A search for prognostic biomarkers in diffuse large B-cell lymphoma with proteomics and immunohistochemistry

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“The future depends on what you do today” Mahatma Gandhi

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ABSTRACT

Diffuse large B-cell lymphoma (DLBCL), the most common lymphoma in the Western world, can by gene expression profiling or immunohistochemistry (IHC), be divided into two subgroups according to its "cell-of-origin". The subgroup ABC (or non-GCB) with similarities to active post-germinal centre B-cells, is associated with worse outcome. In addition, patients with primary refractory disease or early relapse have a very dismal prognosis. The aim of this thesis has been to identify novel prognostic biomarkers in a large retrospective DLBCL patient cohort by mass-spectrometry (MS)-based proteomics and IHC. Quantitative MS-based proteomics (QMS) revealed several differentially expressed proteins between refractory/relapsed patients (REF/REL) and patients with progression-free survival ≥5 years (CURED). Many ribosomal proteins were up-regulated in REF/REL patients while numerous proteins associated with the actin cytoskeleton were up-regulated in CURED patients. By using QMS we also found several up-regulated proteins in non-GCB DLBCL related to the tumour microenvironment, including interferon (IFN)-stimulated proteins. By using IHC we found a prognostic association for two proteins (CREBBP and TBLR1) that are frequently mutated in DLBCL, and for IFI16 and MNDA, both belonging to the pyrin and hematopoietic IFN-inducible nuclear (PYHIN) family. In conclusion, we have found increased expression of several proteins or groups of proteins not previously described in DLBCL and with potential prognostic impact. Further functional studies are warranted to elucidate their role in immunochemotherapy resistance.

Keywords: Diffuse large B-cell lymphoma, cell-of-origin, proteomics, immunohistochemistry, ribosomal, PYHIN

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SAMMANFATTNING PÅ SVENSKA

Diffust storcelligt B-cellslymfom (DLBCL) är det vanligaste lymfomet med ca 600 nya fall per år i Sverige. Lymfom är en tumör med ursprung i lymfatiska celler, vars normala uppgift är att delta i kroppens immunförsvar. DLBCL delas in i två undergrupper efter ursprungscell, s.k. "cell-of-origin". Denna uppdelning baseras ursprungligen på studier av genuttryck och den ena gruppen som liknar aktiva B-lymfocyter som träffat på antigen (post-germinalcenter B-celler) kallas ABC eller non-GCB och har sämre prognos. Standardbehandling för DLBCL är en antikropp mot B-celler samt en kombination av cellgifter. Trots att ca 60 % av alla patienter botas så har framför allt de som inte svarar på primär behandling eller återfaller tidigt en mycket dålig prognos. I den här avhandlingen har vi därför sökt efter proteiner som kan fungera som prognostiska biomarkörer vid DLBCL. Materialet utgörs av formalinfixerad paraffinbäddad tumörvävnad från en retrospektiv kohort av DLBCL-patienter som diagnostiserats mellan 2004 och 2016. I två delarbeten har vi använt kvantitativ masspektrometri (QMS) för att studera det globala proteinuttrycket vid DLBCL. Vi fann att lymfomcellerna hos patienter som inte svarat på behandling eller återfallit tidigt i sin sjukdom hade ett överuttryck av ribosomala proteiner medan det hos dem som överlevt minst 5 år istället förelåg ett ökat uttryck av proteiner relaterade till cellskelettet. Det kanske mest intressanta fyndet sågs hos patienter tillhörande gruppen non-GCB där vi fann ett ökat uttryck av proteiner relaterade till lymfomcellernas mikromiljö, dvs. de inflammatoriska celler som förutom lymfomcellerna är en del av tumören. Bland annat sågs flera interferon (IFN)-stimulerade proteiner och i delarbete IV fann vi sämre prognos hos patienter med ökat immunhistokemiskt uttryck av de IFN-stimulerade proteinerna IFI16 och MNDA, vilka båda tillhör den s.k. PYHIN-familjen. Vi använde också immunhistokemi i arbete II och fann en sämre prognos för patienter med ökat uttryck av proteinerna TBLR1 och CREBBP vars gener ofta beskrivits muterade vid DLBCL.

Sammanfattningsvis drar vi slutsatsen att DLBCL är en komplex sjukdom och att det finns flera olika proteiner eller grupper av proteiner som kan ha prognostisk betydelse. Ökat uttryck av ribosomala eller interferonstimulerade proteiner vid DLBCL är inte tidigare beskrivet. Fortsatta funktionella studier krävs för att ytterligare studera dessa proteiner och deras relation till behandlingsresistens

LIST OF PAPERS

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

I. Bram Ednersson S, Stenson M, Stern M, Enblad G, Fagman H, Nilsson-Ehle H, Hasselblom S, Andersson P-O.
Expression of ribosomal and actin network proteins and immunochemotherapy resistance in diffuse large B cell lymphoma patients.
British Journal of Haematology 2018; 181: 770-781.

II. Bram Ednersson S, Stern M, Fagman H, Nilsson-Ehle H, Hasselblom S, Andersson P-O.
TBLR1 and CREBBP as potential novel prognostic immunohistochemical biomarkers in diffuse large B-cell lymphoma.
Leukemia & Lymphoma 2020; 61: 2595-2604.

III. Bram Ednersson S, Stern M, Fagman H, Nilsson-Ehle H, Hasselblom S, Thorsell A, Andersson P-O.
Proteomic analysis in diffuse large B-cell lymphoma identifies dysregulated tumor microenvironment proteins in non-GCB/ABC subtype patients.
Leukemia & Lymphoma 2021; 62: 2360-2373.

IV. Bram Ednersson S, Stern M, Fagman H, Nilsson-Ehle H, Hasselblom S, Andersson P-O.
Increased expression of the interferon-inducible PYHIN proteins IFI16 and MNDA show prognostic impact in diffuse large B-cell lymphoma.
Manuscript.

CONTENT

ABBREVIATIONS

aaIPI	Age adjusted International Prognostic Index
ABC	Activated B-cell
BCR	B-cell receptor
BTK	Bruton's thyrosine kinase
COO	Cell-of-origin
CSR	Class switch recombination
DE	Double-expresser
DH/TH	Double hit/triple hit
DLBCL	Diffuse large B-cell lymphoma
FFPE	Formalin fixed, paraffin embedded
GC	Germinal centre
GCB	Germinal centre B-cell
IFN	Interferon
IGH	Immunoglobulin heavy chain
IHC	Immunohistochemistry
MS/QMS	Mass spectrometry/quantitative mass spectrometry
Non-GCB	Non-Germinal centre B-cell
OS	Overall survival
PFS	Progression-free survival

R-CHOP	Rituximab, Cyclophosphamide, Doxorubicin (Hydroxydaunorubicin), Vincristine (Oncovine), Prednisolone
RP	Ribosomal protein
SH	Single hit
SHM	Somatic hypermutation
TMA	Tissue microarray
TME	Tumour microenvironment
TMT	Tandem-mass-tag
WHO	World Health Organization

1 INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma in Sweden and the rest of the Western world with approximately 600 new cases each year in Sweden (1, 2). It consists of a clonal expansion of mature B-lymphocytes (B-cells) with the morphology of predominantly large blasts that proliferate in a diffuse manner (3).

A B-cell lymphoma with centroblastic/large non-cleaved or immunoblastic morphology was first described in 1974 in the European Kiel Classification for Lymphoma developed by Lennert and co-workers (4) and in the American Lukes and Collins classification (5). In 1982 the REAL classification (Revised European American Lymphoma) was proposed by the International Lymphoma Study Group (6), which gave the lymphoma its name "Diffuse large B-cell lymphoma" as a subgroup of mature (peripheral) B-cell neoplasms. In the latest World Health Organisation (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues from 2017, the diagnosis DLBCL has become vastly comprehensive as several specific entities are named based on localization and morphological, immunohistochemical or cytogenetic findings and the principal group of DLBCL *not otherwise specified* in addition contains several subgroups (3).

DLBCL mainly affects lymph nodes but 30 – 40% of cases primarily affect extranodal organs such as the gastrointestinal tract, skin or genitourinary organs (3, 7). It is mainly a disease of the elderly population with a median age of around 70 years but can occur at all ages (3, 8) and is slightly more common in men (2). Other risk factors are chronic inflammatory disease and ethnicity with e.g. a higher risk in the white population in the United States (9). Another risk factor is heredity, with increasing molecular investigations of the disease revealing germ line mutations in genes important for DLBCL pathogenesis (10).

Prognostic factors used in a clinical setting are disease stage according to Ann Arbor, serum lactate dehydrogenase and performance status, all incorporated into the age-adjusted International Prognostic Index (aaIPI) used for the allocation of patients to different treatment protocols (11).

Immunochemotherapy using the R-CHOP regimen (the anti-CD20 antibody *R*ituximab, the cytostatic agents *C*yclophosphamide, *H*ydroxydaunorubicin and *O*ncovine (Vincristine) and the corticosteroid *P*rednisone), is standard for

patients treated with curative intent and cures up to 60% of the patients (12-14).

1.1 THE B-CELL

B-cells originate from pluripotent haematopoietic stem cells in the bone marrow and pass through different stages of development from the early pro-, pre- and immature B-cell stages in the bone marrow to mature B-cells in peripheral lymphoid organs (15).

1.1.1 The B-cell receptor and B-cell maturation

The B-cell receptor (BCR), also called immunoglobulin (IG), for recognition of antigens consists of two heavy and two light chains. During the pro B-cell stage the gene encoding for the *heavy* chain of the immunoglobulin (*IGH*) undergoes the so called V(D)J recombination process during which different coding sequences for variable (V) and constant regions are linked together with joining (J) and diversity (D) segments (15, 16). The VJ rearrangements of the immunoglobulin receptor *light* chain genes (*IGL*) kappa or lambda occur as a second step at the late pre B-cell stage (15, 17). These processes, enabled by the introduction of double strand DNA breaks by the recombination activating genes RAG1 and RAG2, lead to the primary, innate, immune repertoire (18).

Antigen naïve B-cells can migrate to peripheral lymphoid organs such as lymph nodes (19), where they encounter antigens presented to them by dendritic cells or macrophages, leading to T-cell mediated B-cell activation (20). In these activated B-cells two additional genetic events occur which further sharpen the immune repertoire. One mechanism is somatic hypermutation (SHM) of both heavy and light chain variable regions. This process takes place in the dark zone of the lymph node germinal centres (GCs), mainly populated by highly proliferating centroblasts (Figure 1), and is triggered by Activation induced deaminase (AID) (16, 21). The other mechanism, which is also triggered by AID, is class switch recombination (CSR). During CSR the heavy chain class switches from the IgM or IgD of the innate immune repertoire to one of the classes IgG, IgA or IgE of the secondary, adaptive immune response. The class switch enables the B-cells to interact with different effector molecules and can occur in the GC as well as prior to GC-formation (22). B-cells pass through several cycles between the dark zone for SHM and the centrocyte rich light zone (Figure 1) of the GC where antigen recognition takes place. B-cells with high affinity BCR can

exit the GC light zone as plasmablasts that further mature to plasmacells or memory B-cells (21).

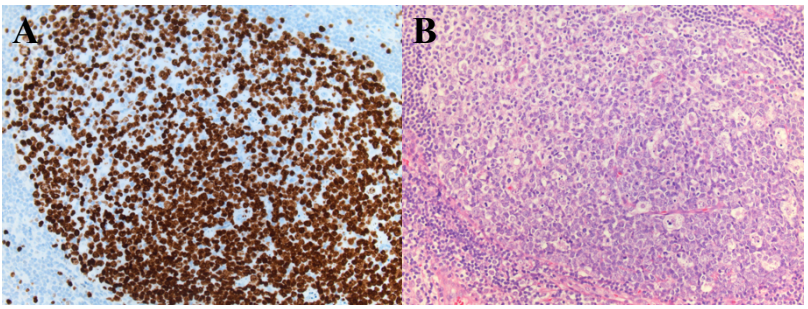

Figure 1. Polarization of a germinal centre (GC) in dark and light zones. A) Immunohistochemical staining with the proliferation marker Ki67 shows the dark zone with high proliferation index at the bottom right corner and the light zone at the top left. B) Haematoxylin-eosin staining of the same GC shows the dark zone with predominance of large centroblasts and the light zone with predominance of small centrocytes. Copyright Susanne Bram Ednersson 2021.

1.1.2 B-cell receptor signalling

When an antigen binds to the membrane bound BCR a number of signalling pathways are triggered. The membrane proteins CD79a and CD79b are bound to the immunoglobulin structure and their immunoreceptor tyrosine-based activation motifs (ITAMs) become phosphorylated upon antigen binding of the BCR. This results in binding of the kinase SYK to the ITAMs, which through the recruitment of other signal proteins can propagate the signal through different pro-survival pathways. Among these are PI3K/AKT/mTOR and NF-κB pathways that both require the activation of Bruton's thyrosine kinase (BTK), which is recruited by PI3K, in turn recruited by the membrane bound B-cell antigen CD19 (23, 24).

Active and tonic B-cell signalling

In its non-active state, the membrane bound immunoglobulin structure consists of two monomer complexes of one heavy and one light chain. Antigen binding causes the two monomers to dimerize and triggers active B-cell signalling through the PI3K-AKT, NF-κB, MAPK and NFAT pathways. In resting B-cells however, the immunoglobulin molecule remains in a monomer state and a tonic, antigen independent signal necessary to maintain B-cell viability, is produced through the PI3K pathway (23, 24).

1.2 CELL-OF-ORIGIN

Originating from gene expression profiling studies (GEP) of fresh DLBCL tumour tissue, which were first published by Alizadeh *et al.* in 2000 (25), the cell-of-origin concept (COO) divides DLBCL into two main subgroups depending on the cell stage in the development of mature normal B-cells, from which the lymphoma is presumed to originate. The study showed that CHOP-treated DLBCL patients with a lymphoma gene expression profile similar to that of activated B-cells (ABC) had a worse prognosis as compared to those with a germinal centre B-cell (GCB) expression profile. This finding was later confirmed in R-CHOP treated patients and a small unclassified group has additionally been identified (26, 27).

Among the genes characterizing the GCB group in the original GEP study were the germinal centre B-cell marker CD10 and the transcription factor BCL6. CD10 is a marker of reactive follicles (28) and BCL6 is necessary for GC-formation and SHM (29). In the ABC group the transcription factor IRF4 (MUM1) was among the most interesting overexpressed genes. IRF4 is normally predominantly expressed by plasma cells but also in a subset of GC B-cells and plays a role in CSR (30). The proteins corresponding to these genes were later translated into an immunohistochemical (IHC) algorithm by Hans and co-workers, which separates DLBCL into GCB and non-GCB subtypes, with shorter survival for the non-GCB group and a good correlation with gene expression data (31).

Over the years several other IHC algorithms have been developed for the analysis of COO. Still, the correlation between immunohistochemistry-based classifications and prognosis has been ambiguous, especially in R-CHOP treated patients and it has varied between different algorithms (32-34).

In 2014, Scott *et al.* developed the NanoString-based Lymph2Cx assay, which classifies DLBCL into ABC, GCB and "unclassified" based on 15 discriminating transcripts and 5 house-keeping transcripts (35). The method correlates well with the GEP classification by Lenz *et al.* from 2008 (26). The original study by Scott *et al*. showed worse outcome for ABC patients classified with Lymph2Cx. Some later studies have supported this correlation with survival (36, 37) but others have failed to show a significant correlation with prognosis for COO analysed with the Lymph2Cx alone (34, 38).

B-cell signalling in ABC vs. GCB

The type of active signalling present in some types of B-cell lymphomas has been termed *chronic* active B-cell signalling, since it depends on different types of activating mutations rather than on antigen-stimulation (Figure 2A). This type of signalling is seen in e.g. ABC-type of DLBCL, is dependent on BTK and strongly relies on NF-κB signalling. Activation of the NF-κB pathway stimulates the expression of IRF4 and suppression of BCL6 and is thus an underlying mechanism for the different protein expression patterns used to separate DLBCL into GCB and non-GCB with immunohisto-chemistry. In ABC, activating mutations in the NF-κB pathway protein CARD11 are seen in about 15% of cases. Mutations in the ITAM motives of CD79a/b are present in about 1/3 of ABC cases, which increases BCR expression and reduces negative feedback on BCR-signalling, further potentiating lymphomagenesis (39-41).

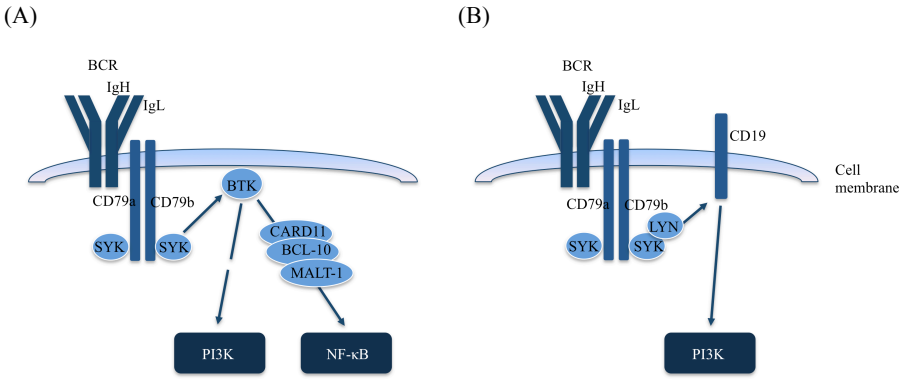

Figure 2. A) Chronic active B-cell signalling in ABC DLBCL relies on activation of Bruton's thyrosine kinase (BTK) to activate PI3K and NF-κB stimulatory signalling pathways. B) Tonic B-cell signalling in GCB DLBCL requires activation of CD19 but not BTK and relies on the PI3K pathway. (Based on data from Young, et al.; Targeting pathological B cell receptor signalling in lymphoid malignancies; Nat Rev Drug Discov; 2013;12(3):229-43 and Efremov, et al.; Mechanisms of B Cell Receptor Activation and Responses to B Cell Receptor Inhibitors in B Cell Malignancies; Cancers (Basel); 2020;12(6)).

GCB-type DLBCL, by contrast, relies on tonic B-cell signalling, which otherwise is the hallmark of Burkitt lymphoma. This type of signalling relies on the PI3K/AKT/mTOR pathway and is dependent on SYK and CD19 but independent of BTK and the stimulation of PI3K is propagated directly from CD79a (40-42) (Figure 2B).

1.3 EXTENSIVELY STUDIED BIOMARKERS IN DLBCL

1.3.1 Gene rearrangements

A translocation between chromosomes 14 and 18, t(14;18)(q32;q21) (*IGH-BCL2*), which juxtaposes the anti-apoptotic gene *BCL2* with the promoter of the immunoglobulin heavy chain (*IGH*), is frequent in DLBCL, predominantly in the GCB type. In GCB it is present in approximately 35% of cases (36, 43, 44). Since this aberration is the hallmark of follicular lymphoma some of these cases probably represent transformed low-grade lymphomas (45).

The transcription factor MYC is under normal conditions necessary for B-cell development in the bone marrow as well as for maintaining the GC reaction in reactive lymph nodes (46). In normal cells MYC has a dual role in both stimulating proliferation and increasing apoptosis (47). A rearrangement of *MYC* (8q24), such as t(8;14)(q24;q32) (*MYC-IGH*) or with other translocation partners (48) is seen in up to 14% of DLBCL and also more commonly reported in GCB (49-53).

In 2007, a cohort of 16 DLBCL patients with the presence of concurrent t(14;18) and *MYC* rearrangement was described clinically and all showed aggressive features (54). This “double-hit” (DH) concept that had previously only been described in case-reports of DLBCL, constitutes a separate subgroup in the current WHO classification named High-grade B-cell lymphoma (HGBL) with *MYC* and *BCL2* and/or *BCL6* rearrangements, which is distinct from DLBCL *not otherwise specified*. This group of HGBL also contains a “triple hit” lymphoma (TH) with t(14;18), *MYC* rearrangement as well as a rearrangement of *BCL6* (3q27), the latter also a possible participant in a DH lymphoma together with *MYC* rearrangement (3). A rearrangement of the transcription factor *BCL6* (3q27) is more common in ABC and is present in up to about 30% of all DLBCL, which makes it the most common gene rearrangement (36, 38, 55). The translocation (14;18)(q32;q21) (*IGH-BCL2*) is suggested to occur in the process of V(D)J recombination in the bone marrow while the other two aberrations (*MYC* and *BCL6*) seem to occur at a later stage in the germinal centre during the process of CSR (56).

DH or TH have been reported in about 7% of lymphomas with DLBCL morphology and have been associated with a shorter survival (50, 52, 57). Especially DH involving *MYC* and *BCL2* are more frequently of the GCB

type (51, 52). The prognostic impacts of *MYC* or *BCL2* or *BCL6* rearrangements as single hits (SH) however are divergent. Some studies have reported a negative prognostic effect predominantly for *MYC* rearrangements although several studies have not shown a significant impact on survival for the rearrangements of *MYC* as SH (50, 52, 58). The translocation partner for *MYC* also seems to be important for the association with prognosis both in SH and DH/TH cases. Data indicate a worse prognosis for cases in which *MYC* is translocated to either heavy or light chain immunoglobulin genes than with a non-IG translocation partner (48, 50, 52). Rearrangements of *BCL2* or *BCL6* as SHs do not seem to affect outcome (58).

Double hit gene signature

A double hit gene signature (DHITsig) was suggested among GCB DLBCL and has been associated with inferior outcome more similar to ABC patients but that only in part encompasses cases that are DH with FISH analysis. The DLBCL90 assay developed 2019 by Ennishi *et al.* contains the 30 most discriminating genes from the DHITsig and the model was later validated as showing negative prognostic impact, at least for overall survival (OS) (59, 60).

1.3.2 Proteins

Over the years, the prognostic impact of the expression patterns of several protein biomarkers as detected by immunohistochemistry has been evaluated for DLBCL. Some of the most frequently studied are:

BCL2, MYC and BCL2/MYC double-expresser

Both BCL2 and MYC have been extensively studied as single protein biomarkers in DLBCL. Inferior prognosis related to BCL2 expression was shown by some studies (61, 62) before the R-CHOP era but its value as a prognostic marker has greatly diminished in R-CHOP treated patients (63-66). Most of the studies used ≥ 50% positive cells as a cut-off for BCL2 positivity. MYC overexpression has been independently associated with inferior prognosis in R-CHOP treated patients in some studies (44, 67, 68) with cut-off for positive staining varying from 40 to 50% but in other studies the results have been inconclusive (38, 69).

In 2012, two studies showed that the combined expression of BCL2 (cut-off 50% or 70%) and MYC (cut-off 40%) was independently associated with inferior outcome in R-CHOP treated DLBCL patients but that MYC or BCL2 overexpression alone was not (70, 71). This concept of “immunohisto-

chemical double-hit" (70) or "double-expressor" (72), in the 2017 WHO classification named "double-expresser" (DE) with cut off 50% for BCL2 positivity (3), constitute about 30% of DLBCL and is more frequently encountered in ABC (36, 38). DE has remained an independent negative prognostic factor in many subsequent studies although with variable cut-off levels for the percentage of cells to signify positive staining (38, 53, 69, 73, 74).

For both BCL2 and MYC protein expression a positive correlation has been shown with the presence of t(14;18) and *MYC* rearrangements respectively (75-77). In addition, at least for BCL2, copy number gains can be a mechanism underlying elevated protein expression and gains of *BCL2, BCL6* or *MYC* have also been shown to contribute to outcome in combination with rearrangements as single hits (77-79).

BCL6

Protein expression of the transcription factor BCL6 does not seem to correlate with the presence of *BCL6* rearrangements (76, 80) and the relationship between BCL6 expression and survival is probably obscured by the fact that its expression is tightly associated with GCB DLBCL, a group frequently reported to have better prognosis. Horn *et al.* 2013 found that low BCL6 expression is associated with shorter survival but their analysis was not adjusted for COO (44) and a subgroup analysis based on COO conducted for primary gastric DLBCL showed no correlation with BCL6 expression and survival for non-GCB patients (81).

CD5

The membrane protein CD5 is normally expressed by T-lymphocytes. De novo CD5 positive DLBCL has a gene signature that is separate from that of transformed chronic lymphocytic leukaemia (CLL), an indolent B-cell lymphoma with expression of CD5 as a hallmark (82). CD5 positive DLBCL constitutes around 10% of DLBCL cases and CD5 positivity has been reported as an independent negative prognostic factor or to present with aggressive features, in one study in combination with MYC positivity (83-85).

TP53

Overexpression of the tumour suppressor protein TP53 has been associated with a negative prognostic impact in DLBCL that seems to be further

increased by the coexpression of other proteins such as CD5, MYC or DE or presence of *MYC* or *BCL2* rearrangements (86, 87).

CD30

CD30 is expressed by a population of activated lymphocytes as well as in several types of lymphomas, e.g. Hodgkin Lymphoma (88). IHC positivity for CD30 is variably seen in DLBCL and is sometimes associated with an anaplastic morphology or positivity for Epstein-Barr virus (89, 90). CD30 positivity has been shown to be associated with a favourable outcome in some studies but the prognostic effect could be influenced by other factors such as low IPI and absence of DE (90-92).

Ki67

Ki67 binds to a nuclear protein expressed by actively proliferating cells (93) and is used as an immunohistochemical marker for cycling cells. The prognostic effect of the proportion of Ki67 positive cells in DLBCL has varied among different studies, from no prognostic effect to high Ki67% being an independent negative factor. The results are however complicated by the fact that different cut-off levels have been used for the designation of Ki67 high (69, 94-96).

1.4 MOLECULAR SUBGROUPS OF DLBCL WITH POSSIBLE PROGNOSTIC IMPACT

Some of the most commonly mutated genes in DLBCL are *MYD88*, the tumour suppressor *TP53*, the cell cycle regulator *PIM1* and the epigenetic regulator *KMT2D* (56). *MYD88* mutation is predominantly seen in ABC DLBCL in which it stimulates NF-κB signalling independent of BCR-signalling (41).

During recent years attempts have been made to classify DLBCL into subgroups based on the mutational pattern and relate these molecular subgroups to patient outcome and COO. Especially the works by Reddy *et al.* 2017, Schmitz *et al.* 2018 and Chapuy *et al.* 2018 have gained much attention (97-99) and illuminate the genetic heterogeneity of the lymphoma.

Reddy *et al.* (97) identified the 150 most frequently mutated genetic drivers of DLBCL and related their expression to COO and outcome. Of these, the mutational frequency of twenty genes significantly differed between ABC

and GCB. In ABC, mutations were seen in e.g. *MYD88, CD79B*, *PIM1,* and the transcriptional repressor *TBL1XR1* and in GCB in e.g. *BCL2*, the epigenetic regulators *CREBBP* and *EZH2* and the interferon regulatory factor *IRF8*. The epigenetic regulator EZH2 is necessary for GC-formation together with BCL6 and mutated EZH2 seems to cooperate with BCL6 to drive lymphoma development (100). *CREBBP* has been described as a tumour suppressor gene whose inactivation stimulates growth of GCB cells (101). In the context of DLBCL, an important function for TBL1XR1 (TBLR1) is the association with the NF-κB pathway for the activation of downstream target genes (102).

Chapuy *et al.* (99) identified five molecular subgroups of DLBCL, Cluster 1 – 5 (C1 – C5) based on clusters of significant driver mutations that were related both to COO and prognosis. C1, mostly harbouring ABC patients with structural variants of *BCL6* and mutations of *NOTCH2* pathway genes and C4 with mutations in e.g. histone genes, immune evasion molecules and NF-κB modifiers and mostly harbouring GCB patients had better prognosis. A worse prognosis was seen for group C3 containing mostly GCB patients with t(14;18)(*IGH-BCL2*), mutations in *BCL2* and epigenetic regulators *KMT2D*, *CREBBP* and *EZH2* and for group C5 with 18q (*BCL2*) gains, *CD79B* and *MYD88* mutations and mainly consisting of ABC patients. C2 showed inactivation of TP53 but was not associated with a specific COO. The outcome for this group was similar to C3 and C5. A C0 group was also identified that lacked significant driver mutations.

Schmitz *et al.* (98) presented four genetic subtypes of DLBCL that partly overlap with the clusters identified by Chapuy *et al*. (figure 3). The MCD subtype with mutations in *MYD88* and *CD79B* overlaps with C5 and similar to this subgroup mainly consists of ABC patients and was associated with worse prognosis. Also the N1 group had unfavourable outcome and harbours *NOTCH1* mutations and mainly ABC patients. The BN2 group with *BCL6* fusions and *NOTCH2* mutations overlaps with C1 and had a better outcome and so did the EZB group with mutations in *EZH2* and *BCL2* translocations. BN2 was not associated with a particular COO while EZB mainly consisted of GCB patients and overlaps with C3 with reference to the pattern of genetic aberrations but not to outcome for the whole cohort. In GCB patients however EZB showed a trend for worse outcome.

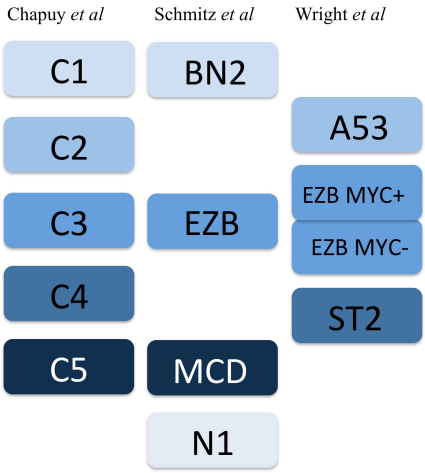

Figure 3. Overlap is seen in the mutation profile between some of the DLBCL subgroups proposed by Chapuy et al., Schmitz et al. and Wright et al. as exemplified by the same colour shade. (Based on Chapuy et al; Molecular subtypes of diffuse large B cell lymphoma are associated with distinct pathogenic mechanisms and outcomes; Nat Med; 2018;24(5):679-90 and Schmitz et al; Genetics and Pathogenesis of Diffuse Large B-Cell Lymphoma; N Engl J Med; 2018;378(15):1396-407 and Wright, et al; A Probabilistic Classification Tool for Genetic Subtypes of Diffuse Large B Cell Lymphoma with Therapeutic Implications; Cancer Cell; 2020;37(4):551-68 e14).

In 2020 Wright *et al.* (103) further developed the classification by Schmitz *et al*. by identifying two more genetic subgroups; A53 with aneuploidy and inactivation of *TP53*, and ST2 with mutations in *SGK1* and *TET2*. Additionally, the EZB subgroup (98) was further divided into MYC^{+} or MYC^{-}, based on the presence or absence of the previously mentioned double-hit signature (DHITsig) (figure 3) (59). This genetic subtype classifier (LymphGen) thus identifies seven subgroups encompassing about 60% of DLBCL cases (Figure 4)

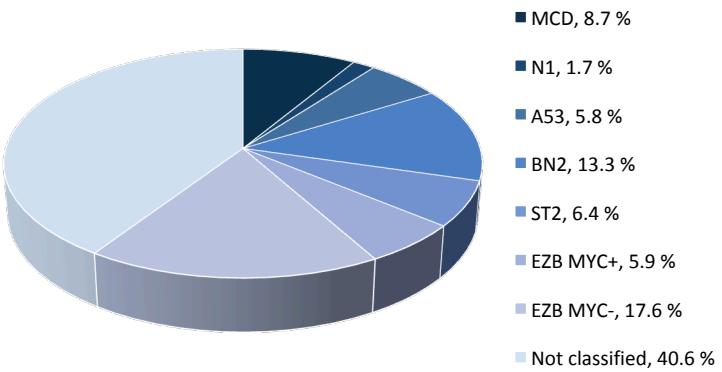

Figure 4. The seven molecular subtypes described by Wright et al encompass ≈60% of DLBCL cases, leaving approximately 40% still unclassified. (Based on data from Wright, et al; A Probabilistic Classification Tool for Genetic Subtypes of Diffuse Large B Cell Lymphoma with Therapeutic Implications; Cancer Cell; 2020;37(4):551-68 e14).

1.5 TUMOUR MICROENVIRONMENT OF DLBCL

In the era of a heterotypic view on tumour development and progression rather than a reductionist, the tumour microenvironment (TME) has become increasingly in focus (104). The TME refers to the stromal and inflammatory cells, such as T-cells and macrophages that infiltrate between the neoplastic cells. Interactions between neoplastic cells and the cells of the microenvironment can be both tumour suppressive and stimulate tumour growth (105). Among lymphomas the best example of this concept is probably classic Hodgkin lymphoma with few neoplastic cells and the bulk of the tumour consisting of non-neoplastic inflammatory cells (106).

1.5.1 Interactions between tumour cells and the microenvironment

Immune suppression and *immune evasion* are two partly overlapping mechanisms for the interplay between neoplastic cells and the microenvironment, both occurring in DLBCL (107). Both mechanisms belong to what has been named an *inflamed* tumour phenotype with a high proportion of infiltrating T-cells and often activation of NF-κB pathway in the lymphoma cells, opposed to a *noninflamed* phenotype (108).

Immune evasion or immune escape is a result of so called *immunoediting*. During this process malignant cells, under pressure from the immune system, acquire means to avoid the anti-neoplastic effects exerted by the surrounding immune cells. The process has three phases: during the *elimination phase* the identification and elimination of neoplastic cells by effector cells of the innate immune system leads to release of tumour antigens which trigger the adaptive immune system and the generation of tumour specific CD8-positive cytotoxic T-cells. The second phase is *equilibrium* during which neoplastic cells by clonal evolution can gain resistance to anti-tumour immune mechanisms and as long as a balance exists between anti-tumour immunity and immune suppression the surviving neoplastic clones remain dormant. Lastly, during the *escape* phase neoplastic cells that have gained enough advantages opposed to the immune system and also gained other pro-survival functions can escape dormancy and develop a clinically manifest tumour (109, 110).

Several mechanisms of immune evasion have been described in lymphomas including DLBCL. Upregulation of the immune checkpoint programmed cell death 1 ligand (PD-L1) on lymphoma cells and predominantly on

macrophages of the TME, leads to suppression of PD1-expressing cytotoxic CD8-positive T-cells and reduced inflammatory response. Other mechanisms include reduced expression of HLA proteins caused by alterations in e.g. the genes *CD58* and *CREBBP*, increased proportion of the pro-tumoural macrophage type M2 as opposed to pro-inflammatory M1 macrophages and overexpression of the anti-phagocytic protein CD47 (107, 111).

Immune suppression is important under normal conditions to avoid immune reactions to self-antigens and is largely maintained by a CD4-positive T-lymphocyte population named regulatory T-cells (112). In tumours these T-cells release factors like interleukins and transforming growth factor β that have an inhibitory effect on effector T-cells and thus promote tumour growth (113). The PD1/PD-L1 checkpoint is also involved in this process since PD-L1 can stimulate the proliferation of immunosuppressive regulatory T-cells in addition to its suppression of cytotoxic T-cells (107, 111).

1.5.2 Molecular classification of DLBCL related to the microenvironment

Attempts have been made to identify molecular subgroups in DLBCL that also encompass gene expression by the TME. In 2008, Lenz *et al.* published a highly acclaimed study on the prognostic influence of gene expression profiles that also includes the DLBCL microenvironment and in which B-cells and non B-cells were analysed separately (26). This work was based on and further developed the gene expression profiling study by Rosenwald *et al.* from 2002 (27) and describes three molecular subgroups with prognostic impact. A gene expression profile found in a subset of the lymphoma cells was associated with a favourable outcome and was named the *germinal-center B-cell signature*. The *stromal-1* and *stromal-2* gene signatures however were predominantly features of the cells in the tumour microenvironment. The stromal-1 signature was enriched for components of the extracellular matrix, such as collagen and high infiltration of histiocytes and was associated with better prognosis. The stromal-2 signature was associated with endothelial cells markers and regulators of angiogenesis and was associated with a high density of blood vessels in the lymphomas. The outcome for stromal-2 was worse than for the other two groups and the signature was encountered both in GCB and in ABC DLBCL.

The aforementioned molecular subgroups of DLBCL also relate to different aspects of the TME. Inflamed phenotype DLBCL mostly consists of ABC lymphomas and the molecular subtypes of C1, BN2 and N1 are all associated with immune escape (98, 99, 107). Mutations in immune escape molecules

were however in addition seen in C4 with mainly GCB-like DLBCL (99). The MCD subtype of mainly ABC DLBCL (98) showed prominent aberrations in *HLA* genes and *CD58*, contributing to immune escape. One study also found a higher expression of both PD1 and PD-L1 in the lymphoma cells and higher PD-L1 expression on macrophages in ABC vs. GCB patients (114). Nevertheless, most DLBCLs and especially GCB-like DLBCL including double-hit, belong to the noninflamed phenotype with a paucity of immune cells and reduced anti-tumour immune effects. The diminished expression of genes associated with immune cells in DLBCL with alterations in e.g. the genes *EZH2* and *TP53* (97, 108) could explain the low expression of immune system related gene signatures in the molecular subgroups of A53 and EZB-MYC^{+} (103).

Most recently a study was published that presents a gene expression characterisation system that integrates five different states of DLBCL B-cells with 39 states of the cells from the TME (immune- and stroma cells). Dissection of the TME in ABC and GCB lymphomas showed enrichment for TME states associated with adverse outcome in ABC, such as increased expression genes associated with M2 macrophages. Contrarily in GCB, TME states associated with a favourable outcome dominated, such as a gene signature associated with metabolically active regulatory T-cells. The B-cell and TME data were further integrated into nine lymphoma ecotypes of which most showed association with adverse or favourable outcome (115).

To integrate prognostic information from both the malignant B-cells and the cells of the TME thus seems to be important to fully understand the clinical behaviour of the disease.

1.6 PROTEOMICS FOR THE STUDY OF GLOBAL PROTEIN EXPRESSION

Several key studies on DLBCL mentioned in the previous sections focus on the lymphoma gene expression and/or the mutational profile. Even though genetic classifiers are becoming increasingly in focus it is nevertheless proteins that exert the effects of the encoded genes.

The term *proteomics* refers to the study of the *proteome* that has been defined as “The entire PROTein complement expressed by a genOME, or by a cell or tissue type” (116, 117). Studies of the tumour proteome, as opposed to its genome, could possibly give a more accurate picture of the actual functions of the cell, since not all transcribed genes are translated into proteins and

aberrant protein expression could further be the cause of epigenetic or posttranslational modifications (118).

The presence of putatively prognostic protein biomarkers for malignant tumours can be investigated with both focused diagnostic methods such as immunohistochemistry and large-scale methods examining the whole tumour proteome such as mass spectrometry.

1.6.1 Proteomic mass spectrometry-based studies on DLBCL

The early mass spectrometry studies of the proteome of DLBCL cells were most often performed on cell lines or mouse models or in small patient cohorts. For instance, Romesser *et al* 2009 compared the proteome of malignant splenic B-cells to normal B-cells in a mouse model. The malignant splenic B-cells served as a model for ABC DLBCL with which it shares a similar transcriptional signature. Proteins that were differentially expressed in malignant B-cells compared to normal resting or proliferating B-cells were mainly associated with energy metabolism, regulation of the cell cycle, were ribonucleoproteins or were important for cell structure (119). Deeb *et al* 2012 compared the global protein expression between ABC and GCB DLBCL cell lines. Among proteins overexpressed in ABC were IRF4 and other proteins associated with constitutive NF-κB activity (120). These findings were in 2015 confirmed by Deeb *et al* in a small DLBCL patient cohort (121). Liu *et al* 2013 instead compared the proteome of DLBCL cells, cultured from patient tissue samples, with high or low sensitivity to CHOP. 19 proteins were differentially expressed between these two groups (122). Ruetschi *et al* 2015 performed a study with a similar approach comparing the proteome of five DLBCL patients with refractory disease or early relapse to five cured DLBCL patients with at least five years' follow up. None of the 35 most functionally relevant proteins however overlapped with the 19 proteins described by Liu *et al*. In the progression-free group proteins associated with the actin cytoskeleton were overrepresented (123). Thus there are implications in these early studies that groups of potential prognostic biomarkers, as well as biomarkers related to the two COO groups, can be identified from the study of the DLBCL proteome.

1.7 TREATMENT OF DLBCL

1.7.1 Standard treatment and response

Immunochemotherapy with the R-CHOP regimen consisting of the anti-CD20 antibody *R*ituximab and the cytostatic agents *C*yclophosphamide, *H*ydroxydaunorubicin (doxorubicin) and *O*ncovine (vincristine) and the corticosteroid *P*rednisone is standard primary treatment for DLBCL patients treated with curative intent (12).

The precise function of the transmembrane B-cell antigen CD20 is not entirely clear but it appears to be necessary for proper BCR-signalling. Rituximab is an antibody aimed at CD20 and kills B-cells by several mechanisms, both through a direct effect from the CD20-inhibition leading to apoptosis and indirect effects mediated by complement activation as well as by cytotoxicity and phagocytosis exerted by immune cells (124).

Cyclophosphamide is an alkylating cytostatic drug that causes cross-links by adding alkyl groups to the DNA strand. This inhibits the proliferation of the malignant cell but the drug also exerts both stimulatory and inhibitory effects on the cells of the immune system (125).

Doxorubicin is an anthracycline that exerts its effects by inhibiting the progression of topoisomerases and subsequently causes DNA damage and apoptosis (126).

Vincristine belongs to a group of drugs named Vinca alcaloids, derived from plants that interfere with microtubule formation necessary for the separation of the chromosomes during mitosis (127).

The glucocorticoid Prednisone induces death of lymphoid cells mainly by apoptosis (128).

With the combination of R-CHOP, 60% to 70% of the patients are cured (14). Two-years follow up is standard in treatment protocols as patients at this time point have the same OS as the general population and thus can be considered cured (129).

However, despite a high cure rate about 30 - 40% of the patients do not respond well to primary treatment (130). For relapses there are several standard high-dose salvage regimens available together with supportive autologous stem cell transplantation (ASCT) and including cytostatic agents

such as etoposide, cytarabine, cisplatin and gemcitabine (131). However, for patients with refractory disease, as defined as progressive or stable disease during immunochemotherapy or relapse within a year after ASCT, the prognosis is dismal with a 2-year OS of about 20% (132).

1.7.2 Resistance to cytostatic agents

Several mechanisms may mediate resistance of neoplastic cells against cytostatic agents. One well known such mechanism against the CHOP-regimen elements doxorubicin and vincristine is the use of ATP-dependent pumps. The first of these to be described was P-glycoprotein, which pumps the drug out from the cell (133). This mechanism belongs to a group of resistance mechanisms known as *multidrug resistance* since cross-resistance can develop towards other drugs with different structure and mechanisms of action (134). Another important resistance mechanism in DLBCL is defective apoptosis that is also a multidrug resistance mechanism. This may be acquired by up-regulation of the anti-apoptotic protein BCL2 and is sometimes associated with rituximab treatment. Down-regulation of TP53 has been associated with resistance against several agents used for treatment of DLBCL such as doxorubicin and the salvage therapy agents cisplatin and cytarabine (134, 135). Resistance mechanisms can either be inherent in the neoplastic cells, so called *intrinsic* resistance or be due to mutational events occurring during treatment, so called *treatment aquired* resistance. Resistance mechanisms can also stem from the microenvironment, e.g. by increased adhesion of lymphoma cells to stromal cells, which can protect the neoplastic cells from cytotoxic effects of treatment (135, 136).

1.7.3 Novel therapies

Inhibitors of NF-κB: lenalidomide, ibrutinib and bortezomib

The concept of cell-of-origin and the more thorough understanding of the different molecular mechanisms of the GCB and ABC subgroups, has shed light on several potential candidate proteins for direct inhibition and a more tailored approach in the treatment of DLBCL. A number of specific inhibitors have been investigated, especially for ABC DLBCL, that target proteins active in B-cell signalling and the activation of NF-κB. The drugs lenalidomide, ibrutinib and bortezomib all inhibit NF-κB pathway and some promising results have been seen in combination with R-CHOP in phase II-studies for lenalidomide (137, 138). A phase III study however, did not show improvement in PFS for the combination of R-CHOP and lenalidomide compared to R-CHOP as primary treatment for ABC DLBCL (139). Additionally, no benefit has been proved in phase III trials for the BTK-

inhibitor ibrutinib in combination with R-CHOP (140, 141). The proteasome inhibitor bortezomib has been shown to inhibit NF-κB activity in ABC but has not shown any benefits compared to R-CHOP alone in phase II studies (137) nor in a larger phase III study (140).

So, even though initially promising effects with these agents in combination with R-CHOP, R-CHOP still remains the standard drug combination.

CAR-T, BiTE, new antibodies and immunoconjugates

Chimeric antigen receptor T-cells (CAR-T) are autologous T-cells, collected with apheresis and engineered to express a synthetic chimeric T-cell receptor directed against a specific antigen, such as the B-cell antigen CD19. The binding of these CD19-specific T-cells leads to death of CD19-positive lymphoma cells. The therapy has been studied as an alternative for treatment of refractory DLBCL with promising results indicating long-term progression-free survival for about 40% of the patients (142-144). This therapy alternative is approved by the U.S. Food and Drug Administration and the European Medicines Agency and is included in the latest up-date of the Swedish national care program for aggressive B-cell lymphomas 2021 as treatment for refractory or relapsing DLBCL after two lines of treatment.

Bispecific T-cell engager (BiTE®) is another means of T-cell mediated targeted antibody therapy in which free antibodies are constructed that react both to CD3ε of the T-cell receptor and a tumour specific antigen, such as CD19 in the case of B-cell malignancies, linking lymphoma cells to cytotoxic T-cells for their destruction (145). Blinatumomab showed an overall response rate (ORR) of around 40% in phase II studies as second or third line therapy for refractive DLBCL (146, 147). Newer BiTE® antibodies, e.g. epcoritamab, glofitamab and mosunetuzumab appear to be more effective with ORR of 50-70% and with a high percentage of complete remissions (148-150).

Polatuzumab-vedotin and loncastuximab are two drugs aimed at CD79b and CD19 respectively and that in addition contain an antimitotic or cytotoxic part for the specific killing of B-cells (151, 152). A phase II study on polatuzumab-vedotin in combination with R-bendamustine showed a substantial reduction in risk of death (58%) compared to R-bendamustine alone in refractory or relapsed patients (151). In addition, a press release from Roche in August 2021, announced that the primary endpoint of PFS was met in the ongoing phase III Polarix trial with polatuzumab-vedotin combined with R-CHP opposed to R-CHOP as primary treatment for DLBCL patients. Promising results in refractory/relapsed patients have also been shown for

loncastuximab as a single agent (152). Tafasitamab instead mediates direct cytotoxicity via CD19-binding and results for refractory/relapsed DLBCL patients are promising also for this drug when combined with lenalidomide (153).

2 AIM

The overall aim of this thesis was to identify new biomarkers with relation to patients' outcome and chemoresistance in a large retrospective patient cohort of DLBCL.

2.1 SPECIFIC AIMS

Paper I

To elucidate the different global protein expression patterns in DLBCL patients with early relapse or primary refractory disease vs. cured patients with quantitative mass spectrometry, in order to find prognostic or predictive biomarkers for the early identification of patients with especially dismal prognosis.

Paper II

To, with immunohistochemistry, search for the prognostic potential of a number of proteins encoded by frequently mutated genes with previously demonstrated prognostic potential in DLBCL.

Paper III

To, with quantitative mass spectrometry, investigate the global protein profiles in the two cell-of-origin groups GCB and non-GCB/ABC.

Paper IV

To, with immunohistochemistry, investigate the prognostic impact of proteins belonging to the interferon-inducible PYHIN protein family.

3 PATIENTS AND METHODS

3.1 PATIENT SAMPLES

Paper I

From the western Sweden lymphoma register (Västsvenska lymfomregistret), all patients in Västra Götalandsregionen (VGR) and Halland who were diagnosed with DLBCL from 2004 to 2014 and had received R-CHOP with a curative intent were identified. A total of 270 patients fulfilled the criteria a) primarily progressive disease / relapse within 1 year from diagnosis (REF/REL) or b) cured with a minimum follow up of 5 years (CURED). Although most patients are considered cured after 2 years follow-up (129), a time limit of 5 years was chosen to minimise the number of later relapses. Specific subgroups of DLBCL, such as that of the central nervous system and primary mediastinal large B-cell lymphoma, were excluded.

After re-evaluation of paraffin slides 91 cases remained (38 REF/REL and 53 CURED) from which sufficient archived FFPE material was available with large enough areas of lymphoma cells. Six patients from Uppsala, belonging to the REF/REL group were also included summing up to 97 patients in total (44 REF/REL and 53 CURED) for proteomic analyses and inclusion in tissue micro array (TMA)-blocks.

Paper II, III and IV

An additional 552 DLBCL patients treated with R-CHOP with curative intent diagnosed in VGR between 2006 and 2016 were evaluated for these studies, some of whom did not fulfil the criteria of REF/REL or CURED at the time for paper I and the rest not belonging to either of these two groups. After microscopic and clinical re-evaluation, FFPE tumour material from 133 patients remained for proteomic analyses for paper III, giving a total number of 202 patients for this paper including 69 patients from paper I with sufficient FFPE material available. For inclusion in TMA-blocks, material remained from 134 patients rendering a total number of 208 patients for paper II and 211 patients for paper IV, depending on variable loss of core biopsy material from TMA sections.

3.2 TMT-BASED QUANTITATIVE MASS SPECTROMETRY

Mass spectrometry (MS)-based proteomic analysis can be applied to cells, fresh tissue as well as to FFPE tissue. Proteins are extracted from the cells or tissue and enzymatically digested, usually by trypsin into shorter peptides to create molecules of an ideal size to fit the mass range of the mass spectrometer (154). The peptides can be identified by combining their mass over charge ratio (*m/z*) and the amino acid sequence information found in the peptide fragmentation mass-spectra.

The basic components of the mass spectrometer are the ion source, the mass analyser and the detector unit. In the Electro-spray ion source the liquid sample is exposed to high voltage, which produces an aerosol of electrically charged droplets that subsequently are transferred into gas phase for the following analysis steps (155).

In the mass analyser the electrically charged peptides are separated depending on their *m/z*. In the Orbitrap mass analyser, the ionised peptides are trapped in an electric field and oscillate between inner and outer electrodes. The oscillating signals from the ions are transformed into a mass spectrum, as the oscillation frequency is dependent on the *m/z*. In the first mass spectrum (MS^1), the *m/z* of all ions are measured. The intensities of the peptides in MS^1 spectra only reflect how well they have been ionised and not the actual abundance or concentration of the different peptides in the sample (156).

Through a subsequent step of peptide fragmentation the amino acid sequence of each peptide can be identified by the comparison of the experimental fragment spectrum for each peptide to theoretical spectra in a protein database. This step is called MS/MS or MS^2 (Figure 5b) and mediates identification of the peptides and their corresponding proteins in the sample (156, 157).

The MS-analysis is data dependent, in that the mass analyser will select the peptides with highest intensities in a scan for fragmentation. This means that for a sample with high complexity (containing a large number of proteins) there is a risk that peptides with weaker signals will not be identified. To reduce the sample complexity and improve the number of identified proteins, one or more separation steps are normally included before final nano liquid chromatography (nLC) MS analysis (158). Separation with nLC is based on hydrophobicity. The peptides interact with the hydrophobic C18-material in

the stationary phase and are subsequently eluted with a gradient of increasing concentration of an organic solvent such as acetonitrile (159, 160).

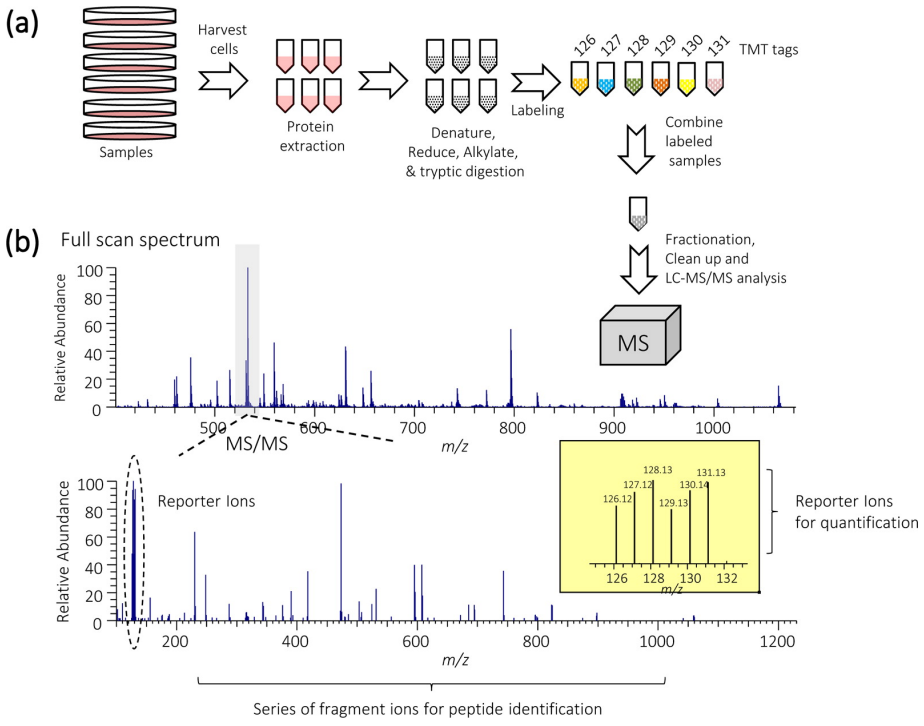

Figure 5. Workflow for quantitative mass spectrometry (QMS). a) Samples are collected followed by protein extraction, trypsin digestion and labelling with isobarically labelled TMT tags for identification and relative quantification. b) MS^2 fragmentation spectra for identification of peptides and (yellow box) relative quantification based on relative abundance of reporter tags for each peptide. (Reproduced with permission from Rauniyar, et al; Isobaric labeling-based relative quantification in shotgun proteomics; J Proteome Res; 2014;13(12):5293-309. Copyright American Chemical Society, AuthorChoice licence).

MS is not in itself a quantitative method but by unique labelling of the peptides it is possible to measure the relative abundance of the same peptide in different samples and relatively quantify the corresponding protein.

In Tandem-Mass-Tag (TMT) based quantitative mass spectrometry (QMS) the peptides in each sample are labelled with a unique isobaric tag, after which the samples are combined into one sample (Figure 5a). The tag consists of a reporter part (mass tag) with a unique mass used in the quantification step and a normaliser part that ensures that the masses of the

tags are identical and have the same chemical/physical properties in the identification step. Identical peptides, derived from different samples, co-elute in the nLC-separation and are indistinguishable in MS^1. Upon fragmentation in the mass spectrometer, a unique reporter ion is produced for each peptide variant, and is used for relative quantification of the peptide and its protein (Figure 5b) (161, 162).

Tissue microdissection with laser catapulting

Tissue microdissection with laser catapulting (163, 164) was used for paper I to purify the samples to predominantly contain neoplastic cells and to secure an equal amount of total protein content from each FFPE tumour sample used for TMT-based QMS. The method is performed in an inverted microscope with the objectives placed under a haematoxylin-eosin-stained tissue section, which is mounted on a membrane-covered slide. Areas of interest are delineated and subsequently cut by laser from the tissue section and catapulted into a tube placed above the slide. Microdissection was performed for paper I on 10-µm thick tissue sections with a PALM MicroBeam laser microscope (Zeiss) and the PALM RoboSoftware 4.6. Two million μm^2 of tumour tissue were dissected from all cases and from case 1-9 a additional 2 million μm^2 each were microdissected, pooled and used as a control in all sets in the QMS experiments.

3.2.1 TMT-based QMS in paper I

TMT-based QMS was performed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Nine TMT-labelled micro-dissected FFPE samples were analysed in each set together with one TMT-labelled control, which consisted of a mixture of micro-dissected tissue from five CURED and four REF/REL patients. The analysis included one separation step. A total of twelve sets were analysed. For each sample in a set, the relative abundance of each protein was calculated in relation to the control sample of that set. Since theoretically the protein abundance in the control sample should be the same across all analysed sets, this enabled all samples from all sets to be compared at a later stage. For protein identification and relative quantification, which was performed with Proteome Discoverer version 1.4 (Thermo Fisher Scientific), the data-files from all sets were merged. The search was performed in the Human Swissprot Database (version August 2016) using Mascot 2.3 (Matrix Science) as a search engine.

3.2.2 TMT-based QMS in paper III

For paper III, instead of micro-dissection, core biopsies were cut from the FFPE blocks with the aim to include both neoplastic cells and cells from the tumour microenvironment, as well as to obtain an equal tumour volume from each sample. For the use as control in all sets additional biopsies were cut from 25 of the samples (non-GCB and GCB), pooled and the results from this pooled control was used for calculating ratios as described above (3.2.1). Two separation steps were included and a total of 23 11-plex TMT-sets were analysed. The proteomic analyses were performed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) with ten TMT-labelled FFPE samples together with one TMT-labelled control. Proteome Discoverer version 2.2 (Thermo Fisher Scientific) was used for protein identification on the merged data-files. The data processing included a normalisation step to compensate for unequal amounts of tumour tissue in different samples. The protein search was performed in the Human Swissprot Database (version September 2018) with Mascot 2.5.1 (Matrix Science) as search engine.

3.2.3 Protein network and interaction analyses

For papers I and III, protein network analyses were performed with version 10.0 or 11.0 of the web-based tool STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, https://string-db.org). Protein accession numbers as per the database UniProt (https://www.uniprot.org) were used in STRING to identify protein networks. The web-based tool KEGG (Kyoto Encyclopaedia of Genes and Genomes, https://www.genome.jp/kegg) was used to identify enriched pathways.

3.3 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is used in routine diagnostic pathology for the study of specific cellular antigens. The method can be used for various purposes such as defining the lineage of a morphologically undifferentiated tumour, for diagnostic sub-classification of e.g. lymphomas, to elucidate the origin of metastatic lesions and to evaluate prognostic biomarkers such as the proliferative index. In brief, pre-processed FFPE slides are incubated with primary antibodies specific to membranous, cytoplasmic and/or nuclear antigens. Visualization and signal amplification are obtained by secondary antibodies linked to an enzyme that catalyses the generation of a chromogenic reaction product such as diaminobenzidine (DAB). Haematoxylin is used as a counterstain for the visualisation of the cells. The

method allows for direct visualisation of where the protein of interest is expressed, i.e. both the type of cell and the subcellular localization (165).

TMA blocks

For the construction of a Tissue microarray block (TMA), cores are punched from several FFPE blocks and embedded together in a new paraffin block. This enables an analysis such as IHC to be performed on many samples on one slide, which reduces technical variation and costs (166).

For paper I, three TMAs were constructed, two with 0.6 mm diameter cores in duplicate from each patient and one, containing the material from Uppsala, with 1 mm diameter cores in duplicate for each patient. For paper II four new TMAs were constructed containing 1 mm cores in duplicate from each case. All TMA blocks, except for the Uppsala cases, were also used for the IHC stainings for paper III and IV.

Patients with different clinical outcome and with different COO categories were mixed in all TMA blocks.

3.3.1 IHC stainings

IHC stainings for BCL2, MUM1, BCL6, CD10 and Ki67 from routine diagnostic workup were evaluated on archived slides for all patients in all papers except for a minority of cases that were evaluated on TMAs. Four µm sections from TMA blocks were stained with ready-to-use antibodies from Dako, Agilent, Santa Clare, CA, United States (BCL2, clone 124; MUM1, clone MUMp; BCL6, clone PG-B6p; CD10, clone 56C6; Ki67, clone MIB-1). MYC antibody (clone Y69) was purchased from Abcam, Cambridge, UK and evaluated on TMA sections for all cases in all papers.

Ready-to-use IHC antibodies for CD3 and CD68 from Dako, Agilent, Santa Clare, CA, United States were evaluated on 4 µm sections from TMA blocks for paper III and CD3 also for paper IV.

Specific antibodies stained on 4 µm sections from TMA blocks for the different papers were:

Paper I

RPS5, RPL17, Enah/Vasp-like antibody and MARCKS like protein antibody, all purchased from Abcam, Cambridge, UK.

Paper II

CREBBP (anti-KAT3A/CBP), IRF8, EZH2, TBLR1, purchased from Abcam, Cambridge, UK and NCOR1 from ThermoFisher Scientific, Rockford, IL, USA.

Paper III

GBP1, IRF8, MLKL, MNDA, SOD1, SWAP70, and WEE1 from Abcam, Cambridge, UK.

Paper IV

IFI16 and MNDA from Abcam, Cambridge, UK and AIM2 from Sigma-Aldrich, St. Louis, MO, USA.

All IHC stainings for all papers were performed in an AutostainerLink using routine protocols. Heat-induced epitope retrieval was performed with EnVision™ FLEX Target Retreival Solution (50x) in 95°C (Dako, Agilent, K8000, Santa Clare, CA, United States). Controls were used according to the manufacturers instructions.

3.3.2 Evaluation of IHC stainings

All papers

The Hans algoritm (31) was used for the determination of COO based on CD10, BCL6 and MUM1 in all papers.

IHC cut-off values used for BCL2 and MYC positivity were ≥ 50% and ≥ 40% respectively (3, 71). Cases positive for both BCL2 and MYC were designated double-expressers (DE).

Paper I

Ki67% was evaluated in “hot spot” areas and the fraction of positive nuclei was evaluated in approximately 1000 cells at 40x magnification using a grid.

IHC stainings for RPS5, RPL17, Enah/VASP-like protein and MARCKS-like protein were evaluated on tissue slides. The evaluation was performed by two pathologists, independently and blinded to the clinical outcome. Based on the global cytoplasmic staining intensity, cases were divided into two categories: 1) strong cytoplasmic staining intensity in the majority of the cells and 2) no

or weak or intermediate cytoplasmic staining intensity in the majority of the cells. As the global staining intensity may vary between different slides, the limits for the intensity categories were decided by comparing all cases in a specific TMA slide. With this visual approach, concordance of categorization was > 80%. Discordant cases were re-examined in a double head microscope to reach consensus.

Paper II - IV

For papers II – IV digital image analysis (DIA) was used for the evaluation of IHC stainings, conducted with the software Visiopharm 2019.02 (Hoersholm, Danmark) on TMA slides scanned in 40x magnification with a NanoZoomer S210 (Hamamatsu Photonics, Hamamatsu City, Japan).

For each IHC antibody an individual Analysis Protocol Package (APP) was created for the recognition of nuclei. The same APP could, with some adjustments of the settings, be applied also for the interpretation of cytoplasmic IHC staining patterns. The program evaluates the staining intensity in a defined object area that can be a whole cell or nucleus or part of a cell or nucleus. Based on the intensity thresholds set in the APP, the object is designated a certain staining intensity category. The proportions of each category in an analysed region (region of interest, ROI) of a sample were obtained by dividing the total area of that category within the ROI by the total area of all objects (positive or negative) within the ROI. IHC staining intensity was evaluated in three or four categories depending on the global staining pattern for each antibody: negative/weak/strong or negative/ weak/intermediate/strong.

GBP1-expression was mainly observed in macrophages and seldom in the lymphoma cells. These two cell types differ significantly in size and the proportion of each staining intensity category for this antibody was instead calculated as the total area of positive cells in each category divided by the *total area of the analysed ROI.*

IHC staining for AIM2 in paper IV was evaluated manually on scanned TMA slides. This was due to a granular cytoplasmic staining pattern found in several cases, which impaired a reliable digital evaluation. Two pathologists first evaluated the stainings independently in Visiopharm and categorised cases into <50% or ≥ 50% positive cells and of weak or strong staining intensity for the cases with ≥ 50% positive cells. Cases for which the evaluation disagreed were later re-evaluated to reach consensus.

3.4 COO WITH LYMPH2CX

For paper III, COO was determined with both immunohistochemistry and the NanoString based Lymph2Cx assay for 87 of the patients for which ≥ 60% of the area of the sections consisted of neoplastic cells (35).

3.5 STATISTICAL METHODS

For all papers Mann-Whitney U-test and Pearson's chi-squared test or Fisher's exact test were used to compare background variables or the expression patterns of immunohistochemical biomarkers between two independent groups.

Paper I

For comparison of log-transformed peptide expression average between the two groups REF/REL and CURED, a two-tailed Welch's t-test was used with and without Benjamini-Hochberg (B-H) procedure for correction of multiple comparisons. Pearson's chi-squared test was used to analyse the proportions of expressed peptides.

Paper II

For prognostic analyses progression-free (PFS) and overall survival (OS) were used. PFS was defined as time from diagnosis until the date of progression, relapse, death or last follow-up and the definition of OS was time from diagnosis to the date of death or last follow-up. Univariable and multivariable analyses were performed with the Cox proportional hazard regression model. The Kaplan-Meier method and log-rank test were further used to calculate PFS and OS.

Paper III

A two-way ANOVA was used for the comparison of log-transformed values of protein abundance between COO groups with TMT set number as covariate. Also, proteins with missing values in some sets were compared and the analyses were performed with and without adjustment for multiple comparisons.

Paper IV

PFS and OS were used for prognostic analyses as defined for paper II. Univariable and multivariable analyses were performed with the Cox proportional hazard regression model. The Kaplan-Meier method and log-rank test were used to calculate PFS and OS.

Statistical analyses were performed with SPSS, versions 22 - 26 (IBM Corp., Armonk, NY) or R version 3.3.2 or 3.6.1 (R Foundation, Vienna, Austria) or Stata for Macintosh, version 13.1 (StataCorp, College Station TX). Normalisation of data for paper III was performed with NormalyzerDE version 1.2.0 using Loess from the LIMMA package.

4 RESULTS

4.1 PAPER I

4.1.1 Background data

The REF/REL group had a higher median age (71 vs. 64 years), a higher proportion of high-risk aaIPI (59 vs. 36%) as well as higher proportion of MYC-positive (41 vs. 13%) and of MYC/BCL2 DE lymphomas (40 vs. 12%).

4.1.2 Proteomic analyses

2127 proteins were identified in total and 442 proteins were present in all samples, of which 102 ($p<0.05$, t-test) and 20 ($p<0.05$, B-H adjusted t-test) were differentially expressed between the groups REF/REL and CURED. REF/REL had a higher expression of ribosomal proteins (RPs) (46 of 65 up regulated, $p=7.6 \times 10^{-10}$) while CURED had a higher expression of proteins that are associated with the actin cytoskeleton (20 of 37 up regulated, $p=1.4 \times 10^{-9}$) (Figure 6).

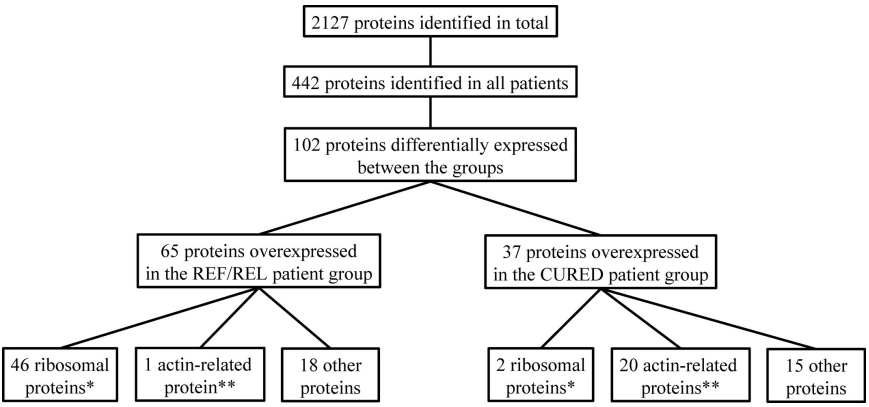

Figure 6. Flow chart of protein groups differentially expressed between REF/REL patients with refractory disease or relapse within one year from diagnosis and CURED patients with at least five years progression free survival. (Reproduced with permission from Bram Ednersson, et al; Expression of ribosomal and actin network proteins and immunochemotherapy resistance in diffuse large B cell lymphoma patients; Br J Haematol; 2018;181(6):770-81. Copyright John Wiley and Sons, licence no 5139231091075).

Among the 20 proteins that remained significant after B-H adjustment 15 were up-regulated in REF/REL of which twelve were ribosomal proteins and none were actin-related. Of the 5 proteins up-regulated in CURED two were actin-related and none ribosomal.

STRING database network analysis (enrichment p-value = $< 10^{-16}$) resulted in two prominent clusters: ribosomal proteins and proteins involved in the regulation of the actin cytoskeleton. In the KEGG pathway analysis as well, RP interactions (false discovery rate (FDR) 9.0×10^{-70}) and regulation of the actin cytoskeleton (FDR 0.0079) were the most significant pathways.

For REF/REL, subgroup analyses were performed to investigate the correlation of the expression pattern of RPs related to the expression of MYC, BCL2 and MYC/BCL2 (i.e. DE). In the REF/REL MYC+ subgroup 13 of 46 RPs were up-regulated. For MYC-, BCL2- and DE-negative patients no RPs were up-regulated compared to the corresponding positive groups.

4.1.3 Immunohistochemistry

Two ribosomal (RPL17 and RPS5) and two actin-related proteins (Enah/Vasp-like protein and MARCKS-like protein), which were among the most differentially expressed in the proteomic analyses, were chosen for IHC evaluation. All except MARCKS-like protein had B-H-adjusted p-values <0.05 in the proteomic analyses. MARCKS-like protein was however included since it had the lowest fold change (REF/REL vs. CURED) of the 102 differentially expressed proteins with p-value 0.0096, but with a B-H-adjusted p-value of 0.21. All IHC antibodies showed a cytoplasmic staining pattern in the lymphoma cells. The proportions of cases with strong global IHC staining for the four proteins are shown in Table 1.

Table 1. Percentage of cases with strong global IHC positivity for two ribosomal and two actin related proteins in REF/REL vs. CURED.

	Strong global cytoplasmic IHC positivity			
IHC antibody	% REF/REL, n=44	% CURED, n=53	P-value	Fold change REF/REL vs. CURED
RPS5	39	12	0.003	1.21
RPL17	37	12	0.004	1.23
Enah/Vasp	20	16	0.70	0.73
MARCKS	5	20	0.03	0.68

4.2 PAPER II

4.2.1 Background data

The numbers of male and female patients and patients belonging to each COO group were equal. The median age was 67 years. BCL2 was expressed in 74.0% of the tumours while MYC was expressed in 16.8% and the proportion of DE was 12.0%.

4.2.2 Immunohistochemistry

DIA results presented as median percentage of all nuclei and comparison between GCB and non-GCB for the nuclear IHC stainings of CREBBP and TBLR1 are shown in Table 2. Cases for which one of the TMA duplicate cores was missing were omitted for that specific IHC antibody, as were also cases for which one of the cores stained with strong intensity and the duplicate core was negative. For both CREBBP and TBLR1 there was a clear difference between the IHC staining pattern for GCB and non-GCB patients.

Table 2. Nuclear IHC staining pattern as median percentage and range for all patients and per cell-of-origin.

IHC antibody, categories of nuclear staining pattern	Median % nuclei (range)	Median % nuclei (range)	Median % nuclei (range)	P-value
	All patients	GCB	Non-GCB	
CREBBP	(n=195)	(n=95)	(n=100)	
Positive weak	32.8 (0 – 94.8)	36.3 (0.5 – 72.9)	29.2 (0 – 94.8)	0.19
Positive intermediate	23.5 (0 – 99.2)	9.9 (0 – 89.4)	34.8 (0 – 99.2)	<0.001
Positive strong	0.0 (0 – 35.9)	0 (0 – 1.6)	0 (0 – 35.9)	0.006
Positive total	69.0 (0.5 – 99.2)	60.8 (0.5 – 97.1)	75.6 (2.4 – 99.2)	<0.001
TBLR1	(n=192)	(n=93)	(n=99)	
Positive weak	19.1 (0.5 – 89.1)	20.7 (1.4 – 89.1)	14.5 (0.5 – 83.7)	0.015
Positive intermediate	45.5 (1.9 – 89.9)	49.2 (4.3 – 89.2)	41.6 (1.9 – 72.6)	0.001
Positive strong	28.3 (0 – 97.6)	23.3 (0 – 94.0)	36.3 (0 – 97.6)	0.005
Positive total	99.7 (77.4 – 100)	99.5 (77.4 – 100)	99.8 (82.7 – 100)	0.022

4.2.3 Prognostic analyses

Background variables

For the whole cohort follow-up time was 1-166 months. PFS was 75% at 3 years and 70% at 5 years. OS was 81% at 3 years and 76% at 5 years. Patients with high aaIPI and with DE had both lower PFS (p=0.003, p=0.010) and OS (p=0.001, p=0.001). For COO, no difference was seen in PFS or OS between non-GCB and GCB.

IHC stainings, univariable analyses

Of the four proteins analysed with IHC and DIA, TBLR1 and CREBBP were significantly associated with survival.

For TBLR1, since nearly all nuclei in all cases were positive, prognostic analyses were performed on percentage of nuclei with *strong* staining intensity since this parameter had a larger variation between cases (Table 2). For CREBBP, the percentage of nuclei with strong staining intensity was very small in most of the cases and therefore prognostic analysis was performed for *strong* and *intermediate* staining intensity analysed together as one variable (i/s). For CREBBP, prognostic analysis was in addition performed for the percentage of all positive nuclei (Table 2).

In univariable Cox regression analyses for continuous variables, TBLR1 strong and CREBBP i/s were associated with both shorter PFS and OS and CREBBP positive was associated with shorter PFS (Table 3).

Table 3. Association with PFS and OS for the percentage of nuclei with TBLR1 strong intensity, CREBBP strong/intermediate (i/s) intensity and CREBBP positive nuclei (Univariable Cox analysis, continuous variables).

	Progression free survival		Overall survival	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value
TBLR1 strong	3.19 (1.35 – 7.52)	0.008	2.94 (1.14 – 7.56)	0.025
CREBBP i/s	3.53 (1.49 – 8.35)	0.004	3.52 (1.36 – 9.14)	0.010
CREBBP positive	3.86 (1.06 – 14.0)	0.04	3.51 (0.85 – 14.4)	0.081

HR=Hazard Ratio. CI=Confidence Interval.

TBLR1 strong and CREBBP i/s were then categorised with cut-off on the median values (28.3% for TBLR1 strong, 23.0% for CREBBP i/s). Significantly worse PFS and OS were seen for TBLR1 strong above median

while a percentage of CREBBP i/s nuclei above the median was associated with shorter PFS but not with OS (Figure 7).

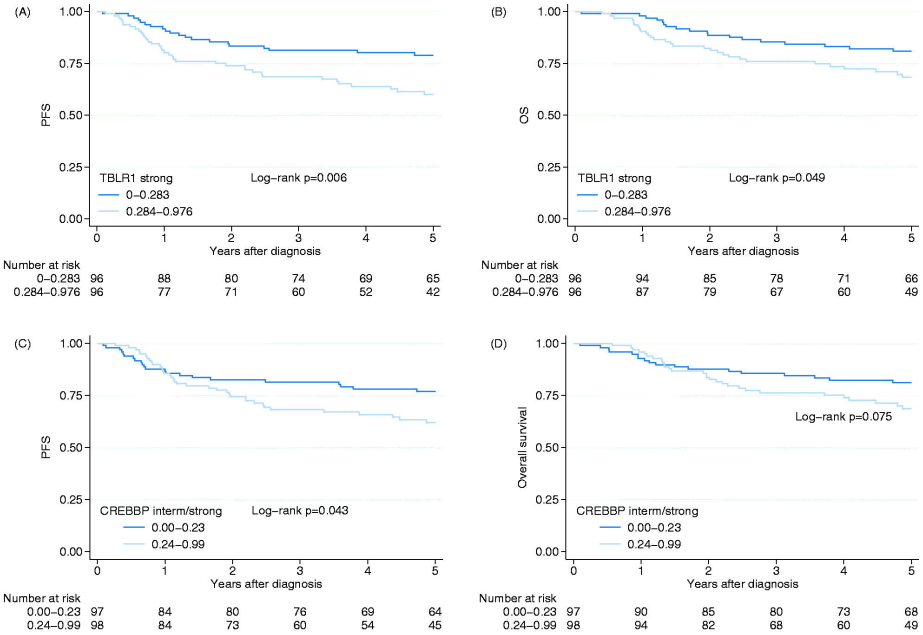

Figure 7. Kaplan-Meier and log-rank tests for progression free survival (PFS) and overall survival (OS) for A, B) TBLR1 strong with cut-off on median and C, D) CREBBP with cut-off on median. (Reproduced with permission from Ednersson SB, et al; TBLR1 and CREBBP as potential novel prognostic immunohistochemical biomarkers in diffuse large B-cell lymphoma; Leuk Lymphoma. 2020:1-10. Copyright, The Author(s)).

Prognostic analyses related to COO

High percentage of CREBBP i/s nuclei as a continuous variable was significantly associated with shorter PFS and OS (p=0.004, p=0.001) in non-GCB patients but not in GCB in univariable Cox analysis. Among non-GCB patients with CREBBP i/s above median, 25 events were observed of which 19 were relapses, four progressive disease (PD) and two deaths. Among patients with CREBBP i/s below median only seven events were observed (three relapses and four PD).

Multivariable analyses

A percentage of TBLR1 strong nuclei above median remained significant for worse PFS in multivariable Cox regression analysis (p=0.017) when analysed together with aaIPI high, DE and COO. Only DE remained significant for OS in the multivariable analysis. CREBBP i/s was not significant in multivariable analysis (Table 4).

Table 4. Multivariate Cox analyses for TBLR1 strong and CREBBP intermediate/strong with cut-off on median values. (Reproduced with permission and adapted from Ednersson SB, et al; TBLR1 and CREBBP as potential novel prognostic immunohistochemical biomarkers in diffuse large B-cell lymphoma; Leuk Lymphoma; 2020:1-10. Copyright, The Author(s)). HR=Hazard Ratio. CI=Confidence Interval.

	Progression-free survival		Overall survival	
Variables	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
TBLR1 strong: >median vs. <median	1.96 (1.13 – 3.40)	.017	1.65 (0.91 – 3.01)	.10
aaIPI: 2–3 vs. 0–1	2.01 (1.17 – 3.43)	.011	1.79 (0.99 – 3.24)	.053
COO: non-GCB vs. GCB	1.06 (0.62 – 1.82)	.82	1.00 (0.55 – 1.82)	.99
DE: yes vs. no	2.43 (1.30 – 4.53)	.005	2.52 (1.27 – 4.97)	.008
CREBBP intermediate/strong: >median vs. <median	1.54 (0.89 – 2.67)	.13		
aaIPI: 2–3 vs. 0–1	1.81 (1.06 – 3.10)	.029		
COO: non-GCB vs. GCB	0.93 (0.54 – 1.60)	.80		
DE: yes vs. no	2.71 (1.47 – 5.00)	.001		

aaIPI: age-adjusted international prognostic index; COO: cell-of origin; GCB: germinal centre B-cell like; DE: double-expresser.

4.3 PAPER III

4.3.1 Background data

The median age was 67 years and the numbers of men and women as well as patients in each COO group were equal. The proportion of DE was 12.9%, MYC-positive cases 15.3% and BCL2-positive cases 76.2%. COO classification by IHC and Lymph2cx were concordant in 70% of cases. The

majority of the discordant cases were classified as non-GCB by IHC and unclassifiable by Lymph2cx.

4.3.2 Proteomic analyses

Proteomic analyses identified 6430 proteins in total of which 498 were significantly differentially expressed between non-GCB and GCB as classified by IHC after adjustment for multiple comparisons ($p<0.05$, fold change ≥ 1.20). In non-GCB patients 224 of these proteins were up-regulated and in GCB patients 274 proteins were up-regulated.

Several B-cell antigens were identified (e.g. CD19, CD20 and CD22). Among the up-regulated proteins in non-GCB and GCB respectively, several were previously associated with the respective groups, including the proteins used in the Hans algorithm (IRF4/MUM1 in non-GCB and CD10 and BCL6 in GCB).

Further, in the non-GCB group, several proteins were up-regulated that previously have not been described in this subtype and that seem to be involved in interactions with the tumour microenvironment. Among these are the monocytic markers CD163 and CD64, the lymphocyte/monocyte marker CD85c, the T-cell marker CD44 and the interferon inducible proteins MLKL, MNDA, IFIT2, IFIT3 and GBP1. STRING network analysis of the proteins up-regulated in non-GCB further showed, both for all 224 proteins (enrichment p-value $< 1 \times 10^{-16}$) and the 40 topmost up-regulated proteins (enrichment p-value $< 1 \times 10^{-16}$) that the most enriched pathways were involved in interferon signalling and interactions with the immune system (Figure 8).

The differentially expressed proteins in ABC vs. GCB with COO analysed by Lymph2cx were to a great extent overlapping with the differentially expressed proteins in non-GCB vs. GCB with COO analysed by IHC. Sixteen of the 20 most up-regulated proteins in ABC were found among the most up-regulated proteins in non-GCB.

The proteomic results were also compared between the clinical groups REF/REL (n=29) and CURED (n=113) as for paper I. No significantly differentially expressed proteins were however found between these groups after adjustment for multiple comparisons. Among the 6430 proteins totally identified, about 160 ribosomal proteins were present which was about half the proportion of ribosomal proteins found for paper I. The number of actin-related proteins was 35 of 6430.

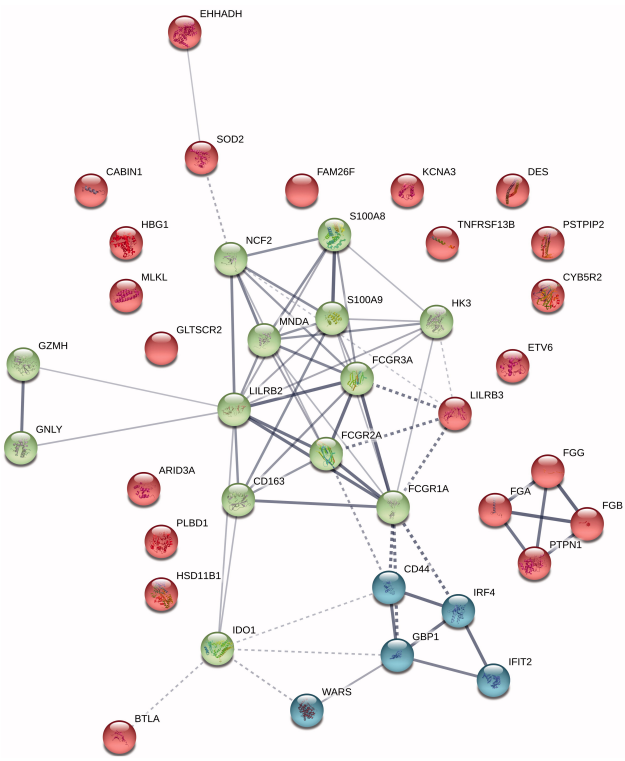

Figure 8. A STRING network analysis using k-means cluster analysis showing the 40 most overexpressed proteins in non-GCB. The dominant (light green) cluster shows proteins involved in immune response. (Reproduced with permission from Bram Ednersson S, et al.; Proteomic analysis in diffuse large B-cell lymphoma identifies dysregulated tumor microenvironment proteins in non-GCB/ABC subtype patients; Leuk Lymphoma; 2021:1-14. Copyright, The Author(s)).

4.3.3 Immunohistochemistry

For all proteins except for SOD1 that were evaluated by IHC, significantly different expression patterns were seen between non-GCB and GCB that were concordant with the proteomic analyses results: GBP1, MLKL and MNDA (all up-regulated in non-GCB in the proteomic analysis) had both a significantly larger proportion of positive cells and stronger staining intensity in non-GCB than GCB with IHC. The opposite was found for IRF8, SWAP70 and WEE1 (up-regulated in GCB in the proteomic analysis) with a significantly larger proportion of positive cells and/or significantly higher staining intensity in GCB than non-GCB with IHC. Parallel stainings with CD68 and CD3 showed that GBP1 mainly stained what was interpreted as macrophages and MNDA mainly stained what was interpreted as T-cells.

4.4 PAPER IV

4.4.1 Background data

The median age of the cohort was 66 years. The number of patients in each COO group was equal as well as the number of men and women. The percentage of DE tumours was 13.3%.

4.4.2 Immunohistochemistry

To summarise, IHC expression of IFI16 and AIM2 was mainly observed in lymphoma cells while MNDA expression, as reported also in paper III, was primarily observed in what was interpreted as T-lymphocytes.

Digital image analysis of IFI16 staining showed positive nuclear staining of the majority of the lymphoma cells in most cases (median 97.0% positive cells) and the intensity was mainly intermediate. For the nuclear antigen MNDA the results were the reverse, with most cases containing few positive cells (median 11.6% positive cells) for which the intensity mostly was weak. For AIM2, manual evaluation showed a positive but weak cytoplasmic staining as the predominant pattern (86% of the cases).

4.4.3 Prognostic analyses

Background variables

PFS for the whole cohort was 74% at 3 years and 69% at 5 years. OS for the whole cohort was 81% at 3 years and 75% at 5 years. Lower PFS and OS were observed for high aaIPI ($p=0.004$ and 0.023) and DE ($p<0.001$ and $p<0.001$). No prognostic impact was observed for COO.

IHC stainings, univariable analyses

A significant association with survival was seen for IFI16 and MNDA but not for AIM2. The main findings were a significant association with shorter PFS and OS for the variables IFI16 intermediate/strong (i/s) ≥ median (≥ 84.1%) (PFS: Hazard Ratio (HR) 2.2; 95% CI 1.3 – 3.7; $p=0.005$. OS: HR 2.1; 95% CI 1.2 – 3.8; $p=0.012$) and MNDA positive ≥ the 4th quartile (≥ 23.9%) (PFS: HR 2.4; 95% CI 1.1 – 5.5; $p=0.029$. OS: HR 4.2; 95% CI 1.6 – 11.5; $p=0.005$) in univariable Cox-analyses. IHC stainings for IFI16 i/s ≥ median and MNDA positive nuclei ≥ 4th quartile are exemplified in Figure 9 together with pseudo-coloured images from the digital analyses.

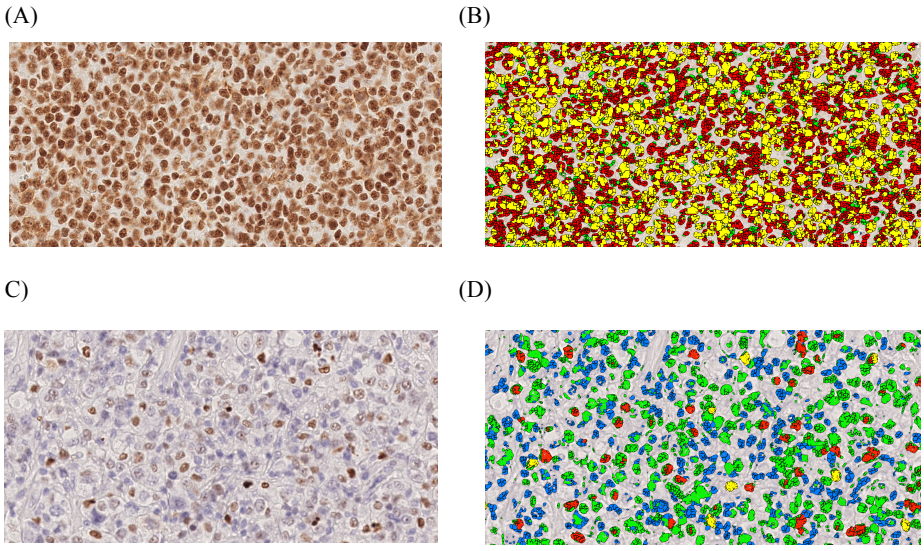

Figure 9. Immunohistochemistry (IHC) for (A, B) IFI16 and (C, D) MNDA with paired pseudo-coloured images from digital analysis, 40x magnification. Blue colour=negative, green=weak staining intensity, red=intermediate staining intensity, yellow=strong staining intensity. A, B: IHC for IFI16 with a percentage of nuclei with intermediate and strong staining intensity above the median (84.1%). B, C: IHC for MNDA with a percentage of positive nuclei above the 4th percentile (23.9%).

For IFI16, the association with survival for IFI16 i/s split at median is also illustrated by the Kaplan-Meier curves in Figure 10.

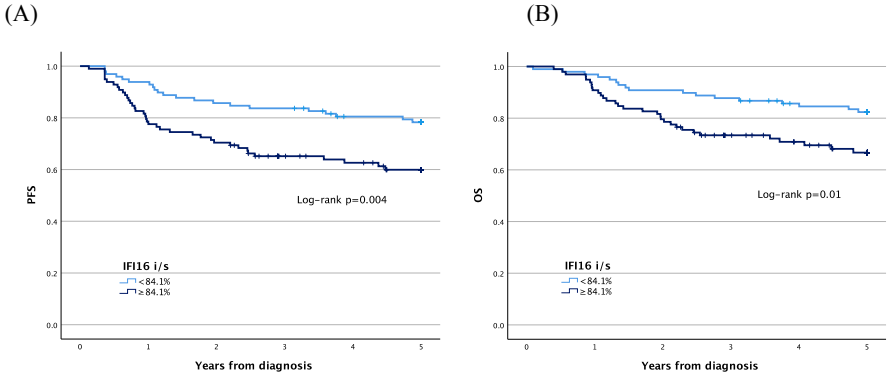

Figure 10. Kaplan-Meier curves and log-rank tests for A) PFS and B) OS for IFI16 intermediate/strong with cut-off on median.

Multivariable analyses

IFI16 i/s ≥ median was an independent factor for both shorter PFS (HR 2.0; 95% CI 1.2 – 3.4; p=0.010) and OS (HR 2.0; 95% CI 1.1 – 3.7; p=0.021) in multivariable Cox-analysis with aaIPI and DE. Also, multivariable Cox-analysis showed a significant association with MNDA positive ≥ the 4th quartile for shorter PFS (HR 2.2; 95% CI 1.0 – 5.0; p=0.049) and OS (HR 4.1; 95% CI 1.5 – 11.2; p=0.005) when analysed with aaIPI and DE. COO was not significant for either PFS or OS in any of the multivariable Cox analyses.

Although a positive correlation was seen between the expression of IFI16 and MNDA, with the majority of cases with MNDA positive ≥ the 4th quartile also showing IFI16 i/s ≥ median (p=0.008), these variables showed independent negative impact on PFS when analysed together in multivariable analysis (IFI16 i/s ≥ median: HR 1.9; 95% CI 1.0 – 3.3; p=0.039 and MNDA positive ≥ 4th quartile: HR 2.5; 95% CI 1.1 – 5.9; p=0.035) and MNDA also for OS (HR 3.8; 95% CI 1.4 – 10.6; p=0.010). Patients (n=30) with both IFI16 i/s ≥ median and MNDA positive ≥ the 4th quartile also had the worst outcome (Figure 11).

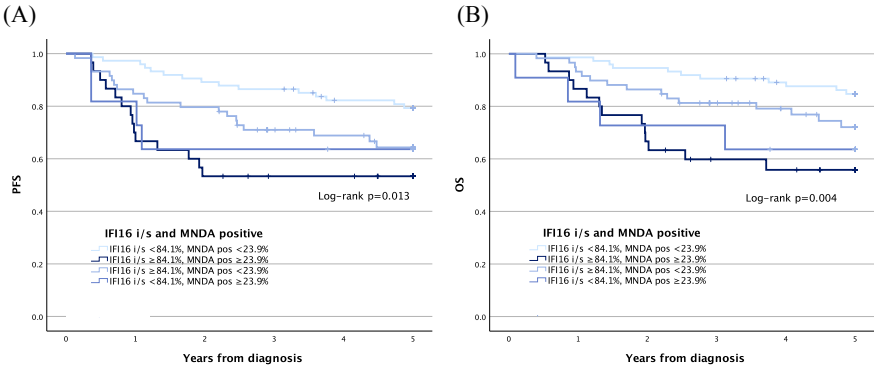

Figure 11. Kaplan-Meier curves and log-rank tests for A) PFS and B) OS illustrate that the shortest survival was seen for the group of patients (n=30) with both IFI16 intermediate/strong above median in combination with MNDA positive above 4th quartile.

5 DISCUSSION

In this thesis several possible new prognostic biomarkers have been identified for DLBCL, either as single proteins or functionally related groups of proteins. All studies are of an exploratory nature and a common feature is that they provide a rationale for further investigation of these putative biomarkers in relation to drug resistance and prognosis in DLBCL.

5.1 GLOBAL PROTEOMICS IN SEARCH FOR PROGNOSTIC BIOMARKERS

Mass spectrometry-based proteomics can detect a vast number of proteins in a sample and the challenge lies in the search for significant patterns in the large quantity of information produced by the analysis (154). Since QMS provides a relative quantitation, the proteome of two or more specific groups need to be compared (161). A general drawback of all proteomic methods as opposed to genetic studies is of course that they do not give information on potential mutations of the detected proteins and silencing mutations of importance for tumour behaviour will pass undetected since they are not translated. However, since proteomic methods measure the effector molecules and not their "blueprints", they could potentially give more information than genetic studies about actual functional cellular processes that are important for tumour aggressiveness and drug resistance.

The approaches in paper I and III respectively were different with respect to the material used for proteomic analyses. For paper I the material was purified by microdissection in order to include as few cells as possible from the microenvironment, while for paper III the microenvironment was deliberately included. Furthermore, the total amount of tissue used for the experiments was larger in paper III and both these factors could probably explain the higher number of proteins identified in that study.

In paper I we compared REF/REL and CURED patients resulting in two main groups of differentially expressed proteins, namely ribosomal proteins and actin-related proteins. In paper III, the cell-of-origin (COO) group non-GCB/ABC DLBCL was compared to the other COO group; GCB and the main finding was up-regulation in non-GCB of proteins related to interactions with the tumour microenvironment (TME).

5.1.1 Ribosomal proteins are potential prognostic biomarkers in cancer

Ribosomal proteins (RPs) form complexes with ribosomal RNA (rRNA) to constitute the ribosomes where protein translation takes place. Eighty different RPs exist, of which 33 are constituents of the small subunit of the ribosomes (RPS) and 47 of the large (RPL). Three RNA polymerase enzymes (RNA pol) are involved in the synthesis of RPs and rRNA: RNA pol I and III synthesize the four different types of rRNA and RNA pol II synthesizes the mRNA encoding the RPs (167).

Over the years there have been many indications that RPs not only function as parts of the protein translation machinery but also have important *extra-ribosomal* functions that are exerted outside the ribosomes. One important such function of RPs is the inactivation of the TP53-binding protein MDM2 leading to TP53 activation. This is triggered by nucleolar stress, a state that can be caused by e.g. UV radiation, hypoxia, or exposure to mutational agents that all lead to impaired ribosomal biogenesis and accumulation of ribosome-free RPs (168-170). In this context, the effect of RPs is tumour suppressive but it can in fact be both oncogenic and tumour suppressive leading to different prognostic impact in different types of cancer (171) and an association with resistance against e.g. doxorubicin or vincristine has been shown for some RPs in previous studies (172, 173).

In 2018, Derenzini *et al*. analysed several previously published whole exome sequencing data sets on DLBCL and showed that several RPs were mutated, which was mutually exclusive to mutations in TP53. Patients harbouring RP mutations had worse prognosis than patients without either TP53 or RP mutations but the functional effects of the mutations were not investigated (174).

The transcription factor MYC is involved in ribosomal biogenesis through stimulation of all three RNA-polymerases and thus stimulates RP synthesis (175). In the genetic classification system of DLBCL suggested by Wright *et al.* 2020, the molecular group EZB-MYC+ showing the double hit gene signature (DHITsig) and enriched for mutations, amplifications and rearrangements of MYC, indeed also showed high expression of RPs (103). This finding probably partly reflects the DHITsig in which five of the 48 overexpressed genes are RPs but no RPs are found among the underexpressed genes (59). One explanation for the enrichment of ribosomal proteins in tumours with up-regulated MYC could of course be that a highly proliferative tumour also up-regulates ribosomal biogenesis due to a need for

increased protein synthesis and that the relation to outcome is caused by MYC up-regulation or DH/TH. On the other hand, the number of RPs overexpressed in MYC+ lymphomas in our material in paper I was rather small (13 of 46 RPs overexpressed) and thus the overexpression of RPs in REF/REL did not seem to be solely dependent on MYC overexpression. GCB patients belonging to the EZB-MYC+ group did show significantly worse survival than EZB-MYC- (103) but since EZB-MYC+ DLBCL apparently harbour both increased MYC activity and an increased expression of ribosomal proteins, identifying the major factor causing inferior survival is challenging. Moreover, since only about half of the patients that were shown to be positive for the DHITsig also showed DH/TH or were double-expressers (59), additional factors other than MYC, DH/TH or DE could be responsible for the worse outcome and one such factor could perhaps be extra-ribosomal functions of the RPs.

In this light, the high proportion of ribosomal proteins among the twenty proteins that remained significantly higher expressed in REF/REL after B-H adjusted t-test in paper I, still could imply an important function for this group of proteins in relation to outcome and it could possibly be one explanation to the lack of response to routine treatment regimen for these patients.

5.1.2 Actin-related proteins can be associated to drug sensitivity in cancer

The actin cytoskeleton plays three important roles in normal as well as malignant cells: It preserves the cell's three-dimensional shape, mediates cell motility and during mitosis mediates the separation of the cell (176). In some previous reports, expression of various actin-related proteins has been detected in lower levels in drug resistant cells. For instance HSP27, which was found up-regulated in the CURED group in paper I, was underexpressed in cell-lines resistant to vincristine and doxorubicin (177, 178). Human germinal center associated lymphoma protein (HGAL) is of importance for the pathogenesis of DLBCL and its expression in DLBCL has been described as a favourable prognostic biomarker. A recent study showed that HGAL interacts indirectly or directly with several cytoskeletal proteins, among them RhoA and tubulin, to reduce B-cell motility in DLBCL which could reduce lymphoma dissemination (179). On the other hand, increased expression of cofilin-1, also up-regulated in CURED, was associated with increased activity of multidrug-resistance protein in prostate cancer cells (180) and in one study on DLBCL, fibrilin-1 mRNA was found together with non-GCB subtype and higher aaIPI and Ann Arbor stage (181). Another recent study

investigated the presence of mutations in genes coding for cytoplasmic actin in different malignancies and found that mutations in the gene ACTB coding for a type of cytoplasmic actin, found as significantly overexpressed after B-H adjustment in the CURED group, were strongly associated with DLBCL. Neither an evaluation of the protein expression pattern resulting from these mutations, nor a prognostic evaluation were performed in that study however, which was based on a public gene database (182). Nevertheless, there are implications that actin-related proteins could be of prognostic or pathogenic importance in DLBCL, although whether the effect is stimulatory or inhibitory for tumour progress seems to depend on the specific protein and context.

5.1.3 Ribosomal and actin-related proteins in REF/REL vs. CURED – differences between paper I and III

In the expanded patient material used for proteomic studies in paper III, no significant differences in protein expression were found between the clinical groups REF/REL and CURED. Reasons for these results can be several. The higher proportion of cells from the microenvironment in the samples for paper III increases their complexity, which could possibly lead to some proteins of low abundance not being detected in the analyses. Since several B-cell antigens were found in the analyses for paper III however, one can be certain that lymphoma cell proteins were present to a substantial extent. Nevertheless, there is still a risk for less abundant proteins to be overshadowed in a more complex sample. Another reason for the deviating results could be that the balance between the REF/REL and CURED groups for paper III was more uneven than for paper I, with only 29 patients belonging to REF/REL and 113 to CURED. Indeed, for paper II to IV, the expanded cohort had a favourable survival, as reflected by the 3- and 5-years' PFS and OS reported for the whole cohort in paper II and IV. The reason for this imbalance was a difficulty to obtain sufficient tumour material from several REF/REL patients since the diagnoses in these cases to a larger extent than for CURED patients were made on core biopsies instead of whole lymph nodes. It can be speculated that the small number of REF/REL patients compared to CURED could affect the possibility to find prognostically negative biomarkers and one way to avoid this in further studies would be to include REF/REL patients from another diagnostic centre.

Since elevated levels of ribosomal proteins have shown prognostic impact in other malignancies, mechanistic studies would be an important next step in investigating their actual role in DLBCL. Very few actin-related proteins were detected in paper III but as mentioned above, there are a couple of

studies in recent years that point to a role in pathogenesis or stimulated tumour progression or reduced lymphoma dissemination for this protein group in DLBCL which could deserve further investigation.

5.1.4 MS-based proteomics confirm non-GCB- and GCB-specific proteins

In paper III, 224 proteins were significantly up-regulated in non-GCB DLBCL compared to GCB, among them the classic non-GCB protein MUM1, while CD10 and BCL6 were up-regulated in GCB. This confirms that the originally molecularly defined groups of COO are distinctly separated also on the protein level and is in agreement with these three proteins being robust differential markers for COO. The results are additionally supported by at least two previous proteomic studies on DLBCL (121, 183). To relate to the section above, the results also point to that the two COO groups are functionally more homogeneous than the clinical groups of REF/REL and CURED.

5.1.5 Expression of tumour microenvironment associated proteins differ between non-GCB and GCB

Apart from the classic COO-associated proteins, the main finding in paper III was an up-regulation in non-GCB DLBCL of proteins previously reported as associated with the tumour microenvironment (TME). Among them were CD163, a marker of tumour promoting M2 macrophages and one study found an association with worse prognosis in DLBCL for an increased number of CD163+ M2 macrophages (184). The antiphagocytic membrane protein CD47 was also up-regulated in non-GCB and a high expression of CD47 was a negative prognostic factor for non-GCB patients in another study (185). As mentioned, the microenvironment seems to be more important for ABC/non-GCB DLBCL patients than for GCB (107). Indeed, one recent study showed higher prevalence of both T-cells and macrophages in ABC DLBCL vs. GCB and these cells also showed a different infiltration pattern that possibly could be associated with stimulation of lymphoma cell proliferation (186).

5.2 SEVERAL INTERFERON-STIMULATED PROTEINS DIFFER BETWEEN NON-GCB AND GCB AND MNDA AND IFI16 SHOW PROGNOSTIC POTENTIAL IN DLBCL

Several of the TME-associated proteins up-regulated in non-GCB in paper III are interferon (IFN)-stimulated. IFNs produced by NK-cells, macrophages, helper- and cytotoxic T-cells have important functions in the defence against viruses and both the type II IFN (IFN-γ) and type I IFNs such as IFN-α and IFN-β are important mediators of the immune response against tumour antigens (109, 187, 188). However, the effect on neoplastic cells seems to be double-sided as e.g. constant exposure to IFN-γ has been shown to facilitate immune escape and promote tumour growth through increased genomic instability and the inducement of e.g. PD-L1 expression on tumour and immune cells (189).

Among the IFN-stimulated proteins found up-regulated in non-GBC with proteomics is GBP1, known to participate in autophagocytosis of engulfed microorganisms (190). A doxorubicin-resistant myeloma cell-line showed overexpression of GBP1 RNA (191) and in breast cancer its overexpression was correlated to resistance to the drug paclitaxel (192). Yet, a favourable outcome has been associated with GBP1 expression in some tumours such as high grade serous ovarian carcinoma (193) and thus, as for many biomarkers, its pro- or suppressive functions on tumours seem to be context-dependent. Of interest is also our IHC finding that GBP1 was predominantly expressed by macrophages of the TME, suggesting an effect mainly derived from the microenvironment in DLBCL.

Three types of interferon-induced IFIT proteins (-2, -3 and -5) were up-regulated in non-GCB in the proteomic studies in paper III. These are proteins that apart from their primarily described function in antiviral response in addition appear to play a role in promoting or reducing tumour progression, mostly described in carcinomas (194).

Human myeloid cell nuclear differentiation antigen (MNDA), another interferon-stimulated protein, was also up-regulated in non-GCB. As demonstrated in paper IV, MNDA and interferon-inducible protein 16 (IFI16), were also significantly associated with worse survival in DLBCL. Both IFI16 and MNDA belong to the hematopoietic interferon-inducible nuclear (PYHIN) family of proteins, of which one important function is to act as pattern recognition receptors that sense foreign DNA from viruses as well

as damaged host DNA (195). MNDA, together with the IFN-inducible protein SP140 were also found as up-regulated in ABC in another proteomic study, which further support an important pathogenetic role for IFN-inducible proteins in this DLBCL subtype (121). MNDA is mainly thought to act as a transcription factor and to be involved in stimulating apoptosis in neutrophils (195). An association between elevated MNDA with reduced levels of antiapoptotic proteins has been shown in chronic lymphocytic leukaemia (196) and in osteosarcoma in which increased proliferation was seen with inhibition of MNDA (197), thus suggesting a tumour suppressive role. On the other hand, a study on myelodysplastic syndrome, a hematologic malignancy associated with increased apoptosis, showed lower IHC expression of MNDA compared to control bone marrows (198). Taken together, also the function of MNDA thus appears to be context-related.

The STING-protein pathway activated by IFI16 when sensing foreign or damaged DNA, leads to expression of other interferon-inducible proteins and can serve as a cellular protection system against malignancy, e.g. in alerting anti-tumour inflammatory response. Yet, there is also increasing evidence that STING stimulation can promote tumour growth (199). Furthermore, STING exerts negative feedback on IFI16 (200) and disruption of the STING pathway, as seen in some other malignancies (199) could hypothetically be an underlying mechanism for accumulation of IFI16 in DLBCL. In B-cells, IFI16 exerts its effects through the NF-κB pathway, a pathway that in addition plays an important role in driving lymphomagenesis in ABC/non-GCB DLBCL (39, 201), and it is tempting to speculate that this pathway is of particular importance for the negative prognostic effect of IFI16 in DLBCL. IFI16 was also shown to stimulate PD-L1-expression in cervix cancer cells through the NF-κB pathway (202) and therapeutic blockage of IFI16 could possibly affect interactions between DLBCL and the microenvironment in a prognostically favourable way. Another way for IFI16 to exert a negative prognostic effect has been described in pancreatic adenocarcinoma in which activation of an inflammasome, under normal conditions used in defence against microbes (203), was proposed to increase the population of tumour-stimulating macrophages in the TME (204).

Given the connection between IFNs and the tumour microenvironment and immune escape, interferon-induced proteins would be interesting to explore further in DLBCL, supported by the potential prognostic effect shown for MNDA and IFI16 in paper IV and by the up-regulation of IFN-stimulated proteins in non-GCB patients in paper III.

5.3 IHC ANALYSES INDICATE PROGNOSTIC IMPACT FOR OVEREXPRESSION OF PROTEINS WHOSE GENES ARE FREQUENTLY MUTATED IN DLBCL

In paper II, strong or intermediate/strong IHC expression of the transcriptional repressor TBLR1 and the histone modifier CREBBP were associated with inferior survival and recurrent mutations in both corresponding genes have been shown in genetic studies on DLBCL (97-99).

CREBBP mutations are more commonly seen in GCB DLBCL and seem to be of an inactivating type (205) and in the study by Reddy *et al.* from 2017 CREBBP mutations were seen together with better outcome in ABC DLBCL (97). In our material, CREBBP IHC expression was significantly higher in the non-GCB group compared to GCB. When we compared the impact of CREBBP IHC expression pattern between the COO groups, the negative prognostic effect was seen solely in non-GCB. One explanation for this finding might be that the higher expression observed could be a result of fewer silencing CREBBP mutations in that group. Also in other malignancies, such as acute myeloid leukaemia or small cell lung cancer, high expression of CREBBP has been reported with poorer outcome (206, 207), supporting our findings that it could be a negative prognostic marker, although one report instead showed worse prognosis in acute lymphatic leukaemia for low expression of CREBBP (208). One recent study investigated the relation between mutations in epigenetic regulators such as CREBBP and features of the microenvironment in DLBCL and found an inferior prognosis associated with silencing CREBBP mutations, presumably through pro-tumour effects exerted by the microenvironment (209). Thus, full understanding of the effects of CREBBP protein over- or under-expression and the effects from CREBBP mutations needs further exploration with functional studies.

TBLR1 is a transcriptional repressor but is also known to activate the NF-κB pathway (102) which possibly could explain some of the association with worse prognosis in our material at least for non-GCB cases, since the NF-κB pathway is an important mediator of lymphoma growth in that group (39). Both Schmitz *et al.* (98) and Chapuy *et al.* (99) found an association with worse outcome for mutations in *TBL1XR1* and increased TBLR1 IHC expression has further been correlated to inferior survival in e.g. ovarian and gastric carcinoma (210, 211).

5.4 CELL-OF-ORIGIN WITH IHC FAILS TO SHOW PROGNOSTIC IMPACT

COO defined with IHC did not prove to be a prognostic factor, neither in paper II nor in paper IV and likewise the proportions of non-GCB and GCB in REF/REL and CURED patients were similar, all in agreement with many previous studies on R-CHOP treated patients (32-34, 53, 66, 96). Despite COO being less informative as to patient outcome, substantial amounts of data however, including the findings in paper III, imply that the non-GCB group harbours many interesting focus points for further search for biomarkers. Interactions between lymphoma cells and the microenvironment seem to be more important for the pathogenesis in these patients than in GCB and could give new suggestions on specific therapies to be added to standard treatment.

6 CONCLUSION

To conclude, these exploratory studies show that global proteomics as well as IHC with digital image analysis can be used for the investigation of potential prognostic or functional groups of proteins in DLBCL. Even though the results from the proteomic studies in paper I could not be reproduced in paper III, there is increasing evidence in the literature that particularly ribosomal proteins could be interesting to investigate further in relation to cancer and drug resistance. Additionally, some genes in which mutations are previously described as prognostic in DLBCL, are informative to study also on the protein level and our IHC studies show that the protein expression patterns of these genes could be used as prognostic factors as well. Also, the long standing genetic or immunohistochemical subdivision of COO into non-GCB and GCB still remains the basis for a definition of functional subgroups of DLBCL. Even though many studies, including ours, have failed to show a prognostic difference between the two COO groups they are still important in identifying and interpreting important tumour mechanisms in DLBCL. Further, interactions with the microenvironment, seemingly important for tumourigenesis in non-GCB DLBCL, can give us ideas to which protein interactions to target in experimental treatment of DLBCL.

7 FUTURE PERSPECTIVES

The studies in this thesis are exploratory in character and performed on a rather limited number of patients. To further confirm our findings it would be necessary to expand the material or better still repeat the analyses in an independent cohort. Prospective studies, either with MS-based proteomics or IHC could give additional information on the prognostic importance of the up- or down-regulation of the protein groups or single proteins identified in our material. Another important aspect is the need to investigate the functional aspects of these proteins in different signalling pathways in DLBCL and how resistance to the drugs included in the R-CHOP regimen is affected by the turning off or on of the corresponding genes. Indeed, functional studies on DLBCL cell-lines with the gene modification tool CRISPR have been discussed in our group but not yet been put into action. Lastly, since our cohort is retrospective with the oldest cases included being from 2004, we have no information on the proportion of double or triple hit (DH or TH) lymphomas. DH/TH is a feature that has proved to be important for patient survival and the molecular mechanisms resulting from those gene rearrangements could be of interest to study in relation to the different protein groups identified here, such as e.g. ribosomal proteins, known to interact with MYC.

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