G., Thunberg, U., Book, M., Sundstrom, C., Rosenquist, R., Roos, G., Erlanson, M., Amini, RM., Enblad, G. (2007) Mast cell infiltration is a favourable prognostic factor in diffuse large B-cell lymphoma. *Br. J. Haematol.*, 138(1):68–71

II Hedström, G., Thunberg, U., Berglund, M., Simonsson, M., Amini, RM., Enblad, G. (2013) Low expression of microRNA-129-5p predicts poor clinical outcome in diffuse large B-cell lymphoma (DLBCL). *Int. J. Hematol.*, 97(4):465–71

III Hedström, G.*, Thunberg, U.*, Amini, RM., Zainuddin, N., Enblad, G., Berglund, M. (2014) The MDM2 polymorphism SNP309 is associated with clinical characteristics and outcome in diffuse large B-cell lymphoma. *Submitted manuscript*
* These authors contributed equally to the work

IV Hedström, G., Hagberg, O., Jerkeman, M., Enblad, G. (2014) Impact of age in an unselected population of Diffuse large B-cell lymphomas. *Submitted manuscript*

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Related publications (not included in the thesis)

I Berglund, M., Hedström, G., Amini, RM., Enblad, G., Thunberg, U. (2013) High expression of microRNA-200c predicts poor clinical outcome in diffuse large B-cell lymphoma. *Oncol. Rep.*, 29(2):720–4

Contents

Abbreviations

aaIPI	Age adjusted IPI
ABC	Activated B-cell - like
Akt	Protein Kinase B
ALK	Anaplastic lymphoma kinase
AML	Acute myeloid leukemia
ARF	p19ARF
ASCT	Autologous stem cell transplantation
ATM	Ataxia telangiectasia mutated protein
BAFF-R	B-cell activator of the TNF-a family - receptor
BCL2	B-cell lymphoma 2
BCL6	B-cell lymphoma 6
BCR	B-cell antigen receptor
c-Myc	Myelocytomatosis oncogene isoform c
CD	Cluster of Differentiation
Cdk6	Cyclin-dependent kinase 6
cDNA	Complementary DNA
CLL	Chronic lymphocytic leukemia
CNS	Central nervous system
CSR	Class switch recombination
CT	Computed tomography
DFS	Disease specific survival
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
E-IPI	Elderly-IPI
EBER	EBV-encoded small RNA
EBV	Epstein-Barr virus
ECOG	Eastern Cooperative Oncology Group
EFS	Event free survival
ESMO	European Society for Medical Oncology
FDG	Fluorodeoxyglucose
FGF-2	Fibroblast growth factor 2
FO	Follicular B-cells
FOXP1	Forkhead box-P1
GC	Germinal centre
GCET1	Germinal center B-cell expressed transcript 1
GEP	Gene expression profiling

HDCT	High dose chemotherapy treatment
HHV	Human herpesvirus
HL	Hodgkin's lymphoma
HPF	High power field (x400)
HRS	Hodgkin and Reed-Sternberg
HSC	Haematopoietic stem cell
Ig	Immunoglobulin
IL	Interleukin
IPI	International Prognostic Index
IRF4	interferon regulatory factor 4
kDa	kiloDalton
LSS	Lymphoma specific survival
LT-HSC	Long-term HSC
MC	Mast cell
MCT	Tryptase-only MC
MCTC	Tryptase and chymase containing MC
MDM2	Murine double minute 2
miRNA	MicroRNA
MPP	Multipotent progenitor cell
mRNA	Messenger RNA
MUM1	Multiple myeloma oncogene 1
NGF	Nerve growth factor
NK	Natural killer (cells)
non-GC	not GC
NOS	Not otherwise specified
OS	Overall survival
P1	Promoter region 1
P2	Promoter region 2
p53	Protein 53
PCR	Polymerase chain reaction
PET	Positron emission tomography
PI3K	Phosphatidylinositol-3-kinase
PK	Protein kinase
QPCR	Quantitative
R-IPI	Revised IPI
REAL	Revised European American Lymphoma (classification)
RFLP	Restricted length polymorphism
RING	Really interesting new gene
RNA	Ribonucleic acid
RNU6B	RNA U6 small nuclear 2
RR	Relative risk ratio
RS	Relative survival
RT	Radiotherapy

RT-	Real time-
s-LDH	Serum lactate dehydrogenase
SHM	Somatic hypermutation
SLR	Swedish lymphoma registry
SNP	Single nucleotide polymorphism
SOX4	Sex determining region Y - box 4
Sp1	Sp1 transcription factor
ST-HSC	Short-term HSC
TD	T-cell dependent
TI	T-cell independent
TNF-α	Tumour necrosis factor alpha
TP53	p53 gene
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

Introduction

Cancer

Life is defined as the ability to grow, change and ultimately to die. Without death we would not value life as we do. In a multicellular organism, such as the human body, the individual cell has put aside it's own interest to maximise cell growth and proliferation in favour of the entire individuals interest to survive and propagate. To maintain the balance, every phase in the cell cycle is tightly monitored and controlled. Unfortunately, control mechanisms can cease to maintain their proper function due to a vast number of causes. At best, a single cell will die and the individual will continue to prosper. At worst, the single cell will turn malignant.

Cancer as a disease has been known since ancient times, but it was not until Watson and Crick published data regarding the DNA double helix, in the early 1950s, that researchers were able to pinpoint the exact genetic mutations underlying the malignification of cells. To date, our understanding of mechanisms driving a cell to malignant transformation has increased substantially and we have accepted the key role of potential oncogenes and suppressor genes in cancer development [1-3]. Other regulatory mechanisms have not been equally elucidated and research about specific regulatory pathways and epigenetic regulation is only in its infancy, although it seems that defects in a few pathways and regulatory mechanisms are responsible for malignant transformation in a broad range of cancers [4, 5].

But cancer cells are no solo players. As Hanahan *et al.* so excellently summarised in 'Hallmarks of cancer: The next generation', cancer cells function in a complex interplay with the microenvironment [6]. Normal cells from the immune system can be found in the tumour microenvironment, altering the conditions for tumour cells [7]. Taken together, malignancies with similar presentations clinically and morphologically could thus have aberrations in different regulatory pathways but also be under influence from differing microenvironments.

Diffuse large B-cell lymphoma (DLBCL) is a distinct example of a malignancy comprising of very heterogeneous cases with different clinical outcome [8] even though all DLBCL originates from transformation of lympho-

cytes, normally existing as a part of our immune system. Therefore, a search for better prognostic markers may help us differentiate DLBCL into sub-entities with different prognoses, for which further optimisation of treatment could be warranted.

This thesis has come to concentrate on different descriptive aspects of DLBCL biology, from an overall perspective of epidemiological analyses to a more tapered description of parts of the microenvironment and certain important regulatory processes on intracellular and gene level.

The first edition of the 'WHO classification of lymphoid neoplasms' described the entity 'diffuse large B-cell lymphoma' (DLBCL). The second edition of the WHO classification from 2008 includes an entity named diffuse large B-cell lymphoma not otherwise specified (DLBCL-NOS) [8]. In this thesis 'diffuse large B-cell lymphoma', or DLBCL, is used synonymously with both these WHO classification terms.

The history of diffuse large B-cell lymphoma

The early history of lymphomas is essentially a history of Hodgkin's lymphoma. It began in 1832 when Thomas Hodgkin published a paper entitled 'On Some Morbid Appearances of the Absorbent Glands and Spleen', and described the disease that later came to bear his name [9]. He performed his work as 'Inspector of the Dead and Curator of the Museum' at the medical department of Guy's Hospital. Without microscopic studies, Hodgkin described a few patients with lymphoma, identified at 'cadaveric inspection', differentiating these from the more common disorders of lymphadenopathy and splenomegaly (e.g. syphilis, tuberculosis, amyloidosis, carcinoma, leukaemia and other). However, during the following years Hodgkin's disease fell into oblivion and it wasn't until Samuel Wilks, some decades later (1865) included tissue samples from some of Hodgkin's cases in his own research, and provided a more detailed pathological description, that Hodgkin's early contribution was acknowledged by the naming of Hodgkin's disease [10]. In 1878 Greenfield described the pathognomonic large cells of Hodgkin's disease even though Steinberg (1898) and Reed (1902) are acknowledged with the first full description of the cells [11].

If the history of Hodgkin's lymphoma and classification is a clear path, the historical path of other lymphomas is winding and impenetrable. During the same time-period as Thomas Hodgkin investigated lymphoma, Rudolph Virchow described the first cases of leukaemia in 1845 and later (1863-67) expanded his scientific contribution when he named a subdivision 'lymphosarcoma' [12, 13]. During the following decades many names were used, not

always in coherence, but in 1893 Kundrat reinstated the term lymphosarcoma in the sense used by Virchow [13].

Follicular lymphoma was the first sub-entity of the lymphosarcomas clearly described (in 1925) [14], although the malignant character was recognised first later. However, little further progress was made in defining different subsets of lymphomas and to clarify the confusion in names used. During this time-period, and in the following decades, several pathologists also advocated a 'fluid' view of lymphoma, stating that malignant lymphoma was not several diseases but one disease with the ability to express different microscopic appearances.

In 1942, Gall and Mallory introduced a classification based on clinicopathologic criteria, attempting to bring forward a systematic classification of lymphomas [15]. Rappaport *et al.* carried the work forward with their classification whereas Lennert *et al.* later proposed the Kiel classification, followed by others [16-19]. Another milestone in lymphoma classification came from advancements in immunology during the 1960s, and the understanding of lymphoid differentiation along two different tracks. One path for T (thymus derived) cells and one path for B (bursa derived) cells [20, 21]. When it later - during the 1970's - became possible to immunophenotype B- and T-lymphocytes, the diagnosis and classification could take another leap forward with further subdivision of lymphomas as seen in the revised European-American lymphoma (REAL) classification [22].

More refined diagnostics lead to the 2001 WHO classification which, with the updated WHO classification in 2008, described a current state of more than 50 different types of lymphoma [8, 23, 24]. Long gone is the time when lymphomas were believed to be one single disease with multiple expression patterns. There is no doubt that lymphomas are several, distinctly separate, diseases, and that diffuse large B-cell lymphoma (DLBCL) is one, or perhaps many, of them. DLBCL, can now be further subdivided into T-cell rich large B-cell lymphoma, DLBCL associated with inflammation, EBV+ DLBCL of the elderly and DLBCL not otherwise specified (DLBCL-NOS) [23].

Normal lymphocyte development

Long-term haematopoietic stem cells (LT-HSC) in the bone marrow have the ability for self-renewal and reconstruction of the entire haematocyte system. LT-HSC differentiate into short term HSC (ST-HSC) which in turn differentiate further into multipotent progenitor cells (MPP) [25]. Knowledge of the steps that follow is mostly based on murine models and all intermediate stages have not been found in humans [26], but through a series of intermediate steps MPP differentiate down different paths [25]. One of these paths will end up in the pro-B cells, representing the first irrevocably committed B-cell precursor. It is during these early stages of B-cell development that rearrangement starts of the B-cell receptor (BCR) heavy chain locus as well as up-regulation of B-cell specific genes such as PAX5, CD19, Igα and Igβ [27, 28]. When pro-B cells develop into pre-B cells the immunoglobulin (Ig) heavy chain (IgH) locus undergoes random recombination of the diversity (D) and joining (J) gene segments on both alleles, followed by recombination of the variable (V) segment to the DJ locus on one allele and, if non-functional, also on the second. Once the B-cell carries functional rearrangement, the cell assemble these and express a pre-B-cell receptor (pre-BCR) on the surface [29].

The expression of a pre-B-cell receptor is the first requirement of a functional BCR in a B cell for further development towards a mature B-lymphocyte. Appropriate pre-BCR signalling leads to cell proliferation, as well as further phenotypic changes towards B-cells. The cells then enter the small-pre-B stage and begin the rearrangement of the Ig light chain locus. Together with Igα and Igβ the light and heavy chain form an antigen-specific cell surface receptor. Through a receptor mediated negative selection, where most (about 80%) of immature B-cells succumb due to auto-reactivity, those surviving leave the bone marrow and migrate to the spleen where they become transitional B-cells [27, 30]. In the spleen, functional B-cells, upon BAFF-R (a TNF-α family B-cell activator receptor) signalling, develop further into either marginal zone B cells, which later have the ability to become short-lived plasma cells, or mature naïve B-cells [25, 31]. However, the mandatory involvement of spleen in naïve B-cell development is under debate [32].

Naïve B-cells are capable of interaction with foreign antigen in either a T-cell dependent (TD) or T-cell independent (TI) manner. Following TD activation, B-cells usually enter the follicle of secondary lymphoid organs, including lymph nodes. Here B-cells undergo heavy clonal expansion and form structures called germinal centres (GCs). The next step in B-cell maturation takes place in GCs where B-cells undergo somatic hypermutation (SHM) and class switch recombination (CSR) [33]. SHM produces point

mutations in the Ig V gene and CSR switch of the constant region of the antibody to a secondary one, thus producing different antibody classes (e.g. IgG, IgE and IgA) and altering effector function. The ultimate goal is production of high-affinity antigen-specific antibodies [34]. In the end, GC B-cells have three possible fates: Apoptosis, when CSR and SHM leads to reduced antigen binding for the CBR or autoimmunity; Undergo further rounds of SHM and CSR; Differentiation into either memory B cell or plasma cells and exiting of the GC [33, 35] (Figure 1).

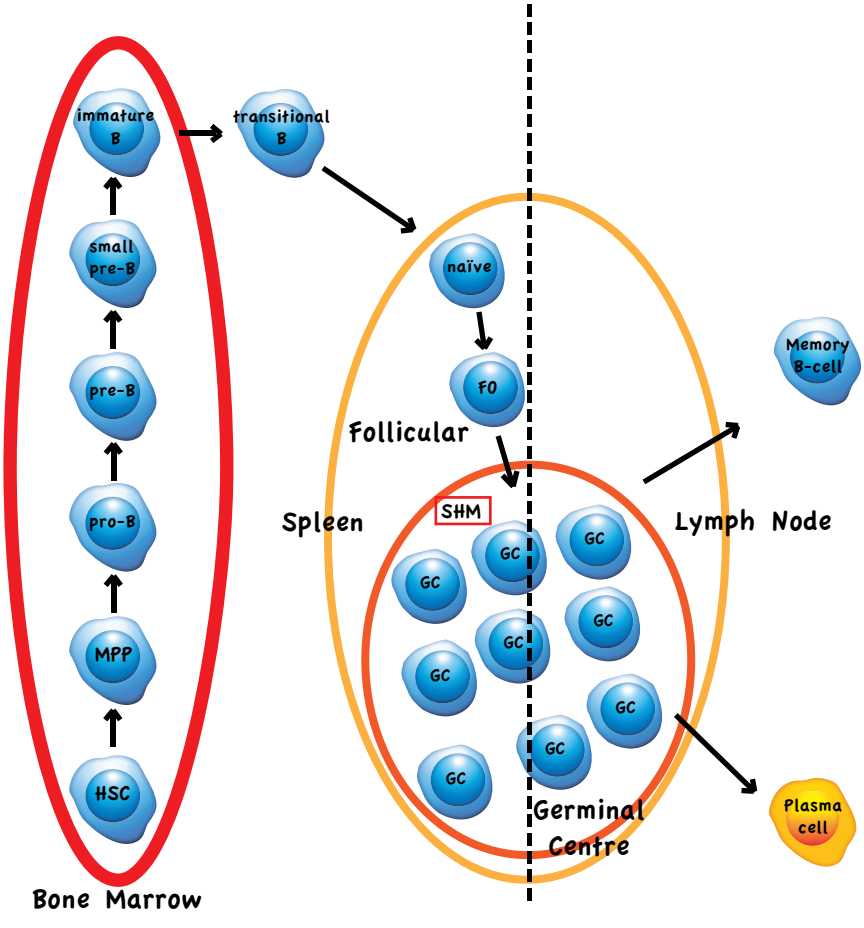

Figure 1. Haematopoietic Stem Cells (HSC) differentiate in bone marrow (MPP: multipotent progenitor cells) while undergoing Ig-gene recombination and finally leave the bone marrow as Transitional B-cells. These travel to the spleen where they become naïve B-cells in the marginal zone and further differentiate into follicular B-cells (FO) and enter germinal centre (GC), either in spleen or other lymphoid organs where they undergo somatic hypermutation (SHM). In the end, the B-cell can differentiate further into long-lived plasma cells or memory B-cells. Modified from Kuppers [36] and Pieper [31].

Epidemiology

With an annual incidence of about 4% of all new cancers in USA and about 3% in Sweden, malignant lymphoma (Hodgkin's lymphomas excluded) is one of the most common cancer forms [37-39]. However, as a group, lymphomas consist of many different types [8]. The most common type is DLBCL, accounting for about 30% to 40% of all B- and T-cell lymphomas [37, 39-41]. Men are slightly more susceptible than women [40, 42] and the median age at diagnosis in Sweden is approximately 70 years, although DLBCL can occur in all age groups [42].

DLBCL can arise *de novo*, without a previous history of indolent lymphoma, or from a transformation of a previous low-malignant lymphoma such as follicular lymphoma, chronic lymphocytic leukaemia (so called Richter's transformation), marginal zone lymphoma or nodular lymphocyte predominant Hodgkin's lymphoma [8, 43]. The great number of subtypes of DLBCL and the clinical and biological heterogeneity still existing within each respective subtype, has given rise to questions about the meaning of classification and the practical clinical implications [44, 45]. Regardless, it reflects the complexity of DLBCL biology and the consequent difficulties in finding clinically relevant markers for subgrouping.

Diagnosis of DLBCL

Diagnostic criteria

Most commonly, DLBCL present with clinically detectable enlarged lymph nodes, but sometimes not [8]. B-symptoms (Fever > 38 ºC; night sweating; unintentional weight loss > 10% in less than 6 months) may be present [46]. Lymphoma diagnostics rely solely on histopathological examination of immunohistochemical stainings of tumour material, either from removed lymph node or needle biopsy sufficient for formalin fixation and paraffin embedding [47]. Histopathological examination should be done according to the WHO classification [8]. A thorough clinical examination, medical history, laboratory investigations together with computed tomography (CT) scan is essential for diagnosis and risk determination. Depending on lymphoma type and clinical characteristics, a bone marrow biopsy may be warranted before initiation of treatment. A diagnostic spinal tap should be considered in certain high-risk patients [47]. FDG-positron emission tomography (PET)/CT can be performed prior to treatment, but the exact role of PET in a clinical setting is yet to be determined [47-49].

Sites of involvement

At the time of diagnosis, patients present with nodal or extra nodal disease. Thirty to forty percent present a solely extra-nodal disease with the gastrointestinal tract as the most common extra-nodal site [8, 50, 51]. Other extranodal sites include bone, testis, spleen, liver and kidney. Bone marrow is affected in up to 70% of patients and involvement may be concordant with DLBCL, but is more commonly concordant with a low-grade B-cell lymphoma in the bone [8].

Staging and clinical prognostic factors of DLBCL

The biological heterogeneity of DLBCLs is reflected in the clinic where DLBCL patients may have markedly divergent outcome. Despite efforts made, few prognostic factors have found their way into every day clinical practice. One major tool for prognostication and stratification of treatment of DLBCL patients is the International Prognostic Index (IPI), first described in 1993. The IPI is based upon data from more than 2000 patients, treated between 1982 and 1987. Five clinical factors were found to have adverse prognostic influence: age > 60 years, elevated serum lactate dehydrogenase (s-LDH), Eastern Cooperative Oncology Group (ECOG) performance status 2-4, Ann Arbour stage III-IV and involvement of more than one extra-nodal site. According to IPI, DLBCL patients can be categorized into four risk groups: low risk, intermediate low risk, intermediate high-risk and high-risk groups, each group having different survival rates (73%, 51%, 43% and 26% respectively for 5-year survival). In the same article, an age-adjusted IPI (aaIPI - adverse risk factors: Ann Arbour stage III-IV, elevated s-LDH and performance status 2-4) was proposed. Unlike the intention with aaIPI directed mainly at patients younger than 60 years, the aaIPI was also found to be of use for older patients [52]. However, both IPI and aaIPI were composed for patients treated with an anthracycline-containing regiment (predominantly CHOP) and accordingly have been found less useful in rituximab (e.g. R-CHOP) treated patients [53].

Instead, a revised IPI (R-IPI) has been found more accurate in an R-CHOP treated cohort. R-IPI reallocates patients from the initial four risk groups of IPI into R-IPIs three risk groups ‘very good’, ‘good’ and ‘poor’, respectively. Unfortunately, neither IPI nor R-IPI manage to identify a patient group with 50% chance, or less, of survival [54]. Therefore other prognostic variables to identify those most in need of advanced therapies must be considered. Attempts have been made with alternative prognostic indexes applicable for certain groups. The Elderly Prognostic Index (E-IPI) has since been constructed due to the distribution of IPI and aaIPI being skewed to-

wards higher risk groups among the elderly (older than 70 years), thus failing to distinguish those within low IPI risk groups [55].

Nevertheless, with increased understanding of the molecular pathobiology of DLBCL, it is evident that IPI, aaIPI and R-IPI fail to identify certain groups of patients. Even though several alternative propositions to IPI and aaIPI have been made, the original prognostic indexes are still major clinical tools for prognostication. But by assessing gene expression risk scores, a sub-classification system entirely independent of IPI has emerged (see 'Molecular Classification of DLBCL') [56, 57]. Bret *et al.* combined a gene expression profile and IPI, thus distinguishing multiple subgroups with markedly different survival ratios [58]. Nevertheless, the combined risk score must be validated in a prospective study and gene expression profiling must be implemented in clinical practice before a combined risk score based on gene expression profiling will be widely employed.

Cell of origin

According to the current dogma of lymphomagenesis, malignant transformation of a B cell lymphocyte occurs at a particular stage during normal B-cell differentiation, freezing the differentiation stage. In line with this, different lymphoma types represent a particular maturation step in lymphocyte maturation. Each stage during B-cell differentiation has a number of stage-specific cell characteristics. The malignant B-cell and its subsequent clones carry these specific morphological, immunophenotypic and gene expression properties, enabling a backward tracing of the features of malignant B-cells to their origin and clonal history. At least in theory [59].

Through studies of particular structures of the B-cell receptor and expression of differentiation markers, the origins of various B-cell lymphomas have been determined. Most types of B-cell lymphomas are derived from GC or post-GC B-cells [60, 61]. Gene expression profiling (GEP) of DLBCL has found a B-cell gene expression profile associated with B-cells derived from GC (GC-type), and another gene expression pattern resembling that of activated B-lymphocytes in peripheral blood (ABC-type), i.e. post-GC/non-GC (described in other section of this thesis) [56]. Thus, DLBCL contains either ongoing somatic hypermutation of Ig genes (GC-type) or no ongoing hypermutation but evidence of a previous passage through germinal centre (ABC-type/non-GC) [62]. The somatic hypermutation that occurs in germinal centres, the massive clonal expansion of GC B-cells and the special microenvironment of the GC, may all be risk factors for initiation of, or full transformation to, a malignant B-cell clone in the GC [60].

These findings support the theory that DLBCL arises from specific stages of normal B-cell development, GC or non-GC, and present a frozen differentiation stage with typical morphological, immunophenotypic and gene expression profiles representative of the stage of origin. However, as it turns out, the frozen stage of malignant B-cells might not be entirely frozen, since further changes in the genome may occur after the lymphomagenesis has been initiated [59].

WHO classification

With the last revision of 'WHO classification of Tumours of Haematopoietic and Lymphoid Tissues' in 2008, came an expanded spectrum of aggressive B-cell lymphoma subtype classifications, several now recognised as distinct entities. DLBCL can be classified as DLBCL-NOS (an exclusion diagnosis distinct from the subtypes), specific subtypes of DLBCL, or other lymphomas of large B-cells [8]. Classification according to the WHO 2008 table (Table 1) is primarily based upon distinct morphological, biological, immunophenotypic and/or clinical parameters [45].

2008 WHO classification of DLBCL variants and subtypes
Diffuse large B-cell lymphoma not otherwise specified (DLBCL-NOS)
Common morphological variants
Centroblastic
Immunoblastic
Anaplastic
Rare morphological variants
Diffuse large B-cell lymphoma subtypes
T-cell/histocyte rich large B-cell lymphoma
Primary DLBCL of the CNS
Primary cutaneous DLBCL, leg type
EBV-positive DLBCL of the elderly
Other lymphomas of large B-cells
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
ALK-positive large B-cell lymphoma
Plasmablastic lymphoma
Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
Primary effusion lymphoma
Borderline B-cell lymphomas
B-cell lymphoma, unclassifiable, with features between DLBCL and Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features between DLBCL and classical Hodgkin's lymphoma

Table 1: WHO 2008 Classification of DLBCL variants and subtypes [8]

Immunohistochemistry

Morphology

Most commonly, the entire lymph node architecture is obliterated by diffuse proliferation of large lymphoid cells with nuclei size equal to or exceeding the nuclei in normal macrophages, or more than double nuclei size than normal lymphocytes. Tumour cells may also aggregate in one/or several parts of the lymph node. Partial nodal intrusion of tumour cells may be interfollicular and/or sinusoidal. Often, the perinodal tissue is infiltrated and bands of sclerosis may be observed. Due to the heterogeneity seen in DLBCL, three common morphological sub-variants of DLBCL are recognised as well as additional rare variants. All variants may exist with high number of histocytes and/or T-cells, not to be confused with histocyte/T-cell rich DLBCL, unless all inclusion criteria are fulfilled.

- The centroblastic variant is the most common variant of DLBCL. Centroblasts are medium to large lymphoid cells with oval to round vesicular nuclei, containing fine chromatin. The cytoplasm is amphophilic to basophilic. However, most commonly a mixture of centroblasts and immunoblasts is evident in DLBCLs.

- The immunoblastic variant has more than 90% immunoblastic cells in the tumour. The cells have a single, centrally located nucleolus and basophilic cytoplasm. This variant of DLBCL may be confused with extramedullary involvement of plasmablastic lymphoma or immature plasma cell myeloma.

- The anaplastic variant of DLBCL is characterised by large cells with bizarre pleomorphic nuclei and may mimic undifferentiated carcinoma [8].

Immunophenotype

DLBCL tumour cells express characteristic B-cell markers such as CD19, CD20, CD22, CD79a and PAX5. They also express surface and/or cytoplasmic immunoglobulin (IgM>IgG>IgA) in 50% to 75% of all cases. An exception is ALK-positive DLBCL that uniformly expresses clonal cytoplasmic IgA. Expression of CD30 varies but with positive CD30 expression, demarcation is required from ALK-negative anaplastic large cell lymphomas which are T/natural killer cell (T/NK cell) neoplasms [63].

DLBCL further express transcription factors such as OCT-2 and BOB.1 from the B-cell program. LM02 expression is associated with GC phenotype

whereas FOXP1 is associated with ABC phenotype [63]. Inter-individual expression varies for CD10, BCL6, MUM1/IRF4, which is reflected in their role in different classification algorithms [64, 65]. On average, about 40% of DLBCL express CD10, 60% BCL6, 50% BCL2, 40% MUM1, 30% p53, 20% CD43, 10% CD30 and CD5 <10% [63]. Ki67 reflects proliferation activity, which is usually high (40% to 90%) and an index of 80% or more has been found to correlate with poor prognosis [8, 63, 66]. T-cell markers are usually absent, although DLBCL variants with positive CD5 and CD3 have been described [63]. Other variable markers in DLBCL are GCET1 and c-Myc (Table 2) [65, 67]. However, DLBCL may lack one or several B-cell markers (CD19, CD20, CD22 or CD79a), may (unlike normal GC cells) coexpress MUM1 and BCL6, or express the t(14;18) translocation [63, 68]. DLBCL seldom expresses cyclin D1 although it occasionally occurs [63].

	Immunohistochemical marker	
Type	**Marker**	**Description**
B-cell markers	CD19, CD20, CD22, CD79a, PAX5	
Immunoglobulin	IgM>IgG>IgA	
Possible anaplastic	CD30 (10%)	
Other markers	BCL6 (60%)	GC-antigens
	BCL2 (50%)	Involved in apoptosis
	CD10 (40%)	GC-antigens
	MUM1 (40%)	Associated with ABC subtype
	P53 (30%)	Involved in cell cycle control
	CD43 (20%)	T-cell lineage antigen
	CD5 (<10%)	T-cell lineage antigen
	Ki67 (40-90%)	Proliferation
	OCT-2	Marker of complete B-cell program
	BOB.1	Marker of complete B-cell program
	GCET1	GC-marker
	c-Myc	Involved in cell cycle control
	FOXP1	Associated with ABC subtype
	LM02	Associated with GC subtype

Table 2: Immunohistochemical markers in DLBCL [28, 63, 65, 67, 69]

Molecular classification of DLBCL

Gene expression profiling (GEP) has been a priceless tool in the research and establishment of the different DLBCL types. GEP of DLBCL has identified several signature patterns resembling normal B-cell development as well as different GEP signatures correlating with prognosis [56, 70-73]. Based upon GEP, DLBCL can be distinguished into three major subgroups according to profiles resembling normal B-cell stages, a subdivision originally based on a cell-of-origin model. One subgroup expresses genes resembling germinal centre B-cells (GC group) whereas another group expresses genes resembling activated B-cells (ABC group) [56, 57]. Rosenwald *et al.* also identified a third subgroup, type 3 DLBCL, with a heterogeneous gene expression pattern, indicating the possibility that type 3 DLBCL might consist of more than one type of diffuse large B-cell lymphoma. Notably, patients with type 3 DLBCL retrospectively had similar clinical outcome as patients with ABC type (5 year survival rate 39% and 35% respectively whereas 60% for the GC type) [70]. However, these findings are based upon GEP. Since GEP is neither free nor resource efficient, investigators have attempted to translate the findings made by GEP regarding subdivision of DLBCL to an interconvertible immunohistochemical expression pattern [64, 65, 74-76]. Unfortunately no single immunohistochemical staining has sufficient accuracy to subdivide DLBCL into GC or ABC or type 3 DLBCL with preserved conformity to GEP [77]. Instead, several immunohistochemical stainings must be considered in an algorithm. Hans *et al.* described an algorithm including immunohistochemical stainings against CD10, BCL6 and MUM1 to subdivide DLBCL into GC or non-GC subtypes (due to similar survival rates for ABC subtype and type 3 DLBCL, Hans *et al.* incorporated type 3 DLBCL into the ABC subtype, creating the non-GC subtype) [64].

The possibility of using immunohistochemical stainings for DLBCL subdivision and prognostication was re-confirmed by Berglund *et al.* [76]. However, the value of an immunohistochemical based algorithm has been questioned since studies have failed to replicate earlier results when patients are treated with rituximab [78, 79] whereas the GEP model seems to still apply [80]. Nevertheless, by the refinement of already existing algorithms and description of new ones, immunohistochemical stainings can still be a useful tool for subdividing DLBCL. Choi *et al.* have proposed an algorithm based on GCET1, CD10, BCL6, MUM1 and FOXP1 with 93% concordance with GEP classification of DLBCL [65] and the newly described Tally algorithm, which has a slightly different approach, is based on CD10, GCET1, MUM1, LMO2 and FOXP1 [81] whereas Nyman *et al.* proposed a simplified algorithm based only on MUM1 and FOXP1 [82] (Figure 2).

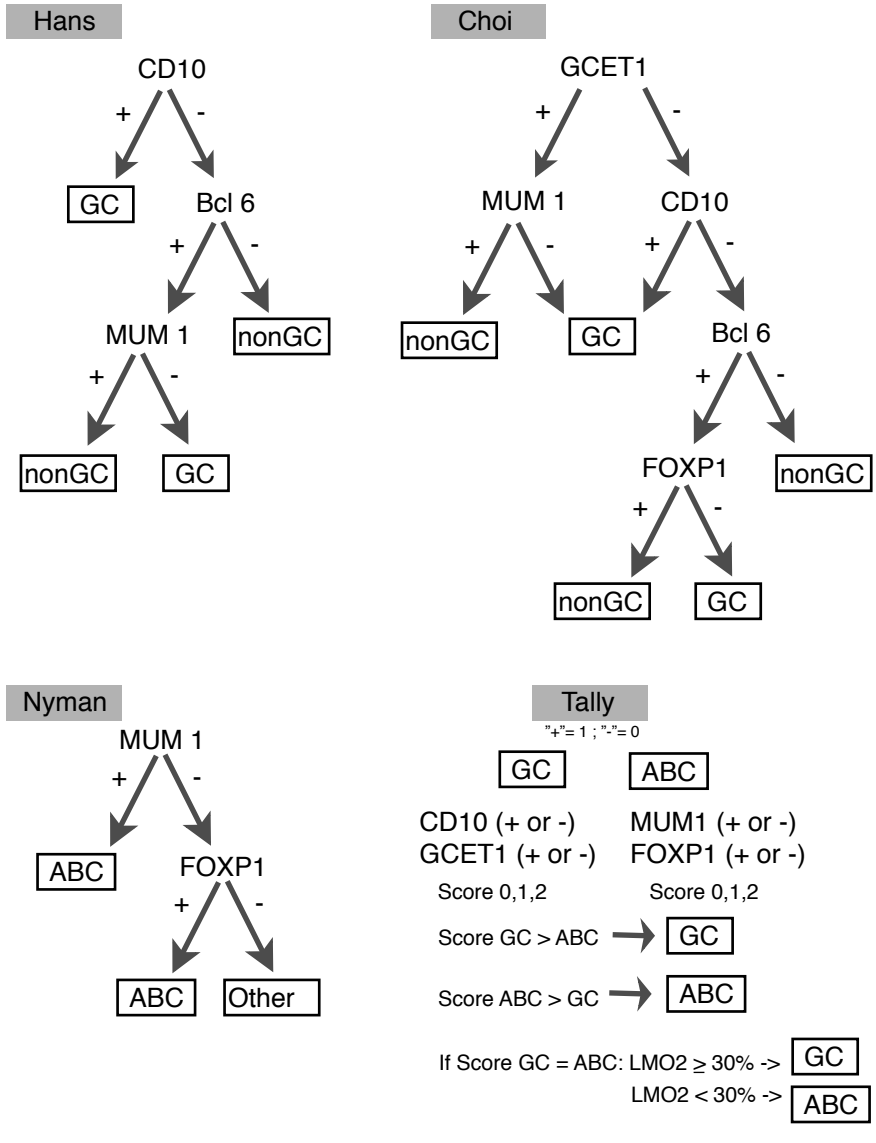

Figure 2. Classification of DLBCL by immunohistochemical algorithms. In the Tally algorithm, antibody results are not examined in a particular order, instead they are summarized and the weight of the score determines the outcome.

However, immunohistochemical stainings and GEP are not the only techniques available for molecular classification of DLBCL. Fluorescence In Situ Hybridisation (FISH) is commonly used to detect chromosomal translocation in lymphomas, where the most common in DLBCL are t(14;18)(q32;q21) and t(3;14)(q27;q32), involving rearrangement of the BCL2 and BCL6 genes respectively. The translocation of BCL2 juxtaposes

regulatory elements from the Ig locus close to the BCL2 locus, but also disrupts the negative suppression of BCL6 [83, 84]. Increased levels of BCL2 have been found to correlate with worse outcome in DLBCL. Another potential translocation in DLBCL involves the c-Myc oncogene located at region 8q24. It is deregulated in about 10% of DLBCL cases, most commonly due to t(8;14)(q24;q32) translocation which places it under control of the IgH promoter, leading to over-expression of the transcription factor c-Myc [83].

c-Myc participates in regulation of cell proliferation and differentiation but also in cell apoptosis, with multiple control mechanisms at these levels. It is present during certain stages of B-cell development, but not all, and a constant over-expression in B-cells leads to lymphoma development [3]. Sometimes DLBCL harbours a simultaneous rearrangement of c-Myc and BCL2 or/and BCL6 leading to a very aggressive clinical course in these so called 'double-hit' lymphomas [85].

Unfortunately the progress in translating molecular classification into effective treatment strategies has met many challenges, although there may be light ahead with new drugs directed against small molecules [86-89].

Treatment

An international perspective

Treatment recommendations for DLBCL are continuously revised. Although improvements have been made in the treatment armamentarium, especially with the introduction of immunotherapy (the first substance being an anti-CD 20 antibody known as rituximab) and haemopoietic growth factors, which have allowed us to treat older patients, differentiation and individualisation of treatment is still based upon a few rough clinical factors. An age above 60 years is included as a parameter in the IPI and is still one of the most important factors when it comes to decisions about treatment regimens [90].

Chemotherapy

From the first description in 1978 of the combined chemotherapy regimen with Cyclophosphamide, Doxorubicine, Vincristine and Prednisone (CHOP; Table 3) several other regimens have been investigated [91-96]. Despite the multitude of tested regimens throughout the years, CHOP still remains key in the treatment-arsenal and is often used with tolerable side-effects even in an elderly population [97]. Recently, more intense chemotherapy regimens (LNH03-2B trial) have been found to improve outcome in young patients and have been incorporated in new treatment strategies (Table 4) [47, 98].

The ESMO guidelines from 2012 are summarised in Table 5. However, the establishment of these strategies in clinical practice may not yet have been fully implemented globally.

CHOP chemotherapy		
Drug	**Group of drug**	**Major side effect**
Cyclophosphamide (C)	Alkylating	Haematologic, nausea
Doxorubicine (H)	Anthracycline	Haematologic, cardiac
Vincristine (O)	Vinca-alkaloid	Nausea, neuropathy
Prednisone (P)	Cortico-steroid	Endocrine (diabetes etc.)

Table 3: CHOP chemotherapy

ACVBP chemotherapy		
Drug	**Group of drug**	**Major side effect**
Doxorubicine (A)	Anthracycline	Haematologic, cardiac
Cyclophosphamide (C)	Alkylating	Haematologic, nausea
Vindesine (V)	Vinca-alkaloid	Myelosuppression, neuropathy
Bleomycin (B)	Other	Pulmonary
Prednisone (P)	Cortico-steroid	Endocrine (diabetes etc.)

Table 4: ACVBP chemotherapy

2012 ESMO guidelines for treatment of DLBCL		
DLBCL < 61 years		
low risk IPI, no bulk	**low risk IPI with bulk; low-intermediate risk IPI**	**Intermediate-high risk IPI; high risk IPI**
R-CHOP 21 x 6	R-ACVBP with sequential consolidation Or R-CHOP 21 x 6 + IF-RT on bulk	R-CHOP 21 x 8 or R-CHOP 14 x 6 with 8 R Or R-CHOEP 14 x 6 Or R-ACVBP + HDCT with ASCT Or R-dose-dense (R-CHOP 14 like) + R-HDCT with ASCT
DLBCL > 60 years		
Healthy	**> 80 years without cardiac dysfunction**	**Unfit or frail or > 60 years with cardiac dysfunction**
R-CHOP 21 x 8 (R-CHOP 21 x 6 for low risk IPI) Or R-CHOP 14 x 6 with 8 R	attenuated regimens: R-miniCHOP 21 x 6	Doxorubicine substitution with Etoposide or liposomal doxorubicine or others: R-C(X)OP 21 x 6 Or palliative care

Table 5: ESMO guidelines [47]

Immunotherapy

The addition of rituximab to chemotherapy has significantly improved both progression-free and overall survival for DLBCL patients [78, 97, 99, 100]. The number of cycles administered typically depends on clinical prognostic factors included in IPI (Table 5) [47, 101].

The effect of rituximab is mainly through chemo-/immuno-sensitisation where rituximab triggers tumour cell death through antibody dependent cell dependent cytotoxicity, complement-dependent cytotoxicity and apoptosis [102, 103]. Rituximab interacts with several regulatory cell-signalling pathways, resulting in synergistic activity with chemotherapeutic drugs [103]. However, tumour cells must express CD20 for the anti-CD20 antibody rituximab to exert its effect. Unfortunately not all patients respond, a phenomenon that has not yet been fully elucidated [102].

Radiotherapy

Inclusion of radiotherapy (RT) in treatment of DLBCL seems to have some supporting evidence in the literature, but not yet sufficiently for general recommendation, although there is an ongoing debate regarding the applicability of RT [47, 101, 104]. One exception is primary DLBCL in testis and CNS where RT could play a role in treatment [47].

Stem cell transplantation

Allogeneic stem cell transplantation is a transplantation where the patient receives cells from another person (a donor), in contrast to autologous stem cell transplantation (ASCT), where the patients' own stem cells are harvested before high dose chemotherapy is administered. While ASCT is standard treatment for patients with relapsed DLBCL the recently published ESMO guidelines has found - due to varying results in clinical trials - ASCT to still be considered experimental in first-line therapy, although it may be used in first-line therapy selectively for high-risk patients [47].

CNS-prophylaxis

DLBCL patients with involvement of more than one extranodal site are at higher risk of relapse in the central nervous system (CNS) if they have high-intermediate or high-risk IPI, especially if s-LDH is elevated [105]. CNS prophylaxis should be administered to this group [106] but it is possible that intrathecal injection of methotrexate is not the optimal method. Intravenous high-dose methotrexate could be a relatively safe alternative with efficient disease control [107].

Treatment in Sweden

Treatment in Sweden has changed over the last decade but present treatment recommendations are R-CHOP-21 x 6 for patients with aaIPI = 0 (evidence 1), or R-CHOP-21 x 3 with additional RT if stage I A and tumour size < 10 cm (evidence 2-3). For patients with aaIPI = 1 recommendations are use of R-CHOP-14 x 6 or R-CHOP-21 x 8 (evidence 1). Patients with aaIPI = 2-3 are recommended R-CHOP-14 x 6 (evidence 5) or R-CHOEP-14 x 6 (evidence 4) if they are < 66 years whereas R-CHOP-14 x 6 is recommended (evidence 1) for those > 65 years. Special recommendations exist for primary CNS lymphoma, bulky disease and other, but are beyond the scope of this text.

Inflammation and cancer / the microenvironment

In 'Hallmarks of Cancer' from 2000, and emphasised in 'Hallmarks of Cancer: The Next Generation' from 2011, Hanahan and Weinberg postulate that tumours are complex tissues, not only masses of cancer cells, containing a heterogeneity of integrative cells. The 'normal cells' recruited to the tumour form the tumour-associated stroma and are active participants in tumourigenesis and should therefore not be described as passive bystanders [5, 6]. The last couple of decades, we have known that the tumour consists of networks of tumour cells, inflammatory cells, fibroblasts and microvessels [6, 108, 109]. In 'Hallmarks of Cancer: The Next Generation' Hanahan and Weinberg add two emerging Hallmarks to the original six. One is the capability to modify cellular metabolism and the other is the ability of cancer cells to evade immunological destruction [6].

The immune system has the potential to destroy cancer cells or inhibit further growth, but conversely also has the capability to facilitate their growth. Full activation of adaptive immune cells (which express diverse, somatically generated, antigen-specific receptors) might result in eradication of tumour cells whereas chronic activation of innate immune cells (which normally forms the first line of defence against pathogens) might actually enhance tumour development [110]. Two potential pathways linking inflammation and cancer have schematically been drawn. The intrinsic pathway, where genetic events cause tumour development, initiate the expression of a pro-inflammatory program guiding the construction of the inflammatory microenvironment. In the extrinsic pathway, chronic inflammation triggered by events such as infections (e.g. Helicobacter pylori, Hepatitis viruses and more), autoimmune diseases (e.g. Inflammatory bowel disease), or other inflammatory conditions, facilitate cancer development [111].

In chronic inflammation, with potential evolvement of malignant transformations, the interaction between adaptive and innate immune response is perturbed [110]. Innate immunity has traditionally been defined as consisting of those functions that are nonspecific in nature and with which the host is born [112]. Mast cells (MC), eosinophils, basophils, neutrophils, macrophages, natural killer (NK) cells and dendritic cells, are considered innate immune cells [110].

The adaptive immune cells differ from the innate immune cells in their expression of generated, diverse, antigen-specific receptors. Individually the cells express structures directed against a specific antigen and upon recognition a clonal expansion is necessary to obtain sufficient response to combat the infection. B-lymphocytes, CD4+ helper T lymphocytes and CD8+ cytotoxic T lymphocyte are traditionally considered a part of the adaptive, or acquired, immune response [110, 112].

More recently, innate and adaptive immune cells have been found to interplay in a more sophisticated way than previously believed [113]. However, the regulatory effect exerted by certain immune cells is not restricted within the immune system but has the potential to bridge the gap to cells of other origins. The immune system might play an important role in almost every step of cancer development, including tumour initiation and tumour progression but also in tumour response to therapeutics, as reviewed by Bachireddy *et al.* [114].

Inflammation has the potential to contribute to a more favourable microenvironment in the tumour, containing growth factors which sustain proliferation, survival factors that limit cell death, pro-angiogenic factors and enzymes that facilitate angiogenesis, invasion and metastasis [6]. In colorectal cancer, a classification based upon the tumour-specific immune cell profile has been found superior to TNM classification in assessing prognosis [115]. In Hodgkin's lymphoma the number of infiltrating macrophages [116] and eosinophils [117] have been found to correlate to prognosis. Also, mast cells have been found to promote growth of tumour cells [118, 119] and correlate with poor prognosis in Hodgkin's lymphoma [120].

Mast cells

Overview

Mast cells (MCs) are evolutionarily conserved inflammatory cells and MC-like cells are believed to have appeared approximately 450-500 million years ago [121]. Over the time MCs have come to possess capacity to act in a 'Dr Jekyll and Mr Hyde'-nature, with MC response sometimes beneficial, sometimes noxious for the organism [122].

Mast cells derive from CD34+ multipotent haematopoietic progenitor cells in bone marrow [123], but unlike many other haematopoietic cells, MCs exit the bone marrow in an immature state and circulate in the bloodstream before they migrate into the tissue, where they ultimately will reside [124]. MCs are abundantly found in tissues near surfaces exposed to the environment, such as skin, airways, gastro-intestinal tract, and near blood and lymphatic vessels [125, 126]. A suitable place for the first-line of defence against environmental organisms [127].

Under the influence of local cytokines and the microenvironment, MCs mature in peripheral tissue, forming a broad spectre of heterogeneity [126, 128]. Roughly, human MCs can be classified according to their content of proteases and proteoglycans in granules, as tryptase-only (MCT) or both tryptase and chymase containing (MCTC) [129].

MCs are activated by a broad set of stimuli such as, whole bacteria, parasite products, structures of certain viruses, as well as emanating danger signals from surrounding tissue, including epithelial, endothelial, and tissue-resident immune cells [127]. Depending on the mixture of activating signals and activating pathway (MCs can be activated through toll-like receptors, complement receptors or Fc receptors), different patterns of inflammatory mediators are released from cytoplasmic granules [126].

MC granules contain tens of thousands of different compounds, including histamine and tryptase [122]. Degranulation and the release of mediators occur within seconds of activation. In addition to this immediate response, MCs have the capability to synthesise inflammatory mediators, such as cytokines and chemokines de-novo in a process requiring transcriptional changes. Hence, the slower release [127].

But the role of MCs may be more complex than only acting as secretory cells. Emerging evidence indicates that MCs also function as antigen-presenting cells, both indirectly and directly [130-132]. MCs also have the unusual capability to, following appropriate stimuli, re-enter the cell cycle

and proliferate, thus contributing to the expansion of MC population in the tissue [125].

Mast cells in tumours

It is well known that there usually are low numbers of infiltrating MCs in tumours and that MCs exist in close conjunction to tumour cells (Figure 3) [133]. MCs are found in all steps of tumour development. In pre-malignant lesions MCs are predominantly clustered at the margin, and are believed to facilitate angiogenesis associated with tumour growth [6].

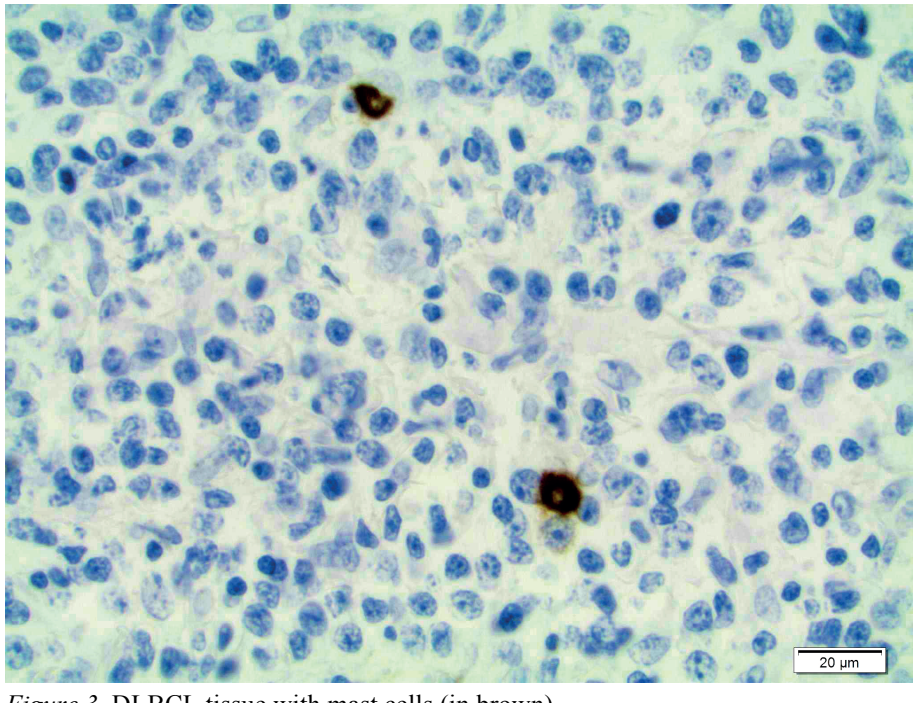

Figure 3. DLBCL tissue with mast cells (in brown)

MCs produce many pro-angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor 2 (FGF-2). MC granules also contain tryptase, which stimulate proliferation of endothelial cells, promote vascular tube formation [134] and help degrade extracellular matrix for neovascularisation [109, 134]. Other secretory components from MCs are chymase, histamine, heparin and other cytokines such as Interleukin 8 (IL-8), Tumour necrosis factor α (TNF-α), Tumour growth factor β (TGF-β) and Nerve growth factor (NGF), which all are involved in angiogenesis [134]. In Hodgkin's lymphoma a high number of MCs correlate negatively with prog-

nosis [120] but not with microvessel count [135], implying angiogenesis, at least in part, be non-MC driven. In DLBCL, a higher blood vessel density is associated with worse prognosis [136, 137], but no data have been reported regarding the correlation between MC count and blood vessel density.

p53

In 1979, several independent groups published data regarding protein 53 (p53), a protein named after its molecular weight of ~53kDa that bind to the large T-antigen of the Simian Virus 40 (SV40 adenovirus), a DNA tumour virus [138-141]. The p53 gene (TP53) was first believed to be an oncogene since p53 was elevated in many human tumours. It was finally ten years later that the true nature of p53 began to be resolved, when it was found that the original studies had been performed on cells with a TP53 gene harbouring a mutation at codon 135, inducing its oncogenic effect [142]. In contrast, the non-mutated form of p53 could inhibit transformation of cells [1] and actually acted as a tumour suppressor gene in such an essential way that it was named 'the guardian of the genome' [143]. The p53 protein has ability to regulate the expression of a large group of important genes for cell-cycle regulation [144-147].

Under normal conditions the p53 protein is constantly produced and maintained at low levels by ubiquitylation and proteasomal degradation. The former is mediated by several E3 ubiquitin ligases but mainly the murine double minute 2 (MDM2) ligase [148-151]. The regulatory machinery of p53 makes it very sensitive to a range of cellular stress signals, such as DNA damage, nucleotide depletion, hypoxia and more [152, 153]. Upon response to stress signals, p53 is stabilised, undergoes post-translational changes and exerts its multi-facetted effect [153-155], including regulation of autophagy, senescence or apoptosis, glucose metabolism and mitochondrial respiration [156-158]. High levels of accumulated p53 can also induce cell-cycle arrest in G1 and/or G2 phase [159-161].

The importance of a viable p53 function for normal cell survival is understood in relation to the TP53 gene itself, which is mutated or deleted in about 50% of all human cancers. However, other regulatory mechanisms of the p53 pathway may be corrupt in many tumours with wild-type TP53 [162]. Multiple studies have demonstrated that mutations in the TP53 gene are associated with poor prognosis in lymphomas [163-167], especially in the GC-subtype [168, 169]. But some studies have failed to demonstrate this association [170].

However, mutations in the p53 gene are less common in haematological malignancies compared to solid tumours, and in B-cell lymphomas TP53-mutations are found in only 10-20% of all tumours [162, 171]. Here, other disruptions of the p53 pathway may influence p53 function, such as the key regulatory factor MDM2 [172].

MDM2

MDM2 belongs to the family of E3 ubiquitin ligases containing a RING (Really Interesting New Gene) domain [173] and functions as the main ligase for p53 degradation [174]. The significance of MDM2 as a p53 regulator is underlined by the fact that MDM2 amplification is present in up to 10% of cancers [175]. Classically, the mechanisms by which MDM2 suppresses p53 follow two paths: binding to the N-terminal domain of TP53 and thereby masking TP53's access for transcription, or by ubiquinating p53, thereby targeting it for proteasomal degradation [176-179]. However, the presence of E3 ubiquitin ligase activity might be necessary to suppress p53 activity, since MDM2-p53 binding alone has been found to be insufficient [180].

The MDM2 gene is located on chromosome 12q13-14 and encodes a 90kDa (491 amino acids) phosphoprotein [181]. Transcription can start from two promoter regions. Promoter region 1 (P1) is located upstream of exon 1, and P2 lies within the first intron, upstream of exon 2 [182]. P1 is responsible for regulation of basal MDM2 levels in unstressed cells whereas P2 is triggered by activated wild-type p53 [145]. The MDM2 protein is regulated by a positive feed-back loop of p53. The p53 binds to a response element in intron 2 and transactivates MDM2 transcription from P2, thus generating a feed-back loop where p53 controls its own degradation and return to basal levels after stress response [183].

Post transcript modifications of MDM2 include ubiquitination, sumoylation and phosphorylation [184]. Phosphorylation can occur by means of several different proteins. DNA-dependent protein kinase (DNA-PK) has the ability to inhibit MDM2-p53 interaction through phosphorylation of MDM2 in vitro, which is suggested to weaken MDM2-p53 binding [185, 186]. Another phosphatidylinositol 3-kinase family member is ATM, which phosphorylates MDM2 in response to gamma radiation or carcinogenesis [187]. However, MDM2 phosphorylation by several components of the Akt (serine-threonine specific protein kinase B) signalling pathway, one being protein kinase B Akt [188, 189], promotes MDM2 nuclear entry and degradation of p53 [190]. The PI3K/Akt pathway is crucial in regulation of cell proliferation, differentiation, metabolism and survival [191].

In response to stress signals such as ionising radiation, ATM phosphorylates p53 and thereby promotes its activity, stability, and impairs interaction with MDM2 [192]. However, before p53 accumulation, MDM2 is phosphorylated in an ATM-dependent, DNA-PK-independent manner, thus contributing to the diminished MDM2-p53 interaction [187].

A complex regulatory network exists around p53 and MDM2, one which is not yet fully elucidated. Activated oncogenes can induce ARF protein interaction with MDM2. ARF is a direct inhibitor of ubiquitin ligase activity of MDM2 [193] and blocks the MDM2 export of p53 from the nucleus to the cytoplasm where it normally undergoes degradation. This leads to increased levels of p53 protein in the nucleus [194] where p53 has the ability to upregulate certain genes that will carry out the p53 effector response [144].

Three essential p53 target genes for cell-cycle arrest have been identified: p21(WAF), GADD45 and 14-3-3σIs [144, 146, 195]. The p53-target-genes with pro-apoptotic activity can be divided into three categories depending on their subcellular location. The first group includes genes encoding cell surface proteins (e.g. Killer/DR5, PERP, CD95). The second group includes genes encoding mitochondrial proteins (e.g. Bax, Noxa, Puma, p53Aip1). The third group includes genes encoding for proteins located in the cytoplasm (e.g. PIDD and PIGs) [196]. However, the main downstream pathway of p53 response differs in a tissue-specific manner, reflecting the complex network in which p53 functions [147].

Single nucleotide polymorphism

According to the Hardy Weinberg law, in a random-mating population, the frequencies of two occurring alleles are expected to remain in equilibrium between generations [197]. DNA polymorphisms are usually defined as variations present in more than 1% of the population. A Single Nucleotide Polymorphism (SNP) is where one nucleotide in the DNA sequence is substituted in one particular spot. The majority of DNA polymorphisms are not important despite some altering the peptide structure through amino acid changes. SNPs are most likely a reflection of natural selection acting against deleterious changes in the human genome [198]. Although it has been long hypothesised that polymorphisms occurring outside of exons, e.g. in the regulatory regions, are important modulators of gene expression and evolutionary change [199], convincing evidence for this has only recently emerged [200].

More than 4.5 million SNPs have been mapped, representing about 73% of the total estimated SNPs in humans [201]. One SNP important in regula-

tion of MDM2 gene transcription is the MDM2 SNP 309 (rs2279744). The polymorphism in MDM2 SNP309 is a T to G substitution at nucleotide 309 in the first intron of the intronic promoter P1 and occurs in heterozygous state (T/G) in about 40% of the population and in homozygous (G/G) state in about 12% [172].

The T to G change extends one of the binding sites for the transcription factor Sp1 [172], resulting in enhanced affinity for Sp1 by 122% [202] and consequently enhanced Sp1 binding and higher levels of MDM2 [172]. However, the results have not been reproducible in all investigated populations, resulting in a hypothesis that polymorphism in another SNP (MDM2 SNP285) located close to MDM2 SNP309 could counteract the effect of a T to G change in SNP309. The prevalence of the polymorphism in SNP285 differs between populations around the world and would thus explain the variance in impact of SNP309 [202, 203].

Another possibility could be that polymorphisms in both MDM2 SNP309 and SNP 285 act in a hormone-dependent way. Bond *et al.* described the polymorphism in SNP309 to be important for premenopausal women but not for men. There was a higher proportion homozygous for the SNP309 G allele in premenopausal women compared to postmenopausal women or men, implicating that premenopausal women homozygous for G allele were diagnosed at a younger age compared to men [204]. However, another study in a German DLBCL population has not been able to confirm these findings [205]. This is in line with another recent study where a polymorphism in SNP285 only counter-affected a T to G change in SNP309 in premenopausal women [206, 207]. Therefore it seems fitting that oestrogen can enhance transcription of MDM2 from a SNP309G allele compared to SNP30T allele, resulting in higher levels of MDM2 in cells with G allele [208].

Micro-RNA

The first findings of small RNA molecules with the potential to regulate gene transcription dates back to 1993 [209, 210]. However, it would take almost a decade before the description of the small RNA subclass 'microRNA' (miRNA) and understanding was reached that miRNA expression is an important regulatory factor for gene expression. First described in Caenorhabditis elegans [211] in 2001 and later in B cell lymphoma in 2005 [212], the research field of miRNAs is now profound. In contrast to early estimates of the number of miRNAs in the human genome of about 255 [213], 1594 miRNAs were identified in the human genome by August 19th 2012 according to miRBase.org, a database of published miRNA sequences and annotations [214, 215].

MicroRNAs are endogenous small noncoding RNAs containing about 18 to 24 nucleotides that regulate gene expression, mostly through interaction with productive translation products and mRNA stability [216-218]. Most human miRNAs lie between protein-coding genes whereas about one third lie in introns of annotated mRNA [219]. MicroRNAs can be transcribed from the promoter of an associating gene but approximately 25-30% of intronic miRNA are transcribed from independent promoters [220, 221].

Clusters of miRNAs occur and different miRNAs within a cluster are often transcribed together from a single polycistronic transcription unit [222], a promoter that can be located several kilobases away, implying that miRNA transcription is more complex than for protein coding genes [220]. MicroRNA transcription may be regulated by factors binding directly to miRNA promoter elements and regulating their expression. One such factor is c-Myc (with its second ability to function as an oncogene), which binds close to the miRNA-17-5p cluster and up-regulates transcription of the entire miRNA-17-92 polycistron. This polycistron has tumour-promoting abilities [223]. But the picture is not simple and c-Myc exerts a regulatory control over more miRNA clusters than the miRNA-17-92 cluster [224] in a complicated manner that is not yet fully elucidated.

Another possible regulator of miRNAs is p53, which has the ability to bind to conserved sequences in the promoter region of the miRNA-34 family and activate their expression. This results in miRNA-34a over-expression, which leads to cell-cycle arrest and apoptosis in a similar manner as seen with enhanced p53 levels [216, 225-230].

After the transcription, miRNAs are generated from local hairpin structures of pri-miRNA by two different RNase-III-type proteins (Drosha and Dicer) [231, 232]. Mature miRNA then recognise and bind to specific sites of mRNA, altering further refinement towards functional proteins [233-237]. But miRNA can also affect the overall mRNA stability, leading to mRNA degradation [238]. If miRNA previously was believed to follow a few simple rules to regulate target gene expression, it is now clear that miRNA-mediated activities are complex and yet not fully understood [239-241].

The complex activity of miRNAs position them as regulators in many cellular processes such as, cell differentiation, apoptosis, as well as tumourigenesis [242-246]. Thus, miRNAs have been reported to predict outcome in many different tumour types, such as AML, CLL as well as solid tumours and DLBCL [247-253].

One specific miRNA, described to be excessively expressed in lymphoid haematopoietic stem cells compared to myeloid haematopoietic stem cells, is

miRNA-129-5p [254]. It has been found that a Hodgkin's lymphoma cell line has low expression of miRNA-129-5p [255] and that low expression of miRNA-129-5p could be of prognostic value. Low expression also leads to up-regulation of the oncogene SOX4, which potentially leads to more metastasis and possibly involvement in disease onset in endometrial and gastric cancers [256-259]. In contrast, in urinary bladder cancer, a high expression of miRNA-129-5p was found to be associated with progression and poor prognosis [260]. To date, miRNA-129-expression levels are poorly investigated in DLBCL.

Overall Aim

The main objective of this thesis was to study the clinical presentation of DLBCL and the importance of tumour microenvironment and molecular regulation in DLBCL tumour cells in order to determine potential prognostic factors for clinical implementation in future treatment decisions. We aimed to use technologies accessible in the clinical laboratory to facilitate transition of any research results into a clinical setting.

Specific Aims

I To investigate the prognostic role of tumour microenvironment with emphasise on inflammatory responses in the tumour by exploring the relationship between the number of mast cells and clinical characteristics and prognosis in DLBCL.

II To explore the impact of miRNA-129 deregulation in DLBCL by analysis of miRNA-129-5p expression levels in correlation with clinical characteristics and prognosis.

III To analyse the role of the MDM2 SNP309 polymorphism in DLBCL patients and correlate genotypic data with histopathological and clinical characteristics.

IV To examine the impact of age on clinical characteristics and survival from DLBCL in the Swedish population.

Methods

Patients

All patients in the study were diagnosed according to criteria valid at time for diagnosis, namely the REAL or WHO classification systems.

In Paper I, the study population consisted of 154 patients with *de novo* DLBCL diagnosed at two major hospitals in Sweden (Uppsala and Umeå) between 1984 and 2004.

In Paper II, the study population consisted of 61 patients with *de novo* DLBCL, diagnosed at one major Swedish hospital (Uppsala) between 1986 and 2008.

In Paper III, the study population consisted of 169 patients with *de novo* DLBCL diagnosed at two major Swedish hospitals (Uppsala and Umeå) between 1984 and 2011.

In Paper IV, the study population consisted of all DLBCL cases 20 years or older, registered in the Swedish lymphoma registry (SLR) between 2000 and 2013.

Treatment recommendations in Sweden have changed over time from when the first patient was diagnosed until the present day. Rituximab was widely included in treatment over the time period 2003-2005.

All studies were approved by the ethical review board of Uppsala University.

Tumour material

All tumour tissue used was diagnostic lymph nodes or diagnostic needle biopsies from lymph nodes. All samples were formalin-fixed, paraffin-embedded and sectioned in 3-4 μm thick sections for further immunohistochemistry staining and evaluation (Paper I and II) or micro-dissection and DNA/RNA extraction (Paper II and III).

Immunohistochemistry and evaluation

In addition to routine immunohistochemical stainings performed at the diagnosing pathology department, additional stainings were performed as follows: In Paper I, sections were immunostained with a monoclonal G3-antibody recognising tryptase. Tryptase-positive MCs were counted in 10 randomly selected high power fields (HPF; 400x), using an ocular with a lattice square net; In Paper II, sections were stained for SOX4, CDK6 and EBER.

DNA/RNA preparations

Sections of formalin fixed, paraffin embedded tumour material were microdissected into areas with dense tumour cell infiltration and surrounding areas. DNA and RNA were purified according to the manufacturers protocol using the 'Recover All Total Nucleic Acid Isolation Kit' (Ambion).

RT-QPCR

Real time quantitative PCR (RT-QPCR) is a method to determine the amount of DNA or cDNA in a sample. The method used in the work presented here, is probe-based, stem-loop RT-QPCR. The reaction consists of two steps: Step one – synthesizing of cDNA from miRNA using a stem loop RT primer specific for the 3' portion of the miRNA molecule aimed for study, and reversed transcribed with reverse transcriptase, producing a strand of cDNA. Step two – quantification of the RT product using conventional TaqMan PCR. This includes miRNA specific forward primer, reverse primer and labelled TaqMan probes.

During the PCR the TaqMan probe anneals specifically to the target between the forward and reverse primer. The TaqMan probe contains a reporter dye (FAM-dye) at the 5' end and a non-fluorescent quencher at the 3' end. Thus, the fluorescence is diminished in intact probes. However, during the PCR amplification step, DNA polymerase cleaves off the quencher from annealed probes, causing an increase in fluorescence (Figure 4), which can be measured.

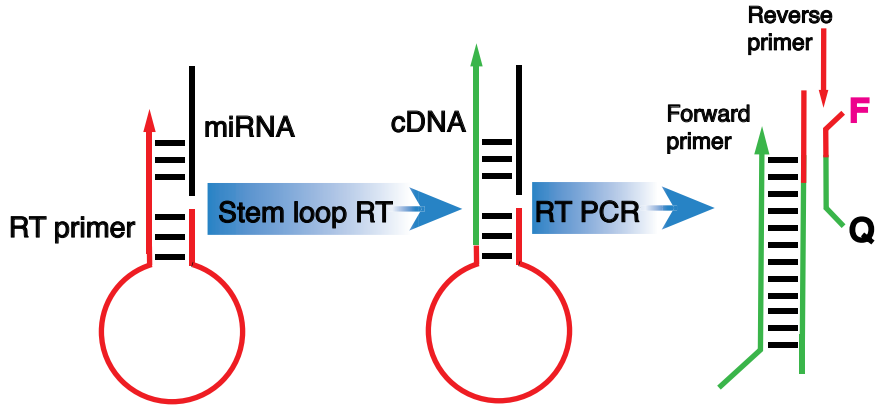

Figure 4. Schematic description of TaqMan real-time (RT) quantification of miRNAs. Stem loop RT-primers bind to 3' of miRNA and is reversed transcribed. The RT-PCR TaqMan probe binds to the target between forward and reverse primers. During subsequent transcription, DNA polymerase separates the quencher (Q) and the fluorescent reporter dye (F). This results in increased fluorescence, detectable at a threshold level.

By measurements of the fluorescence and the number of PCR-cycles, the initial amount of miRNA can be determined. The threshold cycle (CT) is defined as the fractional cycle at which fluorescence passes through a set threshold [261]. Thereafter each miRNA analysis was normalised according to expression levels of RNU6B (RNA U6 small nuclear 2) and calculated according to the Pfaffl-method [262], although in this case a modified Pfaffl-formula was used due to the lack of available controls from each corresponding patient, as described in Paper II.

For the RT reaction, approximately 5 ng of RNA was used and the amplification carried out in a Gene Amp 9700 PCR machine according to the manufacturers protocol (Applied Biosystems, CA, USA). For the quantification of miRNA-129-5p, all samples were run in triplicate and amplifications carried out in a TaqMan Real-Time PCR machine according to the manufacturers protocol (Applied Biosystem, CA, USA). Results were analysed with the software ABI Prism 7000 (Applied Biosystem, CA, USA).

SNP Genotyping Analysis

Determination of the MDM2 SNP309 polymorphism was performed by genotyping using the PCR-restricted length polymorphism (PCR-RFLP) technique. The PCR was carried out in a volume consisting of 0.8 µM of each primer (SNP309-Forward: 5'..GAGGTCTCCGCGGGAGTTC..3' and

SNP309-Reverse: 5'..CTGCCCACTGAACCGGC..3'), 1.5 mM $MgCl_2$, 8% DMSO, 800 µM deoxynucleotide triphosphate, 5 units of Taq gold polymerase, 200 ng genomic DNA and water up to a total volume of 50 µL. The PCR-program for MDM2 amplification was carried out by 40 cycles of 60 sec denaturation at 94 ºC, 60 sec annealing at 60 ºC, 120 sec extension at 72 ºC. Finally, an extension period of 10 min at 72 ºC followed the final cycle and then 4 ºC until end. The RFLP was carried out with digestion of the 155 bp PCR-product with the MspA1I endonuclease (and NEB4 buffer according to the manufacturers protocol - New England Biolabs) (1 hour at 37 ºC, 20 min at 65 ºC, then 4 ºC until end) and the strands separated on a 12.5% GeneGel Excel polyacrylamide gel (100 minutes, 400V, 25mA, 6W at 14 ºC). The end result was visualised with silver staining according to the manufacturers protocol (Pharmacia LKB Biothechnology, Uppsala, Sweden). The MspA1I cleaved the G allele into three products (10 bp, 46 bp and 99 bp) and the T allele into two products (10 bp and 145 bp).

Statistics

Survival curves were plotted according to Kaplan-Meier and survival differences between curves were estimated with log-rank test. Disease-free survival (DFS) was calculated from date of diagnosis until date of relapse or last follow-up. Lymphoma specific survival (LSS) was calculated from the date of diagnosis until death directly due to lymphoma or due to other reasons but with persistent lymphoma, or last follow-up. Overall survival (OS) was calculated from date of diagnosis until date of death (from any cause) or last follow-up. Patients who never attained remission were set to a DFS of zero days. A cox-regression model was applied to investigate impact of differences in clinical variables between groups.

In Paper I event-free survival (EFS) was used synonymously with DFS. Differences in proportions and continuous variables were calculated with chi-square test and Mann-Whitney U-test. All statistical calculations were performed with the software Statistica 7.0.

In Paper II and III were differences in proportions and continuous variables calculated with Fishers exact test and t-test. All statistical calculations were performed with the software R (The R Foundation for Statistical Computing, www.r-project.org).

In Paper IV relative survival (RS) analysis was used as an additional method to measure DLBCL survival. Relative survival is a commonly used method for capturing net survival in population-based cancer studies, where it computes mortality directly or indirectly correlated to the disease without

requiring data about actual cause of death [263, 264]. In contrast to observed overall survival, which takes into consideration all deaths regardless of cause and gives a crude measurement of survival, RS can be expressed as a ratio between observed survival in the study population and the expected survival in a comparable group from the general population [265]. Ideally, the group drawn from the general population should exclude the specific disease studied (in this case DLBCL), however, this is rarely practically manageable and makes little difference in outcome [263, 265]. An RS regression model was expressed as a risk ratio (RR) [266]. All statistical calculations were performed with the software R.

Results and Discussion

The heterogeneity seen in DLBCL is reflected at multiple levels from the macroscopic view [8] with different clinical presentations to a microscopic view of the tumour microenvironment [7, 267] and furthermore, to a subcellular level with differences in protein expression [56, 64]. Even though many patients with DLBCL can be cured from the disease, successful treatment fails for a proportion of patients. It is therefore of utmost interest to not only find new treatment strategies but also define new prognostic markers that can help clinicians to guide more intense treatments to those whom would benefit the most.

Paper I

Mast cell infiltration is a favourable prognostic factor in diffuse large B-cell lymphoma

In this paper we investigated heterogeneity in tumour microenvironment by using mast cells as a marker for immune system response to DLBCL. The study population consisted of 154 DLBCL patients and tryptase-positive MCs were detected in 79% of all cases. No difference was found between the GC and non-GC subgroups. After dichotomisation 71% had up to 7 MC / 10 HPF and 29% had 8 MCs or more / 10 HPF. Patients with high MC count had a better DFS and a tendency for a better LSS than patients with few MCs ($p < 0.05$ and $p = 0.055$ respectively).

MCs are known to have an active role in the host inflammatory response for other haematological malignancies [268] and our findings strengthen the hypothesis that this is also true for DLBCL and that MCs are related to a favourable outcome. In addition to a potential immune response there may also be a direct interaction between the tumour cells and the MCs. Such a bi-directional inter-relationship has been suggested to occur in Hodgkin's lymphoma (HL) [119, 269] where MCs and 'Hodgkin and Reed-Sternberg' (HRS) cells interact via CD30–CD30 ligand. This probably leads to an increased proliferation of the HRS cells [119] and shorter time to relapse [120]. However, DLBCL do not express CD30 and the correlation between

MC and outcome is opposite to that found in HL, which implies that such an interaction must follow another path. One possibility is interaction via CD40 since DLBCL has been described to express CD 40 and mast cells express CD 40 ligand [270]. Interestingly, the CD40–CD40 ligand interaction has different effects on the B-cell during different stages of B-cell development [271].

When the GC and non-GC subgroups were analysed separately, GC patients with high MC count had a better LSS compared to GC patients with few MCs, whereas no difference was seen in the non-GC group (Figure 5).

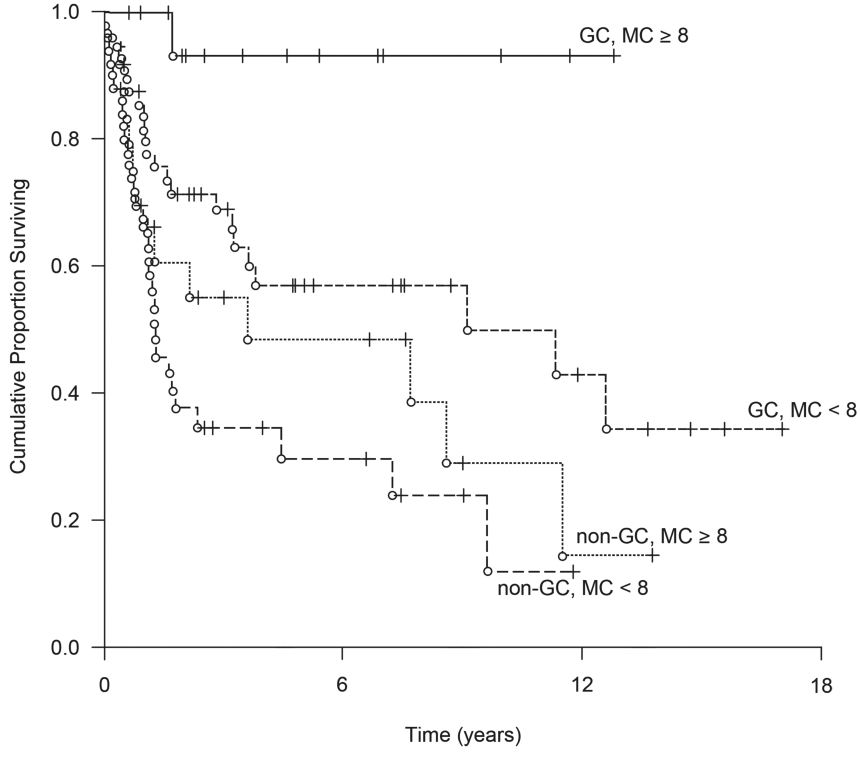

Figure 5. Lymphoma specific survival (LSS). Patients with the GC subgroup and a high number of MCs had a better LSS ($p < 0.01$) compared to patients with few MCs.

In a multivariate analyses adjusted for aaIPI (≤1 vs. ≥2), GC vs. non-GC and age (<65 vs. ≥65 years), MC count persisted as being significantly related to LSS and all variables but age were significantly related to EFS. Inter-

estingly, only MC was significantly related to longer EFS when the GC subgroup was analysed separately by univariate analyses. In contrast to the non-GC subgroup where univariate analysis for longer EFS was significant for aaIPI, age and MC, the multivariate analysis including the same factors found only aaIPI and MC to retain a significant correlation with EFS. Therefore, the effect of MCs on prognosis appear to be independent of other known risk factors for DLBCL.

In paper I we described the correlation between MCs and DLBCL survival. MCs are known to interact with the microenvironment in a tumour promoting way [118]. Tumour cells are under direct influence from different MC released mediators with potential for altering regulatory mechanisms within the tumour cell. In paper II we investigate another regulatory mechanism on the molecular level in tumour cells in relation to microscopic, macroscopic and clinical characteristics.

Paper II

Low expression of microRNA-129-5p predicts poor clinical outcome in diffuse large B cell lymphoma (DLBCL)

The role of miRNAs in tumourigenesis and tumour regulation is only beginning to emerge and the role for miRNA-129-5p in DLBCL is not well investigated. In this paper we investigated a cohort of 59 patients. The patient group was dichotomised with a miR-129-5p level cut-off equal to mean Pfaffl value. Fifty-nine percent of the patients had low relative expression of miRNA-129-5p (below the mean Pfaffl value) and 41 percent had high relative expression (above the mean Pfaffl). DLBCL patients with low relative expression had a shorter OS compared to patients with high relative expression (Figure 6A). Also, for patients treated with rituximab (n = 25), there was a difference in OS between patients with low and high expression of miRNA-129-5p (Figure 6B).

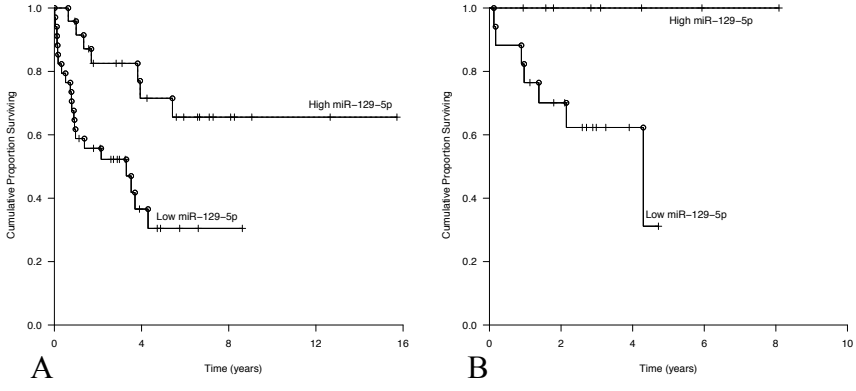

Figure 6. A) MiRNA-129-5p expression and overall survival in DLBCL. After dichotomisation upon mean Pfaffl-value, patients with high relative expression of miRNA-129-5p had significantly longer overall survival ($p = 0.004$). B) High miRNA-129-5p expression had a significant impact on longer overall survival for DLBCL patients treated with rituximab ($p = 0.04$).

With the exception of a significant difference in proportions of GC/non-GC between the low and high miRNA-129-5p expression groups, clinical characteristics were similar between the groups. This is in concordance with other findings where miRNA-129-5p is up-regulated in the GC subtype [251]. The observation that patients within the GC subtype of DLBCL were more common in the group with high miRNA-129-5p may provide an addi-

tional explanation for the differences in prognosis observed, which is consistent with previously published results [272].

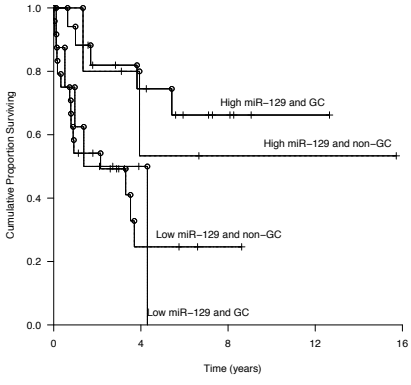

Figure 7. MiRNA-129 expression in GC/non-GC DLBCL patients and overall survival. Only in the GC subtype of DLBCL did high miRNA-129-5p levels correlate to a significantly longer overall survival ($p = 0.02$ vs. $p = 0.16$).

Additionally, only patients with the GC subtype of DLBCL and low expression of miRNA-129-5p had a significantly shorter overall survival compared to those with a high miRNA-129-5p expression. This was not seen for the non-GC subtype (Figure 7). Therefore we can not rule out the possibility that the survival difference is partly related to DLBCL subtype which, in that case, poses interesting questions about the role of miRNA-129-5p in different DLBCL subtypes. Specific miRNA-profiles have been found to be related to GC and non-GC subtypes and correlated with survival [251]. Even though this study used another immunohistochemical algorithm to subdivide DLBCL into GC and ABC, as well as tissue microarray [65], their results are consistent with the results presented in Paper II, a higher miRNA-129-5p expression in the GC subtype [251]. This strengthens our hypothesis of differences in miRNA-129-5p expression within DLBCL. Unfortunately, different methods of microRNA analysis produce very different expression data [273] and as long as a common validation process between methods is lacking, only relative expression levels can be compared.

MiRNA-129-5p is differently expressed in different tissues as well as different malignancies. Gastric cancer express lower amount of miRNA-129-5p compared to normal tissues [274] and probably low amounts of miRNA-129-5p can induce elevated SOX4 expression. SOX4 is a transcription factor involved in cell progenitor development and migration. Recently it has been described that low expression of miRNA-129 results in over-expression of

SOX4 [256]. High expression of SOX4 is associated with shorter median time to metastatic relapse in breast cancer [254, 257, 275]. However, we found no difference in the ratio of cells expressing SOX4 when the low and high miRNA-129-5p expression groups were compared. But our analysis was limited to the ratio of SOX4 expressing cells in each tumour and we may therefore have missed if cells were still expressing SOX4 to a lower degree. Moreover, miRNA-129-5p has been found to regulate the expression of Cdk6. Low levels of Cdk6 result in G1 arrest and cell death [255]. However, a relationship between the number of Cdk6 expressing cells and miRNA-129-5p levels was not found in our material.

In Paper II, we described how DLBCL patients with low expression of miRNA-129-5p had a significantly worse prognosis compared to patients with high miRNA-129-5p expression. Although miRNAs form small regulatory units in the cell, in Paper III we investigated an even smaller unit. Single nucleotide polymorphisms (SNP) at certain key sites in the genome have the potential to alter the regulatory machinery and thereby comprise the cell cycle. One such polymorphism is the one found at position 309 in the promoter region of the MDM2 gene.

Paper III

The MDM2 polymorphism SNP309 is associated with clinical characteristics and outcome in diffuse large B-cell lymphoma.

In paper III we investigated the prognostic role of a polymorphism in MDM2 SNP309 in 201 patients with DLBCL. The distribution of 38,3% with the TT genotype, 9.5% GG and 52.2% TG genotype is similar to other studies and comes close to the Hardy Weinberg equilibrium [204].

Patients homozygous for the T allele were diagnosed at a younger age than patients with at least one G allele even though the mean age for both groups was above 60 years, in contrast to other studies where the G allele has been found associated with elevated cancer risk and younger age at diagnosis [276, 277]. In one study women with a GG genotype were, on average, diagnosed with DLBCL 13 years earlier than women with TT genotype [204]. However, the results are inconsistent [205]. For malignant melanoma there is data in line with our findings, that double T allele carriers are diagnosed at a younger age compared to those homozygous for the G allele [278]. Taken together, this indicates different effects of the MDM2 SNP309 polymorphism on age in different sub-settings.

A T to G change in MDM2 SNP309 increases the affinity for the transcription factor Sp1, resulting in higher levels of both MDM2 RNA and MDM2 protein [172]. Consistently, non-small-cell lung cancers homozygous for the T allele contain lower expression levels of MDM2 mRNA compared to tumours with at least one G allele [279]. High levels of MDM2 lead to down-regulation of p53 [174].

Patients homozygous for the T allele in MDM2 SNP309 had both significantly longer OS, LSS (Figure 8A) and DFS. In a cox-regression model, the MDM2 SNP309 polymorphism was found as a significant contributor to the survival differences and the longer LSS indicates the differences were not only due to the age differences between the groups. However, the difference was most pronounced for patients with low-stage and low-aaIPI disease (Figure 8B).

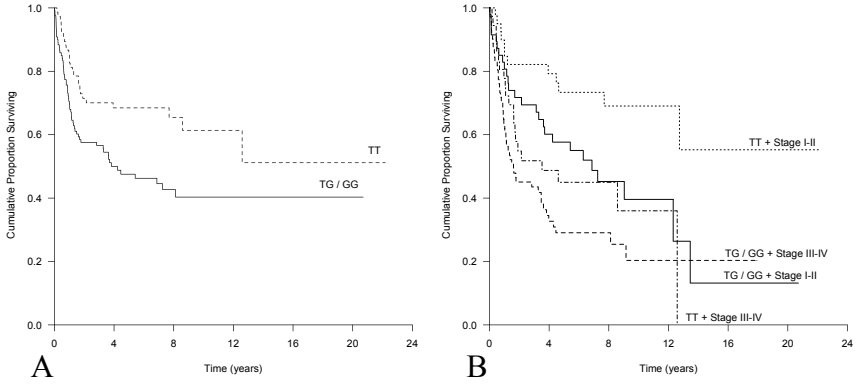

Figure 8. A) DLBCL patients with MDM2 SNP309 TT genotype have a significantly longer lymphoma specific survival compared to patients with TG or GG genotype ($p = 0.006$). B) The longer overall survival for DLBCL patients with TT-genotype was only found among patients with low-stage (I-II) disease ($p = 0.015$).

Treatment protocols have changed during the time period since the first study patient was diagnosed with DLBCL. The aforementioned survival difference was only seen in the subgroup of patients not treated with rituximab whereas there were no significant survival differences among rituximab treated patients.

Several studies in recent years have investigated the impact of the T to G polymorphism in MDM2 SNP309 on cancer prognosis [276, 277, 280-282] and the G allele in MDM2 SNP309 has been found to significantly reduce long term survival in pancreatic cancer [283], hepatocellular carcinoma [284] and chronic lymphocytic leukaemia [285], among others [286-288]. These findings are in line with some of our findings and strengthen the hypothesis that the MDM2 SNP309 polymorphism could exert a plausible impact on DLBCL prognosis. However, the impact seems to be removed by the addition of rituximab to administered treatment.

Conclusively, in Paper III we describe how a polymorphism in MDM2 SNP309 (T to G change), previously correlated to impaired survival for DLBCL patients, and how this correlation is undetected for R-CHOP treated patients. Even though this is a germline change in DNA, other changes on a molecular level in DLBCL may be due to age. Therefore, in the last paper presented here, we investigated the impact of age upon prognosis.

Paper IV

Impact of age in an unselected population of Diffuse large B-cell lymphomas

There are several indicators of biological differences between younger and older patients with DLBCL [289-293], together with clinical observations of superior survival for young DLBCL patients this led us to investigate the impact of age upon DLBCL survival. However, DLBCL predominantly affects people older than 60 years, as seen in our study presented in Paper III, and in cancer statistics from the United States, both of which had a mean age above 60 years [37, 294]. Therefore, to obtain a sufficient proportion of young DLBCL patients we conducted a retrospective population-based cohort study of all patients diagnosed with DLBCL between 2000 and 2013 registered in the SLR, which cover approximately 95-97% of all lymphoma cases in Sweden [295].

To our knowledge, this is one of the largest population-based DLBCL studies performed to date. Due to the sheer size of the study could we obtain an adequate number of patients in the lower age range to specifically address the importance of age on survival from DLBCL, in all age groups. We found a decline in both OS and RS for each additional subsequent 10-year age group (Figure 9).

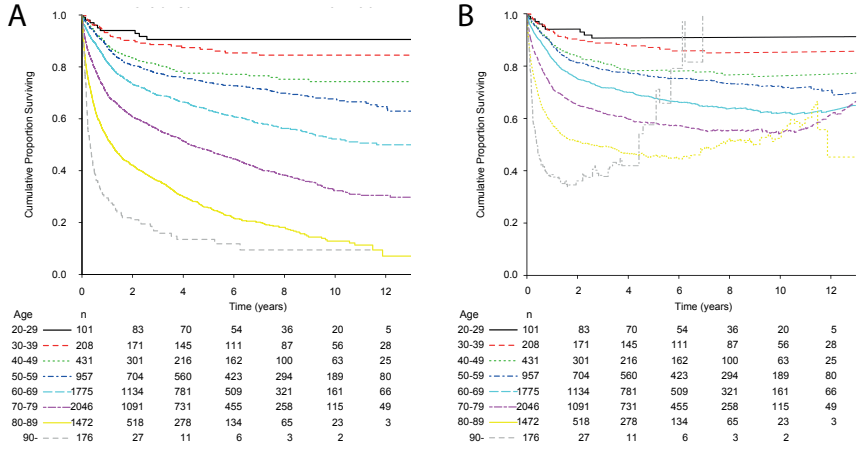

Figure 9. A) Observed overall survival and B) Relative survival from DLBCL declined for each subsequent 10-year age group.

Since a part of the study population was treated prior to the general clinical introduction of rituximab, a sub-analysis was performed, limited to the time period after rituximab was introduced into the national treatment recommendations. The decline in survival persisted for subsequent age groups. In a survival regression model adjusted for stage, performance status, s-LDH and two or more extranodal sites, each 10-year age group above the age of 39 had a significantly higher risk ratio (Table 6), i.e. significantly poorer survival, compared to the reference group of 20-29 year olds.

Relative risk ratio (RR) of DLBCL in Sweden 2000-2013			
DLBCL (n=7166)	**RR**	**95% CI**	**p**
Age - y			
20-29	REF		
30-39	1.44	0.60-2.27	0.39
40-49	2.71	1.96-3.46	0.009
50-59	2.52	1.79-3.25	0.014
60-69	3.38	2.65-4.11	< 0.001
70-79	4.59	3.87-5.32	< 0.001
80-89	7.58	6.85-8.30	< 0.001
90-99	2 e-12	-	-
Stage			
I	REF		
II	1.03	0.85-1.22	0.73
III	1.11	0.92-1.30	0.28
IV	1.37	1.19-1.54	< 0.001
s-LDH			
Normal	REF		
High	1.76	1.63-1.89	< 0.001
ECOG PS.			
0	REF		
1	2.55	2.39-2.72	< 0.001
2	4.54	4.35-4.72	< 0.001
3	7.13	6.94-7.32	< 0.001
4	12.66	12.45-12.87	< 0.001
Extranodal			
No	REF		
Yes	1.08	0.96-1.21	0.22

Table 6: Relative risk ratio for clinical parameters in DLBCL in a Swedish population.

The results presented here are in line with other studies regarding other types of lymphoma, such as Hodgkin's lymphoma where there, in a Swedish population, is a significant survival difference between patients below 50 years of age, 51-65 years of age and above 66 years of age [296]. In an

American population, there were also survival differences between age groups younger than 60 years [297] whereas there is data of survival for follicular lymphoma with differences between patients younger than 40 years compared to those 41-59 years old [298]. An age younger than 60 years have also been suggested to be incorporated in the IPI for better risk classification of DLBCL [299].

On a molecular level it has been described that proportions of non-GC subtype increase with age, and these patients have a worse prognosis [82]. Furthermore, a subgroup with both DLBCL and follicular lymphoma has been described, with an IRF4 translocation [300]. Patients with IRF4 translocations were found to be younger and have GC subtype with an indolent low-stage disease to a higher extent. The proportion of this subgroup in our material is unknown since data on molecular characteristics are not incorporated in the SLR.

Another possibility for an underlying cause for the poor survival among the elderly in our DLBCL population could be due to a greater burden of comorbidities which make effective treatment more difficult to pursue. Elderly patients also experience more toxicity and treatment is more often prematurely ended for this reason [301]. However, the increased toxicity in the elderly sub-population does not explain the survival differences for patients younger than 60 years since the treatment usually is well-tolerated.

Conclusively, in Paper IV we describe a significant decline in survival with each subsequent 10-year age group above the age of 39, also after adjustment for other risk factors. Perhaps it is time to incorporate age groups younger than 60 years in the prognostic model [299] since some of them might benefit from an altered treatment.

Concluding remarks and reflections

To fully understand the biology of the heterogeneity of diffuse large B-cell lymphoma we must incorporate it into a larger context and study all aspects of the disease, from overall epidemiological aspects down to cell-cell interactions in the tumour microenvironment and in turn, further down to the subcellular level to examine its molecular characteristics and regulation of the disease. The aim of all Papers included in this thesis have been dual; to obtain a more detailed understanding of DLBCL as disease and to examine the possibility of sub-grouping into different prognostic categories. Focus persists on improved risk-classification and enhanced knowledge of DLBCL biology, to be able to distinguish the subgroups most in need of intensified treatment. With the aid of epidemiological data such as in Paper IV, there was a possibility to, more clearly than any previous study, illuminate the impact of age upon survival and to distinguish subgroups in both ends of the prognostic risk scale. It could clearly and pedagogically be illustrated that age is an important risk factor and that this increase in risk starts at very young age. Interestingly, the results remained for patients treated during the last time period when rituximab was incorporated into the national treatment recommendations.

Prognostic markers often tend to change when therapy changes, as is described in Paper III. There it could be seen how the prognostic value of MDM2 SNP309 polymorphism in the pre-rituximab era disappeared for patients treated with rituximab. This same reason made any further validation in an independent cohort futile. However, not all prognostic values meet this fate and some persist through several treatment regimens, such as the prognostic impact of age [54]. Nevertheless, our findings about MDM2 SNP309 polymorphism contributed another puzzle-piece in the understanding of DLBCL biology and what important regulatory factors may be at play within the cell machinery.

However, the puzzle of intracellular regulatory machineries is far from solved and there are still many routes to investigate. One such field, recently surfaced, is the complex regulatory machinery of microRNAs [211]. Since more than 1500 microRNAs have been described to date [215], to analyse the intracellular expression of each one would be costly and time and material consuming. However, by analysis of available literature there is a possi-

bility to pinpoint interesting microRNAs for further investigation, as was described in Paper II. Here microRNA-129 levels could be correlated to both clinical characteristics and outcome of DLBCL.

As always, when different expression levels are examined and correlated to tumour biology, caution is warranted: one must consider whether the performed analysis actually reflects the intended biology, otherwise the findings could be only due to co-variance. For example, microRNAs have been described as being differentially expressed in different cell types [254]. Because the DLBCL microenvironment consists of a mixture of malignant B-lymphocytes and non-malignant cells appurtenant to the immune system, one possible error could be due to the actual cell type analysed. However, the analysis of non-malignant cells in the microenvironment is sometimes beneficial and gives us a better understanding of tumour biology [117]. As mentioned in Paper I, non-malignant cells have the potential to interact with malignant cells and thus the possibility of being used as prognostic markers [119]. In Paper I it was described how a simple count of infiltrating tryptase-positive mast cells correlated to DLBCL prognosis in a Swedish cohort.

However, it is not mandatory for a prognostic marker to be directly derived from the tumour biology. Some prognostic markers are, for example the sub-classification derived from GC-status [56], whereas other prognostic markers are not [52]. We are not yet completely aware of all biological steps connecting tumour biology with age but this does not prevent us from using age as a prognostic marker in DLBCL. Therefore, the research must continue to describe both valid and accurate prognostic markers for DLBCL but also shed some light on tumour biology. With the papers presented in this thesis, new insights have emerged regarding the use of new potential markers for prognostication of DLBCL, together with yet another piece of the puzzle of tumour biology. This thesis highlights the important aspects of the DLBCL disease, and the importance of examining the disease from several aspects and with different techniques, to fully understand and connect the biology with practise in the clinic.

Therefore, we continue our research of DLBCL from different angles to better describe the disease. The plan is to continue with epidemiological studies to elucidate the appearance of DLBCL in different sub-groups. Another approach will, via genome analysis of pre-specified pathways, aim to describe specific profiles in relation to tumour biology, clinical characteristics and outcome.

Swedish summary

Populärvetenskaplig sammanfattning på svenska

Allt levande består av celler. Några av de enklaste livsformerna består bara av en cell medan mer avancerade livsformer, som djur och människor, består av många celler. För att cellerna ska fungera optimalt tillsammans finns avancerade kontrollsystem i cellerna som reglerar cellernas tillväxt och delning. Det är detta som bland annat gör att vi slutar växa när vi blir vuxna och att det vid sårläkning inte blir en överdriven ärrbildning. Om det blir fel på dessa regulatoriska mekanismer i cellen och finns kontrollfunktioner som ser till att cellen inte överlever utan dör. Därigenom sätts hela individens fortlevnad före fortlevnaden av en enskild cell.

Ibland blir det dock fel på de regulatoriska mekanismerna och kontrollfunktionerna i en cell så att cellen börjar växa och dela sig ohämmat. Cellen har blivit odödlig och omvandlad till en cancercell – har blivit malign. Den sammanlagda risken att detta ska ske ökar med tiden, vilket gör att alltefter som medellivslängden ökar så ökar risken att någon gång drabbas av cancer.

Om det är vita blodkroppar i lymfsystemet som blir maligna kallas det lymfom (lymfkörtelcancer). Eftersom det finns många typer av vita blodkroppar och i olika utvecklingsstadier, finns det många olika typer av lymfom. Den vanligaste lymfomtypen är Diffust storcelligt B-cellslymfom (Diffuse large B-cell lymphoma; DLBCL). DLBCL kan uttrycka sig väldigt olika hos olika personer, både gällande hur själva cancern ser ut på cellnivå men också vad gäller symtom och överlevnad. Tyvärr kan vi för lite om sjukdomen för att förstå vad det är som gör att det går bättre för vissa drabbade än andra.

I denna avhandling presenteras arbetet med att söka efter nya faktorer som kan hjälpa oss förutse prognosen för de som drabbas av DLBCL och även ge oss ytterligare förståelse om tumörbiologin. Vi vet redan att det i en tumör av DLBCL-typ finns tumörceller men även många olika typer av friska, icke-maligna, celler som normalt tillhör kroppens immunförsvar. Länge trodde man att dessa celler bara var passiva åskådare i tumören men nu vet vi att det finns ett komplicerat samspel mellan tumörcellerna och de

icke-maligna cellerna. Vi har studerat en specifik typ av dessa friska celler som heter mastceller. Dessa har tidigare studerats i andra lymfomtyper än DLBCL och vi har nu kunnat visa att antalet mastceller i DLBCL kan relateras till prognosen. Många mastceller i tumören var prognostiskt gynnsamt och dessa patienter levde i större utsträckning längre än de som hade få mastceller i tumören.

Men det finns även många viktiga regulatoriska funktioner inne i cellen som kan ha betydelse för överlevnad hos de som drabbas av DLBCL. En sådan intracellulär regulatorisk komponent är mikroRNA. MikroRNA är väldigt korta bitar av något som liknar DNA, men som är med och reglerar många viktiga cellulära funktioner. Det finns mer än tusen olika mikroRNA och var och en kan påverka, och påverkas av, många olika intracellulära processer. Vi har specifikt studerat mikroRNA-129 och kunnat visa att halten av detta i DLBCL-cellen kan spela roll för överlevnad. De med hög halt av mikroRNA-129 hade generellt en längre överlevnad jämfört de med låga halter av mikroRNA-129. En möjlig förklaring är att mikroRNA-129 kan nedreglera ett protein som heter Cdk6 och detta i sin tur bidrar till att cellen stannar av i tillväxt och dör.

Det kan även finnas skillnader på DNA-nivå som kan bidra till den heterogenitet i symtombild, överlevnad och tumörbiologi som kan ses för DLBCL. En enstaka förändring på ett ställe i DNA i en cell kallas för mutation, men kallas polymorfism om den specifika förändringen är så spridd att den finns hos en tillräckligt stor andel av befolkningen. Dessa variationer i DNA märks tydligast i att vi ser olika ut som individer men de kan även spela roll för de regulatoriska mekanismer och kontrollfunktioner i en cell som ska se till att allting fungerar normalt. I denna kontrollfunktion är proteinet MDM2 viktigt och där kan en polymorfism göra att det är lättare eller svårare för cellen att läsa DNA-koden och utifrån den bygga proteinet MDM2. Det ger upphov till olika halter av MDM2 i celler vilket i sin tur ger celler olika benägenheter att bli maligna. Vi kunde visa att en polymorfism i MDM2-genen kan relateras till tumörbiologi men även till överlevnad hos vissa patienter. För de som behandlas med cancerläkemedlet rituximab verkar inte den undersökta polymorfin i MDM2-genen spela någon roll för överlevnad. Detta visar på vikten av att undersöka faktorer av prognostisk betydelse utifrån aktuell behandling då nya läkemedel kan påverka vilka faktorer som kan användas.

En prognostisk faktor som funnits med länge och som fortfarande är av betydelse, är inverkan av ålder. Hög ålder ger sämre prognos för de som drabbas av DLBCL. Tidigare har man satt en gräns vid 60 år varefter det skulle vara mer ogynnsamt för överlevnaden i DLBCL. Genom att analysera alla DLBCL-fall i Sverige år 2000-2013 har vi kunnat visa att den försäm-

rade överlevnaden startar vid mycket lägre ålder. Redan i åldersgruppen 40-49 år finns en försämrad överlevnad jämfört med gruppen 20-29 år. Vår analys omfattar en av de största grupperna med DLBCL-fall som hittills beskrivits och det har medfört att vi på ett tydligt sätt kunnat visa ålderns betydelse för överlevnaden efter DLBCL.

Ett mål är att i framtiden kunna erbjuda bättre prognostiska markörer än idag. Markörer som kan hjälpa oss att intensifiera behandlingen mot DLBCL där så är nödvändigt. Denna avhandling belyser DLBCL från flera olika infallsvinklar och med flera olika metoder och förhoppningen är att resultaten kan bidra till en ökad förståelse av sjukdomen DLBCL men även utgöra en grund för fortsatt forskning av faktorer betydelsefulla för utfallet av DLBCL.

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