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# **THE ROLE OF GENETIC ALTERATIONS AND IMMUNE SURVEILLANCE IN DIFFUSE LARGE B-CELL LYMPHOMA**

**BY  
MARIJANA NESIC**

DISSERTATION SUBMITTED 2021



**AALBORG UNIVERSITY**  
DENMARK



# **THE ROLE OF GENETIC ALTERATIONS AND IMMUNE SURVEILLANCE IN DIFFUSE LARGE B-CELL LYMPHOMA**

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**AALBORG UNIVERSITY**  
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# PREFACE

This thesis presents the work carried out from February 2017 to February 2021 at the Department of Hematology, Aalborg University Hospital, and the Department of Clinical Medicine, Aalborg University. The thesis consists of two scientific papers, one systematic review, an introduction to the immunoediting concept in diffuse large B-cell lymphoma, evaluation of material and methods, a summary of main results, and discussion.

During this inspiring journey toward a PhD, I was fortunate to have met enthusiastic and intelligent people that supported and encouraged me to push forward. This thesis would not be possible without them, and to all, a huge thank you.

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My love goes to my family, my parents **Stevan** and **Dragoslava**, and my dearest sister **Emanuela** with her fantastic family. Thank you for believing in me and for all your support, care and love during my whole life. Also, a huge thanks to my father-in-law **Jovica** for cheering me.

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*Marijana Nesic, Aalborg, February 2021*

*For Vilijam and Nikola*



# LIST OF STUDIES

## **I. Mutational landscape of immune surveillance genes in diffuse large B-cell lymphoma**

Marijana Nesic, Tarec Christoffer El-Galaly, Martin Bøgsted, Inge Søkilde Pedersen & Karen Dybkær (2020): Mutational landscape of immune surveillance genes in diffuse large B-cell lymphoma, Expert Review of Hematology, DOI: 10.1080/17474086.2020.1755958

## **II. The mutational profile of immune surveillance genes in diagnostic and refractory/relapsed DLBCLs**

M. Nesic, M. Sønderkær, R. F. Brøndum, T.C. El-Galaly, I. S. Pedersen, M. Bøgsted, K. Dybkær  
Submitted to Molecular Cancer Research, January 2021.

## **III. Optimization of pre-analytical variables for cfDNA processing and detection of ctDNA in archival plasma samples**

M. Nesic, Julie S. Bødker, Simone, K. Terp, K. Dybkær.  
Submitted to Experimental Hematology, February 2021.

## ENGLISH SUMMARY

Diffuse large B-cell lymphoma (DLBCL) is the most prevalent form of non-Hodgkin lymphoma (NHL) among adults, with approximately 450 newly diagnosed patients in Denmark each year. DLBCL remains a significant clinical challenge, as approximately 40% of the patients are not cured by immunochemotherapy treatment consisting of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), who thus suffer from the refractory or relapsed disease. Depending on the risk profile, the relative 5-year overall survival rate range between 50-80%, and treatment failure is most often caused by treatment resistance. In the past ten years, genome-wide sequencing studies of DLBCL made notable progress in revealing the genetic and epigenetic landscape of the disease uncovering genetic alterations that play essential roles in tumor development and maintenance as well as in response to the treatment. The identified genetic alterations result in aberrant activation of several signaling pathways and cellular processes, including immune surveillance, B-cell differentiation, B cell receptor signaling, and epigenetic remodeling. However, only limited understanding exists of the specific molecular features underlying treatment resistance and how it affects progressive carcinogenesis and interaction with immune effector cells in the tumor microenvironment. In theory, at the early stages of carcinogenesis, malignant cells are recognized by natural killer (NK) cells encountering specific ligands expressed on tumor cells. Activated macrophages, dendritic cells, and T- and B-cells promote the production of additional cytokines and further activate tumor-specific T-cells (CD8+ cytotoxic T-cells), generating immune memory for specific tumor components. However, in cases where the immune effector cells cannot eliminate malignant cells, a state of equilibrium develops. Eventually, malignant cells avoid or suppress the antitumor immune response, enabling immune escape and a progression in tumor development. The tumor cells can escape from the host's immune surveillance (IS) by disabling antigen-presentation or by promoting immune exhaustion or suppression of immune effector cells.

The overall aim of this thesis was to investigate the role of genes related to immune surveillance in DLBCL patients and to outline genetic alterations distinguishing diagnostic and refractory/relapsed DLBCLs. Furthermore, to provide a guide for optimal pre-analytical variables for obtaining circulating-free DNA (cfDNA) and assessing the detection of circulating tumor DNA (ctDNA) in the long-term archived DLBCL clinical samples employing digital droplet PCR (ddPCR).

In paper I, we have identified 58 genes involved in immune surveillance of DLBCL by reviewing the literature and cancer databases. From these initially identified genes, further was selected ten the most frequent candidate genes along with the most robustly detected recurrent genetic alterations in these ten candidate genes with predicted effect as highly damaging on protein formation, enabling assessment of their diagnostic and prognostic relevance.

In paper II, the 58 genes related to IS were examined in 30 diagnostic (non-relapsing) and 17 refractory/relapsed biopsies of DLBCL patients by employing whole-exome

sequencing (WES). For 36 out of the 58 genes, genetic alterations were observed in 22 (73%) of the diagnostic DLBCLs (dDLBCL) and 13 (77%) of the relapsed/refractory DLBCLs (rrDLBCL), respectively. We observed a 3.2-fold decreased number of mutated IS genes in rrDLBCLs, but more than 50% of mutated genes were with increased gene mutation frequency compared to dDLBCLs, suggesting the critical role of these genes in the development and progression of the disease and treatment resistance.

Paper III focuses on liquid biopsies as a noninvasive tumor material source in the shape of circulating tumor DNA (ctDNA) and future oncology tools necessary to test molecular biomarkers and stratify cancer patients. Although it is already in use for some cancers, there are still challenges, as pre-analytical clinical practice approaches are not established. Therefore, we investigated pre-analytical variables such as collection tubes and purification of cell-free DNA (cfDNA) after different storage times. We have observed QIAamp Circulating Nucleic Acid Kit (Qiagen) as the most relevant kit for purification of cfDNA, and if blood sample cannot be stored or processed within four hours from blood draw should be used Streck blood collection tube. Additionally, we have demonstrated the feasibility of detecting ctDNA in archival plasma samples over nine years using digital droplet PCR (ddPCR).



# DANSK RESUMÉ

Diffust storcellet B-celle lymfom (DLBCL) er den mest udbredte type af non-Hodgkin lymfom (NHL) blandt voksne med ca. 450 nydiagnosticerede patienter i Danmark hvert år. DLBCL er fortsat en signifikant klinisk udfordring, da ca. 40% af patienterne ikke helbredes ved immunokemoterapi bestående af rituximab, cyclophosphamid, doxorubicin, vincristin og prednison (R-CHOP), og derved lider af den refraktær eller relapserende (R/R) DLBCL. Afhængigt af risikoprofilen er den relative 5-årige samlede overlevelseshastighed mellem 50-80%, og behandlingssvigt er ofte forårsaget af behandlingsresistens. I de sidste ti år har genom-dækkende sekventerings undersøgelser af DLBCL gjort bemærkelsesværdige fremskridt i at afsløre det genetiske og epigenetiske landskab af sygdommen, som afdækker de genetiske ændringer, der spiller vigtige roller i tumorudvikling og vedligeholdelse såvel som responset af behandlingen. De identificerede genetiske ændringer resulterer i afvigende aktivering af flere signalveje og cellulære processer, herunder immunovervågning, B-celle differentiering, B-cellereceptor signalering og epigenetisk remodeling. Imidlertid eksisterer der kun begrænset forståelse af de specifikke molekulære karakteristika, der ligger til grund for behandlingsresistens, og hvordan det påvirker progressiv carcinogenese og interaktion med immuneffektorceller i tumorens mikromiljø. I teorien genkendes maligne celler i de tidlige stadier af carcinogenese af naturlige dræberceller (NK celler), der genkender specifikke ligander udtrykt på tumorceller. Aktiverede makrofager, dendritiske celler og T- og B-celler fremmer produktionen af yderligere cytokiner og aktiverer yderligere tumorspecifikke T-celler (CD8<sup>+</sup> cytotoxiske T-celler), hvilket genererer immunhukommelse for specifikke tumorkomponenter. I tilfælde, hvor immuneffektorcellerne ikke kan eliminere ondartede celler, udvikler der sig imidlertid en ligevægtstilstand (equilibrium). Til sidst undgår eller undertrykker maligne celler antitumorimmunresponsen, hvilket muliggør immunudslip (escape) og en progression i tumorudviklingen. Herved kan tumorcellerne flygte fra værtens immunovervågning (IS) ved at deaktivere antigenpræsentation eller ved at fremme immunudmattelse eller undertrykkelse af immuneffektorceller.

Det overordnede mål med denne afhandling var at undersøge rollen af gener relateret til immunovervågning hos DLBCL-patienter og at skitsere genetiske ændringer, der skelner mellem diagnostiske og R/R DLBCL patienter. Desuden at give en guide til optimale præanalytiske variabler til opnåelse af cirkuleringsfrit DNA (cfDNA) og vurdering af cirkulerende tumor-DNA (ctDNA) ) detektion i de langsigtede

arkiverede DLBCL-kliniske prøver, der anvender digital dråbe-PCR (ddPCR).

I artikel I har vi identificeret 58 gener involveret i immunovervågning af DLBCL ved at gennemgå litteraturen og kræftdatabaser. Fra disse oprindeligt identificerede gener blev der yderligere udvalgt ti af de hyppigste kandidatgener sammen med de mest robust påviste tilbagevendende genetiske ændringer i disse ti kandidatgener med forudsagt virkning som meget skadelige for proteindannelse, hvilket muliggør vurdering af deres diagnostiske og prognostiske relevans.

I artikel II blev de 58 gener, der var relateret til IS, undersøgt i 30 diagnostiske (ikke-tilbagefaldende) og 17 R/R DLBCL biopsier fra patienter, ved at benytte heleksomsekventering (WES). For 36 ud af de 58 gener blev der observeret genetiske ændringer i henholdsvis 22 (73%) af de diagnostiske DLBCL (dDLBCL) patienter og 13 (77%) af R/R DLBCL patienter. Vi observerede et 3,2 gange reduceret antal muterede IS-gener i R/R DLBCL patienter, men mere end 50% af de muterede gener var med øget genmutationsfrekvens sammenlignet med dDLBCL patienter, hvilket tyder på den kritiske rolle disse gener har i udvikling og progression af sygdommen og behandlingsresistens.

Artikel III fokuserer på flydende biopsier som en ikke-invasiv kilde til anskaffelse af tumormateriale i form af cirkulerende tumor-DNA (ctDNA), og som fremtidigt onkologisk værktøj, der er nødvendigt for at teste molekulære biomarkører og stratificere kræftpatienter. Selvom det allerede er i brug for nogle kræftformer, er der stadig udfordringer, siden den præ-analytiske implementering til klinisk praksis ikke er etableret. Derfor har vi undersøgt præ-analytiske variabler såsom opsamlingsrør og oprensning af cellefrit DNA (cfDNA) efter forskellige opbevaringstider. Vi observerede QIAamp Circulating Nucleic Acid Kit (Qiagen) som det mest relevante kit til oprensning af cfDNA, og hvis blodprøve ikke kan opbevares eller behandles inden for fire timer efter blodudtagning, skal der anvendes Streck. Derudover har vi demonstreret muligheden for at detektere ctDNA i arkivplasmaprøver over ni år ved hjælp af ddPCR.



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## ABBREVIATIONS

<b>ABC</b>	Activated B-cell-like
<b>APC</b>	Antigen-presenting cells
<b>ASCT</b>	Autologous stem cell transplantation
<b>BAGS</b>	B-cell associated gene signatures
<b>BCR</b>	B-cell receptor
<b>BCTs</b>	Blood collection tubes
<b>BM</b>	Bone marrow
<b>CAR-T</b>	Chimeric antigen receptor T-cell
<b>CD</b>	Cluster of differentiation
<b>cfDNA</b>	cell-free DNA
<b>CHOP</b>	Cyclophosphamide, doxorubicin, vincristine, prednisone
<b>CSR</b>	Class-switch recombination
<b>CTL</b>	Cytotoxic T lymphocytes (CD8+ T-cell)
<b>CTLA-4</b>	Cytotoxic T-lymphocyte antigen 4
<b>ctDNA</b>	Circular tumor DNA
<b>ddPCR</b>	Droplet digital PCR
<b>DLBCL</b>	Diffuse Large B-Cell Lymphoma
<b>dDLBCLs</b>	Diagnostic DLBCLs
<b>DNA</b>	Deoxyribonucleic acid
<b>ECOG</b>	Eastern Cooperative Oncology Group
<b>FDA</b>	Food and Drug Administration
<b>GC</b>	Germinal center
<b>GCB</b>	Germinal center B-cell-like
<b>GEP</b>	Gene expression profiling
<b>HLA</b>	Human leucocyte antigen
<b>ICI</b>	Immune checkpoint inhibitor
<b>Ig</b>	Immunoglobulin
<b>IHC</b>	Immunohistochemistry
<b>IPI</b>	International Prognostic Index
<b>IS</b>	Immune surveillance
<b>LDH</b>	Lactate dehydrogenase
<b>MHC</b>	Major histocompatibility complex
<b>MHC-I</b>	Major histocompatibility complex class I
<b>MHC-II</b>	Major histocompatibility complex class II
<b>NF-<math>\kappa</math>B</b>	Nuclear factor-kappa B
<b>NGS</b>	Next-generation sequencing
<b>NHL</b>	Non-Hodgkin lymphoma
<b>NK</b>	Natural killer cells
<b>OS</b>	Overall survival
<b>PB</b>	Peripheral blood
<b>PCR</b>	Polymerase chain reaction

<b>PFS</b>	Progression-free survival
<b>PD-L1</b>	Programmed cell death ligand 1
<b>PD-L2</b>	Programmed cell death ligand 2
<b>qPCR</b>	Quantitative PCR
<b>R-CHOP</b>	Rituximab, cyclophosphamide, doxorubicin, prednisone
<b>RNA</b>	Ribonucleic acid
<b>RR</b>	Refractory/Relapse
<b>rrDLBCLs</b>	Refractory/Relapse DLBCLs
<b>RT</b>	Room temperature
<b>SHM</b>	Somatic hypermutation
<b>TCR</b>	T-cell receptor
<b>TME</b>	Tumor microenvironment
<b>UC</b>	Unclassified
<b>WES</b>	Whole-exome sequencing
<b>WGS</b>	Whole-genome sequencing
<b>WHO</b>	World Health Organization
<b>VAF</b>	Variant allele frequency
<b>VCF</b>	Variant call format
<b>VDJ</b>	Variable, Diversity, Joining



# 1. INTRODUCTION

## 1.1. Overview of the immune system

Our immune system is a host defense mechanism encompassing a complex network of organs, immune cells, and specialized molecules, whose ability to discriminate "self" from "non-self" is a fundamental property to attack and eliminate invading bacteria, viruses, cancers, and other pathogens. The immune system branches into the innate and adaptive immune responses, which are firmly connected, each with different mechanisms to recognize the antigens and immune cells involved.

The innate immune response is the first-line defense, where the pathogen's recognition is based on pattern recognition receptors [1]. These receptors detect "non-self" antigens, in other words, structures of pathogens that are not present on normal cells, as well as endogenous damage-associated molecular patterns, which are released in necrotic or damaged cells [2]. The important hallmarks of the innate immune system are the absence of immunological memory and antigen specificity, as well as a quick response after encountering a pathogen. It encompasses different defensive boundaries such as physical (e.g., skin and mucosa), chemical boundaries (e.g., low pH), phagocytotic (e.g., monocytes, neutrophils, tissue macrophages), and inflammatory (e.g., serum proteins) [3]. Specialized cell types involved in the innate immune response are phagocytic cells (macrophages and neutrophils), natural killer (NK) cells, basophils, eosinophils, and mast cells [4]. Moreover, the innate immune system comprises antigen-presentation of a dendritic cell through the major histocompatibility complex (MHC) to T-cells resulting in the activation of the adaptive immune response [3]. Another important segment is a complement system - a biochemical cascade that involves both innate and adaptive immune response, which possess the ability of antibodies and phagocytic cells to kill the pathogen [3].

The adaptive immune response is a slower but highly specific immune response dependent on B-lymphocytes and T-lymphocytes, immune cells of lymphoid origin. These cells are expressing receptors called B-cell receptor (BCR) and T-cell receptor (TCR), which specifically recognize epitopes, antigenic determinants of pathogens [5]. When the adaptive immune system has encountered a pathogen for the first time, immunological memory is formed, making it possible to mediate a fast and efficient response during the second encounter of the same pathogen [6]. The adaptive immune response is divided into the humoral response (antibody-mediated system) related to B-cells and cell-mediated related to T-cells [3].

### *Antigen and antigen-presentation*

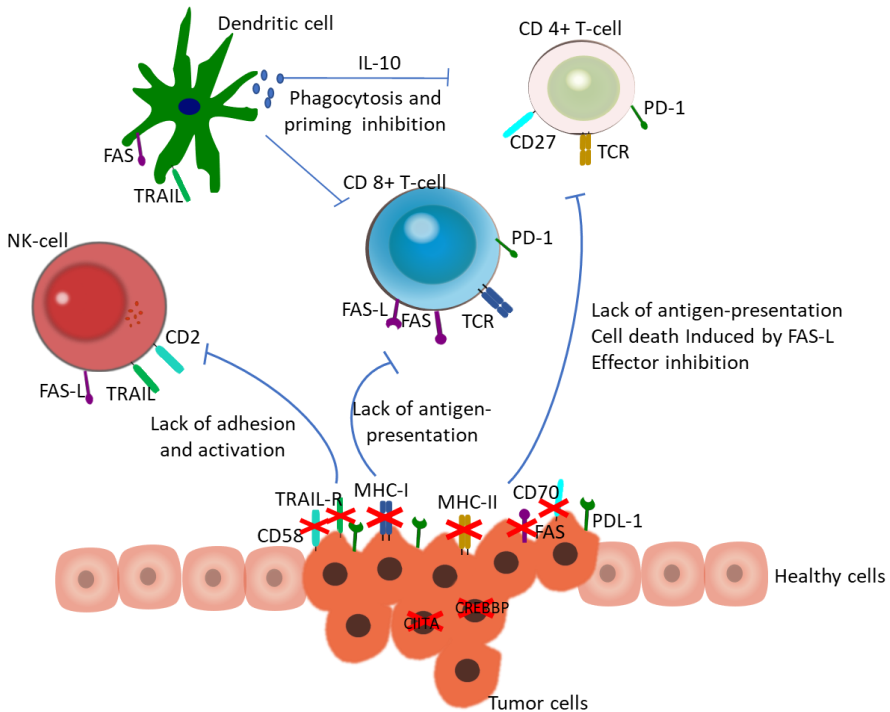
Antigens are molecules such as proteins, peptides, and polysaccharides, which may be presented on the surface of the pathogen or presented by antigen-presenting cells

(APCs), initiating the production of antibodies and immune response. The antigens can be broadly based on origin classified as exogenous from the external environment and endogenously generated within the cytosol of human cells such as tumor antigens (neoantigens) and autoantigen [7].

The antigen-presentation process is based on TCR recognition of antigen molecules, which can be “self-antigen” or “non-self” displayed on a cell surface in association with MHC molecules. The biology of antigen-presentation has significant implications in different pathologic processes, e.g., autoimmunity or tumor immunotherapy [8].

The genomic region of human leucocyte antigen (HLA), so-called MHC, encompasses 3.6 Mb (megabases) in size and harbors more than 100 genes [9]. It is subdivided into two major groups: 1) major histocompatibility complex class I (MHC-I) with human leucocyte antigen *HLA-A*, *-B* and *-C* representing the classical MHC-I genes, and 2) major histocompatibility complex class II (MHC-II) with *HLA-DP*, *-DR*, *-DQ* representing the classical MHC II genes [8,9]. Common for both MHC-I and MHC-II surface molecules is the presentation of antigens to effector T-cells. However, MHC-I and MHC-II have fundamental differences, including the nature and source of peptide fragments presented, tissue-specific expression pattern, and the interacting T-cell population. MHC-I surface molecules are expressed on all nucleated cells and play an essential role in the presentation of “self” and “non-self” antigens to CD8<sup>+</sup> cytotoxic T-cells, where the presentation of “self” antigens does not trigger an immune response. MHC-II molecules are mostly expressed on APCs, presenting exogenous antigens which lead to activation of helper CD4<sup>+</sup> T-cells that release IL-2, activating further expression of receptors on the surface of the CD4<sup>+</sup> T-cells and stimulating B-cells to synthesize antibody [10]. A phenomenon known as cross-presentation can occur where exogenous antigens derived, e.g., from viruses are presented by MHC-I surface proteins to CD8<sup>+</sup> T-cells and endogenous (cytosolic) peptides can be presented by MHC-II surface molecules to CD4<sup>+</sup> T-cells after autophagy [11]. Therefore, cross-presentation plays an important role in bolstering the immune response toward viruses, bacteria, which do not infect antigen-presenting cells, as well as tumors [12].

Some tumor cells can avoid triggering an immune response by losing the expression of MHC-I or MHC-II or both molecules, which can be caused by different mechanisms, including small deletions, mutations, or larger hemizygous deletions [13]. Loss of MHC-I molecule expression caused by mutations in *B2M* and/or *HLA* genes, building blocks of MHC-I molecules, enables avoidance of recognition by CD8<sup>+</sup> T-cells [13–15] (Figure 1). As B-cell lymphomas are tumors of antigen-presenting cells, they can, in addition to the loss of MHC-I expression, lose the MHC-II molecule expression by different mechanisms, including mutations in *CIITA* and *CREBP* preventing activation of CD4<sup>+</sup> T-cells (Figure 1).



**Figure 1. Overview of immune cells and interactions.** The transformed cells can hide from immune effector T-cells through the lack of antigen-presentation because of lost expression of major histocompatibility complex I or II (MHC- I or II) molecules. Lack of adhesion and activation of natural killer (NK) cells occur with inactivation of CD58 allowing tumor cells to escape killing by NK-cells, which are also activated by self-missing signal (loss of MHC-I). Moreover, tumor cells avoid elimination by the immune system through resistance to apoptosis signals or the expression of inhibitory receptors through the loss of FAS or TRAIL receptors. In addition, T-cells can be inhibited by inhibitory programmed cell death ligands (PD-L1) or (PD-L2) expressed by lymphoma cells. The immunosuppressive cytokine IL-10 inhibits phagocytosis, the ability of dendritic cells to stimulate the proliferation of CD4 + T-cells.

### **Macrophages**

Macrophages are professional APCs derived from blood monocytes and play an essential role in the immune system with defined functions in innate and adaptive responses. In the innate immune response, macrophages enable direct defense against foreign pathogens through phagocytosis and subsequent digestion of cellular debris of apoptotic, infected, or neoplastic cells [16]. During the adaptive immune response, they interact with B- and T-cells by releasing cytokines and chemokines [17]. Macrophages have phenotypic diversity and complex functions in homeostasis and disease and can, in general, be divided into recruited macrophages, tissue-resident macrophages, and perivascular macrophages [18].

***Dendritic cells***

Dendritic cells, professional APCs, originate from the bone marrow and circulate in the bloodstream until they reach their target tissues, where pathogens activate them and differentiate into their mature form, able to present antigens through both MHC-I and MHC-II molecules [12]. Furthermore, dendritic cells are found in two different functional states, "immature" and "mature," where the hallmark of mature form is the ability to activate antigen-specific naïve T-cells in secondary lymphoid organs and establish a link between the innate and adaptive immune response [12].

***Natural killer (NK) cells***

NK-cells originate from hematopoietic stem cells with a role in the innate immune response and are unable to rearrange their antigen receptor genes, unlike B- and T-cells [19]. NK-cells have several essential effector functions, including their capacity to spontaneously lyse susceptible pathogens through cytokines such as IFN $\gamma$  and TNF $\alpha$ , which trigger APCs, including macrophages and dendritic cells to boost the immune response [20,21]. Recently it was shown that NK-cells detect and destroy cells that have a loss of MHC-I expression due to the expression of inhibitory receptors for MHC-I on NK-cells [21]. Along with this inhibitory receptor, the detection system of NK-cells includes various cell surface activating (NKG2D) and inhibitory (NKG2A, IL-10R) receptors, and adhesion molecules (CD2), which regulate NK-cell activities [20]. NK-cells can also eliminate target cells via Fas-L and TRAIL-mediated pathways, as shown in Figure 1 [21].

***T-lymphocytes***

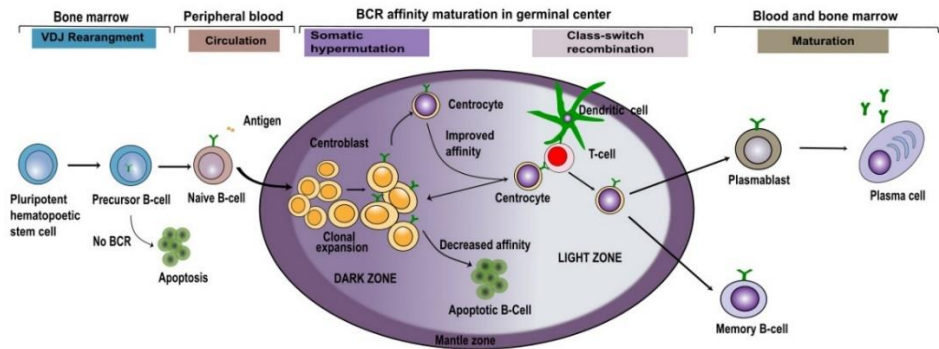
T-cells originate from the bone marrow and mature in the thymus, where they multiply and differentiate into helper (CD4+), regulatory, cytotoxic T-cells (CD8 +), or become memory T-cells. The antigen-binding TCR is expressed on the surface of T-cells and recognizes epitopes mounted on MHC molecules [5]. The MHC-I, expressed by almost every cell type, present antigenic determinants from the cytoplasm. CD8+ T-cells can recognize MHC-I signaling and respond by the secretion of granzyme B and perforin that result in the death of the target cell [22]. As opposed, the CD4+ T-cells are activated upon interaction with MHC-II, expressed by professional APCs leading to a response with the secretion of cytokines that mediate the immune response by other cells, notably B-cells [23]. Therefore, as previously described and depicted in Figure1, if antigen expression via MHC molecules is disabled, T-cells can not destroy tumor cells.



***B-cells and their development***

B-lymphocytes (B-cells) are essential members of the humoral immunity of the adaptive immune response. B-cells are white blood cells that have an essential function in protecting the host from foreign antigens by producing diverse high-affinity antibodies directed against non-self-antigens and provide immunologic memory of previously encountered non-self-antigens. B-cells originate from pluripotent hematopoietic stem cells in the bone marrow that first differentiate into multipotent progenitor cells and then into lymphoid progenitor cells. These undergo several developmental stages, resulting in early pro-B-cells, early pre-B-cell, late pre-B-cells, large pre-B-cells, small pre-B-cells, and finally, immature B-cells. Each stage is marked by various gene expression patterns and immunoglobulin rearrangements [24,25]. Rearrangement and expression of the immunoglobulin (Ig) gene in the bone marrow occur by recombination of V (variable), D (diversity), and J (joining) gene segments of the Ig genes through double-stranded DNA breaks and ligations. Upon transcription and translation, two heavy chain immunoglobulin (Ig) polypeptides and two light chains Ig polypeptides are generated and covalently linked, resulting in the B-cell receptor (BCR), which is expressed on the surface of naïve B-cells in the bone marrow [26]. Before these B-cells leave the bone marrow, their BCR is tested for functionality and specificity to ensure no self-reactivity. If the cells fail this test, they are subjected to apoptosis or undergo anergy [27,28]. Naïve B-cells circulate the peripheral blood and secondary lymphoid tissues/organs. Upon encountering foreign antigens in a T-cell dependent manner, germinal centers (GCs) are formed with a specific structural organization into a light and dark zone, enriched in each of their different cell populations (Figure 2).

In the dark zone of the GC, naïve B-cells differentiate into centroblasts and undergo somatic hypermutation (SHM), in which mutations, primarily changes in a single nucleotide or deletions and duplications, are introduced into the V hypervariable regions of the Ig gene [27,29]. This process can either increase the BCR's affinity for the antigen or produce a non-functional BCR or a BCR with decreased affinity, and the latter two scenarios will lead to apoptosis through a negative selection process [29,30]. Centroblasts expressing BCRs with increased affinity are positively selected and migrate to the light zone of the GC and differentiate into centrocytes, where they reencounter the antigen in a T-cell dependent manner, ensuring increased BCR affinity [30,31]. In the light zone, centrocytes undergo class-switch DNA recombination (CSR) of the constant region of the immunoglobulin heavy chain, resulting in different BCR isotype classes IgE, IgA, or IgG. B-cells travel between the dark and light zone over several rounds, during which several SHM occur, ensuring optimal BCR affinity [32,33]. Mature B-cells with successful affinity maturation of the BCR leave the GC and differentiate into either memory B-cells residing in the bone marrow or secondary lymphoid tissue, or plasmablasts, which continue maturation in peripheral blood into plasma cells, which finally are located back into the bone marrow [27,30].



**Figure 2. B-cell differentiation.** The B-cell maturation process starts in the bone marrow by the differentiation of hematopoietic stem cells into precursor B-cells. Further rearrangement of Variable, Diversity, and Joining (VDJ) segments of the Ig gene generates the B-cell receptor (BCR) that are further tested for autoreactivity. B-cells expressing non-autoreactive BCR circulate in the peripheral blood. By encountering antigen, naïve B-cells are activated by T-cells establishing germinal centers (GCs). In the GCs, the dark and light zone are distinguished (left and right sides, respectively). The dark zone is homing fast-proliferating B-cells, centroblasts that undergo SHM of the variable regions of the Ig gene, and further differentiate into centrocytes placed in the light zone of the GC where class-switch recombination (CSR) takes place. To ensure the optimal BCR affinity, B-cell undergoes several rounds between the dark and light zone. Finally, GC B-cells differentiate into memory B-cells or plasmablast and leave the GCs. The plasmablasts enter the peripheral blood, where they mature and migrate to the bone marrow to become antibody-secreting plasma cells. B-cells that leave the GC as memory B-cells reside in the bone marrow or secondary lymphoid tissue, activated by re-encountering their specific antigen in T-cell independent manner with further proliferation and differentiation into plasma cells.

## 1.2. Cancer and cancer immunoediting

Cancer arises by the accumulation of genetic and epigenetic alterations in a cell leading to survival and growth advantages. These capabilities are acquired during a multistep development and are defined as hallmarks of cancer. They include resistance to cell death, evasion of growth suppressors, ability to sustain proliferative signaling, genome instability, reprogramming of energy metabolism, enabling replicative immortality, inducing angiogenesis, evasion of immune destruction, and active invasion and metastasis [34]. As tumors are complex biological entities themselves, their growth is affected by other types of non-malignant cells infiltrated into the tumor and tumor-associated stroma and vasculature forming tumor microenvironment (TME) [34].

### 1.2.1. Immune surveillance (Elimination)

The tumor cell's ability to avoid destruction by the immune system is considered a cancer hallmark [34]. The complex interactions involving the immune system and tumor cells are referred to as immunoediting, divided into three phases named "three Es": elimination (immune surveillance), equilibrium, and escape (evasion) (Figure 3) [35].

At the beginning of the 20th century, immune surveillance (IS) theory for eradicating nascent transformed cells before they are clinically detected was proposed by Ehrlich [36]. Fifty years later, this theory was refined by Burnet and Thomas, suggesting that the immune system plays a critical role in the surveillance against malignant transformation [37]. Experimental studies supported this idea between the 1970s and 1990s, resulting in the discovery of NK-cells functioning as the effector cells of immune surveillance, yet though not fully confirmed at that time [38]. Further, *in vivo* experiments in gene-targeted and lymphocyte subset-depleted mice emphasized the importance of NK and T-cells in protecting the host from tumor initiation and metastasis [39].

In IS, tumor cells are recognized by the innate and adaptive immune cells, such as dendritic cells, macrophages, NK-cells, CD8+, and CD4+ T-cells, which identify neoantigens presented in MHC molecules. The primary factor of IS is interferon-gamma (IFN $\gamma$ ), which controls tumor growth and amplifies immune responses through chemokine production [40]. Stimulation of NK-cells by stress-induced ligands activates NKG2D receptors expressed on NK-cells, resulting in perforin-, Fas- and TRAIL-mediated killing of tumor cells and subsequent release of neoantigens leading to adaptive immune response. An additional result of IFN $\gamma$  production and NK-cell activation is the maturation of dendritic cells, upregulation of the pathway of antigen processing and presentation, and subsequent activation of the adaptive immune response [41,42].

For an effective antitumor immune response, both CD4+ T-cells and CD8+ T-cells are needed [43]. In particular, naive CD8+ T-cells have to receive three signals to develop effector functions: 1) recognition of antigen presented by APCs through the MHC-I surface molecule, 2) co-stimulation by ligation of CD27 with CD80/CD86, and 3) the presence of an inflammatory cytokine (IL-12) provided by CD4+ T-cells [44]. In addition to providing help to CD8+ cells, CD4+ T-cells can also directly induce apoptosis of tumor cells through the Fas/FasL pathway [44]. Cancer can enter an equilibrium phase if at least one of the transformed cells survives the elimination phase.

### 1.2.2. Equilibrium

In cancer immunoediting, the equilibrium phase can be complete by eliminating tumor cells or incomplete when some tumor cells survive the elimination [45].

In the case of partial elimination, tumor cells can remain dormant or continue to evolve by accumulating further changes such as DNA alterations or changes in gene

expression [45]. During this phase, the immune system strives to eliminate the remaining tumor cells, which are genetically unstable and continually evolve by selecting alterations that enable them to resist, avoid, or suppress the antitumor immune response, resulting in the escape phase [45]. This phase may favor either generation of poorly recognized tumors by the immune effector cells or tumors that have acquired mechanisms for suppressing immune effector functions.

### 1.2.3. Escape (Evasion)

The immune system is not able any longer control tumor progression at this phase, and tumor cells escape from the immune system by the three following strategies. The specific focus is on B-cell lymphomas and Diffuse large-B-cell lymphoma (DLBCL).

#### *(a) Avoiding immune recognition*

Tumor cells reduce their immunogenicity by decreasing the expression of MHC-I and MHC-II molecules on antigen-presenting cells, making them invisible to CD8+ and CD4+ T-cells, respectively [17].

Generally, when a cell presents non-self-antigens, the cytotoxic CD8+ T-cells are activated and destroy the presenting cell, which yet can be inhibited by tumor cell production of immunosuppressive cytokines like transforming growth factor- $\beta$  (TGF- $\beta$ ) or the dismal ratio between T-cells and tumor cells [46,47]

Crucial for MHC-I antigen-presentation is appropriate intracellular processing of proteins, which subsequently will be presented by the MHC-I molecule at the surface. Notably, somatic mutations in *TAP1* and *TAP2* encoding essential proteins of this processing have been detected in cancer, resulting in ineffective antigen-presentation and diminished immune recognition [48]. In addition, loss-of-function mutations of the *B2M* gene are commonly observed in the DLBCL, leading to deficiency of MHC-I molecule expression and reduced immunogenicity [49].

Likewise, MHC-II antigen-presentation is pivotal for adaptive immune response, which in the malignant situation can be disturbed by different genetic mechanisms [50]. Genetic studies revealed that one of the main mechanisms responsible for the loss of MHC- II expression is the inactivation of the *CIITA* transactivator through somatic mutations and gene fusions with *PD-L1* and *PD-L2* genes [51]. Also, genes encoding MHC-II can be downregulated epigenetically through alterations in *CREBBP* and *EZH2* gene [52,53].

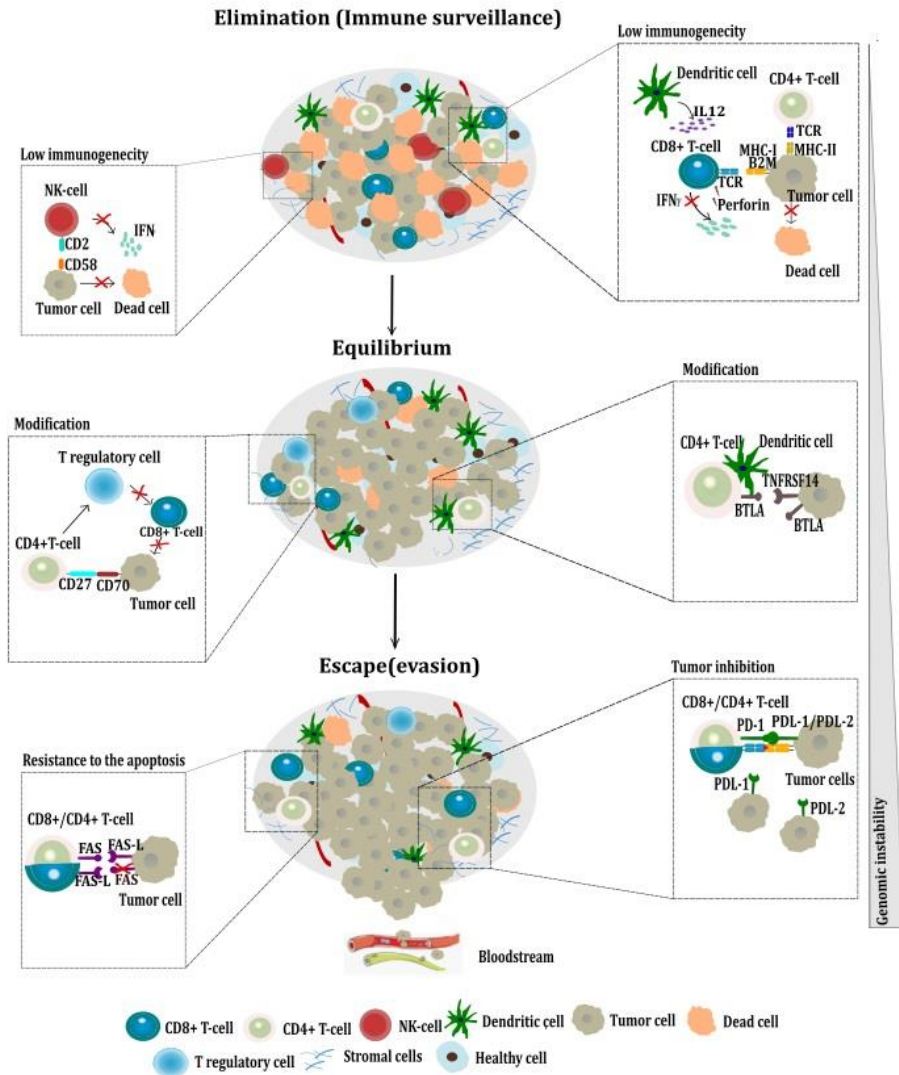
As B-cells are APCs, MHC-II is typically expressed during selection in GC's light zone via antigen-presentation to dendritic cells and T-helper cells [54]. Several studies have demonstrated that loss of MHC-I/MHC-II expression on malignant B-cells is associated with low T-cell infiltration, linked with decreased immune surveillance, and decreased survival time [55–57].

*(b) Suppressing the immune reaction*

High levels of immune-suppressive cytokines in the tumor microenvironment, such as TGF- $\beta$ , and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), as well as overexpression of the immunosuppressive transmembrane protein Programmed cell death ligand 1 (PD-L1)/PD1, have been documented to diminish the tumor immunogenicity [35]. Moreover, the cytolytic capacity of NK-cells was documented to be modulated by CD58 surface expression [14]. Recent studies showed that more than 20% of DLBCLs carry mutations in the *CD58* gene, which is important for NK and T-cell activation and adhesion [49,58]. Loss of TNFRSF14 can trigger B-cell autonomous activation of the lymphoma microenvironment with B- and T-lymphocyte attenuator (BTLA) on CD4+ T-cells [59]. The inactivation of CD70 remains to be investigated in DLBCL, but it is suggested that loss of CD70–CD27 binding reduces tumor B-cells' interaction with potential effector T or NK-cells.

*(c) Resisting immune response*

Tumor cells that do not avoid recognition and suppression by the immune system could circumvent the effect of the immune-induced perforin Fas/Trail-mediated apoptosis by somatic mutations in the *FAS* gene [60]. Although tumor cells acquire many somatic mutations that might be translated into neoantigens, making them visible to the immune system, they also develop mechanisms that make them invisible to immune recognition and block the immune factors in inducing apoptosis [61]. These mechanisms include the expression of MHC molecules previously explained and impairment of immune-checkpoint pathways. In particular, immune-checkpoint pathways, including Cytotoxic T-lymphocyte antigen 4 (CTLA-4) or CD28/ B7 and PD-1 – PD-L1/PD-L2 axis, regulate activity and infiltration of T-cells in the TME [62]. Both B7 and PD-L1/L2 ligands are expressed on APCs, however, the mechanisms of these differ in time of interactions, whereas the CTLA-4/B7 axis regulates early immune responses in lymphoid tissues, while the PD-1/PD-L1 axis is active in the late responses in the peripheral tissues [63]. If these axes are impaired, they inhibit T-cell activity [64].

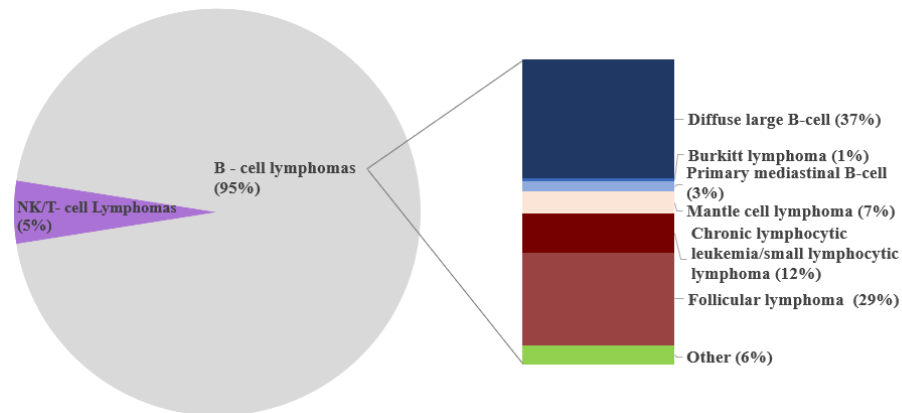


**Figure 3. Cancer immunoediting.** Cancer cells display distinct tumor-specific markers at the early stages triggering innate pro-inflammatory responses followed by the adaptive immune response. In the elimination (immune surveillance) phase, innate and adaptive immune cells recognize and kill tumor cells. Cells that avoid this phase progress to the equilibrium phase, where tumor cells are prevented from expansion by the immune system. However, tumor cells with diminished immunogenicity survive, and increased genomic instability leads to the escape phase resulting in tumor outgrowth.

### 1.3. Lymphoma

Lymphomas are heterogeneous group of malignancies, which arise from the lymphoid lineage cells and are classified as B-cell, T-cell, or NK-cell lymphomas [65]. In accordance with World Health Organization (WHO) guideline, lymphomas are classified into two main classes, Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL), based on clinical, morphological, histological, and genetic features [65]. Most NHLs originate from B-cells (90-95%), which can be indolent (e.g., follicular lymphoma) or aggressive (e.g., Burkitt, Primary Mediastinal B-cell, Mantle Cell Lymphoma, DLBCL), while only 5-10% constitutes mature NK/T-cells lymphoma (Figure 4) [26]. The high incidence of B-cell derived malignancies can be explained by the SHM and CSR processes, which occur in the GCs from where most B-cell lymphomas originate [26,28]. Double-stranded DNA break involved in these processes together with the substantial clonal expansion of germinal B-cells, which is essential for a normal immune response to an antigen, is a high risk that easily leads to malignant transformation due to the introduction of genetic alterations [30,66].

B-cell lymphomas are classified based on the B-cell developmental stage from which the malignant clone arises, as they retain key features of their cell-of-origin. Therefore Burkitt lymphoma, Follicular lymphoma, and germinal center B-cell-like (GCB) DLBCL reassemble GC B-cell origin by showing somatically mutated IgV as a sign of ongoing SHM throughout the clonal expansion, while activated B-cell-like (ABC) DLBCL shows characteristics of post GCs lymphoma [67].



**Figure 4. Lymphoma classification, subclassification of B-cell lymphomas.** Aggressive lymphomas are marked with blue colors; indolent lymphomas are red-colored, frequencies obtained from Swerdlow et al., 2017 [65].

### 1.3.1. Diffuse large B-cell lymphoma (DLBCL)

DLBCL is the most frequent lymphoid neoplasm among adults, with a crude incidence of 3-4/100,000 in the European Union and approximately 450 new patients diagnosed each year in Denmark [68,69]. The incidence rates have increased in the last decades and are increasing with age, affecting more men than women [69,70]. DLBCL can be a primary disease (develop de novo) or transform from an indolent NHL such as follicular lymphoma [70,71]. It can be located in the lymph nodes (nodal) or other tissues (extranodal), where the most common extranodal site is bone marrow [65,72]. According to WHO, DLBCL is a clinically, morphologically, and molecularly heterogeneous group of lymphomas classified into four categories: DLBCL not otherwise specified (DLBCL, NOS), high-grade B-cell lymphoma, other lymphomas of large B-cells, and B-cell lymphoma unclassifiable [65].

### 1.3.2. The tumor microenvironment and DLBCL

TME of B-cell lymphomas is composed of a highly variable number of stromal cells, blood vessels, and extracellular matrix, where tumor cells interact with different cell types in this environment, including tumor-infiltrating immune cells such as B- and T-cells, tumor-associated macrophages, dendritic cells, myeloid-derived suppressor cells, and cancer-associated fibroblasts [73,74].

Recent research by developing technologies allowing high-resolution discrimination of cellular and extracellular determinants at the functional level has uncovered that the TME in B-cell lymphomas is pivotal for providing survival and proliferation signals for disease progression and drug resistance [73,75]. Besides, it was recently revealed that in DLBCL, disordered cross-talk between lymphoma cells and the microenvironment enhances tumor cell ability to escape the host's immune surveillance [76].

### 1.3.3. DLBCL treatment

Currently, standard treatment can cure more than half of the patients suffering from DLBCL [77]. However, the major challenge in DLBCL is that a great number of patients relapse after initial treatment or are refractory to treatment and eventually succumb to their disease, underlying the necessity for establishing new biomarkers and therapeutic options. Here we used the definition of biomarkers as “A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention”[78].

#### ***R-CHOP***

Nowadays, a multidrug regimen consisting of Rituximab (R), Cyclophosphamide (C), Doxorubicin (H), Vincristine (O), and Prednisone (P) (R-CHOP) is the gold standard in the treatment of de novo DLBCL [79]. Rituximab is a chimeric monoclonal



antibody against CD20, a surface protein expressed on all B-cells, and the first immunotherapeutic agent to be used to manage DLBCL [80]. The addition of rituximab to the previously used CHOP regimen increased the five-year overall survival (OS) by 10-15% leading to 50-80% in primary DLBCL [81].

### ***Salvage therapy***

Approximately one-third of DLBCL patients experience a relapse or refractory disease after first-line R-CHOP-treatment. Relapsed/refractory patients, who often have a poor prognosis, receive salvage treatment of high-dose chemotherapy combined with autologous stem-cell transplantation (ASCT), even though treatment has improved, 30-40% of DLBCL patients will eventually die from the relapsed or refractory disease [81,82]. The most common regimens for refractory/relapsed DLBCL patients are R-ICE (rituximab, ifosfamide, etoposide, carboplatin), R-DHAP (rituximab, dexamethasone, high-dose cytarabine, cisplatin), MINE (mesna, ifosfamide, mitoxantrone, etoposide), and GEMOX (gemcitabine, oxaliplatin) [68,83,84]. If the patients are eligible, immunochemotherapy is followed by ASCT, while the patients who are not eligible for ASCT are treated with GEMOX or MINE with the addition of rituximab [68,85]. As most of the patients are not eligible for ASCT due to refractory disease, age, or comorbidities, new therapeutic approaches in DLBCL management need to be established [81].

### ***New therapeutic approaches***

The discovery of rituximab demonstrated a step toward targeted therapy as it specifically targets the B-cell surface antigen, CD20 [80], shifting the field from traditional chemotherapy toward pointing at specific targets on tumor cells. The field is currently in the 'molecular era' of discovering somatic mutations and aberrant intracellular pathways enabling disease stratification into genetic subtypes, revealing great molecular complexity with high variability across patients, which remains a challenge.

The TME plays a role in resistance to chemotherapy and has been widely exploited and utilized for new therapeutic strategies re-educating immune cells of the TME to target the tumor cells [86].

### ***Immune checkpoint inhibitors***

The immune checkpoint molecules are regulators of the immune system, preventing the immune system from attacking cells indiscriminately. In recent years, immunotherapy has made major advances in treating patients by targeting a series of cell surface immune checkpoint molecules [87]. Currently, anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibody drugs, interrupting immune checkpoint signaling and restore immune response, are investigated. CTLA-4 inhibits immune responses in several ways, including attenuation of T-cell activation at an early immune response stage. At

the same time, PD-1 expressed on T-cells inhibits T-cells at later stages of the immune response in peripheral tissues [88]. CTLA-4 is expressed on T-cells, and its engagement downregulates the T-cell response by inhibiting co-stimulation by CD28 [89]. To date, of the two CTLA-4 blocking antibodies, ipilimumab and tremelimumab, only ipilimumab has been approved by the Food and Drug Administration (FDA). Although the CTLA-4 antibody binding is upregulated in lymphoid malignancies, clinical trials have shown relatively low response rates in DLBCL [89].

PD-L1 and PD-L2 are expressed on APCs, which interact with their receptor PD-1 on T-cells, leading to functional exhaustion of T-cells, which promote their apoptosis. Currently used anti-PD-1 antibodies are Nivolumab, Pembrolizumab, and pidilizumab, and for anti-PD-L1 Durvalumab, urelumab, and Atezolizumab Nivolumab [90]. Nivolumab has shown a low overall response rate among patients with DLBCL, particularly in patients ineligible for ASCT or who experienced failure with ASCT [91]. Immune checkpoint inhibitors have a low to moderate toxicity profile where the primary toxicity of CTLA-4 and PD-1 inhibitors is associated with its activity in boosting the immune response [92].

### ***CAR T-cells***

A potential approach for targeting refractory DLBCL is chimeric antigen receptor T-cell (CAR-T) therapy, a revolutionary type of cellular treatment that offers a new curative option. CAR-T cells are generated from patients' T-cells, which are genetically engineered *ex vivo* to express a chimeric antigen receptor (CAR) combined with their natural T-cell receptor (TCR) [93]. CAR is modified to express a synthetic construct that combines an extracellular binding domain, usually an antibody-derived single-chain variable fragment (scFv), and T-cell activating function. The main difference from regular TCR is that CAR enables highly specific antigen targeting in an MHC molecule independent manner [93]. The FDA approved two CD19 CAR-T therapeutic options, axicabtagene ciloleucel (axi-cel) and tisagenlecleucel, to treat adult DLBCL patients with refractory/relapsed disease after two conventional therapies in certified institutions [93,94]. Both of these therapies showed promising results in clinical trials in cases of multiply recurrent DLBCL, but with complications such as fatal neurologic events and cytokine release syndrome [95]. Also, a drawback of CAR-T cell therapies is syndrome B-cell aplasia due to the inability of CD-19 targeting CAR-T cells to discriminate between malignant and normal B-cells [93].

### **1.3.4. DLBCL prognosis**

The International Prognostic Index (IPI) is the gold standard in clinical practice for prognostic stratification of patients [96]. IPI is a scoring system based on five clinical parameters 1) age at diagnosis (>60), 2) elevated serum lactate dehydrogenase (LDH), 3) Ann Arbor stage III or IV (determined by tumor localization), 4) extranodal involvement >1, and 5) Eastern Cooperative Oncology Group (ECOG) performance status  $\geq 2$  [96]. Using the IPI scoring system, R-CHOP treated DLBCL patients can

be stratified into four risk groups (0-1, 2, 3, and 4-5) with different 5-year OS ranging from 26% to 73%, where high IPI being associated with poorer prognostic outcome. This system was developed before the inclusion of rituximab in treatment. With R-IPI, patients can be stratified into three risk groups (0, 1-2, and 3-5) with a 4-year OS ranging from 53-94% [77]. The most recent scoring system developed is the NCCN-IPI system, developed for patients treated with immunotherapy. The system has four risk groups similar to the IPI system and performs better than IPI by discrimination of low- and high-risk patients with a 4-year OS probability between 33% and 96%. It uses the same clinical features as the IPI, however additional age and LHD categories are included [97]. Despite the improvements to the IPI system, neither of them is implemented in standard clinical prognostic evaluation. The ease in using the IPI as a prognostic tool in clinical practice is among its advantages, however, the biological and molecular pathways driving the tumorigenesis and treatment resistance are not reflected in the IPI.

#### **1.4. Molecular subclasses defined by transcriptional classification systems**

Gene expression profiling (GEP) has been the basis for DLBCL classification into two histologically indistinguishable subclasses: the germinal center B-cell-like (GCB) and the activated B-cell-like (ABC), with the remaining cases that can not be classified as ABC or GCB, defined as unclassified (UC) [98,99]. Pathological and clinical outcome differences are observed between the ABC and GCB subclasses, where the ABC subclass is characterized by increased aggressiveness and less favorable disease outcome compared to the GCB subclass [100]. The GCB subclass reassembles GC B-cells with aberrant SHM and CSR and has an overall 45-50% incidence, which is slightly higher than ABC of 40-42% [98,101]. The hallmark of the ABC subclass is genetic alterations (MYD88<sup>L265P</sup> and in CD79B) that evoke a chronically activated BCR signaling pathway and anti-apoptotic nuclear factor kappa B (NF- $\kappa$ B) pathway [102,103]. A gain of function mutation in the *EZH2* gene with hotspot Y641 is a hallmark feature of the GCB subclass [104–106]. The 5-year survival rate for GCB classified patients is approximately 69-79% compared to 52-53% for those classified with ABC-DLBCL when treated with standard immunochemotherapy R-CHOP regimen [107].

The cell-of-origin (COO) classification is based on GEP, using RNA and techniques such as DNA microarrays initially, then RNA-sequencing and nanostring. Further development of transcriptional classification systems led to the development of refined classification, B-cell associated gene signatures (BAGS), an extended cell-of-origin classification with prognostic impact independent of the ABC/GCB classification and IPI [108]. Ennishi and colleagues have recently proposed a GEP based classification system distinguishing high-grade B-cell lymphoma (with MYC and BCL2 and/or BCL6 rearrangements) from GCB referred to as "DHITsig+" and without rearrangements "DHITsig-" [109]. Patients of the DHITsig+ have an inferior

prognosis than those of DHITsig-, where the indicator of inferior prognosis is frequent loss of MHC-I and/or MHC-II expressions [109]. Moreover, the GEP study has revealed molecular signatures originating from inside the malignant cells and the host's TME, both influencing survival [110]. The signatures reflecting the TME are variously present in both ABC and GCB subclasses and are referred to as "Stromal-1" signature (reflects extracellular matrix and active immune response) and "Stromal-2" signature (indicate increased angiogenesis and blood vessel density), which is prognostically unfavorable compared to "Stromal-1" [110]. However, none of these GEP based classifications are widely used but reveal that microenvironment features contribute to the pathogenesis of DLBCL, suggesting a further investigation of other molecular stratification and an integrated approach.

### 1.5. Immunohistochemistry

Immunohistochemistry (IHC) based classification algorithms have been widely applied since every pathology department routinely run IHC but not necessarily the more advanced global mRNA quantitative techniques [65,98]. Hans and colleagues translated the COO classification into an IHC algorithm based on three markers (CD10, BCL6, and MUM1), resulting in the classification of all cases into GCB and non-GCB subclasses [111]. Furthermore, the Choi algorithm is based on BCL6, MUM1, GCET1, CD10, and FOXP1 and has 93% concordance with the GEP ABC/GCB classification of DLBCL [112] as well as the new algorithm described by Tally that has a slightly different approach based on CD10, MUM1, LMO2, GCET1, and FOXP1 [113]. Conversely, Nyman and colleagues suggested a simplified algorithm based only on FOXP1 and MUM1 [114].

The method's advantage is the relatively low cost, while the reproducibility, accuracy, and reliability are disadvantages due to the not standardized IHC stains among laboratories worldwide [115].

### 1.6. Genetic classification systems

In recent years, as the sequencing technologies developed, over 3000 de novo DLBCL cases have been examined, allowing new insight into disease heterogeneity and recurrent genetic alterations as a new parameter allowing classification of DLBCL in distinct subtypes [105,116–120]. Investigation of the genetic landscape of DLBCL started with the discovery of ABC/GCB subclasses and was organized and explored through these subclasses, revealing a remarkable difference in the recurrence of specific genetic alterations, as discussed previously (Figure 5) [105,116,121]. These observations further led to the classification of DLBCL cases based on shared genetic alterations using different sequencing and bioinformatic methods.

Four recent studies analyzing independent cohorts using DNA sequencing methods such as whole-exome sequencing (WES) and targeted sequencing, and different

bioinformatical algorithms classified DLBCL into 5-7 genetic subtypes, which are overlapping to a large extent not only in genetic alterations but also in prognosis (Figure 5) [117–120]. In further discussion, genetic subtypes will be named as they are named initially in the studies and compared (Figure 5).

The MCD [118,119] genetic subtype, which aligns with the cluster 5 (C5) [117] and MYD88 [120] subclasses, is characterized by *MYD88*<sup>L265P</sup> mutation and *CD79A/B* mutations affecting the NF- $\kappa$ B pathway and chronically activate BCR signaling. The MCD is almost exclusively found in ABC-DLBCLs. Besides these hallmark genetic alterations, the MCD subtype is highly enriched with alterations in genes affecting presentations of neoantigens, and it is associated with inferior prognosis [119,120].

The EZB [118,119] genetic subtype, which aligns with cluster 3 (C3) [117] and *BCL2* [120], is characterized by GCB in origin, with *EZH2* alterations and *BCL2* translocations, and is associated with good clinical outcome. Moreover, this subtype includes loss of function mutations in *CREBBP*, affecting the transcription of MHC-II through the epigenetic pathway [122]. In line, *TNFRSF14* loss-of-function mutations were not found in all these three aligned genetic subtypes, suggesting that microenvironment actors are more supportive than essential in classification systems. The BN2 [118,119] genetic subtype, which aligns with cluster 1 (C1) [117] and *NOTCH2* [120], has a favorable outcome, and is defined by *NOTCH2* activating mutations and *BCL6* translocations, and is mostly assigned to ABC and UC subclass [123].

Subtype ST2 [119] aligns to C4 cluster [117] and *SOCS1/SGK1* [120] and is mostly enriched by GCB DLBCLs. The ST2 subtype is associated with a favorable prognosis. Of note, the *SOCS1/SGK1* subtype has a strong similarity with the *TET2/SGK1* subtype [120]. Moreover, C4 and ST2 subtype are enriched with genetic alterations in the *CD58* gene, which is involved in immune recognition by NK-cells and reported as a significant marker of the centrocytes present in the light zone of GC [14,124]. The ST2 subtype also aligns with *TET2/SGK1* [120], indicating the need for further investigation to consider these two subtypes as distinct.

The A53 subtype [119] that aligns with C2 [117] and NEC [120] is characterized by alterations of the *TP53* gene, including an increase in ploidy. Patients of the A53 subtype are both ABC and GCB of origin and are assigned a favorable clinical outcome. The N1 [119] subtype aligns only with NEC [120]. It is defined by *NOTCH1* genetic alterations, consists mainly of ABC DLBCLs of origin, and is associated with a favorable outcome. This subtype is very rare, indicating a discrepancy in classification systems.

Classification of DLBCL into genetic subtypes has improved understanding of disease heterogeneity and patient stratification with the remaining challenge of deciding about accurate allocation of cases to the genetic subtypes. Further efforts have to be made to find the balance in classifiers between getting very few cases unclassified by forcing the classification, which results in genetic subtypes that can be overinclusive [117], and a high percentage of unclassified cases as a result of high requirements for classification into genetic subtype [118,119].



be released by normal and tumor cells by multiple mechanisms such as apoptosis, active secretion, and necrosis [126,127]. The half-life of cfDNA in peripheral blood is from 16 minutes up to 2.5 hours [127,128] when cfDNA is cleared by DNase I activity, kidney, liver, and spleen, followed by macrophagic degradation, but the mechanisms of clearance of cfDNA are still poorly understood [129]. The cfDNA concentration varies in healthy people ranging from 1-10 ng/ml in plasma, which easily can be affected by physiological conditions such as infections and exercise [130,131]. On the other hand, cancer patients display higher levels of cfDNA in plasma ranging from 5-1500 ng/ml [132].

Circulating tumor DNA (ctDNA) are those DNA fragments originating from tumor cells, reflecting the tumor burden. ctDNA constitutes only a small fraction of the total cfDNA (1%) [127,133]. The ctDNA measure approximately 90-150 base pairs in length and encompass tumor-specific alterations in oncogenes, tumor suppressor genes, microsatellite instability, and DNA hypermethylation [134]. ctDNA is released by tumor cells and can be detected by the liquid biopsy method, and increasingly for diagnostic, prognostic, and treatment purposes of cancer [127,133]. A recent study showed that the concentration of ctDNA correlated with tumor size and the stage of disease [135].

The tumor heterogeneity is emphasized with studies sequencing tumor biopsies and showing the differences in mutational status between different tumor regions from the same patient as well as between diagnostic and relapsed tumors from the same patients [136,137]. The current tools for genotyping in DLBCL and other tumors are based on tumor tissue biopsies, and with the current emphasis on tumor heterogeneity, these results may not illustrate the real picture due to the limitations of biopsies. Considering invasiveness and possible inaccessibility for taking tumor biopsy and limited tumor tissue, genotyping of cfDNA is a preferred choice [138]. The current tools for genotyping of cfDNA are next-generation sequencing (NGS) and Droplet Digital PCR (ddPCR), emphasizing high sensitivity and specificity due to the very low content of ctDNA in the background of cfDNA.





## 2. HYPOTHESIS AND AIMS

Every year approximately 450 Danes are diagnosed with Diffuse Large B-cell Lymphoma (DLBCL) and receive immunochemotherapy to eliminate the tumor. However, 30-40% of DLBCL patients suffer from a refractory or relapsed disease, primarily caused by intrinsic or acquired treatment resistance. Treatment resistance can be induced by multiple mechanisms, including the ability of tumor cells to evade immune surveillance of the host by disabling antigen-presentation or by promoting immune exhaustion or suppression. Consequently, it is crucial to identify biomarkers and understand the biological mechanisms of disease initiation and progression, as well as the drug response at the time of diagnosis and relapse. Considering the genetic heterogeneity of DLBCL and that diagnosis is still based on invasive tissue biopsies, which are not feasible for patients' follow-up and sometimes even for relapse due to their invasiveness, it reflects a major obstacle for the development of new treatment approaches and stratification tools. Thus, novel techniques such as liquid biopsy, which is not standardized yet in clinical practice, may replace or complement tissue biopsies to detect somatic mutations in cfDNA purified from patients' blood.

**Hypothesis:** *The main hypothesis in this project is that somatic genetic alterations in specific genes involved in and controlling immune surveillance are important determinants in the DLBCL disease development and response to immunochemotherapy and that we by genetic analysis of primary and refractory/relapsed clinical samples, can develop better tools for disease stratification and simultaneously obtain an improved biological understanding of drug resistance.*

**Objectives:** The overall objective is to examine the presence of mutations of immune surveillance genes in DLBCL at the time of diagnosis and progression or relapse and to assess the impact on disease development and treatment response.

- Aim I** To identify genes involved in major immune surveillance pathways in DLBCL by systematic literature research and cancer database search.
- Aim II** To detect somatic genetic alterations in diagnostic and refractory/relapsed DLBCL patients treated with first-line R-CHOP treatment using Whole-Exome Sequencing (WES) and explore the mutational patterns associated with disease state and treatment response.
- Aim III** To investigate the feasibility of detecting specific tumor genetic alterations in tumor tissue and cfDNA purified from clinical long-term archival plasma samples using WES and single gene ddPCR, respectively.



### 3. EVALUATION OF MATERIALS AND METHODS

In this thesis, the materials and methods used are described in specific sections of the respective manuscripts. This section will elaborate on the main materials and methods and discuss their advantages and disadvantages.

In the second manuscript, whole-exome sequencing (WES) and single gene digital droplet PCR (ddPCR) are used to analyze genetic variants in DNA from diagnostic and refractory/relapsed clinical DLBCL biopsies and DLBCL cell lines. In the third manuscript, different DNA extraction methods are used to optimize cell-free DNA yield from the plasma of peripheral blood from healthy donors, and specific mutations are assessed by ddPCR in tumor biopsies and plasma from DLBCL patients and cell lines.

#### 3.1. Clinical samples

Tissue specimens from DLBCL tumors were stored as fresh-frozen (FF) biopsies making them usable to analyze nucleic acids. Because tissue specimens are subjected to rapid autolysis, they must be stabilized immediately after removal from the patient [139]. The FF biopsies used in this thesis were collected at Aalborg University Hospital, embedded with optimal cutting temperature (OCT), (Tissue Tek®) compound and snap-frozen and stored at  $\leq -80^{\circ}\text{C}$ , allowing extraction of molecules with high quality and yield that is necessary for molecular profiling techniques such Next-Generation Sequencing (NGS) [139]. To minimize the influence of tissue heterogeneity, we made three subsampling of tumor tissue from different locations in the still frozen OCT embedded tissue, homogenized using TissueLyser prior to DNA extraction. A certain degree of contamination by the non-cancerous cell cannot be excluded, even if high-density grey cellular regions were preferred for subsampling. A purer sample composition could be obtained by laser capture microdissection, however, this technique is highly labor-intensive and provides a very small yield. Bioinformatic approaches for estimating tumor purity can be useful but have not been applied to our local clinical samples analyzed since we do not have gold standard pathology estimations of tumor purity to compare against. A paired normal DNA sample from saliva was included to enable subtraction of germline genotype for all diagnostic and refractory/relapsed DLBCL patients analyzed by whole-exome sequencing in the second manuscript, ensuring that only cancer somatic genetic variations were detected.

### 3.2. Cell lines

Five human DLBCL-derived malignant cell lines (SU-DHL-4, FARAGE, NU-DHL-1, DB, and DOHH2) were used to optimize ddPCR assays for rare mutation detection on gDNA and cfDNA.

*In vitro*, human cell lines are a valuable unlimited source of genetic material widely used in cancer research, including studying pathogenesis along with resistance mechanisms. Advantages of using cell lines, besides the mentioned unlimited supply of material, include cost-effectiveness, ease of use, and circumvent ethical concerns associated with the usage of animal and human tissue [140]. An important consideration when using cell lines is that they do not entirely mimic the interindividual heterogeneity of the disease due to their origin from one patient, nor do they reflect the intraindividual genetic heterogeneity of the patients' tumor. Also, the disadvantage of using cell lines for studying biological processes include lack of the tumor microenvironment that often includes interactions with other cell types that may be critical in the studied process, as well as the predisposition of cell lines for genetic drift in the long term culturing resulting with phenotypic alterations and growth patterns [141,142]. Another concern is the risk of cell line cross-contamination with a foreign cell line without remark that could lead to overgrowth of the foreign cell line along with long culturing time which increases the risk of acquiring additional genetic alterations leading to misinterpretation of the experiments [142,143]. Moreover, the metabolism and growth rate can be altered due to mycoplasma infection and can alter experiment results inducing chromosomal aberrations in the cells [142,143]. Therefore, the cultured, as well as frozen cell line stocks, should be tested for mycoplasma regularly. The identity of our cell lines used in this thesis was confirmed using short tandem repeat (STR) profiling of the amelogenin gender-determining marker and eight tetranucleotide repeat loci.

### 3.3. Next-generation sequencing (NGS)

NGS provides a powerful tool widely used in research and clinical practice, enabling sequence information of large regions in the human genome and capturing of the entire genome or transcriptome in a single run. The most significant technological advances, NGS has a variety of applications in both DNA and RNA analysis, such as whole-genome sequencing (WGS), WES, along with gene panel sequencing as main DNA sequencing approaches, and transcriptome profiling (RNA-Seq), mRNA, and small RNA as main RNA sequencing applications. In combination with modified nucleotide analysis, NGS can also be applied to detect DNA-protein interactions in chromatin immunoprecipitation combined with massively parallel DNA sequencing (ChIP-sequencing), epigenome characterization in methylation sequencing (Bisulphite sequencing), and single-cell sequencing. The most commonly used sequencing platforms are Illumina platforms MiSeq, NextSeq, HiSeq, and NovaSeq 6000.

## ***Workflow***

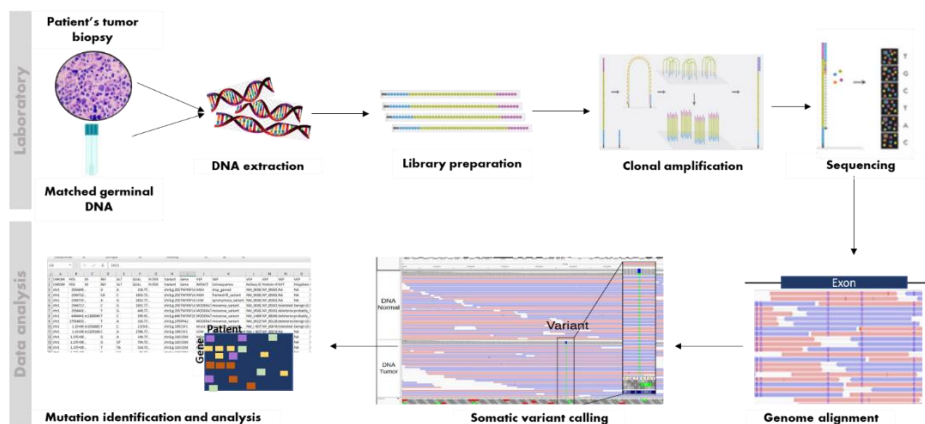
Regardless of the used platform, the principles of NGS can be summarized into four main parts after the DNA extraction: library preparation (construction), amplification, sequencing, and data analysis, as presented in Figure 6. For library construction, DNA needs to be randomly fragmented into 100-200 bp fragments on which adapters consisting of the sequencing primer and barcode oligo are ligated by a DNA ligase. Barcodes (index sequences) are used for discrimination between samples, and they are applied in a second PCR amplification using index specific primers. The library is then loaded into a flow cell containing two types of fixed complementary oligos for adapter hybridization.

The type of amplification differs between the NGS platforms, hence the bridge amplification process, which has been used in this thesis, is described here. In a bridge amplification, a DNA polymerase uses a hybridized fragment to produce a complementary strand. The original strand is then discarded by denaturation, and the newly synthesized strand folds over thus, the second type of fixed oligo on the flow cell hybridizes, forming a bridge, where polymerase produces a double-stranded bridge. The double-stranded bridge is then denatured, resulting in two copies of a single molecule anchored on a flow cell [144]. The process repeats, allowing millions of copies to be generated for each library fragment forming clonal clusters in a predefined number of cycles (Figure 6, top right corner).

For sequencing of the amplified clonal clusters, the reverse strands are denatured, and 3'ends of the forward strands are blocked, preventing unwanted priming. The sequencing process then starts by incorporating nucleotides, labeled by different fluorophores specific for each base, and then blocking chain elongation by the reversible terminator. Non-incorporated nucleotides are washed off, and a camera identifies fluorescence at each chain-end. This sequencing process is named sequencing by synthesis (SBS), and the length of reads is defined by the number of cycles where the new nucleotide is incorporated [144].

For paired-end read sequencing, 3'end of the forward strand is unblocked and hybridizes to a second oligo on the flow cell where the index fragment is sequenced, and by bridge amplification, double-stranded fragments are formed. Whereas "the bridge" formation is linearized, and the forward strand is washed off. The reverse strand is then sequenced in the same manner as the forward strand.

The raw data is processed by bioinformatic removal of the adapter sequences and filtering out low-quality reads. The sequencing reads are aligned against the human GRCh38.d1.vd1 genome reference sequence using Burrows-Wheeler Aligner (BWA) mem v0.7.12 for normal and tumor samples [145]. For detecting somatic variants, we used a combination of Mutect2 and VarScan, and Ensembl variant predictor annotation of variants, which includes SIFT and Polyphen-2 [145]. Analysis of the data is done by the R version 4.0.3 (2020-10-10) programming language.



**Figure 6. Overview of DNA sequencing and analysis workflow.** DNA extraction from the tumor and matched normal sample, DNA is fragmented, allowing adapters and indexes to be ligated to prepare the sequencing library. The library hybridizes to the fixed oligos at the surface of the flow cell and is converted into clusters by bridge amplification. Next, sequencing by synthesis is performed by adding fluorescently labeled nucleotides incorporated in each cycle, and a fluorescent readout is performed. Each DNA fragment sequence is determined and available for subsequent bioinformatics analysis such as genome alignment, somatic variant calling, and final analysis of mutations to determine recurrently mutated genes.

### *Whole-exome sequencing (WES)*

The collection of all exons in a genome are termed 'exome', thus the method for sequencing them is called whole-exome sequencing, which enables simultaneous sequencing of approximately 20,000 protein-coding genes. WES is cost-effective and has a simplified variant analysis and data storage compared to whole-genome sequencing (WGS), thereby being widely used in research and clinical settings [146]. Although the WES approach focuses on protein-coding genes, which are most likely genes to affect the studied phenotype, WGS is a preferable approach for the comprehensive identification of genetic variations, including structural aberrations, point mutations, and small indels. The accuracy of each read is critical for both methods and depends on several factors such as the quality scores of base calling, sequence content, the depth of coverage, and the error rate of the used NGS technology [147]. A quality score, also called a Q score, is assigned to each sequenced base, representing the quantitative measure of the base call's accuracy. It is based on the quality or Phred scores that are used in Sanger sequencing, defining the likelihood of accurate base calling [148]. Q scores range from 10-50, with a  $Q \geq 30$  showing a 1 in 1000 likelihood of an error or 99.9% accuracy of base calling [149]. As the base calling varies between the NGS platforms, and each platform introduces platform-specific errors, the Q scores need to be adjusted as it does not assess the source of the errors, taking into account parameters such as depth of coverage, the error rate of the

sequencing technology, and confidence of the alignment of reads to the reference [147]. The depth of coverage is generally reported as the average number of overlapping reads within the sequenced genomic region, and optimal read depth for sequencing of tumor biopsies is  $\geq 200X$  as sufficient for calling 95% of the mutations for mutation frequency  $\geq 20\%$  [150].

In this Ph.D. thesis, the WES technique was used to identify recurrently mutated genes in diagnostic and refractory/relapsed DLBCL with subselection of genes involved in immune surveillance, as described in detail in paper II. A genetic variant detected with WES (excluding copy number variations) is defined as any position or region in our sample, which differs from the reference genome or germline of the patient. WES was performed by CeGat (Munich, Germany) using DNA extracted from tumor biopsies of DLBCL patients and their individually matched saliva samples as germline control, as shown in Figure 6.

### 3.4. Droplet digital polymerase chain reaction (ddPCR)

ddPCR is improved biotechnology of conventional PCR and enables the absolute quantification of target nucleic acid templates in a sample by counting the actual number of target nucleic acid molecules encapsulated in water-in-oil droplet partitions [151]. It can be used to detect DNA targets such as point mutations, DNA methylation, chromosomal translocations, alternatively spliced mRNA, and copy number variations, which are applied in different fields such as cancer diagnostics, prenatal diagnostics, microbiology, as well as for quantification of NGS libraries [152].

This novel method is used in the presented PhD thesis in paper II and paper III to detect recurrently mutated genetic alterations in gDNA and cfDNA, respectively.

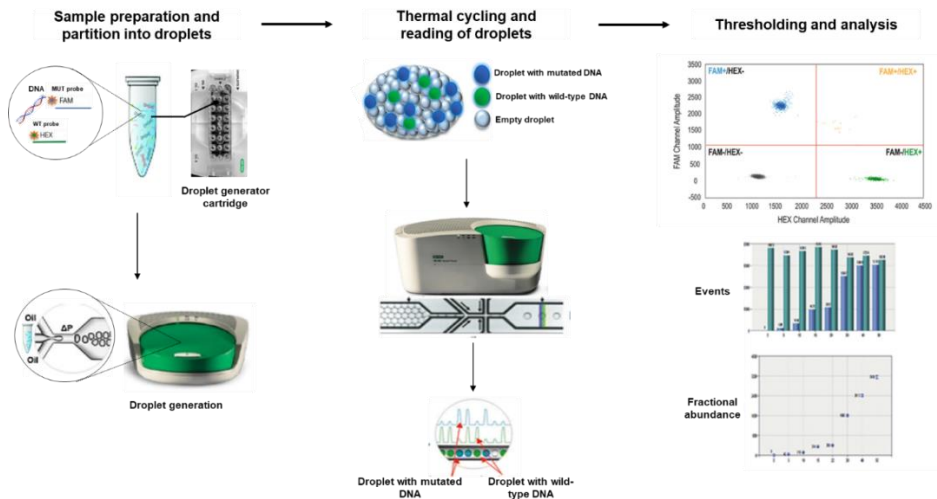
#### *Workflow*

ddPCR relies on PCR principles and offers a simple workflow divided into three major steps: a) Sample preparation and partition into droplets, b) thermal cycling and reading of droplets, and c) thresholding and analyzing the data, as depicted in Figure 7. In the first step, the sample DNA is added to premixed reagents containing 1x ddPCR Supermix for Probes (No dUTP) (Bio-Rad), and 1x wild-type primers/probe (HEX)/1x mutant target primers/probe (FAM) (Bio-Rad) and nuclease-free water. Each 20  $\mu$ L reaction is transferred to a disposable eight-well droplet generator cartridge, which is loaded into a QX200 Droplet Generator (Bio-Rad), which applies vacuum to each well in order to generate nanoliter water-in-oil droplets, which are subsequently transferred to a 96-well PCR plate that is heat-sealed with foil. Each sample is partitioned into approximately 20,000 nanoliter-sized droplets, of which some contain only zero or one copy of the target, while other droplets will contain multiple target copies. After thermal cycling, the plate is transferred into QX200 Droplet Reader (Bio-Rad), which aspirates droplets from the individual wells and measures the

fluorescence in each droplet using the FAM and HEX channels, identifying positive, negative, or empty droplets [151]. The assays are based on two competing hydrolysis probes labeled with the different fluorophores, including a repressive quencher. The wild-type (WT) probe is labeled on the 5' end with the reporter dye HEX and the 3' end with the quencher Iowa Black., while the mutated sequence (MT) is on the 5' end labeled with fluorophore 6-carboxyfluorescein (FAM) reporter dye and on the 3' end non-fluorescent quencher Iowa Black [151]. During the annealing, primers and probes bind to the target sequence, and during the extension, 5'-3' exonuclease activity of the polymerase cleaves off the reporter, separating it from the quencher, thereby releasing a detectable fluorescent signal.

The last step in the workflow is thresholding and data analysis, performed using the QuantaSoft Analysis Pro software program (Bio-Rad). The discrimination of positive droplets (containing the target) and negative droplets (do not contain the target) was conducted by plotting the fluorescence amplitude of each droplet for WT, positive control, and NTC samples leading to the generation of 1D and 2D plots from which the threshold was placed. Droplets with fluorescence amplitude above the threshold are defined as positive, and the fraction of positive droplets is fitted into the Poisson statistic equation (Equation 1) to calculate the target concentration in the sample in copies/ $\mu\text{l}$ , from which absolute target DNA molecules can be calculated [152].  $\lambda$  represents the average number of target molecules per reaction, and  $p$  the fraction of positive reactions [152].

$$\lambda = -\ln(1 - p) \quad (1)$$



**Figure 7. Overview of the ddPCR workflow.** The sample is partitioned into ca. twenty thousand droplets by a droplet generator. Partitioned samples are transferred into a thermal cycler where all the droplets are simultaneously amplified. After amplification, the plate is



transferred into the droplet reader, where end-point fluorescence in each droplet is detected in two channels determining positive and negative droplets. Data is analyzed by the QuantaSoft Analysis Pro software program.

### **3.4.1. Optimization of the assays**

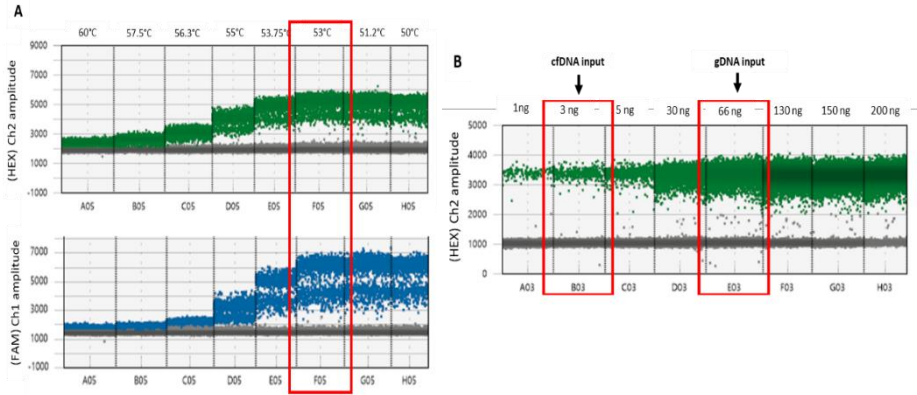
The assays for rare mutation detection were designed by an online Bio-Rad platform representing competing duplex reactions, which contain a single primer pair with two different probes targeting mutant or WT single nucleotide variants. Before testing clinical samples, optimization of DNA input, annealing temperature, and false-positive rate (FPR) was done for each assay using DLBCL cell lines. Moreover, the limit of detection (LOD) was determined by testing the sensitivity of the assay in 30-40 wells containing WT samples [151,153].

#### *Optimization of annealing temperature*

The annealing temperature is a critical factor for reaction specificity, therefore, the optimal temperature was determined by performing a temperature gradient of 55 °C–65 °C (Figure 8 A, as an example). The optimal annealing temperature was selected based on the highest temperature, resulting in the best separation between negative and positive droplets in both channels.

#### *Optimization of input concentration*

To determine the optimal DNA input, a DNA concentration gradient was performed with concentrations ranging from 1-200 ng/well (Figure 8 B). As an input above > 66 ng/20 µL requires digestion by restriction enzymes, 66 ng/well of gDNA input was selected as maximal recommended input of intact DNA that does not affect the quantification accuracy (Figure 8B). For cfDNA recommended input is 5ng/well. However, as it is challenging to obtain enough cfDNA material, we used 3ng/well to be able to perform analyses in duplicates [151]. (Figure 8 B)



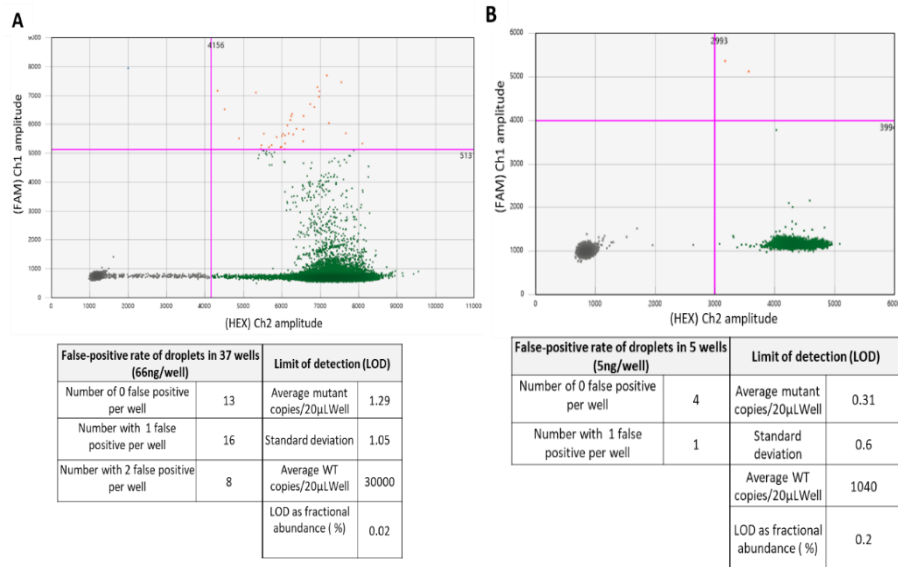
**Figure 8.** Optimization of annealing temperature for ddPCR assays (A) and optimization of input DNA (B)

*Determination of false-positive rate and limit of detection*

For each ddPCR assay, the FPR was determined by testing a minimum of 30-wells of WT-gDNA originating from at least two different DLBCL cell lines to overcome cell-line-specific effects. The DNA input was 66 ng/well, the same as for clinical samples analysis since the DNA input can affect FPR. FPR for analysis of cfDNA was determined by testing five to ten wells of WT-DNA with the input of 3 ng/well. (Figure 9 A and B, an example of the CD58 assays). After determining the FPR, the limit of detection (LOD) was calculated as the average of false-positive rate plus three standard deviations (Equations 2 and 3).

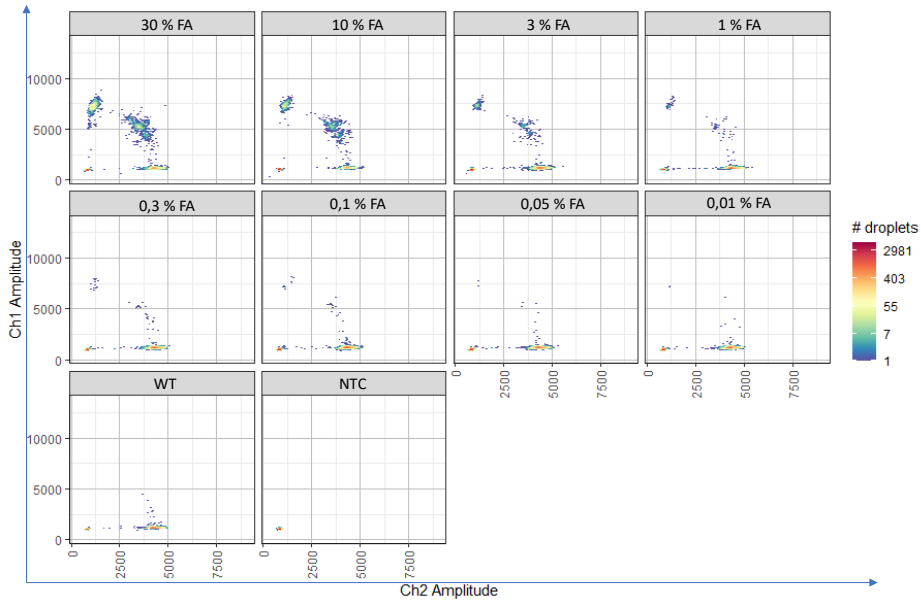
$$\Delta \text{FPR} = \frac{\text{Number of false positives (copies/20}\mu\text{l well)}}{\text{Total number of screened wells}} \quad (2)$$

$$\text{LOD} = \Delta \text{FPR} + 3 \times \text{STD} \quad (3)$$



**Figure 9. False-positive rate and limit of detection.** (A) Calculation of limit of detection (LOD) example on gDNA. (B) calculation of LOD for cfDNA

Furthermore, to test the sensitivity and specificity of the assays, a concentration gradient test was performed with gBlocks, which are gene fragments purchased from Integrated DNA Technologies Integrated DNA Technologies (IDT). These were used as a positive control for assays, spiked into WT DNA in concentrations ranging from 0.01-30% of fractional abundance (FA). (Figure 10)



**Figure 10.** Sensitivity test example (CREBBP assay). FA-fractional abundance; NTC-nontemplate control; WT-wild type DNA (cell line)

To ensure reliable results, each ddPCR setup contained at least three negative template controls (NTCs) that do not contain DNA to identify carry-over contamination. Moreover, at least three wells of WT (background DNA without mutant DNA) and one or two wells of positive control were included to assess the assay specificity and contamination, along with a set of thresholds [153].

The ddPCR method has a relatively easy workflow, however, some steps are critical and can affect assay specificity and sensitivity. A critical step is generating the required number of minimally 10.000 droplets, as a lower number of droplets may affect the results. Moreover, a common problem in Bio-Rad assays is named "rain," representing the positive droplets that appear below or above the main clusters in 1D and 2D plots, making it difficult to discriminate positive and negative droplets. The "rain" effect may be caused by several factors such as poor target accessibility, not optimal annealing temperature, not adequately designed assay, and PCR inhibitors [154]. Studies suggest that "rain" can be reduced by prolonging the elongation time during thermal cycling, determining optimal annealing temperature, using restriction digestion enzymes, or applying tools to reduce the rain droplets [154,155].

Every technology has advantages and disadvantages, and usage depends on the research project. Overall, both NGS and ddPCR have high reproducibility, accuracy, and sensitivity. Of importance, common for both is the applicability in liquid biopsies, which is necessary in order to avoid invasive biopsies. The ddPCR is a cost-effective

technique compared to NGS, and it is suitable for quick testing and interpretation of a single or few markers at the time, while NGS can screen the whole exome or genome at a single run and on a significant number of patients with the costly and time-consuming interpretation of the results [156]. The sensitivity is 5-10% of variant allele frequency (VAF) for WGS and WES and higher for panel sequencing with a sensitivity of 0.1-5%, while the range for ddPCR is 0.001-0.1% [156].





three prediction tools were selected. By search in the Catalog of Somatic Mutations in Cancer (COSMIC) and the Human Gene Mutation Database (HGMD), we observed that 72% of identified point mutations in the ten candidate genes were listed. In addition, all were registered as disease-causing mutations. (Table 1).

Gene ID	Exon	Mutation type	cDNA change	AA changes	Mutation taster	PolyPhen	SIFT	Positive cases in all cohorts	PubMed reference (PIMD)	rs/Cosmic/HGMD reference
<b>CREBBP</b>	E02	Nonsense	c.316C>T	p.Q106*	A	-	-	2/1051	28985567, 28096087	rs587783478
	E26	Missense	c.A336C>T	p.R1446C	D	probably_damaging	deleterious	7/1237	21804550, 28804123, 29713087, 29641966, 21796119, 28096087	rs398124146, COSM88749
	E26	Missense	c.A337G>A	p.R1446H	D	probably_damaging	deleterious	6/579	28804123, 22343534, 29713087, 28554945	rs1057519884, COSM88748
	E25	Missense	c.4223G>A	p.C1408Y	D	probably_damaging	deleterious	3/1028	28804123, 29641966, 29713087	COSM703032
	E27	Missense	c.4478T>A	p.I1493K	D	probably_damaging	deleterious	2/175	28804123, 26647218	COSM5656310
	E05	Nonsense	c.1237C>T	p.R413*	A	-	-	4/1243	28804123, 29641966, 29713087, 26819451	CM021080, rs1302427305, COSM6998605
	E18	Nonsense	c.3517C>T	p.R1173*	D	-	-	2/724	28804123, 29641966	CM065105, COSM88760
	E27	Missense	c.A451T>C	p.F1484S	D	probably_damaging	deleterious	2/724	28804123, 29641966	COSM5651302
	E27	Missense	c.A508A>T	p.Y1503F	D	probably_damaging	deleterious	2/200	28804123, 28096087	COSM88745
	E27	Missense	c.A508A>G	p.Y1503C	D	probably_damaging	deleterious	5/1093	29713087, 29641966, 26819451	rs587783497, COSM88745, CM085345
	E27	Missense	c.A507T>C	p.Y1503H	D	probably_damaging	deleterious	2/387	29713087, 21796119	COSM88744
	E27	Missense	c.A304A>G	p.D1435G	D	probably_damaging	deleterious	2/454	29713087, 28804123	rs200207299, CM053183, COSM88747
	E27	Missense	c.A496T>C	p.L1499P	D	probably_damaging	deleterious	4/476	29713087, 22343534, 21796119, 23699601	COSM88752
	E17	Nonsense	c.3310C>T	p.Q1104*	A	-	-	3/697	29641966, 21796119, 23699601	rs587783479, COSM221505
	E26	Missense	c.A303G>A	p.D1435N	D	probably_damaging	deleterious	3/903	29641966, 29713087, 26647218	COSM703033
	E27	Missense	c.A504T>C	p.W1502R	D	probably_damaging	deleterious	2/789	29641966, 26819451	COSM6932309
	E26	Missense	c.A308T>A	p.S1436R	D	probably_damaging	deleterious	2/789	29641966, 26819451	COSM5948954
	E27	Missense	c.A420T>C	p.C1474R	D	probably_damaging	deleterious	2/789	29713087, 26819451	COSM7338547
<b>TNFRSF14</b>	E01	Nonsense	c.20G>A	p.W7*	D	-	-	4/1708	29641966, 28985567, 21796119, 28096087	COSM6986956
	E01	Nonsense	c.35G>A	p.W12*	D	-	-	16/2072	26819451, 29641966, 28985567, 21796119, 22343534, 25749829	rs768520625, COSM6987205
	E01	Missense	c.3G>A	p.M1I	D	benign	deleterious	4/1050	28985567, 22343534	COSM5947672
	E03	Missense	c.278G>A	p.C93Y	D	probably_damaging	deleterious	3/1001	26819451, 28985567	COSM5949631
	E04	Missense	c.379T>C	p.C127R	D	probably_damaging	deleterious	4/1575	28985567, 29641966	
	E06	Nonsense	c.612G>A	p.W204*	D	-	-	5/1609	29641966, 28985567, 22343534, 25749829	
	E01	Missense	c.2T>A	p.M1K	D	probably_damaging	deleterious	2/1216	26819451, 28985567	COSM6210747
	E02	Missense	c.163C>T	p.P55S	D	probably_damaging	deleterious	3/1879	29641966, 28985567, 29713087	
	E03	Missense	c.296G>A	p.C99Y	D	probably_damaging	deleterious	2/1575	29641966, 28985567	
	E04	Nonsense	c.388C>T	p.Q130*	D	-	-	3/1062	28985567, 22343534, 25749829	rs1232127055
	E04	Missense	c.380G>A	p.C127Y	D	probably_damaging	deleterious	2/1216	26819451, 28985567	COSM6917323
	E05	Nonsense	c.472C>T	p.Q158*	D	-	-	4/1837	29641966, 28985567, 29245897, 26819451	
	E05	Nonsense	c.495C>A	p.C165*	D	-	-	2/365	28804123, 26819451	COSM5949026
	E02	Missense	c.162C>G	p.C54W	D	probably_damaging	deleterious	2/724	29641966, 28804123	
	E06	Nonsense	c.603G>A	p.W201*	D	-	-	2/1084	28985567, 21796119	COSM6986746
	E03	Nonsense	c.283C>T	p.Q95*	D	-	-	3/1131	28985567, 29245897, 21796119	
	E04	Nonsense	c.442C>T	p.Q148*	D	-	-	3/671	29641966, 29245897, 28096087	COSM220659
<b>CD70</b>	E01	Nonsense	c.133C>T	p.Q45*	D	-	-	3/1575	29641966, 28985567	
	E01	Nonsense	c.139C>T	p.Q47*	D	-	-	8/1588	29641966, 28985567, 27835906	rs777583938
	E03	Missense	c.343T>G	p.C115G	D	probably_damaging	deleterious	3/1354	28985567, 22343534, 29713087	
	E03	Nonsense	c.527T>A	p.L176*	D	-	-	2/724	29641966, 2880412	
E01	Nonsense	c.89T>A	p.L30*	D	-	-	-	2/724	29641966, 2880412	



#### 4. SUMMARY OF MAIN RESULTS

B2M	E01	Missense	c.2T>A	p.M1K	D	probably_damaging	deleterious	16/1374	22137796,28985567,26819451,29245897,25749829	COSM144522	
	E01	Missense	c.2T>G	p.M1R	D	probably_damaging	deleterious	9/383	22137796,26647218,26819451,22343534,21796119	COSM144525,rs1057519879	
	E01	Missense	c.1A>G	p.M1V	D	benign	deleterious	9/1262	28985567,26819451,29245897	COSM69783,rs1023835002	
	E01	Missense	c.3G>A	p.M1I	D	probably_damaging	deleterious	4/1216	28985567,26819451	COSM700446,rs1057519877	
	E01	Missense	c.1A>T	p.M1L	D	probably_damaging	deleterious	5/382	26647218,26819451,25749829,21796119,29245897	COSM220667	
	E01	Missense	c.2T>C	p.M1T	D	benign	deleterious	4/264	26819451,22343534,25749829	rs1057519879,COSM144522,COSM144525	
	E02	Nonsense	c.240G>A	p.W80*	D	-	-	4/364	22137796,22343534,29713087		
	E02	Nonsense	c.258C>G	p.Y86*	D	-	-	3/900	22137796,26819451,28985567	COSM144532	
	E01	Nonsense	c.20T>G	p.L7*	D	-	-	12/1900	28985567,26819451,29641966,25123191,25749829	COSM6983511	
	E02	Missense	c.244T>C	p.F82L	D	probably_damaging	deleterious	2/1041	28985567,197104	COSM7338539	
EP300	E27	Missense	c.4370T>A	p.I1457K	D	probably_damaging	deleterious	3/1725	29641966,28985567,28804123		
	E27	Missense	c.4399T>G	p.Y1467D	D	probably_damaging	deleterious	3/1575	29641966,28985567	COSM220521,COSM220522,COSM3357344	
	E29	Missense	c.4659T>G	p.N1553K	D	probably_damaging	deleterious	2/1008	28985567,25123191		
	E05	Missense	c.1244T>C	p.L415P	D	probably_damaging	deleterious	9/1873	29641966,28985567,26819451,21796119		
	E29	Missense	c.4399T>C	p.Y1467H	D	probably_damaging	deleterious	3/807	29641966,28804123,21796119	COSM3357344	
	E29	Missense	c.4399T>A	p.Y1467N	D	probably_damaging	deleterious	4/669	28804123,26819451,29713087	rs200897987,COSM220521	
	E27	Nonsense	c.4384C>T	p.R1462*	D	-	-	2/1041	28985567,28554945	COSM6911759	
	E03	Nonsense	c.454C>T	p.R152*	D	-	-	10/2270	28985567,26819451,29641966,28804123,25749829	COSM1200309	
	E03	Nonsense	c.561T>A	p.C187*	D	-	-	2/1216	28985567,26819451		
	E03	Nonsense	c.605T>A	p.L202*	D	-	-	2/1305	28985567,29713087		
CDS8	E03	Nonsense	c.476G>A	p.W159*	D	-	-	2/262	26819451,29245897	COSM894401	
	E03	Nonsense	c.421C>T	p.Q141*	D	-	-	2/108	21796119,26647218	COSM5704862	
	E03	Nonsense	c.444C>A	p.Y148*	D	-	-	2/724	29641966,28804123	COSM220453	
	E02	Nonsense	c.175C>T	p.Q59*	D	-	-	2/624	29641966,28096087		
	E10	Nonsense	c.1002G>A	p.W334*	D	-	-	2/1216	28985567,26819451		
	E01	Missense	c.52G>C	p.G18R	D	probably_damaging	deleterious	4/229	26608593,26819451	COSM5713843	
	E03	Missense	c.293T>A	p.I98N	D	probably_damaging	deleterious	2/353	22343534,29713087		
	FAS	Nonsense E09	c.826C>T	p.Q276*	D	-	-	2/878	29641966,29713087	CM991192, COSM26157	
	HLA-A	E03	Missense	c.424T>G	p.Y142D	P	probably_damaging	deleterious_low_confidence	2/344	22343534,29713087	rs199474508
		E03	Missense	c.371G>A	p.G124D	D	probably_damaging	deleterious_low_confidence	3/927	22343534,29713087,29641966	
E03		Missense	c.490G>A	p.A164T	D	probably_damaging	deleterious_low_confidence	2/878	29713087,29641966	rs199474538	
E02		Nonsense	c.225G>A	p.W75*	D	-	-	4/927	22343534,29713087,29641966	rs199474408	
E02		Nonsense	c.166C>T	p.Q56*	D	-	-	2/878	29713087,29641966		
E03		Nonsense	c.573G>A	p.W191*	D	-	-	2/878	29713087,29641966	rs199474568	
E02		Nonsense	c.232C>T	p.Q78*	D	-	-	2/878	29713087,29641966	rs281864736	
E03		Nonsense	c.610C>T	p.Q204*	D	-	-	2/878	29713087,29641966	rs199474579	
HLA-B		E03	Nonsense	c.415C>T	p.Q139*	D	-	-	2/878	29713087,29641966	COSM7000206
		E02	Nonsense	c.232C>T	p.Q78*	D	-	-	3/878	29713087,29641966	rs151341159,CM1412116,COSM5710053

**Table 1. The most frequent point mutations detected across included studies.** AA- amino acid change; E- exon; D- disease-causing; A- disease-causing automatic;\* - stop codon; HGMD-Human Gene Mutation Database; SIFT-Sorting intolerant from tolerant; PolyPhen-prediction of functional effects of human nsSNPs, COSMIC - Catalogue of Somatic Mutations in Cancer.

## Paper II

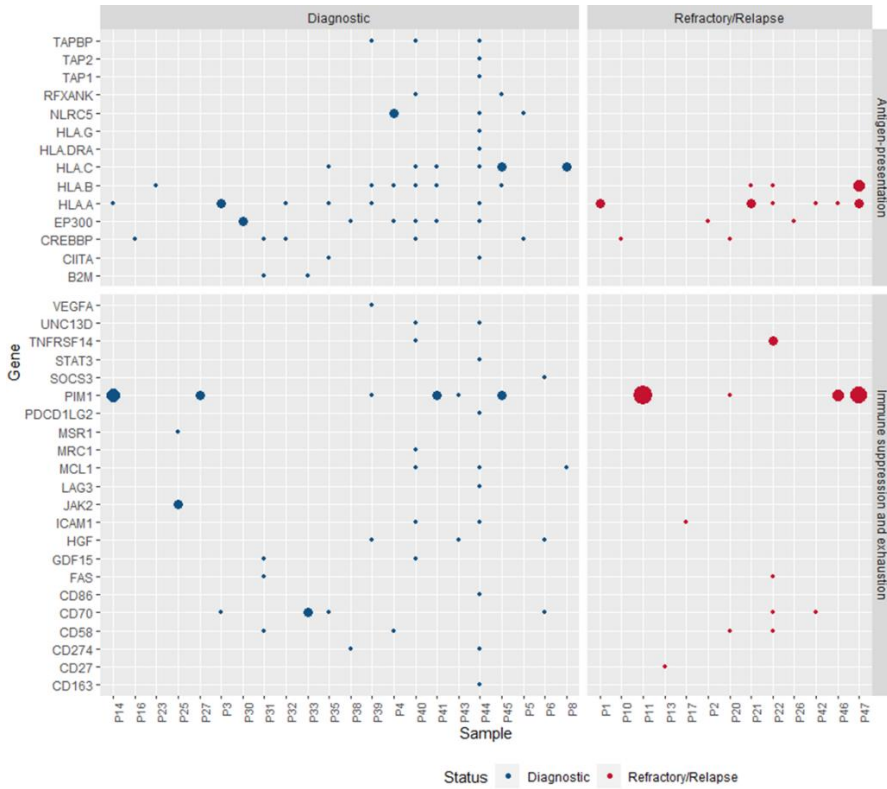
This study aimed to investigate mutational profiles of immune surveillance genes in pre-treatment, dDLBCLs compared to post-treatment, R-CHOP or R-CHOP-like, rrDLBCLs. Based on WES data, analyses were performed on the subset of 58 genes involved in major immune surveillance pathways identified in the previous study [50], from which we have detected genetic alterations in 36 genes in our dDLBCL and rrDLBCL cohorts. Gene mutation frequencies in the 36 genes related to immune surveillance ranged from 3-20 % and 6-35% in dDLBCLs and rrDLBCLs,

respectively [157]. The number of targeted genes varied remarkably, from samples with one or multiple alterations in one gene to samples harboring alterations in multiple genes. A 3.2-fold higher number of mutated genes was found in dDLBCLs (n= 35) than in rrDLBCLs (n=11) (Figure 12), however more than half of the affected genes in rrDLBCLs showed a higher gene mutation frequency in rrDLBCLs than dDLBCLs such as *HLA-A*, *PIM1*, *CD58*, *FAS*, and *TNFRSF14*, though not significantly [157].



**Figure 12. Mutational portrait of 36 immune surveillance genes in dDLBCL vs. rrDLBCL.** On the left side of the figure, genes involved in immune surveillance genes in DLBCL are listed, with gene mutation frequencies shown on the right side. Genes are sorted by the most frequently mutated genes in rrDLBCLs. The size of the circles represents the number of specific mutations ranging from 1-8, while different colors present types of mutations. Gene mutation frequency of individual genes between dDLBCLs and rrDLBCLs does not differ significantly, tested by Fisher's exact test.

Seventy-three percent (22 patients) of dDLBCLs and 77% (13 patients) of rrDLBCL patients harbored genetic alterations detected in at least one of the 36 genes, and the number of genetic alterations ranged from 1-19 and 1-12 per dDLBCL and rrDLBCL patient, respectively (Figure 13) [157]. The distribution of genetic alterations in dDLBCLs and rrDLBCLs was equal, with missense mutations being most frequent, followed by nonsense and frameshift alterations.

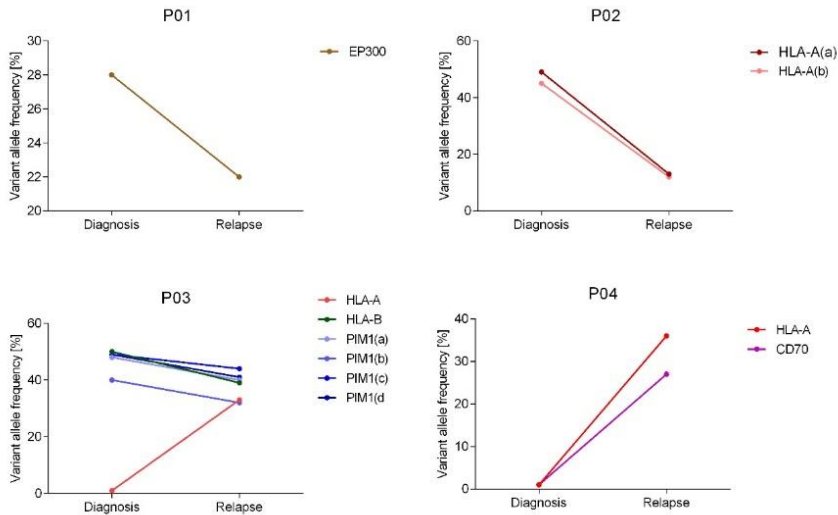


**Figure 13. The mutational pattern of 36 genes affecting antigen-presentation and immune suppression and exhaustion in dDLBCLs and rrDLBCLs.** The blue dots represent mutations in diagnostic (dDLBCLs) patients, and red dots in refractory/relapsed patients (rrDLBCLs); the size of the dots represents the number of variants in a specific gene in the patient range (1- 8). On the left side are genes representing sub group detected in our cohort. Cohorts are named on the top.

Division of immune surveillance genes into those involved in the antigen-presentation and immune suppression and exhaustion showed that rrDLBCL patients had more alterations in the antigen-presentation pathway genes compared to dDLBCL, with 29% and 16% of the patients, respectively (Figure 13) [157]. Conversely, similar frequencies of dDLBCLs and rrDLBCLs (13% and 12%, respectively) had immune suppression and exhaustion affected, and most patients, irrespective of being diagnostic or refractory/relapse, had affected genes in both of the pathways (Figure 13).

Further, we sought to examine the mutational profile of paired diagnostic and relapsed biopsies of the same patients (n=4). As presented in Figure 14, VAFs of individual mutations differed between diagnostic and relapsed biopsies of the same patient. We have observed that VAFs of genetic alterations in all genes except HLA-A in patient

P03 were decreased in relapsed biopsies compared to diagnostic biopsies [157]. Of notice, in P04, diagnosed with progressive disease, no genetic alterations in immune surveillance genes were detected in the diagnostic biopsy, while mutations in *HLA-A* and *CD70* were detected at the time of progression (9 months after diagnosis), indicating expansions of clones with altered immune surveillance genes [157]. All of the matched samples harbored genetic variations in antigen-presenting genes.



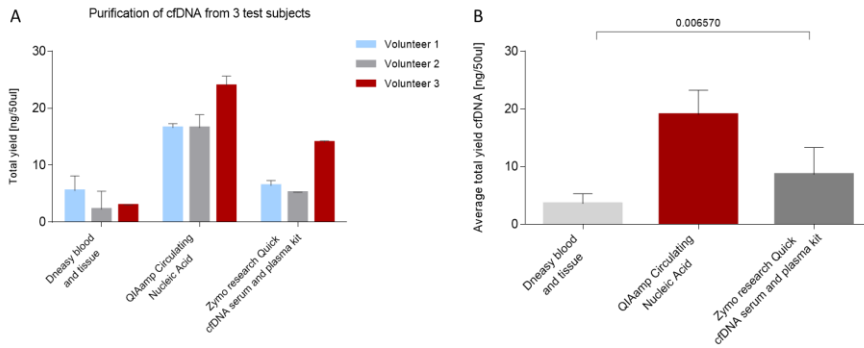
**Figure 14. Exploration of genetic mutations in matched diagnostic and relapsed tumor biopsies of DLBCL patients.** Variant allele frequency is displayed with the line as a percentage of each somatic mutation before and after treatment, showing possible clonal expansion; genes with additional letters (a-d) in parenthesis depict different mutations in the same genes.

### Paper III

In this study, we set out to investigate the impact of pre-analytical variables such as blood collection tubes (BCTs), cfDNA purification, centrifugation regime, and storage time on the yield of cfDNA purified from plasma of healthy donors. Additionally, the usage of long term archival clinical plasma samples was assessed.

To assess the impact of the purification kits on cfDNA yield, three purification kits were compared. A significantly higher yield of cfDNA was obtained by QIAamp Circulating Nucleic Acid purification kit (Figure 15,  $p=0.006570$ , one way ANOVA test).

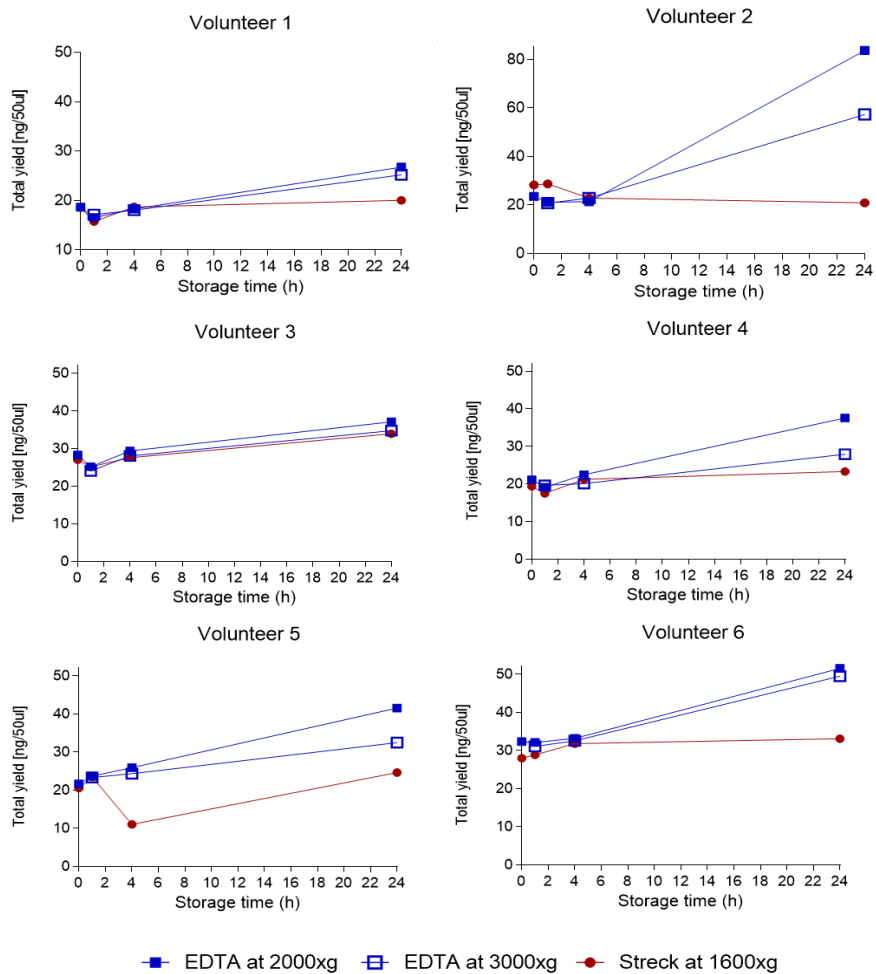
#### 4. SUMMARY OF MAIN RESULTS



**Figure 15.** Comparison of cfDNA purification kits from healthy volunteers (A) The mean with a standard deviation of duplicate purification for each volunteer. (B) The mean yield of cfDNA of all three volunteers with standard deviation. P-value obtained by one way ANOVA test. Bars represent the total yield of cfDNA purified from 1ml of plasma in technical duplicates.

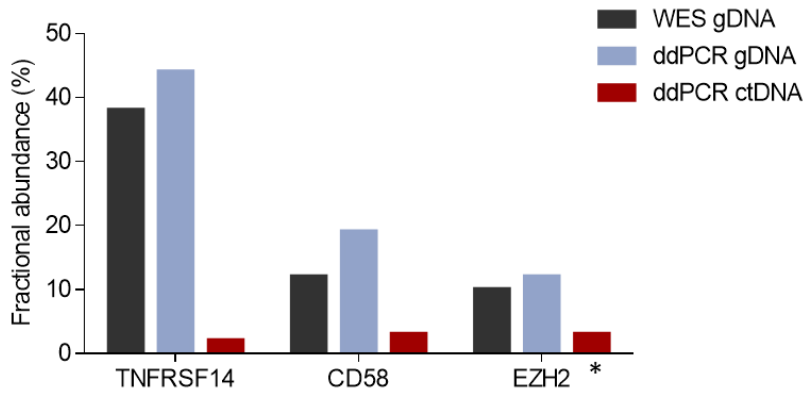
Blood was collected from six healthy volunteers in EDTA and Streck BCTs, and we observed no significant difference in cfDNA yield purified from plasma separated from Streck and EDTA tubes freshly, after 1 h, and 4 h. However, significantly higher cfDNA yield was observed in plasma samples purified from EDTA BCTs after 24 h of storage at 4°C, which could be due to gDNA contamination [158]. Comparison of centrifugation regimens revealed a slightly higher yield of cfDNA extracted from the plasma of blood samples centrifuged at 2000 x g at RT. Therefore, EDTA BCTs can be used if the plasma is separated within 4 h from the blood draw, and centrifugation at 2000 x g is recommended. Streck tube is a better choice for longer storage before plasma separation, particularly when storage occurs at RT (Figure 16) [158].

QIAamp Circulating Nucleic Acid purification method was used to extract cfDNA from long-term (average 8.4 years) archival DLBCL plasma samples (n=15). These samples had matched tumor gDNA that previously was assessed by WES and ddPCR. Patients with tumor biopsies with mutations detected in *CD58*, *TNFRSF14*, or *EZH2* had detectable ctDNA in the cfDNA assessed with ddPCR and mutation-specific assays (Figure 17) [158]. This demonstrates the usability of archival clinical samples, though they were not processed with fully optimal conditions. However, it should be noted that a significantly lower fractional abundance was observed in cfDNA compared to WES and ddPCR assessment of tumor biopsies.



**Figure 16. The yield of cfDNA in blood plasma samples from healthy volunteers** drawn in EDTA and Streck BCTs and plasma separated and purified fresh (0 h) and within less than one hour (1 h), after four (4 h) and 24 h storage time at room temperature for Streck tubes and the same for EDTA BCTs except that for 24h was stored at 4 °C. Each dot represents the yield of cfDNA per 50 µl obtained from 1ml plasma and the mean value from technical duplicates. EDTA samples were centrifugated at 2000 x g and 3000 x g, while blood samples in Streck tubes were centrifugated at 1600 x g at RT; RT- room temperature.

#### 4. SUMMARY OF MAIN RESULTS



**Figure 17. Mutations were identified in ctDNA in all three archival clinical samples with known mutations.** Fractional abundance of mutations is displayed in percentage for gDNA assessed by WES and ddPCR and ctDNA assessed by ddPCR. \*- The patient did not have an available relapse plasma sample in which mutation in *EZH2* was detected in gDNA, but we have detected mutation in the diagnostic archival plasma sample.





## 5. DISCUSSION

In this section, observations and parameters of relevance for the overall project will be discussed as supplementary to results discussed in the individual studies.

### 5.1. Major immune surveillance pathways in DLBCL and interplay with immunotherapy

A considerable number of global and targeted gene panel sequencing studies and clinical evidence have demonstrated the presence of cancer immunoediting, where the tumor cells can gradually gain mechanisms to evade immune surveillance of the host during tumor progression. A significant issue and major mechanism in the development of DLBCL is the evasion from T-cell immune surveillance caused by loss or decreased expression of MHC-I and MHC-II molecules [159,160]. The loss of MHC-I expression is identified in approximately 55-75% of DLBCL patients, while the loss of MHC-II expression characterizes 40% of DLBCL patients. Moreover, both are associated with an impaired immune response while losing MHC-II expression with poor outcome [53,55]. Loss or down-regulation of MHC-I and MHC-II expression can occur at both genetic, transcriptional, and post-transcriptional level in tight interaction with stimulus from the tumor microenvironment where especially T-cells, NK-cells, mast cells, macrophages, dendritic and monocytic myeloid-derived suppressor cells are considered to be important even if neither all inter- nor extracellular mechanisms are entirely understood [161,162]. Thus, expression of MHC-I can be affected by direct somatic mutations in MHC-I encoding genes (*HLA*-genes) or at the post-transcriptional level when for example, mutations in *B2M* prevents the efficient formation of the peptide-binding groove in mature surface-expressed MHC-I molecules whereby recognition by CD8<sup>+</sup> T-cells are avoided [161]. Similarly, mutations in the HLA class II genes are leading to loss of expression of HLA-DR and HLA-DQ in DLBCL [163], or inactivation of *CIITA* by somatic mutations or gene fusion with *PD-L1* and *PD-L2* genes [51], causing loss of antigen-presentation associated with MHC-II and impaired immune response [13]. Impact of tumor microenvironment on immune surveillance evasion in DLBCL is discussed and targeted in several studies since regardless of the presence of numerous immune cells in the tumor microenvironment, tumor cells are not always destroyed due to the overexpression of suppressing ligands on their surface, such as PD-L1/-L2 leading to immune exhaustion or secretion of inhibitory cytokines such as IL-10 resulting in immune suppression [164,165]. The creation of such cancer friendly immune response restricted microenvironment helps the DLBCL to manifest in both nodal and extranodal sites and to reside and survive treatment, thereby being a source for residual disease and relapse [162]. In this thesis, the main focus has been on identifying and investigating the genetic status of genes involved in major immune surveillance pathways in DLBCL, and to a lesser extent decipher the cellular composition of the tumor microenvironment.

The importance of antigen-presentation in both disease initiation, progression, and development of treatment resistance is illustrated in a recent study comparing diagnostic non-relapsed DLBCL samples with ultimately relapsed DLBCL samples showing that antigen-presentation genes appeared to be more mutated in relapsed samples, suggesting it as an intrinsic immune surveillance escape mechanism [166].

Despite the fact that the front-line treatment, R-CHOP, cures approximately 60% of all DLBCL patients, a considerable number of patients do not achieve durable remission due to relapse or primary refractory disease [79,167]. Patients who do not respond to the front-line treatment are offered salvage treatments combined with autologous stem cell transplantation, which unfortunately have limited efficacy and can not be offered to all patients due to comorbidities or performance status [167,168]. For the patients who are not eligible for autologous stem cell transplantation or eventually relapse, novel immunotherapy approaches such as immune checkpoint blockade with antibodies targeting CTLA-4 or PD-L1/PD-1 or CAR-T-cells might be beneficial. However, immune checkpoint blockade targeting PD-L1/PD-1 signaling to prevent the immune suppression mechanisms has been efficient in patients with Hodgkin lymphoma, while it did not show satisfactory response in DLBCL, most likely due to impaired mechanism for antigen-presentation [169,170]. Therefore, it is of critical importance to characterize different genes involved in immune evasion and understand the underlying mechanisms associated with relapse of DLBCL in order to offer new, personalized treatment strategies to patients.

Based on biological function and gene mutation frequency, we conducted a systematic literature review and identified 58 genes involved in major immune surveillance pathways in DLBCL, e.g., antigen processing and presentation, immune suppression, and exhaustion, including epigenetic regulation [50]. The selected 58 genes do not necessarily represent all genes with an impact on immune surveillance since new techniques like CRISPR have documented their potential to select and identify other mechanisms, such as SUGT1, regulating the transcription of *HLA-A* and *HLA-DR*, thereby MHC-I and MHC-II, respectively [13].

Of notice, gene mutation frequencies varied tremendously between studies due to sampling, utilizing different sequencing approaches resulting in different coverage depths, different bioinformatic tools, number of patients, as well as the tumor heterogeneity itself [50]. The tumor heterogeneity can be intratumoral, characterizing the variations between the individual cancer cells of a single tumor and intertumoral, where there are genetic differences between primary and metastasis tumors in the same patient [171,172]. Genetic variations, however, are also frequent at the interpatient level where tumor heterogeneity describes differences among tumors within different patients of the same disease entity, causing variations in immunophenotypes, morphology, therapy responses, and clinical outcomes in different patients [171,172]. From sixteen publicly available sequencing data sources for diagnostic DLBCL samples, 35% recurrently mutated genes were observed to

overlap between the studies, whereas only ten genes (24%) were mutated in more than  $\geq 5\%$  of the DLBCL patients of all included studies [50]. Even though overlap is observed for selected genes involved in immune surveillance, this observation is in agreement with a previous study by Zhang et al., 2013, where they observed overlap of 10-20% among four studies [173]. Of notice, the number of detected mutations increases with the cohort size, and along with low overlap of mutated genes between different studies, the inherent genetic heterogeneity of DLBCL is apparent [173]. The pan-cancer analysis revealed that approximately 400 samples are needed to comprehensively detect genes with  $\geq 5\%$  gene mutation frequency with  $>90\%$  power [174].

To understand the genetic landscape and underlying resistance mechanisms of DLBCL, it is crucial to analyze and understand the difference between dDLBCL and rrDLBCL biopsies and especially the genetic profile of immune surveillance genes of refractory/relapsed DLBCLs is challenging since it has not been widely used to biopsy, collect, register and genetically profile tumor tissue from the tumor regrowth tissues. Only 140 rrDLBCL samples analyzed by global or targeted gene panel sequencing are available from six different studies [166,175–179]. This makes representative findings hard to assess, and biological as well as the clinical impact of specific genetic events remain poorly understood. There are a few studies including in total only 50 matched diagnostic and relapsed samples [166,175,177–180], but they offer a particular opportunity to study clonal selection and development of intra- or intertumoral heterogeneity if regrowth occurs at the same location or an alternative location, respectively. Specific genes including *CD58*, *B2M*, *TNFRSF14*, *HLA-A* were observed in more than two DLBCL sequencing studies to harbor recurrent mutations in the refractory/relapsed disease situation, suggesting they have important roles in the progression and development of treatment resistance in DLBCL [50]. Moreover, genetic alterations in immune surveillance genes, though detected in lower frequencies than *MLL2* or *CARD11* gene, are consistently detected among studies. For example, genetic alterations in *B2M* are detected in all 16 studies examined in our first study, supporting the function and biological role of immune surveillance in DLBCL [50].

In our second study, WES was performed on diagnostic, non-relapsing (dDLBCLs) clinical samples (n=30) and refractory/relapsed (rrDLBCLs) (n=17) treated with R-CHOP or R-CHOP-like we assessed 58 sub-selected immune and surveillance genes [157]. In both dDLBCL and rrDLBCL cohorts, more than 70% of the samples harbor one or more genetic alterations in one or more genes involved in immune surveillance [157]. This observation is concurrent with other studies [13,166], and can also be supported by the recently developed genetic classifiers on dDLBCLs identifying genetic clusters like MCD by Wright et al. or C5 by Chapy et al., where approximately 73% of the patients harbor genetic alterations in genes involved in immune surveillance [117,181].

As one of the limitations of our study was the small cohort size, we have evaluated our observations in external dDLBCLs (Chapuy et al. n=135) compared to external rrDLBCL (Morin et al., n=25, and Greenawalt n=47) [157], corroborating our results of individual gene mutation frequencies in immune surveillance genes between dDLBCLs and rrDLBCLs with trends of increased gene mutation frequencies of *CD58*, *PIMI*, *FAS*, *HLA-A*, and *TNFRSF14* [157,166,182]. The inactivating genetic alterations in antigen-presenting genes such as *HLA-A* can, as earlier mentioned, lead to the loss of MHC-I molecule expression, making tumor cells invisible for CD8+ T-cells [166]. The Fas/FasL system is responsible for activating induced cell death, whereas inactivating genetic alterations in the *FAS* gene in tumor cells can suppress apoptosis of transformed cells [183]. The inactivating mutations in the tumor suppressor gene *TNFRSF14* lead to autonomous B-cell proliferation and extrinsic activation of the lymphoma microenvironment through B- and T-cell attenuator (BTLA attenuator), which is located on CD4+ T-cells [59]. *CD58* has a crucial role in the intracellular signaling pathway for the immune activation of NK- and T-cells [184]. Hence, hiding from the host immune system, defense by resistance to apoptosis, and induction of immunosuppressive tumor microenvironment are possible biological mechanisms that drive rrDLBCL pathogenesis.

The mutational landscape of immune surveillance genes is becoming increasingly important for guiding immunotherapy treatment in DLBCL. Where immune checkpoint blocking antibodies has been successful in other cancer forms, the limited effect in DLBCL is speculated to be closely associated with the loss of antigen-presenting abilities through MHC-I and MHC-II due to mutations or downregulation and activation of immune suppression mechanisms [185,186]. Immune checkpoint blockade with antibodies targeting CTLA-4 or PD-L1/PD-1 aiming at re-educating the immune system by releasing the inhibition of effector cells like T-cells requires an intact antigen-presentation through MHC-I and MHC-II and a pre-existing anti-tumor immune response [161]. Since the malignant B-cells themselves are professional antigen-presenting cells, defects in MHC-I and MHC-II antigen-presentation directly prevent the priming of host CD4+ and CD8+ T-cells and thereby a less efficient response to immune checkpoint blockade. Likewise, an immunosuppressive tumor microenvironment with, for example, the release of IL-10, a potent immunosuppressive cytokine inhibiting the ability of dendritic cells to stimulate the proliferation of CD4+ T-cells, creates a less efficient response to immune checkpoint [187]. If the loss of antigen-presenting abilities is reversible induced re-expression of MHC molecules may be considered using treatment including epigenetic drugs or immunotherapy targeting CD40 [188,189]. Alternative immunotherapy treatment strategies for DLBCL patients with irreversible genetic loss of MHC-I/MHC-II surface expression could be cellular therapy with CAR-T-cell, which does not require functional antigen-presentation [166,190]. Up to date, two different CD19-directed CAR-T-cell regimens are approved for the treatment of rrDLBCL, axicabtagene ciloleucel (Axi-cel), and tisagenlecleucel (CTL109) [191]. Alternative cellular immunotherapies such as CAR-NK-cell are promising for

rrDLBCLs with inactivated *CD58* gene, and where an adoptive transfer of NK-cells, previously expanded, activated, or redirected against tumor cells are required [192].

In our cohort, we have not evaluated rrDLBCL treatment response due to the limited number of samples and not yet any local treatment options, including CAR-T cell components, but our observations of targeted genes and gene mutational frequencies in dDLBCL and rrDLBCL are in overall agreement with related studies within the field. Hence, somatic genetic variations in specific genes involved in and controlling immune surveillance are often targeted and may distinguish important features of DLBCL disease development from diagnosing to progression under the selection that occurs during the response to standard immunochemotherapy. Genetic analysis of primary and refractory/relapsed clinical samples hence can contribute to the development of better tools for disease stratification and improved biological understanding of treatment response.

## 5.2. Liquid biopsies and technical considerations

In DLBCL, the current gold standard for diagnosis is the invasive tumor tissue biopsy that can be obtained either surgically or by needle-core biopsy. In both types of tissue biopsies limitations can be accessing the tumor material, taking follow-up biopsies, and capturing spatiotemporal heterogeneity in the tumor tissues [193]. To overcome these obstacles, liquid biopsies, referred to as the study of tumor-derived markers in body fluid, come into play with the advantage of being non-invasive, enabling serial sampling, and the possibility of capturing the entire genetic and epigenetic profile of the tumor [194]. The major challenge of analyzing ctDNA is that it usually only comprises 1% of the total cfDNA [195], therefore further examinations of techniques are needed to ensure and obtain a stable and high yield of cfDNA.

To address this issue, we investigated pre-analytical variables, including purification kits, type of blood collection tubes, storage times, and plasma centrifugation force [158]. The yield of cfDNA can significantly be affected by the utilization of different purification kits, as we documented by comparing three purification kits, observing the highest cfDNA yield when using QIAamp Circulating Nucleic Acid Kit (Qiagen) [158]. According to our observations from the comparison of EDTA and Streck blood collection tubes stored at room temperature for one, four, and 24 hours before plasma separation, EDTA tubes can be used if the plasma is separated within 4 hours. In contrast, Streck tubes are more appropriate for more extended storage times, especially for storage at room temperature [158]. Ginkel et al. observed no significant difference in cfDNA yield extracted from EDTA tubes stored between various time points up to 24 hours at room temperature before the separation of plasma when using EDTA tubes [196]. Streck tubes are, however, developed to stabilize cells and keep cfDNA stable and usable after a longer storage time at room temperature [197], and as we observed, a more stringent pattern of high-quality cfDNA is obtained using Streck instead of EDTA blood collection tubes after 24 hours.

To avoid degradation of cfDNA, plasma needs to be stored at  $-80^{\circ}\text{C}$ , where short-term storage does not impact the yield, whereas storage above three years can cause reduced cfDNA yield [198]. This was observed in our study when the long-term archival clinical DLBCL samples were investigated for detection of ctDNA, revealing a significantly lower fractional abundance of specific cancer mutations from liquid biopsies than in tumor tissue of the same patients [158]. However, this could also be caused and influenced by contamination with gDNA from lysed cells, the tumor size, localization, or stage. Hence, for analysis of ctDNA, an essential consideration is contamination with the gDNA of lysed cells, estimated by DNA capillary electrophoresis for estimation DNA fragment sizes and generating a quantitative measure of the number of long (cellular DNA) and short (cfDNA) molecules [199,200]. Moreover, quantitative PCR to detect and quantitate specific gDNA fragments can be used for tracking clonotypic immunoglobulin gene rearrangement in cfDNA as used for minimal residual monitoring in DLBCL [201].

The analysis of cfDNA can be affected not only by pre-analytical variables but also by using an adequate method for detecting ctDNA. The content of ctDNA in cfDNA is very low, thus mutant allele frequencies down to  $\leq 0.1\%$  require to be detected reliably. Methods with sufficient sensitivity, such as digital droplet PCR or lower denaturation temperature PCR (COLD-PCR), which can be used before sequencing or genotyping assays, are suitable only for detecting few or single pre-defined targets [202,203]. In contrast, NGS methods allow comprehensive genome detection but fail to detect mutations with an allele frequency of 1-3%. However, new improvements of NGS methods for corrections of sequencing errors using barcoding significantly improve sensitivity [204,205]. A prospective study conducted by Rossi et al. performed targeted ultra-deep sequencing on 50 DLBCL patients and showed a high level of concordance of detected mutations between paired cfDNA and tissue biopsies, where 83% of mutations detected in tissue biopsy were confirmed in cfDNA [206]. A recent study indicated a prognostic value of cfDNA in DLBCL patients, where patients who responded to the treatment had significantly reduced cfDNA concentration compared to non-responding patients [207]. Moreover, relapse was predicted by multiple monitoring of ctDNA in approximately 188 days from detection of ctDNA until clinical relapse in 73% of the cases [208].

In summary, we conclude that the detection of tumor-specific mutations in archival long-term clinical samples is feasible but might not be optimal due to the possible degradation and contamination with gDNA from lysed cells caused by processing times above four hours and suboptimal sample handling. However, together with optimized pre-analytical variables, detection and quantitation of ctDNA definitely provide the basis for further research.

## 5. DISCUSSION

## 6. CONCLUSIVE REMARKS AND FUTURE PERSPECTIVES

Overall, recent years have brought substantial insights into the characteristics of the DLBCL genome and the development of numerous integrative approaches to describe molecular features of DLBCL comprehensively. However, the functional relevance of many genetic features remains poorly understood. A better understanding of the interplay between the tumor and its molecular features and the immune surveillance of the host can help identify which patients would have a better response to immunotherapy and support the selection of the most effective targets for developing such treatments in a patient-specific manner. This PhD study has contributed to identifying genes involved in immune surveillance and further characterized the mutational profile of these genes in diagnostic and refractory/relapsed clinical samples with potential implications in response to immunochemotherapy treatment. However, it will be essential to better understand the tumor microenvironment and overcome the obstacle of the biological heterogeneity of DLBCL, which affects the response towards specific and regimens of treatment drugs.

Advances in next-generation sequencing technologies allow different sequencing approaches on many levels, such as DNA-, RNA-, single-cell-sequencing, and DNA methylation profiling, from which data can be integrated for comprehensive disease understanding and determination of biomarkers. Whole-exome sequencing allows enrichment of interpretable and actionable copy number and sequence variations, while data from whole-genome sequencing adds layers of useful data such as larger structural genomic variations, non-protein-coding sequences, including enhancer and transcription factor binding regions, which can affect cancer development. Furthermore, RNA sequencing provides information on fusion transcripts, the expression levels of genes and functional mutations as well as identification by integrative genome and transcriptome analysis of new potential tumor suppressors and oncogenes with driver roles in lymphomagenesis. Considering that genomic alterations are only present in the lymphoma cells but not in the cells of the tumor microenvironment, bulk and single-cell gene expression analysis may reveal alterations involved in the intrinsic features of the lymphoma cells. It has also been shown that epigenetic regulation of gene expression affects immune response and development of lymphomas, and DNA methylation profiling could help understand some of these biological features.

As the development of liquid biopsies emerges, a non-invasive genetic material source will facilitate genetic analysis and improve current knowledge and development of therapeutic strategies.



## 6. CONCLUSIVE REMARKS AND FUTURE PERSPECTIVES

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