

# **Prognostic significance of tumor cell markers in diffuse large B-cell lymphoma with special emphasis on lymphoma localization**

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## Abstract

Diffuse large B-cell lymphoma (DLBCL) is the most common type of high-grade B-cell lymphoma with different clinical, morphological, immunophenotypical, and molecular features. DLBCL is curable in 60-70% of patients when treated with standard immunochemotherapy R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone).

The main aim of this thesis is to identify prognostic factors in DLBCL by studying tumor markers (**paper I and II**), site of disease (**paper III**) and tumor microenvironment markers in primary DLBCL of the CNS (PCNSL) (**paper IV**) in order to better identify different risk groups of DLBCL patients.

**In papers I-III**, we studied DLBCL patients treated homogeneously with R-CHOP. The negative prognostic impact of double protein expression of MYC and BCL2 so called “double-expressor lymphoma” (DEL) was a common finding in the three papers. In **paper I**, we detected DEL in 27% of patients, distributed with no significant difference between the germinal center derived B-cell subgroup (GCB) in 52% of cases and the non-GCB subgroup in 37% of cases. There was no significant difference in survival between GCB and non-GCB patients. The diagnosis in most of the patients with DEL was made on core needle biopsy in this paper. This finding was more thoroughly investigated in **paper III** with attention paid to the site of biopsy. In **paper II**, we evaluated the concordance of cell of origin (COO) assignment between gene expression profile (GEP) and immunohistochemistry (IHC) to identify the best predictor of survival in a DLBCL cohort including patients from Sweden and Denmark. The overall concordance between the two methods was 83%. We found that ABC/non-GCB subtype identified by both GEP and IHC is associated with the worst outcome. This finding indicates the importance of precise risk stratification in the era of precision medicine. DEL was more common in ABC patients categorized by GEP. In **paper III**, we identified abdominal lymph node involvement by radiological examination in 63% of DLBCL patients with an inferior survival, adverse clinical characteristics and significantly more frequent DEL. These findings may indicate a distinct biological behavior in patients with abdominal nodal disease. In **paper IV**, we demonstrated a significant association between IDO1 and PD-L1 in PCNSL patients. This finding indicates the crucial immunosuppressive role of these two molecules. In addition, in PCNSL low frequencies of *MYC* and *BCL2* translocations and high frequency of *BCL6* translocation was observed and DEL was detected in 49% of cases. Contrary to our results in systemic DLBCL in **papers I-III**, there was no significant prognostic impact of DEL in PCNSL.

*“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less”*

Marie Curie

*Dedicated to all fighters of lymphoma*



# List of Papers

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

- I. Abdulla M, Laszlo S, Triumpf J, Hedström G, Berglund M, Enblad G, Amini RM. A population-based study of cellular markers in R-CHOP treated diffuse large B-cell lymphoma patients. *Acta Oncologica*, 2016 Aug 55(9-10):1126-1131.
- II. Abdulla M\*, Hollander P\*, Pandzic T, Mansouri L, Bram Ednersson S, Andersson P-O, Hultdin M, Fors M, Erlanson M, Degerman S, Munch Petersen H, Asmar F, Grønbaek K, Enblad G, Cavelier L, Rosenquist R\*\*, Amini RM\*\*. Cell-of-origin determined by both gene expression profiling and immunohistochemistry is the strongest predictor of survival in patients with diffuse large B-cell lymphoma. *American Journal Hematology*, 2019 Nov 95 (1):57-67.
- III. Abdulla M, Guglielmo P, Hollander P, Åström G, Ahlström H, Enblad G, Amini RM. Prognostic impact of abdominal lymph node involvement in diffuse large B-cell lymphoma. *Accepted for publication in European Journal of Haematology*. Published online Nov 30<sup>th</sup> 2019.
- IV. Abdulla M, Alexsson A, Sundström C, Ladenvall C, Berglund M, Mansouri L, Lindskog Bergström C, Cavelier L, Enblad G, Hollander P, Amini RM. PD-L1 and IDO1 are important immunosuppressive molecules to consider in primary DLBCL of the CNS. Submitted.

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## Related publications

- I. **Abdulla M**, Laszlo S, Triumf J, Hedström G, Berglund M, Enblad G, Amini RM. Core needle biopsies for the diagnosis of diffuse large B-cell lymphoma - a great concern for research. *Acta Oncologica*, 2016 Oct 56(1):106-109.
  
- II. Mansouri L, Noerenberg D, Young E, Mylonas E, **Abdulla M**, Frick M, Asmar F, Ljungström V, Schneider M, Yoshida K, Skaftason A, Pandzic T, Gonzalez B, Tasidou A, Waldhueter N, Rivas-Delgado A, Angelopoulou M, Ziepert M, Arends CM, Couronné L, Lenze D, Baldus CD, Bastard C, Okosun J, Fitzgibbon J, Dörken B, Drexler HG, Roos-Weil D, Schmitt CA, Munch-Petersen HD, Zenz T, Hansmann ML, Strefford JC, Enblad G, Bernard OA, Ralfkiaer E, Erlanson M, Korkolopoulou P, Hultdin M, Papadaki T, Grønbæk K, Lopez-Guillermo A, Ogawa S, Küppers R, Stamatopoulos K, Stavroyianni N, Kanellis G, Rosenwald A, Campo E, Amini RM, Ott G, Vassilakopoulos TP, Hummel M, Rosenquist R, Damm F. Frequent NFKB1E deletions are associated with poor outcome in primary mediastinal B-cell lymphoma. *Blood*, 2016 Dec 128 (23):2666-2670.





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# Abbreviations

aa-IPI	Age-adjusted International Prognostic Index
ABC	Activated B-cell
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
AS	Alternative splicing
ASCT	Autologous stem cell transplantation
BCLU	B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL
BCL2	B-cell lymphoma 2 protein
BCL6	B-cell lymphoma 6 protein
BL	Burkitt lymphoma
CAR	Chimeric antigen receptor
CARD11	Caspase Recruitment Domain Family Member 11
CHL	Classical Hodgkin Lymphoma
CHOP	Cyclophosphamide, doxorubicin, vincristine and prednisone
CNB	Core-needle biopsy
COO	Cell-of-origin
CR	Complete remission
CT	Computer tomography
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DEL	Double expressor lymphoma
DHL	Double-hit lymphoma
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ESMO	European Society for Medical Oncology
EZH2	Enhancer of zeste homolog 2
FDA	U.S. Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded tissues
FISH	Fluorescent in Situ Hybridization
FNA	Fine needle aspiration
GCB	Germinal centre B-cell
GEP	Gene expression profile
HGBL	High-grade B-cell lymphoma
HCV	Hepatitis C virus
IDO	Indoleamine 2, 3 dioxygenase

IHC	Immunohistochemistry
IPI	International prognostic index
IRF4	Regulatory factor 4 protein
LMO-2	LIM domain only-2
LSS	Lymphoma-specific survival
MAG	Myelin Associated Glycoprotein
MSKCC	Memorial Sloan-Kettering Cancer Center
MYD88	Myeloid differentiation primary response 88
NGS	Next-generation sequencing
NHL	Non-Hodgkin lymphoma
OS	Overall survival
PAX5	Paired Box protein 5
PCNSL	Primary DLBCL of the CNS
PD-1	Programmed Death receptor 1
PD-L	Programmed Death Ligand
PET	Positron Emission Tomography
PFS	Progression-free survival
PMBCL	Primary mediastinal large B-cell lymphoma
PRDM1	PR domain containing 1
PTEN	The phosphatase and tensin homolog gene
R-CHOP	Rituximab-Cyclophosphamide combined with doxorubicin, vincristine and prednisone
RNA	Ribonucleic acid
SEB	Surgical excisional biopsy
SHM	Somatic hypermutation
SSP1	Secreted Phosphoprotein 1
TAM	Tumor associated macrophages
TCR	T-cell receptor
THL	Triple-hit lymphomas
UC	Unclassifiable
WHO	World Health Organization
XBP1	X-box binding protein 1

# Introduction

Malignant lymphomas affect about 2,000 people in Sweden every year. These lymphoid malignancies comprise a heterogeneous group of different disease entities with different features, where some patients are diagnosed with high-grade lymphomas with an aggressive clinical course that require immediate treatment, while others can live for decades without the need for any treatment. The process for lymphoid stem cells in the bone marrow to become highly specialized B- and T-lymphocytes requires several steps of differentiation. The process to become a mature and immunocompetent B- or T-cell is therefore a delicate process, where the lymphocytes are vulnerable to genetic aberrations that may develop along this long pathway of differentiation. Lymphomas arise from genetically transformed and clonally expanded lymphoid cells at different stages of differentiation. B-cells mature and differentiate in the secondary lymphoid organs like lymph nodes, whereas immature T-cells develop in the thymus. The vast majority of lymphomas are derived from transformed B-cells, whereas T-cell lymphomas constitute only about 5% of all lymphomas. All lymphomas are today classified in the updated 2016 World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues, and the different entities are classified mainly based on cell-of-origin (COO) in order to better understand and relate to its normal counterpart for the transformed malignant cells [1].

## Epidemiology

In Sweden, the incidence of DLBCL is approximately 5.5 per 100,000, with slight male predominance [2]. DLBCL is predominant in elderly with a median age of 65 year, although it also occurs in young patients and rarely in children [3].

The etiology of DLBCL is unclear in the majority of patients. However, certain factors may influence the risk of developing lymphoma as genetics, comorbid diseases or their treatments (i.e. immunosuppression), environmental factors such as ultraviolet radiation, pesticides, hair dyes, and diet.

Several infectious organisms have been linked to the risk of lymphomas. Certain subtypes of DLBCL have been highly associated with Epstein-Barr virus (EBV) namely the immunoblastic variant and primary DLBCL of the CNS [4]. A correlation between DLBCL and hepatitis C (HCV) has been shown [5].

An increased risk of DLBCL has been associated with autoimmune diseases such as rheumatoid arthritis, Sjogren syndrome, and autoimmune hemolytic anemia [6].

## DLBCL once upon a time

### Before 1970

The history of malignant lymphoma classification can be traced to the time when malignant lymphoma was divided into four types; Hodgkin's disease, lymphosarcoma, reticulum cell sarcoma, and follicular lymphoblastoma [7]. Hodgkin's disease was named after Thomas Hodgkin, who first described the disease in 1832 [8]. Subsequently, malignant lymphoma was found to be a heterogeneous group of neoplastic diseases of lymph nodes and to differentiate these conditions from Hodgkin's disease, these were named as non-Hodgkin lymphoma (NHL).

Malignant lymphoma was first mentioned by Theodore Billroth in 1871, to describe neoplastic, infectious and miscellaneous causes of lymphadenopathy [9]. However, the disease was first known as "lymphosarcoma" and was recognised by Rudolf Virchow in 1865 [10].

The first attempt to classify malignant lymphoma in modern times was performed by Gall and Mallory in 1942 [11], however, the first well-organized classification of malignant lymphomas was proposed by Rappaport in 1956 and modified in 1966 [12]. The Rappaport classification was based on morphology; cell size and architecture and also included growth pattern such as diffuse, nodular and histiocytic patterns; each with subtypes [12]. DLBCL was recognized as "diffuse histiocytic lymphoma" by Rappaport [13].

### Between 1970-2000

The advances in the science of immunology with the identification of lymphocytes to be B-, T- and natural killer lymphocytes have provided new insights into the classification of lymphoma. The efforts on both sides of the Atlantic Ocean took a step forward and classification of lymphoma based on morphology and immunology was developed in 1974 by Karl Lennart in Europe (Kiel classification) and Lukes and Collin in USA [14, 15]. DLBCL

synonyms were known as centroblastic, B-immunoblastic, large cell anaplastic (B-cell) in Kiel classification and large cleaved or large non-cleaved follicular center cell (FCC), B-immunoblastic in Lukes-Collins classification.

The different terms used by different classifications and the questionable clinical relevance between the clinicians led to the creation of a working formulation in 1982 in order to interpret these classifications for clinical usage [16]. The working formulation was favored in the USA while in Europe, the Kiel classification was still in use [17]. DLBCL was known as diffuse large cell cleaved, non-cleaved or immunoblastic; occasionally diffuse mixed small and large cell in the Working Formulation.

The International Lymphoma Study Group Founded in 1990 by Stein and Isaacson, tried to review and unify the Kiel, Lukes and Collins and the Working Formula classifications. These collaborated efforts led to the development of the revised European-American classification of lymphoid neoplasms (REAL) classification of lymphoma that was published in 1994. The meeting concluded that lymphomas should be classified according to their normal counterpart and include clinical presentation, morphology, immunophenotype and genetic information [18]. Certain clinically relevant subtypes of DLBCL were identified including Large B-Cell Lymphoma Subtype; Primary Mediastinal (Thymic) Large B-Cell Lymphoma, Burkitt's Lymphoma and a Provisional Entity: High-Grade B-Cell Lymphoma, Burkitt-Like.

### After 2000

The World Health Organization (WHO) decided to update the classifications of haematopoietic and lymphoid tissue tumors shortly after the publication of REAL classification. This project was adopted by the Society for Hematopathology and European Association of Hematopathology. The WHO classification was developed over a period of seven years and published in 2001 and updated in 2008 and 2016 [1, 19]. Different entities were established and provisional categories of NHL based on cell lineage and differentiation were described. At least 80 different entities of NHL are described in the WHO classification, each with specific clinical features, immunophenotype, molecular and genetic alterations [1].

## DLBCL in the updated 2016 World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues

The 2016 WHO classification defines DLBCL as the most common type of high-grade B-cell lymphoma with heterogeneous clinical, morphologic, immunophenotypic, cytogenetic and molecular features [20, 21]. Biological and clinical studies have subdivided DLBCLs into specific subtypes and/or variants (Table 1) [22]. Some of these defined subtypes or variants of large B-cell lymphomas represent tumors that arise in a particular site, such as primary mediastinal B-cell lymphoma (PMBCL), primary DLBCL of the CNS (PCNSL), intravascular large B-cell lymphoma and DLBCL, leg type [20, 22].

However, there is still a substantial number of DLBCL cases that have no clearly accepted criteria for subdivision, and these are classified as DLBCL, not otherwise specified (NOS) [3, 22, 23]; DLBCL, NOS account for about 30% of adult non-Hodgkin lymphoma [23].

Few changes of the categories of DLBCL were made in the 2016 WHO classification from 2008 WHO classification; mainly including the COO classification in the pathology report i.e. germinal center B-cell type (GCB-DLBCL) versus activated B-cell type (ABC-DLBCL), and the identification of the double expression of *MYC* and *BCL2* assessed by immunohistochemistry (IHC) as a poor prognostic indicator. Another major change is the emergence of two new categories to replace the provisional category “B-cell lymphoma, unclassifiable, with features between DLBCL and Burkitt lymphoma” (BCLU). These are high grade B-cell lymphoma, with *MYC* and *BCL2* and/or *BCL6* translocations (double-hit or triple-hit lymphoma) and high grade B-cell lymphoma, not otherwise specified.

Table 1. High-grade B-cell lymphomas (2016 WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues)

DLBCL, NOS  
T-cell/histiocyte rich large B cell lymphoma  
Primary DLBCL of the CNS  
Primary cutaneous DLBCL leg-type  
EBV-positive DLBCL, NOS  
Large B cell lymphoma with IRF4 rearrangement  
Primary mediastinal (thymic) large B cell lymphoma  
Intravascular large B cell lymphoma  
DLBCL associated with chronic inflammation  
HHV8 positive diffuse large B cell lymphoma  
Primary effusion lymphoma  
High-grade B-cell lymphoma  
High-grade B-cell lymphoma, with *MYC* and *BCL2* and/or *BCL6*



rearrangements  
High-grade B-cell lymphoma, NOS  
B-cell lymphoma, unclassifiable, with features intermediate between  
DLBCL and classical Hodgkin lymphoma  
Plasmablastic lymphoma  
ALK-positive large B cell lymphoma

## Biology and Pathogenesis

### Normal germinal center

Normal B-cells arise in the bone marrow and undergo primary rearrangement of their immunoglobulin genes before antigen exposure. They migrate from the bone marrow carrying surface immunoglobulin receptors toward the peripheral lymphoid organs where they encounter antigens and produce high-affinity antibodies [24]. The formation of germinal center in the lymphoid organs occurs when the antigen induces a T-dependent antibody response. Two genetic processes take place in the germinal center. These are the somatic hypermutation and switch recombination and both need double-stranded DNA breaks and the presence of activation-induced cytidine deaminase (AID). B-cell differentiation through the germinal center is controlled by a number of key transcription factors, including *BCL6*, *PRDM1*, *IRF4*, and *XBPI*. B-cells that have high-affinity antibodies on their surface survive, they re-express *BCL2* and exit the germinal center and differentiate into either mature plasma cells or long-lived memory B-cells to be part of the humoral immunity [24]. However, this complex biology is not perfect and errors can happen. Unfortunately, some of these errors may lead to the development of B-cell lymphomas [24].

### DLBCL

#### **Morphology**

Morphologically, the 2016 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues defines DLBCL as a diffuse growth of neoplastic large lymphoid cells of B-cell origin with a nuclear size equal to or exceeding normal macrophage nuclei cells that totally or partially efface the normal architecture of lymph node or extranodal tissue (Figure 1) [22]. Variable numbers of reactive T-cells and histiocytes are present. Sclerosis and or geographical necrosis may be present. Apoptosis and increased mitotic rates are high, and about 10% of DLBCL may be associated with starry sky pattern.

The WHO classification describes a number of morphological variants of DLBCL: centroblastic, immunoblastic, and anaplastic variants. In addition, rare morphological variants have been described in the literature: lymphoma

cells may have multilobated nuclei, small size lymphoma cells (small centroblastic), signet ring appearance (like gastric carcinoma), spindle-cell appearance (like sarcoma), or lymphoma cells may have cytoplasmic granules or microvillous projections or intracellular junctions. Inferior prognosis has been reported by some studies in immunoblastic variant [25, 26] and also in certain subsets of anaplastic variants that carry *TP53* [27].

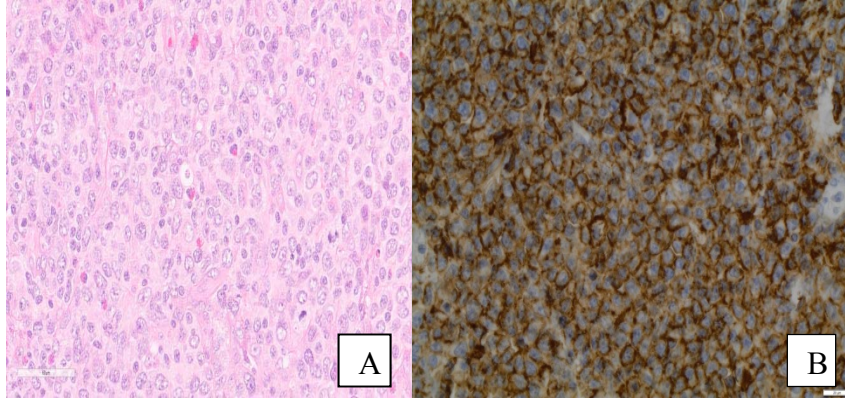


Figure 1. DLBCL characterized by diffuse infiltration of large neoplastic B-cells; A: Haematoxylin and eosin (HE), B: CD20.

#### **Gene expression profile (GEP)**

Alizadeh and colleagues were among the first to use the gene expression profile (GEP) to identify two distinct molecular subtypes of DLBCL, namely GCB, and ABC subtypes, which expressed genes characteristic of their respective COO, with 15% remaining unclassifiable group (UC) [28]. This molecular distinction provides prognostic and predictive information with the GCB subtype exhibiting a better outcome than ABC in DLBCL patients treated with CHOP. This observation was confirmed subsequently in patients treated with R-CHOP [29]. In addition, using the GEP to understand the pathogenesis of DLBCL plays a promising role in the selection of targeted therapies [22, 28-30].

GEP is a reliable method for the identification of the GCB and ABC molecular subtypes of DLBCL; however, it is not a routinely available test for clinical use due to the lack of a standardized commercially available test and the requirement for fresh-frozen tissue specimens.

Several studies assessed the use of IHC as an alternative and practical tool to determine the COO, and different algorithms have been developed with different levels of concordance with GEP [31, 32]. The most commonly used algorithm developed by Hans et al. defines cases as GCB and non-GCB based on three IHC markers (CD10, BCL6 and MUM1) [33].

It is acknowledged that the IHC algorithms do not recognize the UC subtype that represents 10 to 15% of tumors according to GEP, thus they do not correlate exactly with the molecular categories, and are not uniformly reported to have prognostic utility and nor do they determine therapy. However, since GEP is not available as a routine clinical test, the use of IHC algorithms is considered to be acceptable [20].

In the recent years new methods based on the quantification of RNA transcripts extracted from formalin-fixed paraffin-embedded tissues (FFPE) have been developed and provide results concordant with conventional microarray GEP, are reproducible between laboratories, and capture the prognostic impact of the COO classification. The Lymph2X is one of these methods that is based on NanoString technology and includes 20 genes in the panel to determine COO. These methods are still not accessible to most laboratories but may represent a promising alternative to current IHC-based algorithms [30, 34-38].

#### *GCB-DLBCL*

GCB-DLBCLs are believed to originate from lymphoid cells residing in the germinal center and express *CD10*, *BCL6* and *LMO-2* [39]. *BCL6* is characteristically upregulated in GCB-DLBCL. *BCL6* represses the transcription of several genes including *TP53* tumor suppressor, which controls DNA damage-induced apoptotic response, allowing germinal-center cells to tolerate the physiological DNA breaks required for immunoglobulin class switch recombination and somatic hypermutations [40]. Chromosomal translocations involving *BCL6* are found in 40% of GCB-DLBCL cases, but other mechanisms leading to *BCL6* upregulation have been reported, such as somatic mutations [41, 42]. The GCB subtype has more often translocations of *BCL2*, *MYC*, mutations involving histone methylation or acetylation like *EZH2*, *EP300*, *CREBBP*, *KMT2D* (figure 2) [39, 43, 44], and mutations involving B-cell homing like *GNA13*, *GNA12*, *SIPR2* [45], mutations involving *PI3K* pathway signaling, and *JAK-STAT* pathway [46].

### *ABC-DLBCL*

ABC-DLBCLs are considered to originate from B-cells at a plasmablastic stage, just prior to germinal center exit [39] and express a plasma-cell like transcription program.

The ABC subtype has more often genetic abnormalities that activate the B-cell receptor signaling and the Toll-like receptor signaling pathways, resulting in activation of NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B-cells) signaling pathway like mutations of *TNFAIP3*, *CARD11*, *CD79B* and *MYD88* (figure 2) [47-49].

Upon activation, the NF- $\kappa$ B pathway promotes cell survival, proliferation and inhibition of apoptosis, as well as driving the cell towards plasma cell differentiation [47, 50, 51].

Both subtypes of DLBCL share overexpression of antiapoptotic protein BCL2, although due to different mechanisms. In GCB-DLBCL, overexpression of BCL2 is largely due to presence of the t(14;18), whereas in ABC DLBCL it is caused by other mechanisms, such as transcriptional upregulation and gene amplification [52].

### *Unclassifiable (UC)*

The GEP of UC subtype is still obscure. While some studies presented UC as having a similar outcome to ABC-DLBCL [29, 53], a recent study demonstrated that a concomitant *NOTCH2* mutations and *BCL6* translocations is common in this subtype and is associated with a favorable outcome [54].

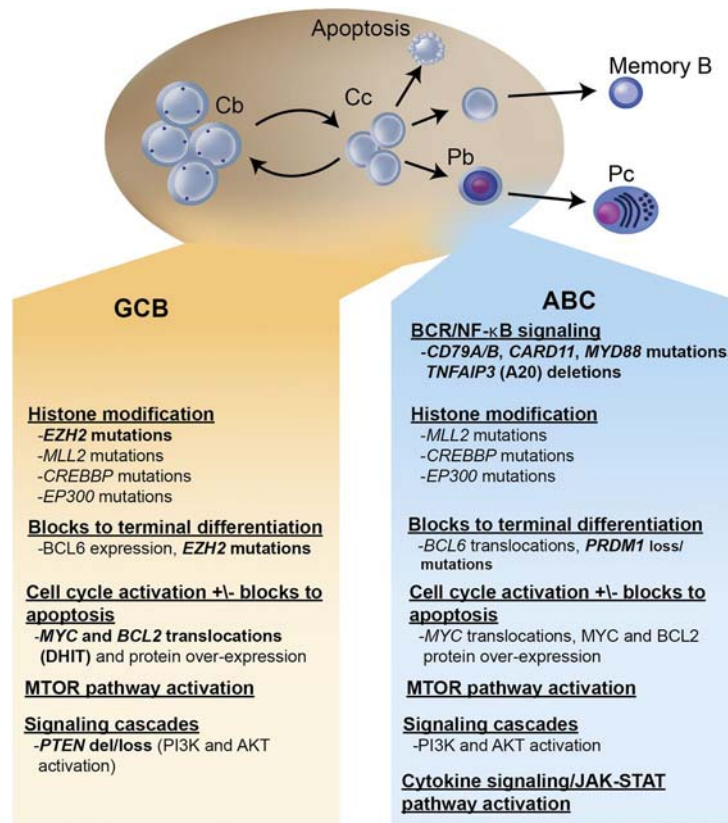


Figure 2. Key oncogenic pathways in DLBCL. Figure adapted from Sehn et al, Blood 2015 [39] with permission.

Monti and colleagues subdivided DLBCL into three groups by using GEP data. These are: oxidative phosphorylation, B-cell receptor/proliferation, and host response [55]. The oxidative phosphorylation group includes tumors carrying t(14; 18) and tumors with apoptotic pathway defects. The B-cell receptor/proliferation includes tumors carrying *BCL6* translocation and may overlap with ABC subtype. The host response group has a T-cell and dendritic cell signature and probably includes cases of T-cell/histiocyte rich large B-cell lymphoma.

Dybkaer and colleagues assigned B-cell-associated gene signature (BAGS) based on normal B-cell subset phenotypes and identified five subtypes: naive, centroblast, centrocyte, memory, and plasmablast B-cell subtypes [56]. These signatures provided additional prognostic significance to the molecular ABC/GCB subtypes where the GCB-centroblast subtype had inferior prognosis compared to the GCB-centrocyte subtyp [56].

### Next generation sequencing (NGS)

The next generation sequencing (NGS) enables our understanding of DLBCL heterogeneous genetic pathogenesis as well as provides potential targets for therapeutic agents. The whole genome sequencing allows high throughput DNA sequencing and provides the opportunity to determine the pattern, frequency, and location of somatic point mutations across the entire genome.

An average of 30 to > 100 genetic mutations per neoplasm have been identified in DLBCL [57-59]. Data indicate that DLBCL cells undergo multiple rounds of clonal expansion and that gene mutations can happen at any time point during this process [45]. These mutations are divided into driver and passenger mutations. The driver mutations occur early in DLBCL pathogenesis and aid in progression of the disease, and they appear in most or all subsequent clones and have therefore the potential to be the best therapeutic targets [60]. On the other hand, the role of passenger gene mutations in DLBCL pathogenesis is not known yet. A challenge that makes highly mutated alleles appear to be drivers when they are in fact passengers mutated by aberrant somatic hypermutation is due to the effect of the enzyme AID [61-65]. The most well-defined AID induced gene mutations by aberrant somatic hypermutation include *BCL2*, *BCL6*, *MYC*, *RHOH/TTF*, *PIM1*, *PAX5*, *IRF4*, *ST6GAL1*, *BCL7A*, *CIITA*, *LRMP*, and *SOCS1* [66].

Chapuy and colleagues have proposed five distinct DLBCL subsets with different pathological mechanisms and outcome (clusters 1-5) [46]. Three of these have previously been undescribed, a low-risk ABC-DLBCL group (C1); poor risk GCB-DLBCLs with *BCL2* SVs and alterations of *PTEN* and epigenetic enzymes (C3), a newly defined group of good-risk GCB-DLBCLs with distinct alterations in *BCR/PI3K*, *JAK/STAT*, and *BRAF* pathway components and multiple histones (C4). In addition, a COO-independent group of tumors with biallelic inactivation of *TP53*, *9p21.3/CDKN2A* and associated genomic instability (C2), and a group in which the genetic signature is associated with extranodal tropism and with frequent *BCL2* gain, concordant *MYD88/CD79B* mutations and additional mutations of *ETV6*, *PIM1*, *GRHPR*, *TBLIXR1* and *BTG1* (C5).

Schmitz and colleagues identified four distinct genetic subtypes in DLBCL. These are: MCD with co-occurrence of *MYD88* and *CD79B* mutations, BN2 with *BCL6* fusions and *NOTCH2* mutations, N1 with *NOTCH1* mutations, and EZB with *EZH2* mutations and *BCL2* translocations. These groups have different gene-expression signatures and respond differently to immunochemotherapy, where the BN2 and EZB subtypes have favorable outcome and the MCD and N1 subtypes are associated with inferior outcomes [54].

### **Rearrangement and expression of MYC, BCL2, and BCL6**

MYC is a transcriptional factor located at 8q24 and is essential for normal B-cell growth, proliferation, and apoptosis [67, 68]. The dysregulation of *MYC* is essential to induce lymphomagenesis in B-cell lymphomas. *MYC* rearrangement is found in 5 to 15% of DLBCL. *BCL2* is an apoptosis regulator factor that inhibits the apoptotic death of cells and is located at 18q21. *BCL2* rearrangement is observed in nearly 30% of DLBCL with GCB subgroup and around 5% in ABC subgroup. *BCL6* is a sequence-specific repressor of transcription, and has been shown to modulate the transcription of STAT-dependent IL-4 responses of B-cells. The *BCL6* gene is located at 3q27.

#### *Double hit lymphoma*

*MYC* translocation can occur in association with *BCL2* translocation and/or, to a lesser extent, *BCL6* translocation, in the so called double-hit lymphoma (DHL) or triple-hit lymphomas (THL), which represent a new category of high-grade B-cell lymphoma in the updated 2016 WHO Classification (High-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements) [69]. These lymphomas which were first defined by Aukema and colleagues in 2011 [70] represent about 10% of high grade B-cell lymphomas and are highly aggressive, with advanced stage disease, extranodal involvement, high serum lactate dehydrogenase (LDH) level, high-intermediate to high International prognostic index (IPI) score and with high failure rate with most treatment protocols [71, 72]. Interestingly, DHL and THL with rearrangement of *MYC/ BCL2* respective *MYC/BCL2/ BCL6* belong almost always to the favorable GCB subtype (figure 3) [73].

DHL with rearrangement of *MYC* and *BCL2* is the most common type of DHL and represents about 65% of all cases, while triple-hit lymphoma (THL) with rearrangement of *MYC*, *BCL2* and *BCL6* represents about 20% and DHL with rearrangement of *MYC* and *BCL6* represents about 15% of DHL cases [71]. There are other types of DHL that have been proposed, of these double rearrangement of *BCL2* and *BCL6* [74], *MYC* and *CCND1* [1], and *MYC* and *TP53* [75, 76].

In addition to rearrangement, extra copies of *MYC/BCL2* have been demonstrated in DLBCL. Poor prognosis is reported in DLBCL with double extra copies of *MYC/BCL2* without rearrangement or when there is rearrangement of *MYC* and extra copies of *BCL2* or the reverse i.e. *MYC* extra copies with rearrangement of *BCL2* and those named atypical double-hit lymphomas to differentiate them from the typical double-hit lymphomas with rearrangement of *MYC* and *BCL2* [77, 78].

### Double expressor lymphoma

The use of reliable antibodies to assess MYC and BCL2 over-expression by IHC with cut-offs of 40% for MYC and 50-70% for BCL2 has identified double expression of these two proteins in 20 to 35% of DLBCL cases and is more common in ABC subtype (figure 3) [79]. Most of these tumors do not carry *MYC/BCL2* chromosomal alterations and have been named double-expressor lymphoma (DEL) in contrast to double-hit lymphoma. DELs have inferior outcomes compared to other DLBCL, NOS, but they are not as aggressive as the HGBL, with rearrangements of *MYC* and *BCL2* and/or *BCL6* [80]. In the WHO 2016 Classification, it is suggested that double expression of MYC and BCL2 proteins without gene aberrations should be considered a prognostic indicator in DLBCL, NOS but not as a separate category [20].

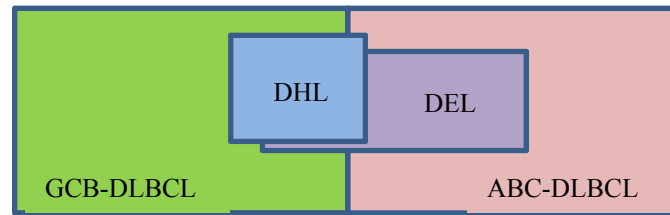


Figure 3. The distribution of DHL and DEL in GCB and ABC subtypes of DLBCL.

### Primary DLBCL of the CNS (PCNSL)

PCNSL is a distinct entity of DLBCL that predominantly affects elderly patients [20]. It accounts for 2-3% of all NHL with increasing incidence in the last three decades [81-83]. Despite improved treatment results for systemic DLBCL, PCNSL still has a dismal outcome [82, 84, 85].

#### Pathogenesis

In contrast to systemic DLBCL in which GEP has identified two main subtypes, the GCB and the ABC, most of the cases of PCNSL belong to the ABC subtype, which may explain the poor prognosis of this distinct entity [86].

Our understanding of the pathogenesis of PCNSL is slowly growing and is challenging due to the rarity of the disease and also on account of the sparse tissue sampling which may limit the powerful work up of most of the single cohort studies and indicates the importance of multicenter collaboration.

A “CNS signature” that can replace the predictive and prognostic role of COO is still largely unclear. Molecular investigations could identify high frequency of mutations in specific genes involved in important pathways that



could be the driver mutations responsible in PCNSL tumorigenesis such as *MYD88*, *CD79A* and the *CARD11* [87-90], dysregulation of factors involved in JAK/STAT pathways such as IL-4, IL-10, and intratumoural *JAK1* [91-94], recurrent chromosomal losses affecting the 6q, 6p21.32 (HLA locus) and copy number alterations (CNAs) such as loss of 9p21.3 (*CDKN2A*) or gain of 9p24.3 [95, 96] and copy number alterations of 9p.24/PD-L1/PD-2 [97].

However, there are still pitfalls that need to be overcome like the mystery why this lymphoma entity develops and is confined to the CNS where very few B-cells, if any, are found under normal circumstances [98], and also whether the B cells home to the CNS in a benign or malignant state.

Malignant transformation may occur in the CNS or outside it. Three hypothesized mechanisms have been suggested by Deckert and colleagues [99]. First, a transformation of B-cells happens outside the CNS and is disseminated but may be eliminated outside the CNS by a specific antitumor immune response, whereas they escape the immune response in the CNS due to the immunoprivileged status of the CNS. Second, transformation of B-cells happens outside the CNS and malignant B-cells acquired a high affinity and tropism for the CNS, which make them home to the CNS. Third, non-malignant B-cells enter the CNS either due to an immune response such as a pathogen inducing inflammation or accidentally when the blood-brain barrier permeability is increased. The B-cells may persist in the brain and they can transform in the CNS leading to the development of lymphoma. The molecular clues of tropism and selective dissemination of DLBCL within the brain have been studied in recent years and probably gene alterations of *SPP1* and *MAG* are two of the most prominent gene alterations that have been shown to be involved in the pathogenesis of PCNSL [100].

*SPP1* is a secreted non-collagenous, chemokine-like glycoprotein and has numerous cellular functions, including cell communication, focal adhesion, immune cell activation, and immune cell migration [91]. *SPP1* is thought to be involved in CNS tropism, B-cell migration, proliferation and aggressive clinical behavior [100]. *MAG* is a cell membrane glycoprotein and member of the immunoglobulin superfamily. *MAG* regulates the interaction of myelin and axons, such as the initiation of myelination and the maintenance of myelin integrity, by expression on periaxonal myelin membrane of the CNS and peripheral nervous system [101, 102]. *MAG* is suggested to play an important role in perineural cancer invasion [100, 103].

## The role of immune system in lymphoma

### Tumor microenvironment in DLBCL

The tumor microenvironment of B-cell lymphomas is characterized by variably robust numbers of immune cells, stromal cells, blood vessels and extracellular matrix [104]. The interaction of tumor cells with their microenvironment is fundamental for tumor cell survival in B-cell lymphoma [105, 106]. The GEP of non-malignant cell populations in DLBCL revealed molecular signatures that correlated significantly with survival after treatment [105]. A stromal-1 gene expression with favorable prognosis reflects extracellular matrix deposition and infiltration of the tumor by macrophages and a non-favorable stromal-2 gene expression was associated with endothelial cells and angiogenesis [105]. These stromal signatures are independent of COO, as they can be seen in both GCB and ABC subtypes [39]. This may explain the inhomogeneous outcome in homogenous cases of GCB and ABC subtypes of DLBCL [28].

Numerous aberrations have been recognized in tumor cells in B-cell lymphoma, which affect interaction between tumor cells and the tumor microenvironment. Recurrent gene mutations, copy number aberrations and translocations in the lymphoma cells are examples of aberrations that reflect the significance of these interactions as important mechanisms of oncogenesis in many of the lymphoma subtypes [104].

### Tumor microenvironment in PCNSL

Tumor cells in PCNSL infiltrate brain parenchyma with a variably heavy inflammatory response with tumor-associated macrophages (TAM) constitute a major stromal component as do reactive T-cells [107]. An interaction between the enhanced production of signal transducer and the activator of transcription 3 (*STAT3*) by malignant B-cells and dense infiltration by CD163-positive macrophages (M2) in the CNS microenvironment has been demonstrated in PCNSL and proposed to have an important role in pathogenesis of PCNSL [107]. *STAT3* plays an important role in tumor progression by inducing anti-apoptotic proteins such as BCL2 [108] and also by allowing tumor cells to escape the immune system by inducing immunosuppressive molecules such as IL-6, IL-10 and transforming growth factor  $\beta$  [109, 110]. It has been reported that higher expression of *STAT3* on lymphoma cells predicts an inferior prognosis in DLBCL [111].

Reactive T-cell infiltrates are present to varying degrees either in the form of scattered lymphocytes or perivascular cuffing occurring alone or in between the vessel wall and malignant cells (reactive perivascular T-cell infiltrate that is shown to be a predictive of favorable outcome (RPVI) [112, 113].

## Immune checkpoint pathways

The immune checkpoint pathways are critical for the maintenance of self-tolerance and also for the protection of tissues from damage when the immune system responds to pathogens under normal physiological conditions [114]. There are different immune checkpoint pathways, of these, Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and Programed Death-1 (PD-1) pathways that block the T cell-mediated immune system at different levels. CTLA-4 essentially regulates initial T-cell activation, whereas PD-1 mainly regulates the immune attack in peripheral tissues at the site of immune effector response [115].

The T-cell is part of the acquired immune system that provides lifelong protection against pathogens. The activation of T-cell reaction is triggered by the recognition of an antigen presented on the major histocompatibility complex (MHC) by the T-cell antigen receptor (TCR). Recognition at the site of inflammation results in an attack against infected tissue. However, in the steady state, all the nucleated cells express self-antigens on MHC and can potentially activate self-reactive T-cells. Most self-reactive T-cells are eliminated in the thymus through a mechanism called “negative selection”; though, many of them escape selection and are present in the periphery and can lead to an attack on self-tissue, leading to autoimmunity. Thus, the importance of immune checkpoint pathways that prevent activation of self-reactive T-cells in the periphery.

To complete the T-cell activation, another set of signals is required, called costimulatory molecules which are mainly provided by activated antigen-presenting cells (APCs). The best-characterized costimulatory system is the CD28 receptor on T-cells and its ligands, namely CD80 and CD86 on activated professional APCs (dendritic cells, macrophages, and B-cells). Upon exposure to antigen, the APCs are activated and CD80 and CD86 are up-regulated which in turn trigger CD28 receptor on T-cells leading to T-cell activation, proliferation, cytokine production, and development of effector functions [116]. The CTLA-4, which has similar structure to CD28, binds to CD80 and CD86 with greater avidity than CD28 and this leads to suppression of T-cell responses [117].

The CTLA-4 is a protein receptor for the APCs protein ligands CD80 and CD86 and it belongs to the immunoglobulin superfamily of proteins. CTLA-4 is encoded by the CTLA-4 gene on chromosome 2 and expressed mainly in T-cells upon activation [118]. The dual physiological role of CTLA-4 by down modulation of helper T-cell activity and enhancement of regulatory T-cell immunosuppressive activity has been a subject of interest for many research studies, to investigate the antitumor effect of CTLA-4 blockade, mainly in solid cancer [119, 120]. Few studies have shown that CTLA-4

may have an essential role for B-cell lymphoma proliferation and survival [121, 122].

PD-1 (CD279) is a protein receptor for the APCs ligands PD-L1 (CD274) and PD-L2 (CD273) and it belongs to the immunoglobulin superfamily of proteins CD28/CTLA-4 [123]. PD-1 is encoded by the PCCD1 gene on chromosome 2 and is expressed on activated CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, NK cells, B-cells, macrophages and some types of dendritic cells (DCs) [123, 124].

Upon activation, PD-1 interacts with its ligands; PD-L1 and/or PD-L2 resulting in inhibition of cell proliferation and production of cytokines, namely IL-2, IL-4, interferon (IFN)- $\gamma$ , and IL-10 [125].

As previously mentioned in this section, the role of PD-1 as an immune checkpoint pathway is mainly to regulate the immune attack in peripheral tissues at the site of immune effector response. However, it is assumed that PD-1 modulates the immune response in initial phases of T-cell activation too. It is believed that T-cells that are stimulated in the absence of the CD28 mediate an incomplete T-cell activation and shift to an unresponsive state called “clonal anergy” and become refractory to further stimulation by the same antigen [126]. However, other studies have demonstrated that the interaction of PD-1 with its ligand PD-L1 results in the so-called “clonal anergy” and maintenance of this phase [127-129]. In addition, PD-1 is suggested to be involved in the regulation of innate immune cells [130].

The PD-1 ligands: PD-L1 and PD-L2 are encoded by CD274 gene and CD273 gene respectively, both located on chromosome 9. PD-L1 is expressed on a wide range of cell types including epithelium, muscle, mesenchymal stem cells, T- and B-cells, DCs, macrophages, and cancer cells, while PD-L2 expression is more restricted and expressed on immune-related cells such as DCs, macrophages, and mast cells [131].

In cancer, the binding of PD-L1 or PD-L2 to its receptor PD-1 inhibits the proliferation of activated T-cells and allows the tumor cells to escape the antitumor adaptive immune response (figure 4) [132].

High expression of PD-1 on leukocytes and PD-L1 on tumor cells is demonstrated to be associated with inferior outcome in solid malignancies [133]. However, the prognostic impact of PD-1 and its ligands PD-L1 and PD-L2 is still unclear in hematological malignancies and several studies with small and large cohorts have been published in the recent years with variable results. Increased numbers of PD-1 positive leukocytes in DLBCL were shown to be associated with superior outcome in two studies [134, 135] whereas in one study on PCNSL, PD-1 was an indicator of poor survival [136]. High expression of PD-L1 on tumor cells was reported to be associated with inferior

outcome in DLBCL [137, 138], while high expression of PD-L1 on leukocytes had no prognostic impact in DLBCL and in PCNSL [137, 139]. Other studies reported favorable prognostic impact associated with high PD-L1 expression on leukocytes [140, 141]. A recent study on DLBCL reported variable results with adverse prognosis associated with increased numbers of PD-1 positive leukocytes and with PD-L1 on leukocytes, while high PD-L2 expression on tumor cells was reported to be associated with better outcome [142]. The diverse results between the above different studies may be partly explained by the use of different antibodies and different methods for evaluation of PD-1, PD-L1 and PD-L2 expression.

In addition to the PD-1 and CTLA-4 pathways, there are numerous other immune checkpoint pathways that can be targeted clinically with monotherapies and in treatment combinations like Lymphocyte activation gene 3 (LAG3), T-cell immunoglobulin-3 (TIM-3) and B- and T- lymphocyte attenuator (BTLA) [131, 143, 144].

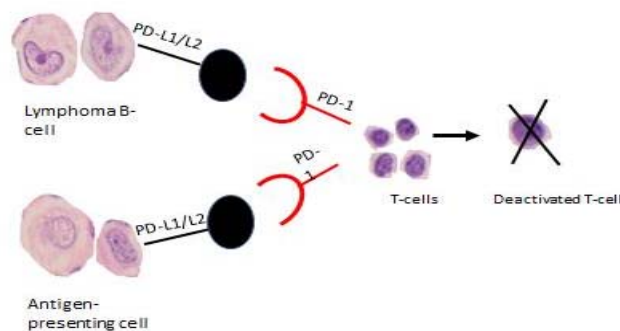


Figure 4. The activated T-cells are deactivated via the PD-1 pathway by interaction of PD-1 with its ligands PD-L1/ PD-L2 on antigen presenting cells (APC) and lymphoma B-cells in DLBCL.

### Indoleamine 2, 3 dioxygenase (IDO)

IDO is an intracellular cytosolic enzyme that is encoded by the *IDO1* gene, located on chromosome 8 [145]. The IDO enzyme is expressed by different types of cells such as endothelial cells, APC, fibroblasts, macrophages and DCs [146, 147]. There are two types of IDO enzyme in the human body, IDO1 and IDO2. IDO2 is more restrictedly expressed than IDO1 and has only 3-5% the enzymatic activity of IDO1 [148, 149] and it is not as well investigated as IDO1.

The role of IDO in immune tolerance was demonstrated by Munn, Mellor and their colleagues who presented IDO expression by placenta cells to prevent maternal T-cells from destroying the fetus during pregnancy, thus preventing fetus rejection *in utero* [150-152]. It is assumed that the same concept might explain the role of IDO in the immune escape of tumor cells based on the preferential sensitivity of T-cells to tryptophan deprivation [153].

The initial observation of IDO association with cancer was first reported in 1956 when catabolism of tryptophan was found to be elevated in bladder cancer [154], but the impact of this association was obscure. IDO is involved in the kynurenine pathway and is responsible for degrading the essential amino acid tryptophan into L-kynurenine [155]. Tryptophan deprivation results in blockade of T-cell proliferation by inducing cell cycle arrest of T-cells and increases their apoptosis [156]. In addition, increasing amounts of tryptophan metabolites, especially Kynurenine, are found to be toxic to lymphocytes and this activates the transcription of the aryl hydrocarbon receptor (AHR)[157] which in turn induces the CD4<sup>+</sup> T-cells differentiation to immunosuppressive regulatory T-cells [158].

## Clinical aspects and diagnostic approach of DLBCL

### Clinical features

Most DLBCL patients present with a rapidly growing mass involving lymph node and or extranodal site [159]. Approximately 40% of cases manifest as extranodal disease [160] and 50% present with stage III-IV disease. About one-third of patients present with at least one of the B-symptoms: night sweats, fever, weight loss. Serum LDH and beta-2-microglobulin are often increased above normal level.

### Radiological examination

Positron emission tomography (PET) in combination with contrast enhanced computer tomography (CT) is superior to either PET or CT alone [161]. PET/CT is a highly sensitive and specific imaging study for the assessment of extent of disease for staging and assessment of response to treatment [162]. However, a contrast enhanced CT scan is also recommended if measuring size of nodes is important [163].

Numerous studies have raised the superior benefit of radiological examination compared to bone marrow biopsy (BMB) for staging of DLBCL [164-166]. However, the histology of bone marrow involvement is of prognostic significance, in which concordant involvement i.e. bone marrow involvement by DLBCL predicts a worse overall survival while discordant involvement i.e. bone marrow involvement by low grade B-cell lymphoma has no prognostic impact [167-169], hence BMB remains to be a standard test for staging of DLBCL particularly when PET is negative or not available. Recent studies have demonstrated that PET is a prognostic indicator irrespective of IPI and COO and can predict survival and response to immunochemotherapy in DLBCL patients [170, 171].

## Histopathological examination

The primary aim of histopathological classification of lymphoma is to identify morphological, immunophenotypical and genetic characteristics that can identify lymphoma subtypes with different clinical behaviors in order to facilitate risk-adapted stratification of treatment [172, 173].

According to the WHO, accurate diagnosis of lymphoma is established on morphology, IHC, and flow cytometry reviewed by an experienced hematopathologist [1]. According to European society for medical oncology (ESMO) guidelines a surgical excisional biopsy (SEB) is preferred to provide adequate tissue for these examinations and for further molecular studies to accurately categorize the lymphoma. However, a core-needle biopsy (CNB) can be considered when excisional biopsy is not possible [174] such in patients with deeply located lymph node or extranodal mass as in the abdominal cavity or mediastinum in which SEB is difficult to perform or in patients with comorbidity and where surgery would entail excessive risk. A fine-needle aspirate (FNA) should not be used as the only primary assessment analysis for diagnosis of DLBCL [174]. In a multi-institution clinical study very poor accuracy between FNA and SEB was shown with only 12% accuracy suggesting that FNA is neither useful for primary diagnosis of lymphoma, nor cost effective, and in addition it may misguide treatment [175]. SEB allows evaluation of lymph node architecture, growth pattern and cellular composition which are mandatory for accurate diagnosis and classification of lymphoma [1].

CNB and FNA are said to be safe, fast, and cost-effective and are regarded by many studies to be an appropriate alternative to SEB to evaluate lymphoma patients. However, the safety discussed in these studies was regarding the procedure itself rather than the safety of acquiring correct diagnosis and establishing appropriate treatment. The cost-effectiveness is also a questionable matter, it is important to evaluate the quality of material obtained by CNB and how often these biopsies are retaken to achieve an accurate judg-

ment. Repeated procedures and delay in diagnosis and treatment are not cost-effective.

Frederiksen and colleagues reviewed 42 publications between 1989 and 2012 which were aimed at studying the effectiveness of CNB and FNA in the diagnosis of lymphoma. The review concluded that FNA and CNB need to be followed by a SEB to achieve definitive diagnosis and classification of lymphoma in 25% to 35% of cases and that the role of FNA and CNB in lymphoma should be reserved for the staging and assessment of recurrent disease rather than primary diagnostic process [176].

The main goal of the accurate primary diagnosis of lymphoma is to establish appropriate treatment which is often urgent. CNB and/or FNA are fast but the more important question is whether they are correct. It is alarming to see that there are hundreds of studies that suggest CNB and FNA as appropriate tools for the diagnosis of lymphoma and they have become the frontline diagnostic procedures in the recent years [177, 178]. However, most of these studies are single-center studies and they lack the discussion of the follow up and outcome of patients included in these studies.

## Prognosis

DLBCL is a heterogeneous group of aggressive B-cell lymphomas with variable clinical course and outcome. In the era of rituximab, the disease is cured in about 60%-70% of patients. The remaining 30-40% patients are either refractory or relapse shortly after remission. The recognition of patients within the curative or refractory group at time of primary diagnosis is of paramount importance for the choice of accurate treatment. There are different clinical and biological prognostic parameters that have been developed to distinguish patients in either group.

The IPI is the most commonly used score since it was designed in 1993 [179]. The IPI score includes five variables: age ( $>60$ ), Eastern Cooperative Oncology Group performance status (ECOG) (0-1, 2-4), serum LDH level ( $>$ normal level), number of extranodal sites (0-1,  $\geq 2$ ), and Ann Arbor stage (stage I-II, III-IV). DLBCL patients were divided into four risk groups according to their IPI score: low, low-intermediate, high-intermediate, and high risk. However, in the era of rituximab, the outcome of the patients improved and the utility of IPI needed to be justified to maintain its powerful stratification. An age adjusted IPI has been suggested (aa-IPI), which includes three parameters for each age category (over or under 60 years): LDH ( $\leq$  the upper normal limit (UNL) vs  $>$  UNL; Ann Arbor stage ( $\leq 2$  vs  $>2$ ) and ECOG ( $\leq 1$  vs  $> 1$ ).



The National Cancer Center Network (NCCN) demonstrated that a more appropriate split of age into four categories (< 40, 40–60, 61–75 and > 75-years-old) and LDH into three categories ( $\leq$  UNL, UNL-3xUNL and  $>$  3xUNL) could better stratify risk DLBCL patients receiving chemoimmunotherapy [180]. In the NCCN-IPI, specific extranodal sites are of more prognostic significance than the number of extranodal sites. Disease in bone marrow, central nervous system, liver, gastrointestinal tract or lung were shown to be relevant parameters associated with prognosis [180].

In PCNSL, Memorial Sloan-Kettering Cancer Center (MSKCC) is used to identify three prognostic groups based on age and Karnofsky performance status (KPS): class 1 includes patients <50 years; class 2 includes patients  $\geq$ 50 years and KPS $\geq$ 70; and class 3 includes patients  $\geq$ 50 years KPS<70 [181].

These score systems are based solely on clinical parameters and new prognostic parameters that reflect tumor heterogeneity need to be incorporated for the stratification of accurate therapy.

## Treatment

### Frontline treatment

Although rapidly fatal if left untreated, DLBCL is potentially curable. Introduced in the 1970s, chemotherapy with (CHOP) definitely changed the treatment options for NHL, prolonging patients' lives and providing better quality of life [182, 183].

In the era of the monoclonal anti-CD20 antibody rituximab, which was introduced in the 1990s, about 60–70% of DLBCL patients treated with a combination of Rituximab and chemotherapy regime (R-CHOP) are cured [184].

### Treatment of patients with refractory/ relapse disease

Despite the significant improvements obtained with R-CHOP, 10 to 15% of patients exhibit primary refractory disease (non-response or relapse within 3 months of therapy) and an additional 20 to 25% relapse following initial response to therapy. Most relapses occur within the first two years, however, about 10% of all progressions occur  $>$ 5 years after treatment [39].

Among patients who progress during initial immunochemotherapy or soon after a brief complete remission (CR), only 30 to 40% will respond to sal-

vage chemotherapy and may subsequently undergo consolidation with autologous stem cell transplantation (ASCT) [185-187].

Dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin with rituximab (DA-EPOCH-R) is another dose-intensive regimen that has shown encouraging results in DLBCL [188, 189].

Chimeric antigen receptor modified T-cell (CAR T-cells) is a revolutionary method for the treatment of malignant tumors. Immune cells (T-cells) from the patient are collected from the blood, engineered in the laboratory and genetically modified to express a chimeric antigen receptor (CAR) that modifies antigen specificity and activates T-cells to better fight the tumor cells, to then be reinfused into the patient [190]. Several clinical studies have shown that B-cell antigen CD19-targeted CAR T-cells form an excellent and potent target with satisfactory achievement in refractory/ relapse DLBCL [191]. CD20-targeted CAR T-cells have been developed and small studies have reported promising results among patients with refractory/relapse DLBCL [192, 193]. However, this type of treatment is highly toxic, expensive and is not available in most of the oncology centers.

Polatuzumab vedotin is a monoclonal antibody against CD79b covalently conjugated to the anti-mitotic cytotoxic agent monomethyl auristatin (MMAE) via a cleavable linker. After infusion, polatuzumab vedotin binds to CD79b on the tumor cell (B-cell surface), is internalized inside the tumor cell and the linker is cleaved to release MMAE, where it inhibits division and induces apoptosis of the tumor cell [194]. Recent studies have presented the beneficial effects of Polatuzumab in combination with chemotherapy and rituximab in DLBCL patients with refractory/ relapse disease [195, 196].

### Precision treatment with novel therapies in DLBCL

As stated, DLBCL is a heterogeneous disease and the standard immunotherapy is not suitable for all patients, which is confirmed by the refractory/relapse rates in about 30 to 40% of patients. Recently, novel therapies have been developed targeting different specific molecular pathways involved in the pathogenesis of DLBCL with the aid of GEP and NGS [197].

#### **Targeted therapy for the GCB-DLBCL**

The GCB-DLBCL subtype is critically dependent on the intrinsic oncogene *BCL6*, making the BCL6 protein an ideal target for specific therapy in this subset of lymphomas. Pre-clinical studies of BCL6 inhibitors are encouraging. A small BCL6 inhibitor, 79-6, was designed with potent inhibitory activity against DLBCL [198]. The combination of this BCL6 inhibitor and R-CHOP has been demonstrated. Other target agents investigated in clinical trials are EZH2 inhibitors since 20 to 25% of GCB-DLBCLs display *EZH2* mutations [199, 200].

### Targeted therapy for the ABC-DLBCL

Multiple therapies targeting the NF- $\kappa$ B pathway or the B-cell receptor signaling pathway under evaluation are promising for ABC-DLBCL [39]. Bortezomib, a proteasome inhibitor that blocks the degradation of I $\kappa$ B $\alpha$  (an inactivating protein for NF- $\kappa$ B), has demonstrated selective benefit within ABC-DLBCL when combined with DA-EPOCH-R in patients with relapsed DLBCL [201]. Drugs targeting various components of the B-cell receptor cascade (including BTK, SYK, PKC $\beta$  and PI3K) are under evaluation. Single agent ibrutinib, a potent BTK inhibitor, showed remarkable activity in patients with relapsed ABC-DLBCL with a response rate of 41% compared to 5% in GCB-DLBCL [197, 202].

Idelaisib, a PI3K  $\delta$  inhibitor, recently showed potent clinical efficacy in indolent refractory or relapsed B-cell lymphoma but has not been fully investigated in DLBCL [203, 204]. However, based on the frequent activation of PI3K/ACT/MTOR pathway in GCB-DLBCL, this drug could be used in the treatment of that subtype [39, 202].

Spleen tyrosine kinase (SYK) is an adaptor that initiates and amplifies the BCR pathway. Fostamatinib disodium (prodrug of R406), a SYK inhibitor, has initially shown promising activity in recurrent lymphoma with various histology subtypes and has demonstrated a response rate of 22% in refractory DLBCL [205, 206].

*MYD88* mutation is found in approximately one-third of ABC-DLBCL, initiating a constitutive NF- $\kappa$ B activation through another pathway, other than that of the BCR signaling cascade. *IRAK4* is one adapter constitutively activated by the mutation responsible for NF- $\kappa$ B activation. Currently, IRAK4 inhibitors have been successfully tested *in vitro* in DLBCL cell lines and might constitute promising treatment in ABC-DLBCL [207].

Another type of drugs showing promising efficacy in the treatment of DLBCL both as a single agent and in combination with immunotherapy or immunochemotherapy is Lenalidomide, which is an immunomodulatory drug with antiangiogenic activity and an inhibitory effect on NF- $\kappa$ B pathway [208]. Interestingly, a preferential activity has been reported in relapsed/refractory ABC-DLBCL as compared to GCB-DLBCL with a 52% vs 9% response rate [209].

### Immune checkpoint blockade

T-cells are regulated in both the priming phase where CTLA-4 has an important impact on regulating the activation of T-cells and the effector phase where the PD-1 present on the T-cell interacts with the ligands PD-L1 and PD-L2 on APC and/or tumor cell. Immune checkpoint molecules on the T-

cell surface upon interaction with their ligands can shut down the activity of the T-cell and help the tumor cell to escape the immune response. The main principle of immune checkpoint inhibitors is to block the receptor or their ligands and thus prevent the negative signal and restore the activity of the T-cells.

Both CTLA-4 and PD-1 pathways are being targeted in an increasing number of solid tumors and hematological malignancies [210].

In 2011, blockade of CTLA-4 using the monoclonal antibody ipilimumab was the first immune checkpoint inhibitor approved by the U.S. Food and Drug Administration (FDA) for the treatment of patients with advanced malignant melanoma [211]. A phase I clinical trial of ipilimumab in 18 patients with relapsed/refractory B-cell lymphomas included 3 patients with DLBCL. Two of these patients had clinical responses and one achieved a complete response that lasted more than 31 months [212]. However, the role of the CTLA-4 pathway in DLBCL is still largely unexplained and further research needs to be evaluated. About 25% of DLBCL express PD-L1 and PD-L2 that correlate with amplification of 9p24.1 making them potential targets for PD-1 inhibitors [213].

High response rate has been demonstrated in relapsed and refractory CHL and encouraging results in PMBCL. The U.S. FDA approved monoclonal anti-PD-1 antibody (nivolumab) for treatment of refractory or relapsed CHL in 2016 [214], and monoclonal anti-PD-1 antibody (pembrolizumab) for the treatment of adult and pediatric patients with refractory or relapsed PMBCL in 2018.

Another study demonstrated that PD-1 blockade restores T-cell function in vitro in EBV-positive DLBCL patients [215]. One study on five patients with PCNSL showed response in all patients when treated with PD-1 blockade [216]. In a phase 1 trial testing nivolumab as monotherapy in R/R lymphoid malignancies, 18% of 11 patients with DLBCL achieved CR and 18% achieved partial remission (PR) [217].

Despite the essential role of the PD-1/PD-L1 pathway and the promising results of PD-1 blockade in DLBCL, clinical trials have demonstrated that there is a subset of patients who did not respond or progress after short response [217]. Another challenging complication is the immune-related adverse events that may affect almost all tissues. Additional research is therefore needed and further clinical trials are required to overcome the disease resistance to the PD-1 blockade and the immune-related adverse effects.

## Future visions

### Diagnosis

#### **“The future is here”**

The heterogeneity of DLBCL may represent the key to improving outcome for patients in the refractory/relapse group. Upon primary diagnosis of DLBCL, the use of GEP and NGS is a future model of diagnostic work and precision therapy can be designed which can suit the patients accordingly. At the present time an important consideration is to ensure adequate tissue samples at the time of primary diagnosis to ensure highly qualified diagnosis and classification and to ensure that there is adequate tumor tissue for the molecular analysis.

New technology has recently been introduced in the research work namely liquid biopsy which is based on detecting tumor DNA particles in blood. There are two major types of research regarding this technology. The first is to detect gene mutations currently detected by tissue analysis that can aid in the design of precision therapy [218] and the second is to assess response to treatment by detecting the presence of residual disease or early relapse [219].

### Treatment

#### **“The war is a trick”**

The war on cancer which started early in the 1970s is continuing and the future is very promising. Again the heterogeneity of DLBCL may represent the key to improving outcome for patients by designing treatment that can efface the different mechanisms involved in the pathogenesis of the disease. The immune system plays an important role in the tumorigenesis and progression of DLBCL. Thus, modulating the immune system is an attractive treatment option, and new concepts have been proposed to manipulate the immune system to preserve its main function, that is to protect the host not the cancer, such as the CAR T-cells, and immune checkpoint inhibitors.

Anti-cancer vaccine therapy is a strategy that is proposed to re-educate host immunity to recognize and target tumor cells. The main principle of vaccine therapy is based on the priming of T-cells that often need co-stimulatory signals from APC. When APC are exposed to large amounts of immunogenic tumor peptides they will present them for T-cells and provide the co-stimulatory signals. This can be done in vitro by fusing APC with tumor cells or by beating APCs with tumor fragments, or in vivo with peptide vaccines [220].

Unfortunately, tumor cells can induce T-cell dysfunction and maintain tumor cell immunosuppressive effects, resulting in failure of clinical trials of tumor

vaccine therapy and adoptive T-cell therapies [221]. Consequently, the most effective strategy may be to use combination therapies such as anti-cancer vaccines with checkpoint blockade to target critical aspects of this environment in an effort to prevent the re-establishment of tumor tolerance while limiting toxicity associated with autoimmunity [220].

Clinical studies on vaccine therapy have demonstrated immune response and evidence of clinical effect in a subset of patients with follicular lymphoma [222]. According to our knowledge, there are no published clinical studies on vaccine therapy in DLBCL.

# Aims

## Overall aim

To identify prognostic factors that can predict survival in DLBCL such as tumor markers, tumor microenvironment markers, site of tumor involvement in order to better identify different risk groups of DLBCL patients.

## Specific aims

I - To determine the COO and double expression of MYC and BCL2 by IHC and assess their prognostic significance within a population-based cohort of DLBCL patients uniformly treated with R-CHOP.

II - To determine the COO by GEP and IHC, to assess the concordance between these two methods and to estimate the best predictor of survival as well as to assess the distribution of double expression of MYC and BCL2 in GCB and non-GCB by IHC and GCB and ABC by GEP in DLBCL patients uniformly treated with R-CHOP.

III - To investigate the clinical and tumor biological characteristics including COO and double expression of MYC and BCL2 in DLBCL patients with abdominal lymph node involvement and to assess the prognostic impact of this site of tumor involvement.

IV - To explore the immunophenotypic features of tumor microenvironment in PCNSL and to identify the significance of these markers in this specific and highly aggressive entity of DLBCL.

# Patient materials and methods

## Patients in papers I-IV

These studies were approved by the Regional Ethical Review Board in Uppsala, Sweden (papers I, III and IV), Sweden and Denmark (paper II). All clinical data were retrieved from patient records. Diagnosis of DLBCL was re-evaluated according to the 2008 WHO classification by at least two expert hematopathologists. Patients with previous history of low grade B-cell lymphoma, PCNSL, immunodeficiency-associated lymphoproliferative disorders (PTLD), BCLU, and PMBCL were excluded. Clinical scoring was based on the aa-IPI with one point for each of the following: (1) Ann Arbor stage III-IV; (2) ECOG performance status 2-3; (3) elevated serum LDH, where 0-1 is considered to be low risk and 2-3 is considered to be high risk. All patients were homogeneously treated with R-CHOP or R-CHOP like regimens.

In paper I [223], a total of 188 patients with diagnosis *de novo* DLBCL between 2002–2012 at the Department of Pathology, Uppsala University hospital were included.

In paper II [224], a pool cohort of 359 patients with diagnosis *de novo* DLBCL between 2004-2015 in Sweden and Denmark were included; with 92 patients from Uppsala from the cohort in papers I and III for whom there was adequate tumor material for assessment of COO by GEP and IHC, 136 patients from Gothenburg, 46 patients from Umea and 85 patients from Denmark.

In paper III [225], the same cohort in paper I was included in addition to newly diagnosed DLBCL patients between 2012-2015 and data of a total of 249 patients were available for radiological re-evaluation.

In paper IV, 132 patients with the diagnosis PCNSL between 1996-2015 at the Department of Pathology, Uppsala University hospital were included. All clinical data were retrieved from patient records. Patients with a history of immunodeficiency syndrome were excluded. Data on clinical characteristics and outcome of 96 of these patients have previously been published [83]. Patients were classified into three prognostic groups according to Memorial Sloan-Kettering Cancer Center (MSKCC) based on age and Karnofsky performance status (KPS): class 1 included patients <50 years; class 2 included



patients  $\geq 50$  years and  $KPS \geq 70$ ; and class 3 included patients  $\geq 50$  years  $KPS < 70$ . In 45/132 cases a block of tissue microarray (TMA) was made from those of whom there was adequate tumor material to perform IHC, FISH, and RNA sequencing. The DLBCL cohorts in paper I-III and PCNSL in paper IV are summarized in a flowchart (figure 5).

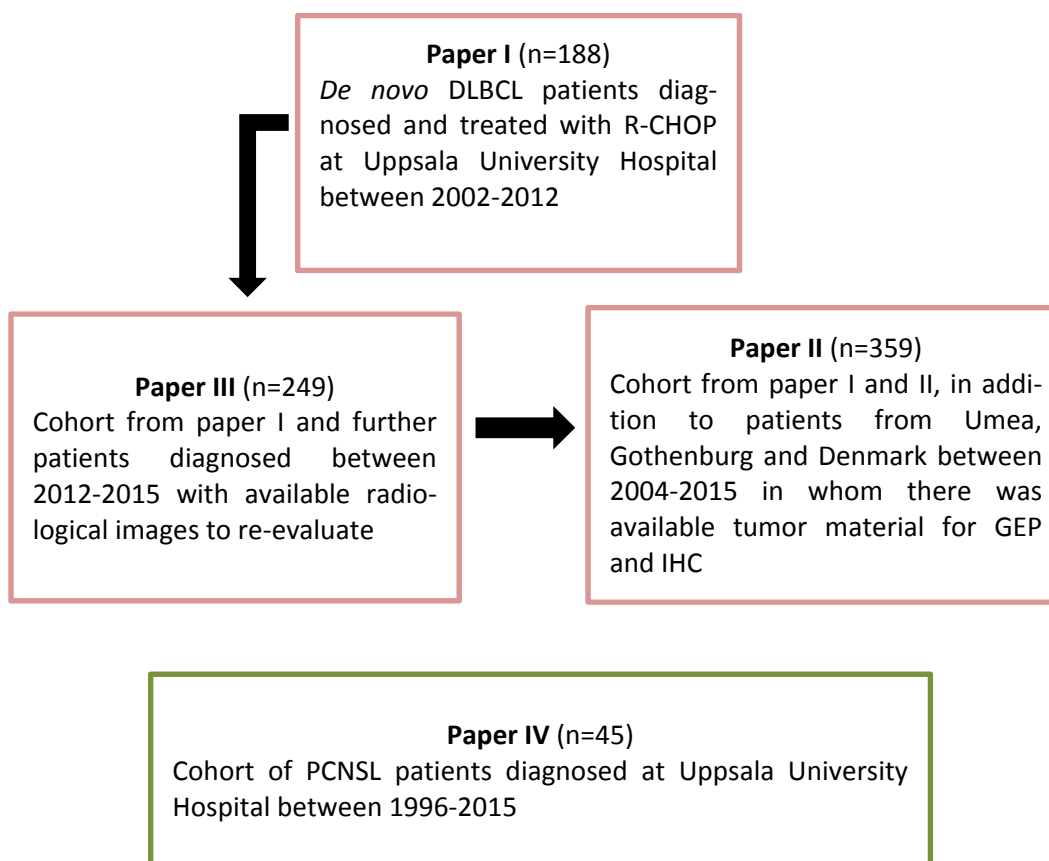


Figure 5. Flowchart of the DLBCL cohorts in paper I-III and of the PCNSL cohort in paper IV.

## Tissue microarray (TMA)

In papers I-IV, TMA construction was performed partly in some cases using a manual tissue array (MTA-1; Estigen OU Tiigi 61b 50410 Tartu Estonia). Briefly, two 1.0 mm tissue cores were taken from areas representative of the tumor and put in a new recipient block.

## Immunohistochemical stainings

IHCs were performed on FFPE material in all papers, using fully automated protocols (DAKO Autostainer Link 48) and evaluated semi-quantitatively. Staining protocols with antibodies to MYC (clone Y69; Abcam), BCL2 (clone 124; DAKO), BCL6 (clone PG-B6p; DAKO), Ki-67 (MIB-1; DAKO), MUM-1 (clone MUM1p; DAKO), CD10 (clone 56C6; DAKO), p53 (DO-7; DAKO), and CD68 (clone KP1; DAKO) were followed.

In paper IV immunostainings for PD-1, PD-L1, PD-L2, and IDO1 were performed manually. Immunostainings for PD-L1 and PD-L2 were performed using double staining for PD-L1/PAX5 and PD-L2/PAX5 to differentiate the expression of PD-L1 and PDL-2 on leukocytes and tumor cells.

## Evaluation of immunohistochemical stainings

All markers were evaluated manually under microscope. In **papers I-IV**, classification of cases into GCB and non-GCB was based on the Hans algorithm using three immunostainings; CD10, BCL6 and MUM1 with cutoff of 30% for each. Cutoff values of 70% and 50% were used for Ki67 and P53 respectively. Optimal cut cutoff values for MYC and BCL2 in **paper I** and for PD-L1 and PD-L2 in **paper IV** were determined by receiver operating characteristic (ROC) curves with Youden's index calculated for each marker. The following cutoff values were used: 40% and 70% for MYC and BCL2 respectively, 85% PD-L1 and 6% PD-L2 for macrophages, and 80% PD-L1 and 60% PD-L2 for tumor cells. In **paper IV**, evaluation of expression of PD-1 in tumor infiltrating lymphocytes (TIL) was manually evaluated semi-quantitatively in 40x magnification in 10 high-power fields (HPF) and grouped as: 0: no positive cells/HPF; 1: <10 positive cells/HPF; 2: 10-30 positive cells/HPF; 3: >30 positive cells/HPF. IDO1 expression was assessed as: negative; focal positive; and diffuse positive expression. Assessment of positive tumor cells and macrophages for IDO1 was based on morphology and comparison with immunostainings for B-cell marker (CD20) and macrophage marker (CD68).

## GEP in paper II

### RNA extraction and NanoString assay

RNA was extracted from FFPE tissue according to the AllPrep DNA/RNA Mini Kit for FFPE protocol (Qiagen, Hilden, Germany). Samples were analyzed with the Lymph2CX assay on a NanoString instrument according to the manufacture's instructions. The dataset was analyzed using the research use only (RUO) version of the NanoString Lymphoma Subtyping Test

(LST), which is based on the Lymph2Cx assay, to determine the COO molecular subtype of each sample. The LST algorithm measures the geometric mean of five housekeeping genes (HK geomean) to ensure RNA quality based on a pre-defined clinical QC threshold of 128. An HK geomean value below 64 was deemed as insufficient RNA quality to provide a subtyping result. A value between 64 and 128 was considered to be borderline quality since it complies with previously published thresholds for RNA quality within clinical research studies, but does not comply with the clinical QC threshold of 128 for individual patients. Each sample surpassing the QC threshold was reported as one of the two molecular subtypes, ABC, GCB, or UC within an equivocal zone.

### Radiological examination in paper III

In paper III, patients were re-evaluated using a contrast-enhanced computed tomography (CT) of the thorax and abdomen. On axial images, the long and short axis of abdominal lymph nodes were measured. They were defined as pathological abdominal lymphadenopathy if two or more of them had a long axis  $\geq 15$ mm and a short axis  $> 10$  mm, and as “bulky” if the single lymph node or the conglomerate mass of lymph nodes was greater than 75 mm in diameter.

### In situ hybridization for Epstein-Barr virus-encoded RNA in paper IV

EBV was detected by *in situ* hybridization with a fluorescein-labeled oligonucleotide probe intended to identify cells expressing Epstein-Barr encoded RNA (EBER) in the TMA cohort.

### FISH for *MYC*, *BCL2* and *BCL6* in paper IV

Interphase FISH was performed on 4  $\mu$ m thick tissue sections using split signal DNA probes for BCL2/18q21 (Vysis BCL2 Break Apart FISH probe kit Ref number 05N51-020) and for MYC/8q24 (Vysis MYC Break Apart FISH probe kit Ref number 01N63-020) and for BCL6 (Vysis LSI BCL6 Dual color Break Apart rearrangement probe kit Ref number 01N23-020) according to the manufacturer’s instruction. Digital images were captured with Olympus BX-51-microscope (Prior Lumen200 light source) and Gen ASIs Capture and Analysis Platform software from Applied Spectral Imaging.

## RNA extraction and quality assessment in paper IV

Extraction of total RNA from FFPE sample was performed using the same kit in paper II (Qiagen, Valencia, CA) and the AllPrep DNA/RNA FFPE Kit (Qiagen, Valencia, CA), respectively. On-column DNase digestion (Qiagen, Valencia, CA) was performed to remove genomic DNA. Total RNA was eluted in 30  $\mu$ l RNase-free water. RNA quantity was measured by fluorometric quantitation using the Qubit RNA HS assay kit (ThermoFisher Scientific, Waltham, MA) while RNA integrity was assessed with the 4200 TapeStation System (Agilent Technologies, Waldbronn, Germany) using the DV<sub>200</sub> metric. Samples were stored in -80°C directly after extraction.

## Library construction and RNA sequencing

The Illumina TruSeq RNA Exome protocol (Illumina, San Diego, CA) was used to prepare RNAseq libraries according to the manufacturer's protocol. For samples with low RNA quality (DV<sub>200</sub> 30-50%), 100 ng input RNA was used for library construction while for medium (DV<sub>200</sub> 50-70%) and high (DV<sub>200</sub> >70%) quality samples, 40 ng and 20 ng input RNA was used respectively. Libraries were sequenced on a HiSeq2500 (Illumina, San Diego, CA) with a 2x126 setup using 'HiSeq SBS Kit v4' chemistry at the SNP&SEQ platform at the Science for Life Laboratory, Uppsala, Sweden. The Bcl to FastQ conversion was performed using bcl2.fastq\_v2.19.1.403 and fastq files were processed using the NGI-RNAseq pipeline (<https://github.com/SciLifeLab/NGI-RNAseq>). In brief, the NGI-RNAseq pipeline is a best-practice analysis pipeline used for RNA sequencing data at the National Genomics Infrastructure at SciLifeLab Stockholm, Sweden. The pipeline uses Nextflow, a bioinformatics workflow tool. It pre-processes raw data from fastq inputs, aligns the reads and performs extensive quality control on the results.

## Statistical analysis

Survival outcomes were defined as overall survival (OS) when time is estimated from diagnosis to death from any cause, lymphoma specific survival (LSS) when time is estimated from diagnosis to death from lymphoma and progression-free survival (PFS) when time is estimated from diagnosis to progression of lymphoma or death from any cause. The Kaplan-Meier method was used for survival estimates with log rank as significance test when comparing survival in different groups. Cox proportional hazard model was used to calculate hazard ratios (HR) with 95% CI as estimates of risk. ROC curves were constructed to find the optimal cutoff values for MYC and BCL2 in paper I and PD-1, PD-L1 and PD-L2 in paper IV. Tabulated values were compared using Chi-square or Fisher's exact test when applicable in papers I- IV. Student's t-test was used to compare means between groups and Pearson's test was applied to determine correlative associations between parameters in paper II. The significance level was set to  $p < 0.05$ .

# Results and discussions

## Paper I

In this retrospective study based on a cohort of R-CHOP treated de novo DLBCL patients, the COO was determined by IHC and GCB subtype was demonstrated in 52% of cases and non-GCB subtype in 37% of cases. There was no significant difference in survival between these two subtypes.

A co-expression of MYC  $\geq 40\%$  and BCL2  $\geq 70\%$  proteins (figure 6) was detected in 27% of patients with no statistically significant difference in co-expression of MYC and BCL2 between the GCB and non-GCB cases. The concurrent expression of MYC and BCL2, known as DEL was correlated significantly with inferior survival (OS, LSS and PFS).

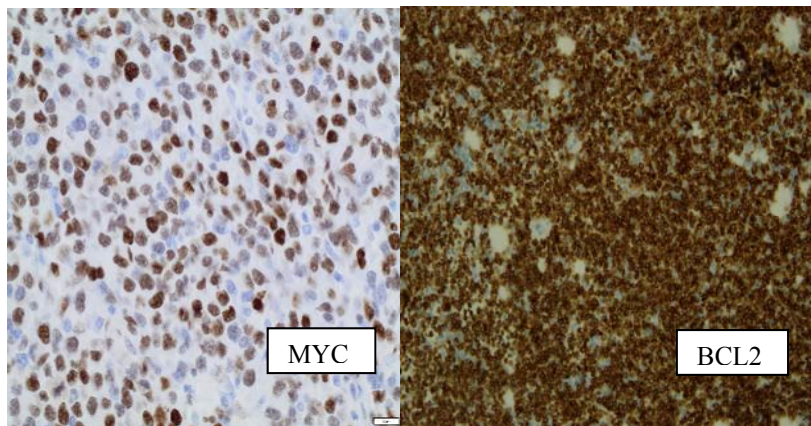


Figure 6. Immunostainings for MYC and BCL2 in a DLBCL patient with concurrent expression of MYC and BCL2.

The five-year OS was 54% in patients with DEL compared to 74% in patients without concurrent expression of MYC and BCL2, and 31% of patients with DEL died within the first two years after diagnosis compared to 12% patients without concurrent expression of MYC and BCL2 who died within the same period (figure 7). This finding is an indicator of the importance of assessment of MYC and BCL2 expression status at time of diagnosis to ensure appropriate treatment and follow up particularly in the first two years after diagnosis.

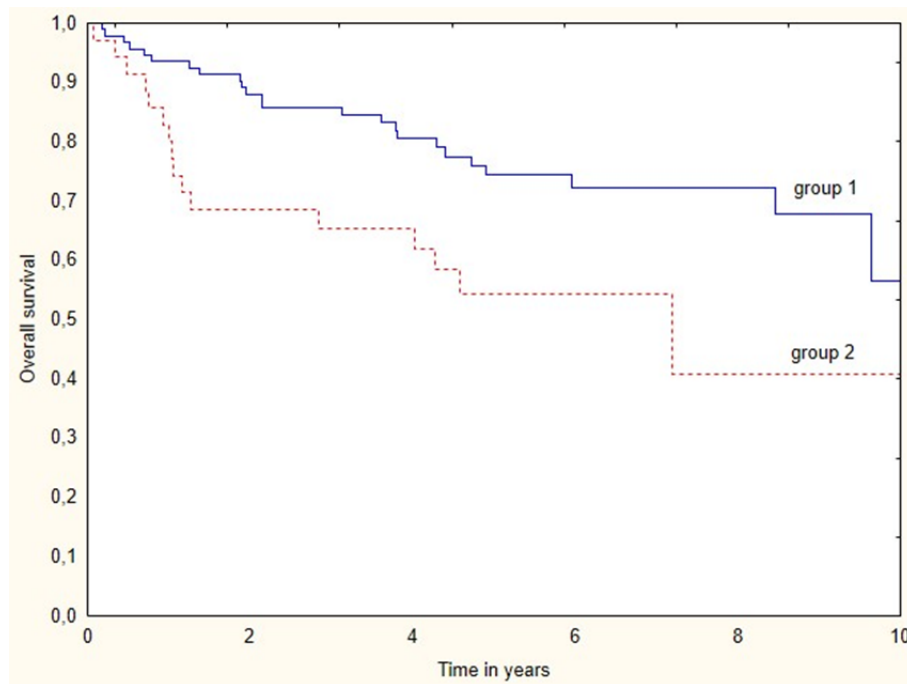


Figure 7. OS in DLBCL patients with double expression of MYC and BCL2 (group 2, n=35) and in DLBCL patients without double expression of MYC and BCL2 (group 1, n= 93).

The inferior outcome among DLBCL patients with concurrent expression of MYC and BCL2 was reported by previous studies [80, 226-228] and was taken into consideration in the updated 2016 WHO Classification which indicates that the status of MYC and BCL2 protein expression has to be described in the pathology report, however, it does not represent a separate entity [20].

Unfortunately, we could not perform FISH to identify DLBCL with translocation of MYC, BCL2 and/or BCL6 which has the worst prognosis and is considered as a separate entity in the 2016 WHO Classification; known as “high grade B–cell lymphoma with rearrangement of *MYC*, *BCL2* and/or *BCL6*” [20]. The main reason was due to the sparse tissue material in most of cases for whom the diagnosis was made on CNB. We searched for the indications of taking CNB and the main reason was due to the challenging site of tumor involvement. With this finding, we proceeded to investigate the site of biopsy in correlation to other clinical and biological parameters (paper III).

In this study we confirmed the importance of IHC for MYC and BCL2 in determining prognosis in DLBCL. As several molecular mechanisms could

drive the overexpression of *MYC*, *BCL2* and *BCL6* at the protein level, IHC is still an advantageous analytic method compared to FISH, even though it cannot identify which patients are harboring *MYC* and *BCL2* translocation. The advantage of using IHC is that the method is rapid, inexpensive and available in most clinical laboratories.

## Paper II

GEP was introduced in early 2000 as an appropriate method to identify two distinct molecular subgroups of DLBCL; i.e. the GCB and ABC and a third small group of cases that are not identical to either GCB or ABC known as UC, based on the COO of tumor cells [28] and that patients within the GCB subgroup show superior outcome compared to patients within the ABC subgroup [229]. The major limitation of using this method in clinical practice is the requirement of fresh frozen material.

Many IHC algorithms have been proposed to replace GEP to identify those two subgroups. However, these IHC algorithms are not fully concordant with GEP mostly because of their inability to recognize UC cases.

NanoString technology with the application of the lymph2Cx assay enables digital GEP on FFEP material with a strong concordance to the original COO model [53, 230]. Thus, the advantage of this method in the better identification of the three molecular types may suggest using NanoString technology in clinical practice instead of the IHC algorithms.

In this retrospective study of a Swedish and Danish cohort of homogeneously treated DLBCL patients we assessed the COO by both GEP using NanoString technology with application of lymph2Cx assay and by IHC using the Hans algorithm on FFEP material, the aim being to evaluate the concordance between these two methods and to identify the best survival predictor.

High correlation between the lymph2Cx assay and the Hans algorithm was observed, particularly when UC were excluded, with 83% overall concordance. Our results are in accordance with previous studies [53, 231]. Also, the concordance between GEP and IHC was more prominent within the GCB subgroup where GEP classified 85% of cases classified as GCB by IHC as GCB, while 58% of non-GCB cases according to IHC were found to be ABC by GEP, probably since most of the UC cases were non-GCB by IHC (table 2). When UC cases were excluded, the concordance between GEP and IHC was 75% regarding the non-GCB and ABC subgroups.



Table 2. Association between the Lymph2Cx assay and the Hans algorithm (IHC)

	ABC according to Lymph2Cx assay (n = 105)	GCB-GEP according to Lymph2Cx assay (n = 168)	UC according to Lymph2Cx assay (n = 42)
IHC			<0.001
Non-GCB (n = 151) (%)	88 (58)	30 (20)	33 (22)
GCB-IHC (n = 157) (%)	15 (9)	133 (85)	9 (6)
Missing (n = 7) (%)	2 (29)	5 (71)	0 (0)

The gene expression profile of UC subgroup is still obscure and different studies demonstrated controversial results regarding survival outcome in DLBCL patients within this subgroup. In our study most of the UC cases were assigned as non-GCB subtype according to IHC, but had a favorable prognosis with survival similar to GCB subtype. Some studies are in agreement with our findings [232-234] while others identified UC group to be associated with unfavorable outcome [53, 235, 236]. A recent study demonstrated concurrent *NOTCH2* mutations and *BCL6* translocations to be prominent in the UC cases and this was associated with a favorable outcome [54].

To determine which method is the best predictor of survival; we compared the outcome of different subgroups of DLBCL patients according to GEP and IHC. A significant difference in outcome was detected on GEP with ABC subgroup having inferior OS and PFS while only PFS was significantly worse among non-GCB subgroup on IHC. More importantly, patients categorized as non-GCB/ABC by both IHC and GEP had the worst prognosis (OS and PFS) in a univariate and multivariate analysis (figure 8).

With this novel finding, we suggest that the determination of COO by using both IHC and GEP to identify the ABC subtype is of superior value, especially in the era of precision medicine when more precise risk stratification is indicated to identify those patients that have greater benefit of targeted interventions, such as lenalidomide and bortezomib, via nuclear factor kB pathway inhibition, and ibrutinib, via Bruton's tyrosine kinase blockade [237].

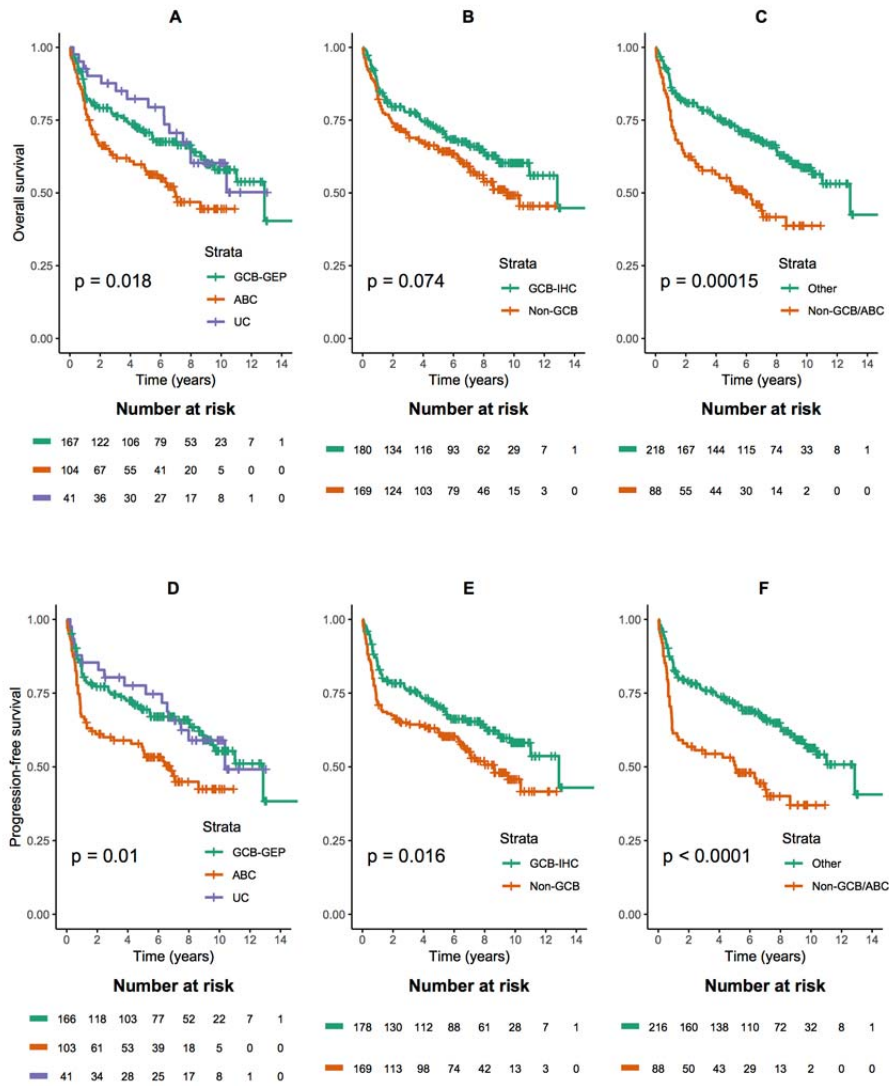


Figure 8. Kaplan-Meier curve for OS according to (A) the Lymph2Cx assay, (B) the Hans algorithm and (C) the Lymph2Cx assay and the Hans algorithm combined, and PFS according to (D) the Lymph2Cx assay, (E) the Hans algorithm and (F) the Lymph2Cx assay and the Hans algorithm combined.

Patients within the ABC subgroup determined by GEP and within non-GCB/ABC subgroup determined by both GEP and IHC were older and had more often double protein expression of MYC and BCL2 than patients within the GCB subgroup while there were no significant differences in age and double protein expression of MYC and BCL2 among GCB and non-GCB cases by IHC.

In addition to the non-GCB/ABC subtype determined by both IHC and GEP, the double protein expression of MYC and BCL2 in this study was associated with inferior outcome in univariate and multivariate analysis. These findings indicate that both COO and double expression of MYC and BCL2 are the most important factors to predict survival in DLBCL patients.

### Paper III

While distinct sites of extranodal DLBCL have been studied extensively and revealed immunophenotypic and genetic differences as well as diverse outcomes, the prognostic value of site of nodal involvement in DLBCL is mainly unknown. A recent study on CHL, an infradiaphragmatic nodal disease was shown to be associated with inferior outcome in patients with early stage disease in comparison to patients with early stage disease with supradiaphragmatic nodal disease [238].

In this study we investigated the clinical and tumor biological characteristics in DLBCL patients with correlation to the site of tumor involvement with particular attention to the abdominal lymph node involvement.

On radiological evaluation we detected abdominal lymph node involvement in 63% (156/249) of patients (figure 9). Those patients had more advanced disease and inferior LSS compared to patients without abdominal lymph node involvement (figure 10). They presented with higher age, higher LDH, more frequent B symptoms, more frequent bulky disease, higher aa-IPI and more advanced stage compared to patients without abdominal lymph node involvement. In addition, they had more frequent concurrent expression of MYC and BCL2 than patients without abdominal lymph node involvement.

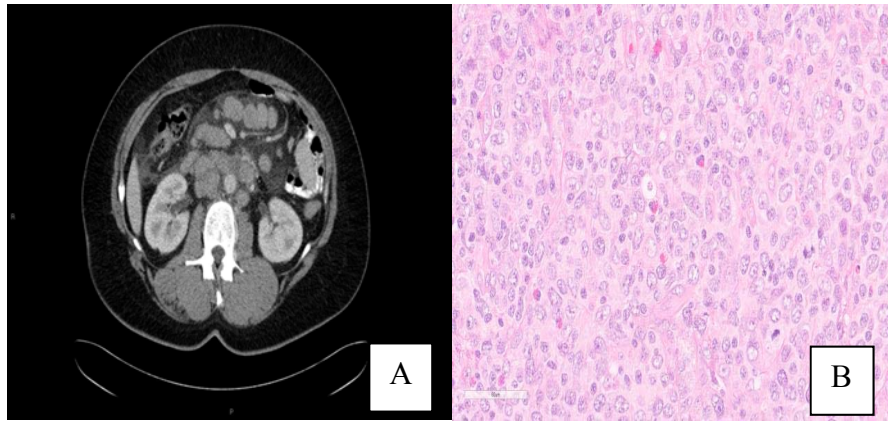


Figure 9. Computed tomography of the abdomen show para aortal lymphadenopathy (A). Histomorphology with HE staining showing involvement of DLBCL (B).

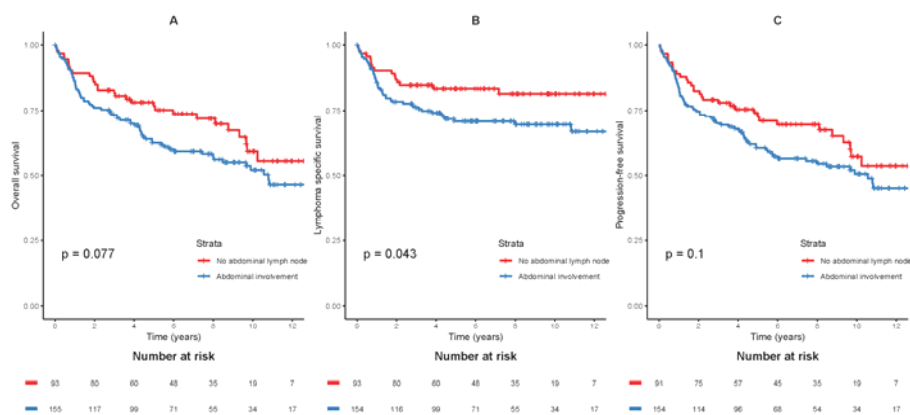


Figure 10. Kaplan-Meier curve for OS (A), LSS (B) and PFS (C) in patients with abdominal lymph node involvement compared to patients without abdominal lymph node involvement.

In 81/156 patients with abdominal lymph node involvement on radiological evaluation, the diagnosis was approved on biopsy from abdominal lymph nodes. Those patients had inferior outcome (OS, LSS and PFS) compared to patients without abdominal lymph node involvement and they did not differ clinically from patients with abdominal lymph node involvement on radiological examination (n=75).

The clinical decision of determining the site for taking biopsy is based on multiple factors, such as comorbidity, anatomical location near large blood vessels, and whether the site represents the most aggressive part of disease

on radiological examination. We believe that these reasons explained the choice of site for taking biopsy in our cohort.

Our findings warrant the risk of underrepresented group of DLBCL patients with abdominal lymph node involvement in research work probably due to the inadequacy of material required for analysis. In addition, this site has not been thoroughly investigated as a separate entity. We encourage further investigations of the abdominal lymph node involvement in DLBCL to explore the accurate explanation for the aggressiveness of this group.

## Paper IV

In a cohort of 45 PCNSL patients, the PD-L1 and PD-L2 proteins were highly expressed on leukocytes in 55.5% and 51% respectively, while high expression of these two proteins on tumor cells was detected with less frequency (11% and 9% respectively) (figure 11).

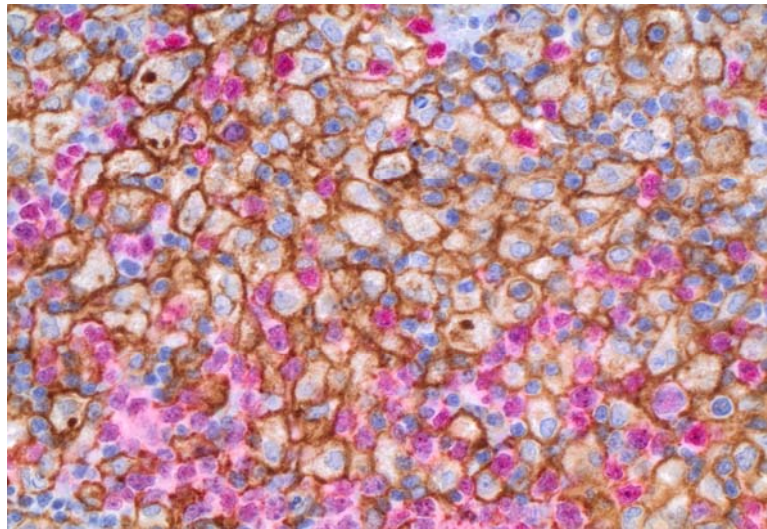


Figure 11. Immunohistochemical double staining with PD-L1 (brown) and PAX5 (red) in a tumor biopsy from a patient with PCNSL.

RNA sequencing revealed that the gene *IDO1* was highly expressed in patients with high expression of PD-L1 on leukocytes. In addition, the positive protein expression of *IDO1* was detected in 79% of cases with high protein expression of PD-L1 on leukocytes. There was no significant difference in expression of the *IDO1* gene in relation to PD-L2 protein expression on leukocytes despite the strong correlation between PD-L1 and PD-L2 protein expression on leukocytes.

The significant association between IDO1 and PD-L1 in our cohort confirms the crucial role of these two proteins as immunosuppressive molecules (figure 12) and indicates the importance of further studies on larger cohorts of PCNSL to explore the role of this correlation in the era of new modalities of treatment including immune checkpoint inhibitors and IDO1 inhibitors.

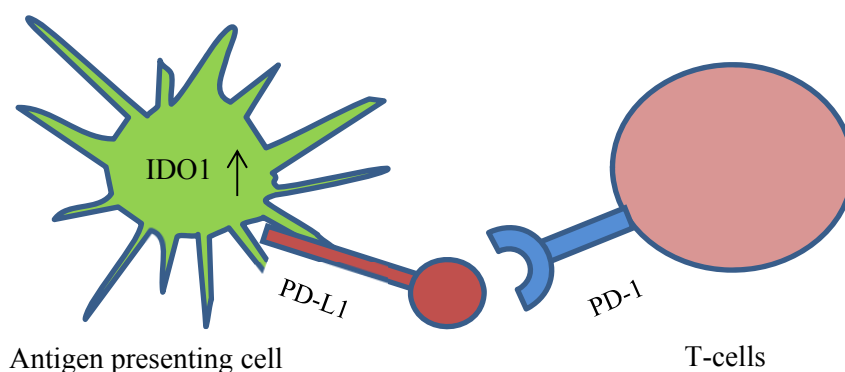


Figure 12. Cartoon diagram representing the interaction between IDO1 and PD-1 pathways in immune system.

Positive EBER was highly correlated with high protein expression of PD-L1 and PD-L2 on tumor cells as well as with gene expression of IDO1. This finding may explain the participant role of EBV infection in the tumorigenesis in EBV-positive PCNSL.

Molecular analysis with FISH revealed that no one case harbored translocation of *MYC*, only one case with *BCL2* translocation was detected, and *BCL6* translocation was detected in 23% of cases. Our findings are in line with others [239]. Not one case was recognized as DHL.

The high frequency of protein expression of *MYC* and *BCL2* in 53% and 89% of cases respectively, indicates a clear discrepancy between translocation and protein expression of *MYC* and *BCL2* and suggests that mechanisms other than translocation could explain the protein expression of *MYC* and *BCL2*.

In our cohort, the non-GCB subgroup was predominant (87%) according to the Hans algorithm and there was no difference in survival between this subgroup and the GCB subgroup. The double expression of *MYC* and *BCL2* which was observed in 49% of the cases did not have any negative prognostic impact. Thus, the significant prognostic role of COO and double expression of *MYC* and *BCL2* that we proposed in papers I-III does not fit patients

with PCNSL and we need another prognostic index to identify the different risk groups within this specific entity.

## Strengths and limitations

A common strength in papers I-III was the homogeneous cohort; all patients included were diagnosed as de novo DLBCL according to the 2008 WHO classification and received R-CHOP or R-CHOP like regimens. Immunostainings in paper I, II and partly in III and IV were done by using the same reagents and protocols. The large cohort in paper II with cases from Sweden and Denmark that were available to perform both GEP and IHC was an advantage. A strength in paper III was the use of radiological evaluation to confirm abdominal lymph node involvement. In paper IV we performed a comprehensive investigation of tumor and tumor microenvironment characteristics in PCNSL patients with numerous robust methods, including immunohistochemistry, FISH, *in situ* hybridization for EBER and RNA sequencing.

The major limitation in papers I-IV was the sparse material in parts of the diagnostic samples which was the reason why many cases were excluded from statistical analysis due to the limited analysis that could be done in these cases despite the availability of the clinical data. This was particularly obvious when we did multivariate analysis. In papers I-III there was not enough material to perform FISH to search for *MYC* and *BCL2* translocations in DEL cases. In paper II the selection of cases depended solely on whether there were enough tissue samples to extract RNA of good quality to perform GEP. However, the clinical characteristics and survival were quite comparable to other studies in DLBCL. In paper III, the radiological examination was based solely on CT although the international guidelines indicate the important role of PET. Unfortunately, PET was only performed in a limited number of patients and we tried to avoid the selection bias by not including PET in our radiological re-evaluation in this paper. In paper IV, the cases with available tissue samples for analysis were less than 50% of the whole cohort (45/132), which somewhat limits the statistical analyses.

## Summary of results

- I- In a cohort of R-CHOP treated DLBCL patients, the COO based on IHC had no association with survival while the double expression of MYC and BCL2 (DEL) was associated with inferior survival in univariate analysis. The DEL cases were distributed with no significant difference between the GCB and non-GCB subgroups.
- II- Determination of COO by IHC was highly correlated with GEP. ABC/non-GCB DLBCL subgroup verified by GEP and IHC and double expression of MYC and BCL2 were both independent discriminators of inferior outcome in univariate and multivariate analysis.
- III- DLBCL patients with abdominal lymph node involvement may represent a distinct entity of DLBCL with prognostic significance. Those patients had more aggressive clinical presentation, presented with higher frequency of double expression of MYC and BCL2 and associated with inferior LSS compared to patients without abdominal lymph node involvement.
- IV- A significant association between both gene and protein expression of IDO1 and PD-L1 in PCNSL patients may reflect the crucial immunosuppressive role of these two molecules, and it is inspiring to study new modalities of treatment in PCNSL with IDO1 inhibitors in combination with other immunotherapies and/or chemotherapy. Double expression of MYC and BCL2 was detected in about half of PCNSL patients and did not show any association with survival.



## Comprehensive discussion and future perspectives

The aims of research in DLBCL are mainly intended to improve the health care among DLBCL patients, minimize the toxic side effects of treatment and to better understand the heterogeneity of the tumor biology of the disease in order to identify different risk groups, especially among patients in the relapsing and/or refractory group, and to seek better options of treatment.

Identification of prognostic status at the time of primary diagnosis of the disease is mandatory to plan for the best care of patients to predict better outcome. Within the IPI, five different risk groups can be identified based on age, Ann Arbor stage, ECOG performance state, LDH level and the presence of extranodal sites of involvement. This score is based solely on clinical parameters and is surrogate of biological markers that can reflect tumor heterogeneity which may explain the inhomogeneous response to treatment among the homogenous risk groups within the IPI score.

The important role of tumor cell markers and tumor microenvironment in the pathogenesis and progression of DLBCL has been highlighted and new possibilities with precision medicine based on better understanding of tumor biology are arising which may give new hope to DLBCL patients with resistant disease. The choice of appropriate treatment in such cases is largely dependent on identifying the targets involved in the pathogenesis in each individual patient.

The prognostic impact of COO is considerable in predicting outcome in DLBCL. For many years now the identification of GCB and non-GCB has been based on different immunohistochemical algorithms that have replaced GEP due to the limitations of using this method on routinely FFPE material. However, none of the IHC algorithms is fully concordant with GEP. Recently, modified GEP on FFPE has shown promising results and could identify GCB and ABC subtypes in addition to the small group of unclassifiable cases.

In **paper II**, we found that ABC/non-GCB subtype identified by both GEP and IHC algorithm is associated with the worst outcome. The identification of this subtype is rewarding especially in the era of precision medicine when ABC subtype shows a greater benefit from certain target interventions such

as lenalidomide and bortezomib. However, our study is based on a retrospective cohort and it will be of interest to investigate this finding on prospective trial studies with targeted interventions. In addition, the dual expression of MYC and BCL2 was also significantly associated with inferior survival both in uni- and multivariate analyses, although not all cases were included due to insufficient material.

**In paper III**, the double expression of MYC and BCL2 was significantly more frequent among patients with abdominal lymph node involvement. These patients had more advanced disease and inferior survival compared to patients without abdominal lymph node involvement and without double expression of MYC and BCL2. In further investigation of larger cohorts of DLBCL it will be interesting to explore the clinical characteristics and tumor biology of abdominal lymph node disease and also to investigate the prognostic impact of localization of lymph node involvement in DLBCL.

**In paper IV**, we detected a high *IDO1* gene expression among patients with high PD-L1 protein expression on leukocytes. This finding supports the crucial immunosuppressive role of both IDO1 and PD-L1 in cancer. Further studies will be of great importance in exploring this correlation and also in investigating the benefit of combined IDO1 inhibitors with immune checkpoint pathway inhibitors in treating lymphoma.

In the same paper we performed survival analysis on only 30 treated patients and found a tendency to superior survival among patients with high protein expression of PD-L1, PD-L2 and IDO1 on leukocytes. The prognostic role of the expression of these immunosuppressive molecules in DLBCL in general and in PCNSL as a specific entity is unclear and further studies are required with perhaps more unified antibodies and methods to explore the prognostic impact of these molecules.

In this thesis, we investigated the prognostic impact of tumor cell markers, markers of tumor microenvironment, and site of involvement that can predict survival in DLBCL and may contribute to the prognostic scale of the disease in the era of new treatment facilities. However, during this journey we confirmed the important role of adequate clinical data, adequate tissue sampling and proper collaboration between the oncologists, hematologists, pathologists, radiologists and clinical geneticists to ensure the best care for patients. From a pathologist point of view an adequate tissue sample is of crucial importance in the delivery of a correct diagnosis and appropriate treatment and to allow for spare tissue samples for research. In addition, we encourage multicenter collaboration in research especially when studying rare entities such as PCNSL.

# Populärvetenskaplig sammanfattning på svenska

Diffust storcelligt B-cellslymfom (DLBCL) är en aggressiv form av malignt lymfom d.v.s en sorts lymfkörtelcancer som utgår från immunsystemets egna celler. Det finns många olika typer och undergrupper av lymfom och diagnosen ställs genom att man undersöker vävnadsprover i mikroskop för morfologi och immunfenotyp och ibland med hjälp av flödescytometrianalys.

Varje år insjuknar ca 500-600 personer i Sverige, vilket gör DLBCL till den vanligaste typen av lymfom (25-30 % av alla lymfom). Medianålder vid diagnos är 70 år. Ålder är en viktig prognostisk faktor och kan därmed vara behandlingstyrande.

DLBCL är något vanligare hos män (M:F 1,4:1). Överlevnad vid DLBCL har kraftigt förbättrats med kombinerad immunoterapi och kemoterapi (R-CHOP). Cirka 60%-70% av patienter blir botade medan 30%-40% av patienter är refraktära till behandling eller kan få återfall efter att tidigare ha gått i remission.

Studier av genuttrycksprofiler (Gene expression profile, GEP) vid DLBCL har identifierat två olika profiler baserat på tumörcellernas ursprung; (cell-of-origin, COO) (1) tumörceller som utgår från B-lymfocyter från germinalcentrum i de sekundära lymffolliklarna -Germinal center B-cell liknande (GCB), s.k. GCB-DLBCL, (2) tumörceller som utgår från B-lymfocyter som passerat germinalcentrum och just påbörjat sin utveckling mot plasmacell -Aktiverade B-cellsliknande, s.k. ABC-DLBCL och en tredje grupp som inte tillhör någon av dessa två subtyper och därför kallas "unclassifiable". ABC-DLBCL har sämre prognos jämfört med GCB-DLBCL med cirka 45% respektive 80% 3-årsöverlevnad efter behandling med R-CHOP.

Genuttrycksprofil analyseras i RNA som extraheras från färsk och/eller fryst vävnad. För närvarande finns inte möjlighet till detta i klinisk diagnostik då diagnostiken vanligen utförs på formalin-fixerat paraffin inbäddat material (FFEP). Olika immunhistokemiska (IHC) algoritmer har använts och som delvis lyckades identifiera GCB och icke-GCB subtyper men ingen metod har visat 100% överensstämmelse med GEP och den prognostiska signifikansen av IHC algoritmer är omtvistad i litteraturen. Nya diagnostiska

tekniker som kan utföras på formalinfixerad vävnad är emellantid under snabb utveckling för genuttrycksprofilering och karakterisering av DLBCL och andra lymfomtyper.

Förutom COO, beskriver 2016 års WHO-klassifikation den prognostiska signifikansen av dubbel expression av proteinerna MYC och BCL2 vid immunhistokemisk färgning. DLBCL, NOS med dubbel expression av MYC och BCL2, s.k. dubbel-expressor lymfom har sämre prognos men är ännu inte en specifik entitet.

DLBCL med rearrangemang av *MYC*- och *BCL2*- och/eller *BCL6*-gener har sämst prognos och är en egen entitet i den senast WHO- klassifikationen - high grade B-cell lymphomas (HGBL) with rearrangements of *MYC* and *BCL2* and/or *BCL6*.

Precisionmedicin är ett nytt koncept som kan bli en ny framtida behandling för DLBCL patienter. Förbättrad diagnostik är viktig för att förstå underliggande genetiska avvikelser som kan individualisera behandling för patienter som har olika genetiska avvikelser även om de har identiska morfologiska och immunfenotypiska egenskaper.

## Delarbete I

I den första studien undersöktes 188 *de novo* DLBCL patienter som var behandlade med R-CHOP mellan 2002-2012 på Akademiska sjukhuset i Uppsala. Tumörbiopsierna eftergranskades och klassificerades enligt 2008 års WHO klassifikation. Äldre patienter hade sämre prognos men det fanns inget skillnad i överlevnad mellan män och kvinnor. Dubbelexpression av MYC  $\geq 40\%$  och BCL2  $\geq 70\%$  fanns i 27% av patienterna och dessa hade sämre överlevnad med en 5-års överlevnad på 54% jämfört med 74% 5-års överlevnad hos patienter som inte hade dubbelexpression av MYC och BCL2. Hög proliferation mätt med Ki67  $\geq 70\%$ , och överuttryck av P53  $\geq 50\%$  var också associerade med sämre överlevnad.

## Delarbete II

I den andra studien undersöktes en kohort av 359 *de novo* DLBCL- patienter från Sverige och Danmark. RNA extraherades från FFPE för att utföra GEP med nanostring teknik. IHC med tre immunfärgningar (CD10, BCL6 och MUM1) analyserades enligt Hans algoritm för att identifiera GCB och icke-GCB subtyper. Överensstämmelsen mellan GEP och IHC var 72% i hela

kohorten (85% för GCB och 58% för ABC). Patienter som klassificerades som icke-GCB enligt IHC algoritm och som ABC enligt GEP hade sämst prognos. Dubbelextion av MYC och BCL2 var vanligare hos patienter med ABC subtyp och var associerat med sämre prognos.

### Delarbete III

I den tredje studien undersöktes samma kohort som i första studien plus ytterligare nya DLBCL fall fram till 2015. Radiologisk eftergranskning med CT av buk och thorax kunde utföras i 249 fall. Därvid konstaterades patologiska lymfkörtlar i buken hos 156 patienter. Dessa patienter hade sämre prognos jämfört med patienter som inte hade patologiska lymfkörtlar i buken (n=93). Dessa patienter (n=156) hade mer aggressiv sjukdom med högre ålder, aa-IPI, stadium, och dessutom fler med dubbel expression av MYC och BCL2 jämfört med patienter som inte hade lymfomengagemang i buken.

### Delarbete IV

I den fjärde studien undersöktes proteinuttrycket av PD-1, PD-L1, PD-L2, EBER, samt RNA sekvensering hos 45 patienter med primärt DLBCL i CNS (PCNSL). Korrelation mellan olika genmutationer och proteinuttrycket av PD-L1, PD-L2 och EBER undersöktes. Resultaten visade att *IDO1* mutation var vanligare hos patienter med högt proteinuttryck av PD-L1 i makrofager. Immunfärgning för IDO1 visade också stark korrelation mellan dessa två proteiner. Den signifikanta korrelationen mellan IDO1- och PD-L1-uttrycket kan vara intressant för framtida behandlingstudier.

Högre uttryck av PD-L1, PD-L2 och IDO1 i makrofager visade tendens till bättre svar på behandling. Högt proteinuttryck av PD-L1 och PD-L2 i tumörceller fanns endast i ett fåtal fall och hade ingen association med överlevnad men det fanns en stark korrelation mellan förekomst av dessa två proteiner i tumörceller med EBV förekomst i tumörcellerna. Högt uttryck av IDO1- protein och *IDO1*- mutation var vanligare hos patienter med EBV förekomst i tumörcellerna.

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