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Recurrent Genetic Mutations in Lymphoid Malignancies

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Abstract
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In recent years, the genetic landscape of B-cell derived lymphoid malignancies, including chronic lymphocytic leukemia (CLL), has been rapidly unraveled, identifying recurrent genetic mutations with potential clinical impact. Interestingly, ~30% of all CLL patients can be assigned to more homogeneous subsets based on the expression of a similar or "stereotyped" B-cell receptor (BcR). Considering that biased distribution of genetic mutations was recently indicated in specific stereotyped subsets, in **paper I**, we screened 565 subset cases, preferentially assigned to clinically aggressive subsets, and confirm the *SF3B1* mutational bias in subset #2 (45%), but also report on similarly marked enrichment in subset #3 (46%). In contrast, *NOTCH1* mutations were predominantly detected in subsets #1, #8, #59 and #99 (22-34%). This data further highlights a subset-biased acquisition of genetic mutations in the pathogenesis of at least certain subsets. Aberrant NF-κB signaling due to a deletion within the *NFKBIE* gene previously reported in CLL warranted extended investigation in other lymphoid malignancies. Therefore, in **paper II**, we screened 1460 patients with various lymphoid malignancies for *NFKBIE* deletions and reported enrichment in classical Hodgkin lymphoma (27%) and primary mediastinal B-cell lymphoma (PMBL) (23%). *NFKBIE*-deleted PMBL cases had higher rates of chemorefractoriness and inferior overall survival (OS). *NFKBIE*-deletion status remained an independent prognostic marker in multivariate analysis. *EGR2* mutations were recently reported in advanced stage CLL patients; thus, in **paper III** we screened 2403 CLL patients for mutations in *EGR2*. An overall mutational frequency of 3.8% was reported and *EGR2* mutations were associated with younger age, advanced stage and del(11q). *EGR2* mutational status remained an independent marker of poor outcome in multivariate analysis, both in the screening and validation cohorts. Whole-genome sequencing (WGS) of 70 CLL cases, assigned to poor-prognostic subsets #1 and #2 and indolent subset #4, were investigated in **Paper IV** and revealed a similar skewing of *SF3B1* mutations in subset #2 and *NOTCH1* mutations in subset #1 to that reported in Paper I. Additionally, an increased frequency of the recently proposed CLL driver gene *RPS15* was observed in subset #1. Finally, novel non-coding mutational biases were detected in both subset #1 and #2 that warrant further investigation.

Keywords: Lymphoid malignancies, mutations, CLL, stereotypy, subsets, PMBL, NFKBIE, EGR2, whole-genome sequencing

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To Mam and Dad

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals. Reprints were made with permission from the respective publishers.

I Sutton LA, **Young E**, Baliakas P, Hadzidimitriou A, Moysiadis T, Plevova K, Rossi D, Kminkova J, Stalika E, Pedersen LB, Malcikova J, Agathangelidis A, Davis Z, Mansouri L, Scarfò L, Boudjoghra M, Navarro A, Muggen A, Yan XJ, Nguyen-Khac F, Larrayoz M, Panagiotidis M, Chiorazzi N, Niemann CU, Belessi C, Campo E, Strefford JC, Langerak AW, Oscier D, Gaidano G, Pospisilova G, Davi F, Ghia P, Stamatopoulos K, Rosenquist R. *Different spectra of recurrent gene mutations in subsets of chronic lymphocytic leukemia harboring stereotyped B-cell receptors*. Haematologica 2016;101(8): 959-967.

II Mansouri L*, Noerenberg D*, **Young E***, Mylonas E, Abdulla M, Frick M, Asmar F, Ljungström V, Schneider M, Yoshida K, Skaftason A, Pandzic T, Gonzalez B, Tasidou A, Waldhueter N, Rivas-Delgado A, Angelopoulou M, Ziepert M, Arends CM, Couronné L, Lenze D, Baldus CD, Bastard C, Okosun J, Fitzgibbon J, Dörken B, Drexler HG, Roos-Weil D, Schmitt CA, Munch-Petersen HD, Zenz T, Hansmann ML, Strefford JC, Enblad G, Bernard OA, Ralfkiaer E, Erlanson M, Korkolopoulou P, Hultdin M, Papadaki T, Grønbæk K, Lopez-Guillermo A, Ogawa, Küppers R, Stamatopoulos K, Stavroyianni N, Kanellis G, Rosenwald R, Campo E, Amini RM, Ott G, Vassilakopoulos TP, Hummel M, Rosenquist R and Damm F. *Frequent NFKBIE deletions are associated with poor outcome in primary mediastinal B-cell lymphoma*. Blood 2016;128(23):2666-2670.

III **Young E***, **Noerenberg D*,** Mansouri L, Ljungström V, Frick M, Sutton LA, Blakemore SJ, Galan-Sousa J, Plevova K, Baliakas P, Rossi D, Clifford R, Roos-Weil D, Navrkalova V, Dörken B, Schmitt CA, Smedby KE, Juliusson G, Giacopelli B, Blachly JS, Belessi C, Panagiotidis P, Chiorazzi N, Davi F, Langerak AW, Oscier D, Schuh A, Gaidano G, Ghia P, Xu W, Fan L, Bernard OA,

Nguyen-Khac F, Rassenti L, Li J, Kipps T J, Stamatopoulos K, Pospisilova S, Zenz T, Oakes CC, Strefford JC, Rosenquist R**, Damm F**. *EGR2 mutations define a new clinically aggressive subgroup of chronic lymphocytic leukemia*. Leukemia. Advanced online publication 3 January 2017.

IV Ljungström V*, **Young E***, Agathangelidis A, Muggen AF, Plevova K, Rossi D, Davis Z, Sutton LA, Baliakas P, Ntoufa S, Davi F, Gaidano G, Oscier D, Pospisilova S, Langerak AW, Stamatopoulos K, Ghia P**, Rosenquist R**. *Whole-genome sequencing in subsets of chronic lymphocytic leukemia harboring stereotyped B-cell receptors*. Manuscript.

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Other papers published by author during PhD period

1. Sutton LA*, Ljungström V*, Mansouri L, **Young E**, Cortese D, Navrkalova V, Malcikova J, Muggen AF, Trbusek M, Panagiotidis P, Davi F, Belessi C, Langerak AW, Ghia P, Pospisilova S, Stamatopoulos K, Rosenquist R. *Targeted next-generation sequencing in chronic lymphocytic leukemia: a high-throughput yet tailored approach will facilitate implementation in a clinical setting.* Haematologica. 2015;100(3):370-6.

2. Mansouri L, Sutton LA, Ljungström V, Bondza S, Arngården L, Bhoi S, Larsson J, Cortese D, Kalushkova A, Plevova K, **Young E**, Gunnarsson R, Falk-Sörqvist E, Lönn P, Muggen AF, Yan XJ, Sander B, Enblad G, Smedby KE, Juliusson G, Belessi C, Rung J, Chiorazzi N, Strefford JC, Langerak AW, Pospisilova S, Davi F, Hellström M, Jernberg-Wiklund H, Ghia P, Söderberg O, Stamatopoulos K**, Nilsson M**, Rosenquist R**. *Functional loss of IκBε leads to NF-κB deregulation in aggressive chronic lymphocytic leukemia*. J Exp Med. 2015;212(6):833-43.

3. Ljungström V*, Cortese D*, **Young E**, Pandzic T, Mansouri L, Plevova K, Ntoufa S, Baliakas P, Clifford R, Sutton LA, Blakemore SJ, Stavroyianni N, Agathangelidis A, Rossi D, Höglund M, Kotaskova J, Juliusson G, Belessi C, Chiorazzi N, Panagiotidis P, Langerak AW, Smedby KE, Oscier D, Gaidano G, Schuh A, Davi F, Pott C, Strefford JC, Trentin L, Pospisilova S, Ghia P, Stamatopoulos K, Sjöblom T**, Rosenquist R**. *Whole-exome sequencing in relapsing chronic lymphocytic leukemia: clinical impact of recurrent RPS15 mutations*. Blood. 2016;127(8):1007-16.

4. Navrkalova V, **Young E**, Baliakas P, Radova L, Sutton LA, Plevova F, Mansouri L, Ljungström V, Ntoufa S, Davies Z, Juliusson G, Smedby KE, Belessi C, Panagiotidis P, Touloumenidou T, Davi F, Langerak AW, Ghia P, Strefford JC, Oscier D, Mayer J, Stamatopoulos K, Pospisilova S, Rosenquist R, Trbusek M. *ATM mutations in major stereotyped subsets of chronic lymphocytic leukemia: enrichment in subset #2 is associated with markedly short telomeres.* Haematologica. 2016;101(9):369-373.

5. Palma M, Gentilcore G, Heimersson K, Mozaffari F, Näsman-Glaser B, **Young E**, Rosenquist R, Hansson L, Österborg A, Mellstedt H. *T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers.* Haematologica. 2017;102(3):562-572.

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Contents

Abbreviations

AID	Activation induced cytidine deaminase
APC	Antigen presenting cell
ASCT	Autologous stem cell transplantation
ATM	Ataxia telangiectasia mutated
BcR	B-cell receptor
BIRC3	Baculoviral IAP repeat containing 3
BM	Bone marrow
BTK	Bruton's tyrosine kinase
C	Constant
CD	Cluster of differentiation
CDR	Complementarity determining region
cHL	Classic Hodgkin lymphoma
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitors
CML	Chronic myeloid leukemia
CSR	Class switch recombination
D	Diversity
DE	Differential expression
del(11q)	Deletion of long arm of chromosome 11
del(13q)	Deletion of long arm of chromosome 13
del(17p)	Deletion of short arm of chromosome 17
DLBCL	Diffuse large B-cell lymphoma
EGR2	Early growth response 2
FASAY	Functional analysis of separated allele in yeast
FCR	Fludarabine, cyclophosphamide and rituximab
FDC	Follicular dendritic cell
FL	Follicular lymphoma
GC	Germinal center
HSC	Hematopoietic stem cell
IG	Immunoglobulin
IGH	Immunoglobulin heavy
IGHV	Immunoglobulin heavy variable
IGK	Immunoglobulin kappa
IGL	Immunoglobulin lambda
iwCLL	International workshop on CLL
J	Joining

JAK	Janus kinase
MBL	Monoclonal B-cell lymphocytosis
MCL	Mantle cell lymphoma
M-CLL	CLL with mutated IGHV genes
MRD	Minimal residual disease
MYD88	Myeloid differentiation primary response 88
MZ	Marginal zone
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NFKBIE	NF-κB inhibitor epsilon
NGS	Next-generation sequencing
NHEJ	Non-homologous end joining
NLC	Nurse-like cells
NOTCH1	Notch homolog 1, translocation-associated
OS	Overall survival
PCNSL	Primary central nervous system lymphoma
PCR	Polymerase chain reaction
PFS	Progression-free survival
PLCϒ2	Phospholipase Cϒ2
PMBL	Primary mediastinal B-cell lymphoma
RAG	Recombination activating gene
RNA-seq	RNA sequencing
RS	Richter syndrome
RSS	Recombination signal sequences
SF3B1	Splicing factor 3b subunit 1
SHM	Somatic hypermutation
SLL	Small lymphocytic lymphoma
SMZL	Splenic marginal zone lymphoma
SNV	Single nucleotide variant
SOCS1	Suppressors of cytokine signaling 1
STAT	Signal transducer and activator of transcription
T-ALL	T-cell acute lymphoblastic leukemia
TdT	Terminal deoxynucleotidyl transferase
TLR	Toll-like receptor
TP53	Tumor protein p53
*TP53*abn	del(17p) and/or *TP53* mutation
TTFT	Time to first treatment
TTT	Time to treatment
U-CLL	CLL with unmutated IGHV genes
V	Variable
VAF	Variant allele frequency
WES	Whole-exome sequencing
WGS	Whole-genome sequencing

Introduction

Lymphoid malignancies arise due to abnormalities in cells sharing a common lineage. They vary greatly in respect to the stage of maturation from which they derive, i.e. precursor or mature lymphoid cells, epidemiology, clinical presentation, morphology, immunophenotype, and molecular features. Malignant lymphomas can broadly be divided into three categories: B-cell lymphoma, T-cell lymphoma and Hodgkin lymphoma. On a global level, these individual entities do not account for high frequencies of cancers, however, when combined they are the seventh most frequently observed group of cancers[1]. According to the 2016 World Health Organization (WHO) classification of mature lymphoid, histiocytic, and dendritic neoplasms, over 80 distinct entities exist[2]. A higher incidence of lymphoma is seen in industrialized areas of the world such as Europe, North America and Australia, with the highest incidence of lymphomas occurring in Israel[3].

This thesis will focus on recurrent genetic mutations in two different entities of B-cell derived lymphomas, i.e. chronic lymphocytic leukemia (CLL) and primary mediastinal B-cell lymphoma (PMBL). Preceding a detailed description of these two malignancies, an introduction to normal B-cell development and maturation is provided.

The B-cell

B-cell development

Differentiation of B-cells begins in the bone marrow (BM), and follows several tightly regulated stages. Asymmetric division of hematopoietic stem cells (HSCs) gives rise to common lymphoid progenitors (CLPs) capable of differentiating into B-cells, T-cells or natural killer cells (NK cells). CLPs destined to follow the T-cell lineage leave the BM and migrate to the thymus for maturation. CLPs compelled to become B-cells remain in the BM to undergo further development before being released.

The first stage of development unique to the B-cell lineage is the progenitor B-cell (pro-B cell), followed by differentiation into precursor B-cells (pre-B-cells). Both of these stages are antigen independent and heavily rely on support from BM stromal cells for successful development. These stromal cells have two main functions in B-cell development, the first, is to keep the HSCs and CLPs in specific BM niches for further differentiation and the second is the production of chemokines. Developing B-cells require signals from chemokines in order to progress to the next stage of differentiation[4,5].

Rearrangements of the immunoglobulin heavy (IGH) locus can be seen at the late pro-B cell stage[6]. While still in the BM the B-cells go through two major check-points. The first occurs between the late pro-B and early pre-B cell stage. If a non-productive IGH gene rearrangement has been produced then development is halted and apoptosis occurs. The pre-B cell expresses a pre-B cell receptor (pre-BcR) consisting of the heavy chain and a surrogate light chain, encoded by the VpreB and λ5 genes and α/β heterodimers[7,8]. Development into an immature B-cell occurs following production of a successful light chain IG rearrangement, which will replace the surrogate light chain. The B-cell goes through the second check-point to assess viability of the newly expressed IG molecule[6]. This cell, now referred to as an immature B-cell, migrates from the BM, through the circulation to secondary lymphoid organs where B-cell maturation occurs (Figure 1).

B-cell maturation

The functional BcR assembled in the previous stages undergoes self-reactivity testing. If the B-cell is autoreactive, the cell will be processed in one of three ways, clonal deletion, receptor editing or induced anergy. Clonal deletion refers to forced apoptosis of the autoreactive B-cells through BcR signaling. Receptor editing involves a secondary rearrangement of the IG light chain, this is the main mechanism utilized in rendering autoreactive B-cells as tolerant[9]. The third possible outcome for autoreactive B-cells is induced anergy; the autoreactive B-cell will exit the BM but will remain unresponsive to antigens[10]. The next stage of maturation is the co-expression of both IgM and IgD molecules on the surface of the B-cell. This results from RNA splicing of the IG heavy chain and the B-cell is now referred to as a mature B-cell or a naïve B-cell[11]. If the naïve B-cell encounters antigen(s), it may undergo affinity maturation followed by differentiation into a memory B-cell or a plasma cell (Figure 1).

Foreign antigens are concentrated in the secondary lymphoid organs i.e. the spleen, lymph nodes and mucosa-associated lymphoid tissue (MALT). These antigens are presented to the circulating B-cell by follicular dendritic cells (FDCs) with assistance from T-cells[12]. If the antigen binds with adequate affinity the activated B-cell migrates into the lymphoid follicle, giving rise to germinal centers (GC), which are specialized microenvironments, and at this point that the B-cell is referred to as a centroblast. Conversely, failure to bind to antigen(s) or inadequate antigen binding results in B-cell apoptosis via Fas signaling[13]. The GC can be divided into three separate zones, the dark zone, the light zone and the mantle zone (Figure 1). Rapid proliferation of clonal centroblasts accompanied by somatic hypermutation (SHM) and class-switch recombination (CSR), discussed below, occur within the dark zone. The centroblasts leave the dark zone and migrate to the light zone, becoming non-proliferating centrocytes. Here, FDCs and T-cells subject the centrocytes to selection. If appropriate, the centrocyte will receive differentiation signals and become either a plasma cell or memory B-cell, subsequently migrating from the GC[14].

B-cells express various specialized proteins that are directly related to the stage of maturation. B-cell lineage commitment, development and maturation are mediated by the transcriptional regulator PAX5[15]. PAX5 is expressed from the pro-B cell stage through to the mature B-cell stage[16]. Expression of the anti-apoptotic protein BCL2 is seen in pro-B cells and mature B-cells but not in pre-B-cells and immature B-cells[17]. BCL6 expression plays a role in inducing proliferation in the GC and in protecting against apoptosis triggered by DNA damage response and is primarily expressed in GC B-cells[18]. Ki-67 is a common immunohistologic-staining

marker of proliferation and high expression is seen in the GC[19]. CD10 expression is first seen at the pro-B cell and immature B-cell stage but is lost by the time the B-cell enters the mature stage[20]. Post-GC B-cells have high expression of the transcription factor MUM/IRF, which is indicative of an antigen stimulated B-cell[21]. MUM1/IRF4 is crucial in lymphoid development with dual roles in T-cell differentiation and GC formation[22]. Plasma cells characteristically express the BLIMP-1 protein, which controls several genes involved in plasma cell differentiation. BLIMP-1 functions as an inhibitor of genes necessary for proliferation allowing the plasma cell to terminal differentiate[23,24].

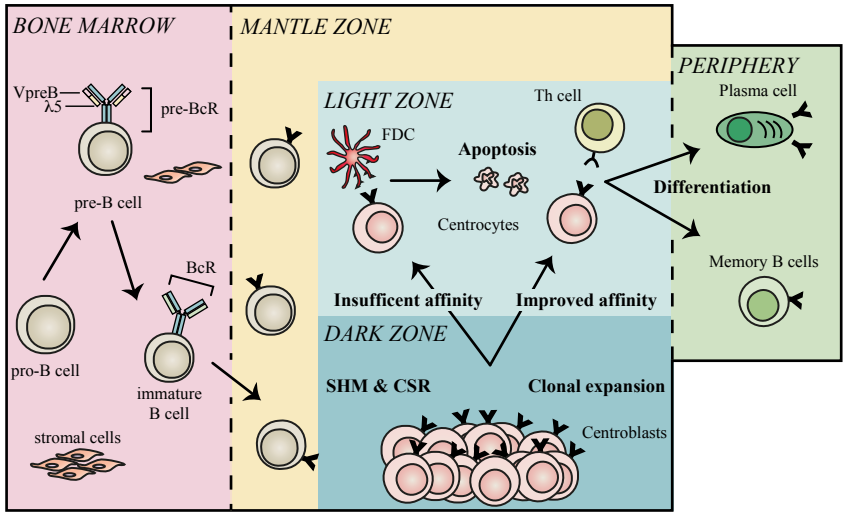


Figure 1. **Cartoon schematic of the stages of B-cell development and maturation through the BM, lymph node and periphery**. Upon exiting the BM the immature B-cell migrates to the lymph nodes (or other secondary lymphoid organs). In the GC dark zones the immature B-cell, now known as a centroblast, undergoes rapid clonal expansion coupled with SHM and CSR. In the light zone the B-cell, at this stage referred to as a centrocyte is exposed to antigens on the surface of FDC, if affinity is insufficient cellular mechanisms are triggered to initiate apoptosis. If the affinity binding is sufficient, the centrocyte can undergo further differentiation into antibody producing plasma cells or memory B-cells with the assistance of T helper cells. Adapted from Rickert *Nature Reviews Immunology* (2013)[25].

V(D)J rearrangement

IG molecules are made up of two identical heavy and light chains, which join together through disulphide bridges and non-covalent interactions to construct a four-chain IG molecule. Each chain has a constant (C) region and a variable (V) region (Figure 2). The V region represents the antigen-binding site and is made up of three complementarity-determining regions (CDRs),

CDR1, CDR2 and CDR3. The variable heavy (VH) CDR3 displays the most variability as it encompasses the diversity (D) and joining (J) genes involved in VDJ recombination[26].

One IGH locus and two IG light chain loci, kappa (IGK) and lambda (IGL), exist and encode numerous genes involved in IG gene rearrangements. While variable (IGHV), diversity (IGHD) and joining (IGHJ) genes are located at the IGH locus, only variable (IGKV/IGLV) and joining (IGKJ/IGLJ) genes are located at the light chain loci (Figure 2)[27-29]. V(D)J rearrangements of genes at the IGH and IGK/IGL loci occur randomly, mediated by the recombination activating genes *RAG1* and *RAG2*[30]. These enzymes introduce double stranded breaks at unique recombination signal sequences (RSS) located 3´ to each IGHV gene, 5´ to each IGHJ and at both ends of the IGHD genes. Non-homologous end joining (NHEJ) factors function to join gene segments together at the sticky ends[31]. DNA repair enzymes remove unpaired-nucleotides through exonuclease activity and the DNA polymerase enzyme terminal deoxynucleotidyl transferase (TdT) inserts N-nucleotides at the junctions of the IGHV, IGHD and IGHJ genes leading to further variability within the IG antigen-binding site[32].

Briefly; an IGHD gene first joins to an IGHJ gene, this IGHD-J rearrangement is then joined by an IGHV gene. This rearranged complex forms the VH CDR3 of the IG molecule. Rearrangements at the light chain loci only involve the joining of IGKV/IGLV genes to IGKJ/IGLJ genes[33].

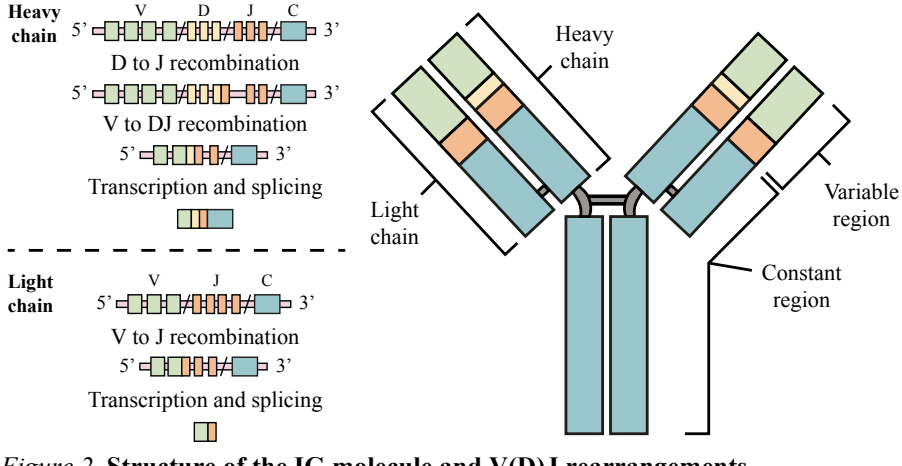


Figure 2. **Structure of the IG molecule and V(D)J rearrangements.** Rearrangements at the IGH and IGK/L loci results in the formation of an antibody molecule comprising of two identical heavy and light chains. Adapted from Küppers *et al. New England Journal of Medicine* (1999)[14].

Somatic hypermutation and class switch recombination

Following antigen stimulation, affinity maturation and CSR of the IG molecule occurs within the GC. Affinity maturation is achieved through SHM, which is triggered by activation induced cytidine deaminase (AID). AID is an enzyme capable of inducing mutations and is highly expressed in activated GCs[34,35]. Mutations acquired through this process target the V region of the IG molecule and increase its specificity[36]. These mutations occur at targeted "hot-spot" regions consisting of specific amino acid motifs e.g. RGYW/WRCY and DGYW/WRCH, and usually concern single nucleotide variations as opposed to deletions or insertions, occurring at a rate of 1 per 1 kilo base pair per cell division[37-40].

CSR is a change in isotypic expression of the IG molecule i.e. IgM to IgG, IgA, or IgE, thus changing the effector function of the IG and its distribution throughout the body[41]. CSR targets the C region of the IG molecule and is achieved through replacement of the expressed heavy chain C region, μ in the case of IgM and δ in the case of IgD to the heavy chain C region of IgG (γ), IgA (α) or IgE (ε). Short nucleotide repeats known as "switch regions" are located upstream of the C region genes. A recombination event takes place whereby DNA between the "switch regions" is excised[42]. AID also plays a role in CSR but its exact mechanism remains unclear[42]. The isotype switched IG molecule retains the same antigen affinity as it had prior to CSR and the processes of SHM and CSR are neither mutually exclusive nor inclusive[42,43].

Rearrangements of IG genes as clonal markers

The IG rearrangement serves as the fingerprint of a B-cell. Analysis of the IG sequence indicates developmental and maturation stage of the B-cell. As discussed in the previous sections the configuration of the BcR reflects the B-cells individual time-line. Pre-GC B-cells experience no SHM, expressing BcRs with a germline configuration, GC derived B-cells exhibit varying degrees of ongoing SHM and in post-GC B-cells the SHM process has ceased altogether. Interrogation of the IG sequence thus indicates its natural history. While healthy humans express a diverse repertoire of B-cells at relatively low levels, monoclonal expansion of a specific B-cell defines several disease entities including the majority of lymphoid malignancies[44]. However, clonal expansion alone is not proof of malignancy as chronic (auto)antigen exposure can also lead to monoclonal populations of B-cells, such is the case in rheumatoid arthritis[45]. Therefore, analysis of the IG genes represents a clonal marker for the malignant B-cell and its progeny also

pinpointing the juncture of malignant transformation aiding in the comprehension of pathogenesis of B-cell derived malignancies.

Extrinsic and intrinsic factors

Microenvironmental interactions

Throughout development and maturation the B-cell is dependent on interactions with the cells and soluble factors in specialized microenvironments within the BM and secondary lymphoid organs. Complex and highly dynamic cross talk in the microenvironment fosters the B-cells ability to survive, proliferate and differentiate. Figure 3 details the typical interactions the B-cell encounters with accessory cells such as T-cells, FDCs or other antigen presenting cells (APCs), monocyte derived nurse-like cells (NLC) and stromal cells in its microenvironment.

In the BM, stromal cells (BMSC) provide specialized niches for precursor B-cell development. This is achieved through secretion of the cytokine CXCL12 and the avid binding of VLA-4 on the precursor B-cell to VCAM-1 on the surface of the BMSC, docking the B-cell in the supportive niche[46,47]. IL-7 secreted from the BMSC promotes commitment to the B-cell lineage and plays a roles in survival, proliferation and maturation of the developing B-cell[48]. B-cell localize to the GC with the assistance of the chemokine CXCL13 produced by FDCs[49,50]. The FcγR receptor on the FDC aids in follicular exclusion of low-affinity autoreactive B-cells and triggers apoptosis[51]. BAFF-R, TACI and BCMA on the surface of the B-cells have roles in survival, proliferation and maturation and also stimulate IgM production[52]. T-cells interact with B-cells through CD40/CD40L binding and stimulate the B-cell to differentiate into plasma cells or memory B-cells[53].

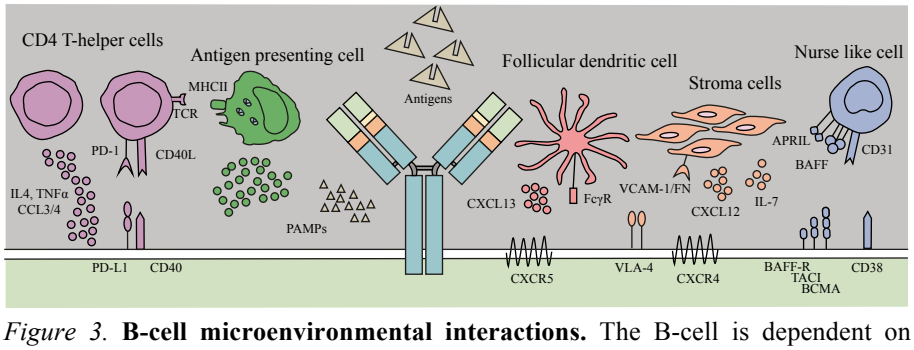


Figure 3. **B-cell microenvironmental interactions.** The B-cell is dependent on various signals from both cell-to-cell interactions and soluble factors from a host of different cell types. These include CD4 T-helper cells, antigen presenting cells, follicular dendritic cells, stromal cells and nurse-like cells.

Signaling pathways

During the life span of the B-cell several signaling pathways are activated depending on the external signals received, inducing a variety of responses (Figure 4).

BcR signaling

The IG molecule is non-covalently coupled with a CD79A/B heterodimer which contains immunoreceptor tyrosine-based activation motifs (ITAMs)[54]. BcR signaling is initiated through antigen binding leading to the phosphorylation of the ITAM tyrosine residues on CD79A/B by the SRC-family kinases. Stemming from this, SYK, a tyrosine kinase, is recruited and phosphorylated and in turn recruits BLNK which then phosphorylates Bruton's tyrosine kinase (BTK) and phospholipase Cϒ2 (PLCϒ2)[55]. PLCϒ2, through the hydrolysis of PIP to DAG with consequential increase calcium levels activates PKCβ, which phosphorylates a CARD11, BCL10 and MALT1 complex leading to NF-κB activation, discussed below[56]. In a similar respect, activation of PKCβ also mediates B-cell differentiation through the activation of MAPK/ERK signaling pathways[57]. BcR signaling can also occur in a "tonic" setting whereby low amplitude maintenance and survival signals are continuously sent through the BcR independent of receptor engagement[58].

NF-κB signaling

Survival and proliferation of the B-cell is promoted through the activation of the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) pathway[59]. Activation occurs downstream of several receptors including BcR, Toll-like receptor (TLR) and CD40. In the canonical pathway the IκB-kinase (IKK) complex, which is composed of IKKα, IKKβ and IKKγ is activated leading to subsequent phosphorylation and degradation of NF-κB inhibitors i.e. IκBα, IκBβ, IκBδ and IκBε. NF-κB transcription factors RelA and p50 translocate to the nucleus where they regulate the expression of various target genes[60]. In the non-canonical NF-κB pathway activation occurs through an IKK complex consisting of two IKKα subunits. The NF-κB transcription factors RelB and p52 travel to the nucleus[61]. The transcription of the BCL2 family members are activated by both canonical and non-canonical NF-κB pathway[62]. Several inhibitors of the pathway have been identified including the protein A20, encoded for by the *TNFAIP3* gene[63].

CD40 signaling regulates B-cell activation and proliferation and upon binding to its ligand it interacts with an E3 ubiquitin ligase complex of TRAF2, TRAF3, c-IAP1 (BIRC2) and c-IAP2 (BIRC3)[61]. The complex is capable of degrading NIK, the main regulator of the non-canonical pathway

and so acts as a negative regulator of NF-κB signaling[61]. TLR signaling relies on adapter proteins such as MYD88; following TLR activation IRAK-1 and IRAK4 become phosphorylated and through TRAF6 activate the NF-κB pathway[64].

JAK/STAT signaling

The IL-4 and IL-13 chemokines have roles in B-cell proliferation, differentiation into plasma cells and class switching to Ig-E antibodies[65]. The cytokine-activated Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is also activated in B-cells through the binding of these cytokines to their receptors. The JAK2 tyrosine kinases is phosphorylated leading to STAT6 activation which targets genes involved in proliferation, differentiation and survival apoptosis[66-68]. This activation is negatively regulated by suppressors of cytokine signaling 1 (*SOCS1*)[69].

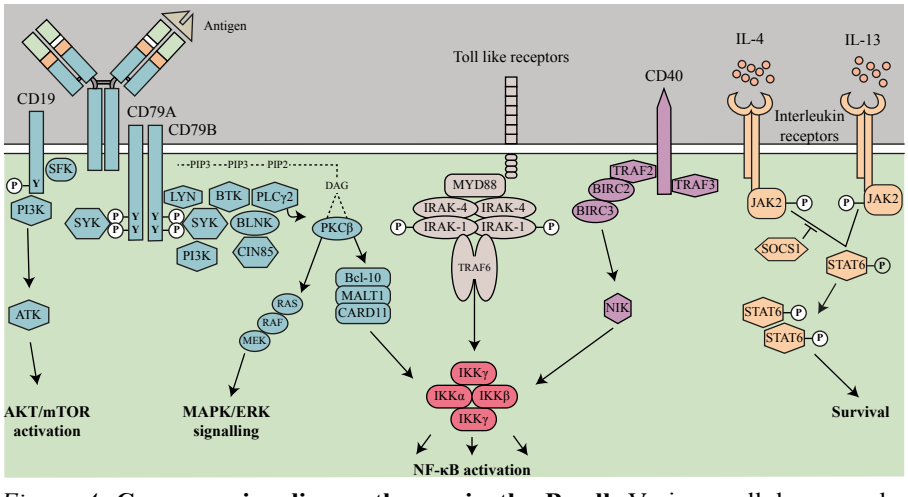


Figure 4. **Common signaling pathways in the B-cell.** Various cellular cascades control different aspects within the B-cell including cell cycle control, differentiation, proliferation, migration and apoptosis.

As detailed above B-cell development and maturation is hugely dependent on both extrinsic and intrinsic factors. For a B-cell to function correctly, a homeostasis between cell extrinsic and intrinsic factors must be maintained. If something disrupts this balance, aberrant responses can occur in the B-cell, resulting in insufficient immune responses, auto-immunity or malignant transformation.

Chronic lymphocytic leukemia

Background

CLL is characterized by the accumulation of clonal B-cells in the peripheral blood, BM and secondary lymphoid organs. CLL cells express a distinct immunophenotypic profile of $CD5^+/CD19^+/CD23^+$ with diminished levels of membrane IgM, IgD, and low CD20 and CD79B[70]. It is the most common form of leukemia found in adults in Western countries, accounting for ~ 30% of all leukemias[71]. The median age of diagnosis is 72 years, with a greater incidence in males than females (ratio 2:1)[72]. Diagnosis is based on a lymphocyte count of $>5x10^9$/L and immunophenotype[73]. Most patients are initially asymptomatic and diagnosed incidentally following a routine blood test. CLL patients exhibit a remarkable clinical heterogeneity with survival times ranging from months to decades. A proportion of patients will experience an indolent disease course and remain untreated, however, patients at the opposite end of the spectrum will require immediate treatment and experience an aggressive disease course. These patients are also at a higher risk to undergo transformation to a high-grade malignancy known as Richter's syndrome (RS) with poor clinical outcome[74].

Small lymphocytic lymphoma (SLL) is regarded as the sister lymphoma to CLL, in so much as it is a non-leukemic manifestation of CLL (accounting for 2.5% of lymphomas in Sweden[75]). A differential diagnosis of SLL is based on the presence of lymphadenopathy and/or splenomegaly and a corresponding low monoclonal B cell lymphocyte count[73,76]. CLL also has a pre-cursor condition known as monoclonal B-cell lymphocytosis (MBL). The diagnosis of which is based on a monoclonal B cell count is less than $5x10^9$/L blood[77]. Screening of healthy individuals older than 40 years revealed a >10% incidence of MBL[78,79]. Low-count ($<0.5 \times 10^9$/L) and high-count ($\leq 0.5 \times 10^9$/L) variations of the condition exist. Low-count MBL rarely progresses to malignancy and express an IGHV gene repertoire divergent to that of CLL[78,80]. On the other hand, 1-2% of high-count MBL cases progress to CLL per year and share similar immunogenetic characteristics[79,81].

Treatment

The majority of CLL patients will not receive treatment at diagnosis (~85-90%). Instead a "wait and watch" policy is employed and decisions regarding therapy are taken only when disease progresses and specific criteria set by the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) are met[73,82]. The "gold-standard" first-line regime for medically fit patients, negative for *TP53* aberrations, is fludarabine, cyclophosphamide and rituximab (FCR)[83]. This combination of a purine analogue, fludarabine, an alkylating agent, cyclophosphamide, and a CD20 monoclonal antibody, rituximab, has achieved a 90% overall response rate, but relapse remains common among these patients[83,84]. However, recently it was shown that M-CLL patients treated with FCR obtained minimal residual disease (MRD) negativity with prolonged remission time and survival[85]. Patients harboring *TP53* aberrations are ideally treated with novel agents targeting kinases of the BcR pathway e.g. ibrutinib (targeting BTK) and idelalisib (targeting PI3K)[86-89]. Medically unfit patients unable to tolerate the toxic affects of FCR are predominantly treated with the alkylating agent, chlorambucil, as a monotherapy or the combination of bendamustine and rituximab[73,90,91].

Prognostic markers

Due to the variability of the clinical course of patients with CLL, it is imperative to have robust prognostic markers to stratify patients accordingly. Classical staging systems, Rai and Binet, are still in use clinically[92,93]. They rely on results from the complete blood count indicating lymphocytosis, anemia and thrombocytopenia and physical manifestations of the disease, such as lymphadenopathy, splenomegaly and hepatomegaly. Patients with low-stage of disease, which today constitute the majority of cases cannot be stratified with enough accuracy using these systems. Several DNA, RNA and protein prognostic markers have been suggested to date but two remain the mainstay for prognostication: genomic aberrations and the IGHV mutational status.

Genomic aberrations

Approximately 80% of CLL patients carry cytogenetic aberrations[94]. The most recurrent aberrations include deletions of 13q14 (~55%), 11q22-23 (12-18%), trisomy of chromosome 12 (11-16%) and deletion of 17p13 (5-10%)[95-97]. Using fluorescence in situ hybridization (FISH), these aberrations are assessed in routine diagnostics to distinguish high risk from low risk patients using the Döhner prognostic model[94]. Deletions of 17p13 (del(17p))

and also *TP53* mutations are associated with advanced disease stage and poor response to therapy. Due to this fact, prior to treatment initiation, patients must be screened for these aberrations[98-100]. Deletion of 11q22-23 (del(11q)) are also associated with poor overall survival (OS). Conversely, deletion of 13q14 (del(13q)) and patients lacking any recurrent aberrations have longer OS[94-97]. Although trisomy 12 is associated with a shorter time to disease progression, this aberration is associated with an intermediate prognosis with considerable heterogeneity[94-97,101]. Of note, MBL cases with a CLL-phenotype are reported as having similar percentages of deletion 13q14 and trisomy 12 as that of CLL cases[81].

IGHV gene mutational status

The significance of SHM status on CLL outcome was reported by two groups in 1999[102,103]. SHM status divided CLL patients into two categories: IGHV mutated (M-CLL) and IGHV unmutated (U-CLL). A ≥98% identity to the germline is required to be considered as U-CLL and vice versa. M-CLL is associated with a markedly longer time to treatment (TTT) and OS in comparison to U-CLL[102,103]. As SHM status of the IGHV gene remains unchanged over the disease course it is particularly suitable as a prognostic marker[104]. On the face of it, IGHV mutational status can stratify CLL patients into two distinct clinical groups. It is important to note however, that this stratification is not a rigid model. Assignment to the M-CLL group does not automatically imply that patients will have a better prognosis. Such is the case with CLL patients belonging to stereotyped subset #2, discussed below[105].

Recurrent mutations in CLL

Analysis of over 1000 whole-exomes and whole-genomes has revealed recurrently mutated genes in CLL[106-109]. A relatively small number of frequently mutated genes were revealed with a very long “tail” of less frequently mutated genes[110]. These mutations targeted various signaling pathways including DNA damage response (*TP53*, *ATM* and *POT1*), Notch signaling (*NOTCH1*, *FBXW7*, *SPEN*, *CREBBP*), NF-κB signaling (*BIRC3*, *NFKBIE*, *TRAF3*) and RNA metabolism (*SF3B1* and *XPO1*). Roles in disease pathogenesis and/or prognosis have been elucidated for a number of these mutations[107-109,111-116]. Several recurrent mutations investigated in this thesis are described briefly below[107-109,111-116].

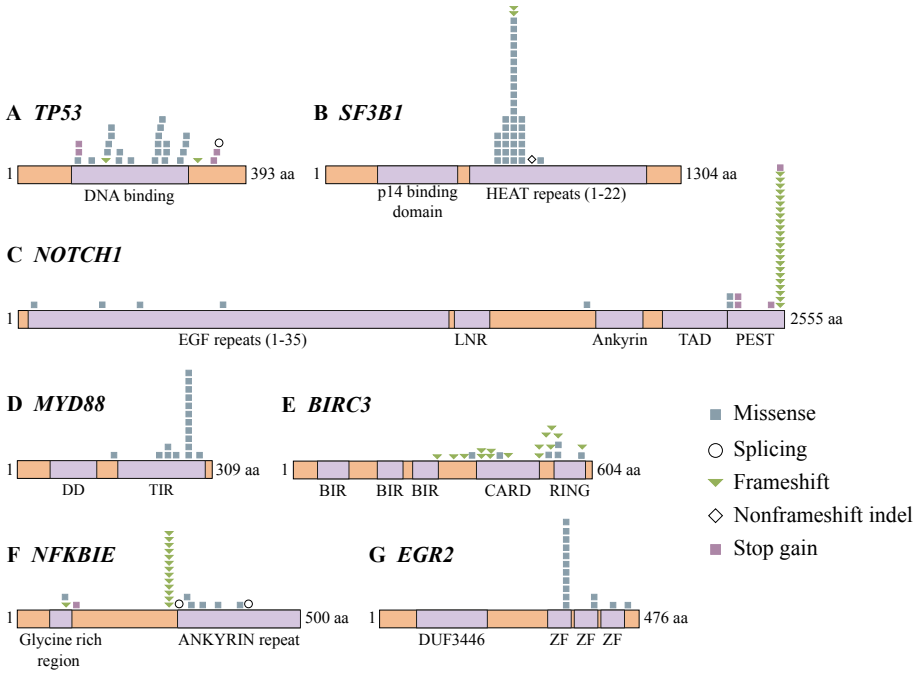


Figure 5. **Localization of recurrent genetic mutations and associated protein domains.** (A) *TP53*, (B) *SF3B1*, (C) *NOTCH1*, (D) *MYD88,* (E) *BIRC3,* (F) *NFKBIE* and (G) *EGR2*.

TP53

TP53 aberrations, i.e. del(17p) and/or *TP53* mutations, are currently the strongest marker indicative of a very poor outcome for CLL patients. The *TP53* gene encodes for the tumor suppressor protein p53 which is activated in response to DNA double stranded breaks (DSBs) and initiates apoptosis or cell cycle arrest[117,118]. Although up to 80% of *TP53* mutated cases carry deletion of 17p, monoallelic mutations of *TP53* also confer a poor prognosis[99]. A mutational hot-spot region in the DNA binding domain of the *TP53* gene (Figure 5A) has been identified in CLL. While *TP53* mutations are infrequently seen at diagnosis (3-6%)[119], they increase in frequencies relative to disease severity with frequencies as high as 37% reported in chemorefractory patient[120,121]. Interestingly, it has recently been reported that subclonal *TP53* mutations have an equally poor survival as their clonal counterparts, highlighting the importance of *TP53* as a driver mutation in CLL progression and chemorefractoriness[122].

SF3B1

SF3B1 encodes for a component in the splicing factor 3b protein complex, which forms part of the catalytic core of the spliceosome, and is involved in

transcription and mRNA processing. Reported mutation frequencies in CLL have varied from 3.6% to as high as 20% and a strong association with disease progression, treatment refractoriness and poor prognosis is evident in *SF3B1* mutated patients[112,113,123-127]. In multivariate analysis, mutations in *SF3B1* were an independent marker for both OS and TTT[112]. An enrichment of *SF3B1* mutations has also been observed in U-CLL patients carrying deletion of 11q[109]. *SF3B1* mutations in CLL are localized to the highly conserved C-terminal HEAT domains. Fifty percent of reported mutations occur at codon K700, wherein a glutamic acid replaces the germline lysine [112,113] (Figure 5B). Mutations within *SF3B1* induce changes in gene expression and splicing and affect key pathways of the CLL cells including DNA damage and Notch signaling, however, their role is not fully understood[128].

NOTCH1

The *NOTCH1* proto-oncogene encodes a class I transmembrane receptor which acts as a ligand activated transcription factor. Notch signaling plays an important role in differentiation, proliferation and apoptosis[129]. The mutational frequency of *NOTCH1* in CLL varies between 4.7-15.1% with higher mutational frequency observed in more aggressive cohorts[107,108,111,112,125,130,131]. CLL cases that undergo subsequent transformation to RS also have higher frequencies of *NOTCH1* mutations[111]. A 2-base pair frameshift deletion within exon 34, located in the c-terminal PEST degradation domain, accounts for the majority of *NOTCH1* mutations reported in CLL (Figure 5C)[111,131]. *NOTCH1* mutations confer a shorter OS and progression-free survival (PFS) and have been shown to be an independent prognostic marker in multivariate analysis, although not in all studies[127,130,132]. Predictive marker analysis indicated patients harboring *NOTCH1* mutations experienced no benefit from the addition of rituximab to their treatment protocol[133,134]. A possible explanation for this is the low CD20 expression reported in *NOTCH1* mutated CLL cases[135]. Trisomy 12 patients with U-CLL are associated with *NOTCH1* mutations[136].

MYD88

The *MYD88* gene encodes a cytosolic adapter protein that functions in signal transduction through IL-1 and the TLR pathways. *MYD88* mutations found in CLL primarily concern a p.L265P substitution within exon 5 (Figure 5D), leading to constitutive stimulation of NF-κB, thereby providing both proliferative and survival advantages to cells carrying the mutation[107,112]. Large-scale mutational screenings have reported a relatively low frequency of *MYD88* mutations (2-5%) in CLL, appearing exclusively in M-CLL and conferring a favorable outcome[112,120,126].

BIRC3

As discussed previously, BIRC3 acts as a negative regulator in the non-canonical NF-κB pathway[61]. Within CLL, frequencies of *BIRC3* mutations are low at diagnosis (2.5-4%), however, frequencies of up to 24% were reported in cases refractory to fludarabine based treatment[120,137]. Inactivating mutations are most frequently observed (90%) in CLL causing the truncation of the C-terminal RING domain of the BIRC3 protein (Figure 5E). *BIRC3* disruption has been proposed to come in second, after *TP53*, as a predictor of poor OS[138], however this has not yet been validated. It should be noted that genetic lesions in *TP53* and *BIRC3* are usually mutually exclusive[137].

NFKBIE

The *NFKBIE* gene encodes IκBε, which acts as a negative regulator of the canonical NF-κB pathway[139]. Mutations within this gene were recently reported in CLL and primarily concerned a 4 base pair frameshift deletion in exon 1 (Figure 5F). Damm *et al* and Mansouri *et al* reported *NFKBIE* mutations at a frequency of 10.7% and 6.2%, respectively, in CLL[115,116]. *NFKBIE* mutations were most commonly seen in patients with U-CLL and poor prognosis. Interestingly, Mansouri *et al* reported an enrichment of *NFKBIE* mutations in the poor-prognostic stereotyped subset #1 (discussed in next section). The paper also delineated the functional consequences of the truncating deletion i.e. reduced IκBε expression and decreased p65 inhibiton leading to increased phosphorylation and nuclear translocation of p65[116].

EGR2

The *EGR2* gene encodes a diverse transcription factor that plays a role in both the development and regulation of B-cells[140,141]. This transcription factor has several target genes and is also a downstream target itself of pre-BcR and BcR signaling, through BRAF signaling and ERK phosphorylation cascades[142-144]. Sustained expression of the EGR2 protein impairs progression of developing B-cell in the BM and also impairs PAX5 expression in pro-B-cells[141]. Expression of the recombinase enzymes RAG1 and RAG2, mediators of V(D)J recombination, are also downregulated upon overexpression of EGR2[145]. In a recent study, *EGR2* mutations were identified in hematopoietic progenitor cells, indicating they are an early initiating event. Furthermore, the same study reported surprisingly high frequency of *EGR2* mutations in advanced stage CLL patients (8.3%)[115]. *EGR2* mutations in CLL appear to cluster among three parallel zinc finger domains (Figure 5G). Further investigation indicated that *EGR2* mutations were not likely to inactivate the protein but rather affect transcription of its

many target downstream genes. By defining a set of genes frequently up-regulated during BcR stimulation and comparing RNA sequencing (RNA-seq) data from *EGR2* mutated and wildtype samples, *EGR2* mutated samples expressed a signature echoing that of BcR stimulation, implying BcR activation in these cases[115].

Immunogenetics in CLL

Immunoglobulin gene repertoire

As discussed in the B-cell introduction, generation of the IG molecule involves the recombination of IGHV, IGHD, IGHJ and IGK/IGL genes followed by SHM and CSR. Due to all the recombination events the potential amount of rearrangements is staggering[146]. Put mathematically, the probability, by chance alone, that two individuals could express clonal B-cell population with an identical BcR IG is 1 in 1 trillion ($1:10^{12}$). Various studies during the 1990s reported that CLL patients utilized a restricted set of IGHV genes, with IGHV1-69, IGHV4-34, IGHV3-7 and IGHV3-21 highly overrepresented[147-149]. SHM within these genes is not uniform, IGHV1-69 has little or no SHM, IGHV4-34 carries a heavy SHM burden and IGHV3-21 has a mix of both low and high presence of SHM. On top of this, it was also discovered preferential coupling of certain IGHV and IGKV/IGLV genes[150].

Stereotyped subsets

Similarities within IGHV, IGHD and IGHJ usage of CLL patients were reported in the early 1990s[151,152]. However, at that point in time CLL was commonly believed to be a disease of naïve B-cells that were antigen inexperienced and had no SHM (U-CLL)[153]. A strong indication pointing towards CLL being an antigen driven disease came in 1998 when Fais *et al* reported CLL SHM in 50% of CLL patients, and patients expressing restricted sets of BcRs with similar IGHV-D-J gene usage and CDR3 characteristics[147]. As mentioned above, a year later it was revealed in CLL could be divided into two categories with distinct clinical prognoses, U-CLL and M-CLL[102,103]. Around this time gene expression profiles in CLL patients revealed an antigen-experienced profile both in U-CLL and M-CLL adding weight to the idea of an antigen driven disease[154,155]. A spate of publications analyzing IG sequence data followed on from these findings and it soon became apparent that unrelated CLL patients could express highly similar IG sequence rearrangements. This phenomenon was referred to as “stereotyped” subsets[156-165].

In a recent multicenter study of 7,424 patients, ~30% of CLL cases could be assigned to a subset based on their IG sequence[156]. Over 200 subsets have been identified to date, however, ~41% of subset cases are represented by 19 subsets, and these are considered "major" subsets. Figure 6 depicts the occurrence of subsets in CLL. The 10 subsets analyzed in this thesis and their relative percentages within CLL are also detailed.

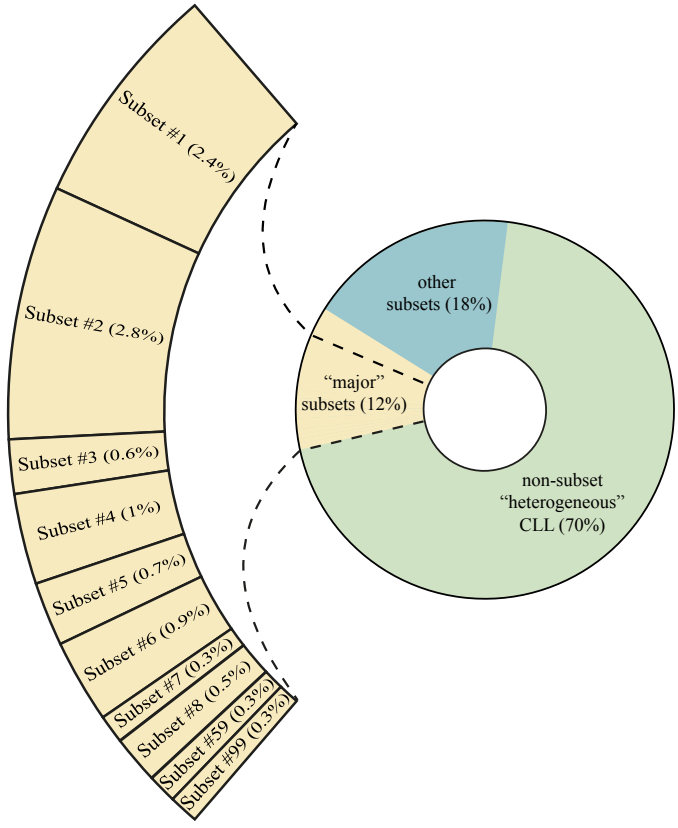


Figure 6. **Frequencies of stereotyped subsets in CLL**. Adapted from Agathangelidis et al *Blood* (2012)[156].

Patients assigned to a specific subset not only share homogenous IG sequences they also experience clinical commonalities, most notably the poor prognostic subset #1, #2 and #8, and the good prognostic subset #4[156,161]. Table 1 briefly outlines the "major" stereotyped subsets included in this thesis, along with their clinical and biological associations[161,166-168]. Clinically aggressive subsets #1, #2 and #8 and the indolent subset #4 are the most extensively studied subsets. Subset #1 has a predilection for both *TP53* and *NOTCH1* mutations, while a striking bias in subset #2 for *SF3B1* mutations has been reported[124,169]. Subset #8 cases often carry mutations within the *NOTCH1* gene and has the greatest potential of all subsets to

transform to RS[166]. One study suggests consolidating classic CLL prognostic models with subset assignment, at least in the case of #1, #2 and #4. These subsets were clinically distinct and statistically significant when analyzed alongside recurrent cytogenetic aberrations[169].

Table 1. **Characteristics of major stereotyped subsets**[124,156,157,160,161,166-171].

Subset	SHM status	IGHV gene(s)	IGHJ gene	VH CDR3 AA length	Cytogenetics association	TTFT (years)
#1	U-CLL	Clan I genes	IGHJ4	13	del(17p) and del(11q)	1.6
#2	U-CLL & M-CLL	IGHV3-21	IGHJ6	9	del(13q) and del(11q)	1.9
#3	U-CLL	IGHV1-69	IGHJ6	22	del(11q) and del(13q)	2.7
#4	M-CLL	IGHV4-34	IGHJ6	20	del(13q)	11
#5	U-CLL	IGHV1-69	IGHJ6	20	del(11q)	1.8
#6	U-CLL	IGHV1-69	IGHJ3	21	del(11q)	1.6
#7	U-CLL	IGHV1-69	IGHJ6	24	del(11q) and del(13q)	2.8
#8	U-CLL	IGHV4-39	IGHJ5	18-19	trisomy 12	1.5
#59	U-CLL	Clan 1 genes	variable	12	trisomy 12	1.0
#99	U-CLL	Clan 1 genes	IGHJ4	14	no data	no data

Cell of origin

While B-cells that initially enter the secondary lymphoid organs express germline IGHV genes and are antigen inexperienced, B-cells that go through the GC encounter antigens, undergo SHM and express mutated IG molecules. Following this logic the point at which malignant transformation occurs can be determined depending on the SHM status of the clonal B-cells. However, in CLL this is a far more complicated endeavor as CLL is made up of populations with both unmutated IG molecules (U-CLL) and mutated IG molecules (M-CLL)[172]. Initial studies speculated that U-CLL cells derived from pre-GC cells and M-CLL from post-GC cells, however, subsequent gene expression profile studies indicated both U-CLL and M-CLL expressed CD27, a phenotype more similar to that of memory B-cells[70,154,155].

In more recent years, transcriptome analysis of CLL and normal B-cell populations suggested that both U-CLL and M-CLL are closely related to mature $CD5^+$ B-cells. Additionally, they reported that a subset of $CD5^+$ B-cell also express CD27 and harbor mutated IGHV genes. The study postulates that the $CD5^+/CD27^-$ population represent the physiological counterpart to U-CLL, while the $CD5^+/CD27^+$ post-GC population equates to the normal counterpart of M-CLL[173].

Staying with the idea that CLL cells derive from $CD5^+$ mature cells and seen to the existence of stereotyped BcRs in CLL it can be postulated that their normal counterpart cells would express such stereotyped patterns. IG sequence analysis of 4 healthy donors revealed a stereotyped pattern in ~7% of $CD5^+$ mature B-cells analysed, whereas <1% of the donors "conventional" B-cells expressed stereotypic features[173]. Another study investigating stereotyped IGHV1-69 rearrangements in unmutated normal B-cells reported a high proportion of CLL specific motifs[174], suggesting a normal counterpart for U-CLL at least. B1 cells, a minor sub-population of B-cells found in mice, located in the peritoneal cavity and spleen, also express stereotyped BcRs[175,176]. Although they seem to make an attractive candidate for the cell of origin in CLL with stereotyped BcRs, two issues must be broached, stereotyped BCRs only exist in 30% of CLL patients and B1 cells have not been characterized in humans yet[156,177,178].

B-cells from the MZ have also been put forward as a postulated cell of origin for the CLL cell[70,179]. MZ cells are predominantly located in the spleen in a diffuse region separating the red and white pulp. They interact with bacterial polysaccharides in a T-cell independent manner. MZ cells can express IgM receptors and can harbor both mutated IGs and unmutated IGs[180-182]. The major counter-argument to this model is in the fact that MZ B-cells do not

express CD5; aberrant expression of CD5 is a key characteristic of the CLL phenotype[183].

A general consensus has yet to be reached among researchers in the field, evaluating the studies thus far it seems that there will not be an unequivocal answer to this CLL cell of origin question.

Culprit antigens

The evidence presented for antigen selection in the natural history of CLL is irrefutable. The search for the culprit antigens is ongoing specifically looking into the nature of the antigen and the binding capacity of the IG molecules.

Through structural and functional analysis of recombinant IG molecules from CLL cells, it was revealed that U-CLL demonstrated polyreactivity and bound with low avidity to molecular motifs on apoptotic cells and bacteria (e.g. cytoskeletal proteins vimentin, filaminB and cofilin-1 and *Streptococcus pneumonia* polysaccharides), while M-CLL patients demonstrated a more restrictive antigen-binding profile[184-186].

Antigen reactivity studies, especially within stereotyped subsets revealed several culprit antigens. Chu *et al.* discovered subset #6 IG molecules bound specifically to non-muscle myosin heavy chain IIA (MYHIIA), an intracellular antigen associated with apoptosis[187,188]. Viral antigens have also been implicated due to the association between subset #4 and Epstein-Barr virus and Cytomegalovirus[189].

The notion that CLL BcRs could induce cell-autonomous signaling independent of extrinsic antigens was recently put forward. This study reported activation of cell-autonomous intracellular signaling in an antigen independent manner mediated by CDR3 auto-recognition of distinct epitopes on near by BcRs[190]. Following on from this, another study reported similar results with an additional facet in that subset #2 interactions were markedly weaker than that of subset #4, indicating the avidity at which different stereotyped IGs recognize intrinsic motifs, potentially underlies the clinical course[191].

Primary mediastinal B-cell lymphoma

Background

According to the 2008 WHO tumor classification, PMBL is a discrete entity within DLBCL[192,197]. However, the revised version of this classification in 2016 describes the disease as a stand-alone lymphoma[2]. It is a rare tumor characterized by a bulky tumor mass in the anterior mediastinum derived from putative thymic B-cells[193]. Patients usually present with advanced symptoms such as superior vena cava syndrome (a blockage of blood flow through the superior vena cava), chest pains, swelling, dysphagia, and a productive cough. These symptoms are caused by direct compression of the mediastinal architecture by the tumor mass[194]. PMBL predominantly occurs in younger females, with a median age of diagnosis of 37 years of age[195].

Diagnosis

Diagnosis is typically made using clinical features, cell morphology and immunophenotype[196]. The malignant B-cells are usually large in size resembling centroblasts or large centrocytes with a pale cytoplasm[197]. PMBLs characteristic immunophenotype includes expression of CD19, CD20, CD22, CD79a and CD45 often with $CD30^{dim}$. The B-cells do not typically express IGs. A challenging feature of diagnosis in PMBL is the procurement of an adequate biopsy due to the location of the tumor mass. Smaller biopsies and needle aspirates may not be representative of the malignant tumor and so a surgical biopsy is preferred.

Prognosis

Even though PMBL is regarded as an entity of DLBCL under the WHO classification in 2008, the current prognostic factors established in DLBCL are not applicable for PMBL patients. Some studies indicated that age-adjusted International Prognostic Index (IPI) used for DLBCL cannot predict treatment outcome in PMBL[198-200]. Low tumor metabolic activity has been suggested as a prognostic marker for favorable disease outcome and can be assessed by measuring flourodeoxyglucose (FDG) uptake using positron

emission tomography (PET) imaging[201,202]. However, further studies are required to validate its prognostic value. Therefore, there is a definite unmet need for molecular prognostic markers in PMBL.

Treatment

A consensus concerning the optimal treatment protocol for PMBL has not been reached. Most guidelines indicate 6 cycles of rituximab containing CHOP therapy with or without radiation therapy. The use of radiation therapy is controversial and depends on both the ability of the patient to tolerate the treatment and the extent of the tumor mass i.e. if it has penetrated outside of the mediastinum. The guidelines in Sweden suggest patients <65 years receive 6 cycles of R-CHOEP-14. Due to toxicity concerns patients >65 years should receive 6 cycles of dose adjusted R-EPOCH[203]. However, recruitment into clinical trials should be investigated for younger patients. Recently, Dunleavy *et al.* published a study on dose-adjusted CHOEP-R treatment (without radiotherapy) in PMBL reporting a 97% overall survival after 5 years[204]. Evaluation to therapy response is analyzed through PET scans. However, PET scans can render false positive results if performed too soon after therapy. Therefore, a PET scan should only be performed 6-8 weeks after treatment conclusion[205].

Molecular features

PMBL shares a number of common molecular, pathological and clinical features with the nodular sclerosing subtype of classical Hodgkin Lymphoma (cHL)[198,206]. Gene expression profiles between cHL and PMBL are strikingly similar showing involvement of the JAK-STAT and NF-κB pathways in disease pathogenesis[206-209]. Like cHL, PMBL cases are reported as having recurrent gains of chromosomes 2p (24-33%) and 9p (70-75%) losses of chromosomes 1p (42%) 3p (29-24%), 6q (24-29%), 7p(24%), and 17p(24%)[210,211]. Recently, whole-exome sequencing (WES) of refractory/relapsed PMBL revealed novel recurrent mutations in *XPO1, MFHAS1* and *ITPKB*[212].

JAK/STAT dysregulation

The *JAK2* locus is located on chromosome 9 and the frequent gains reported in chromosome 9p in PMBL leads to an over expression of *JAK2*, resulting in the constitutive activation of IL-4 and IL13 pathways[206,209,213,214]. However, higher resolution studies suggest that *JAK2* is not the target of 9p amplification, and lack of activating mutations in *JAK2* in PMBL adds

weight to this argument[210,215]. The transcription factor STAT6 is constitutively activated in 72% of PMBL cases and is regulated by IL-4 and IL-13[216]. *SOCS1* negatively regulates the JAK/STAT pathway[69]. In PMBL, *SOCS1* mutations have been reported in 45% of cases[217,218]. Recurrent mutations in *PTPN1*, which leads to increased phosphorylation of members of the JAK/STAT pathway, were identified through WES in PMBL and also in cHL[219].

NF-κB dysregulation

The *REL* proto-oncogene encodes for NF-κB transcription factor and is frequently affected by recurrent gains in chromosome 2p and suspected to play a role in the pathogenesis of PMBL[210,211,220]. Studies of gene expression profiles in PMBL reveal an expression signature enriched for NF-κB target genes with functions in anti-apoptotic TNFα signaling[221]. In the same study, the PMBL cells from the cell line Karpas 1106 were NF-κB dependent[221]. The zinc finger protein A20 is a negative regulator of the NF-κB pathway and is encoded for by the gene *TNFAIP3*[222]. Damaging *TNFAIP3* mutations evoking constitutive activation of the NF-κB pathway were reported in 36% of PMBL patients and 44% of cHL patients[223].

Next-generation sequencing technologies

Major developments in lymphoid malignancy research are paralleled by advancement in sequencing techniques. Sanger sequencing is now being substituted for more advanced next-generation sequencing (NGS) technologies in both research and clinical settings. NGS is capable of generating millions of sequencing reads that can be aligned and analyzed through bioinformatics pipelines, shadowing the one sequencing read output of Sanger. The scope of NGS is vast ranging from entire genomes to targeted regions.

The sequencing techniques used in this thesis utilizes Illumina's solid-phase bridge amplification sequencing technology and broadly follow the four step process chronicled in figure 7.

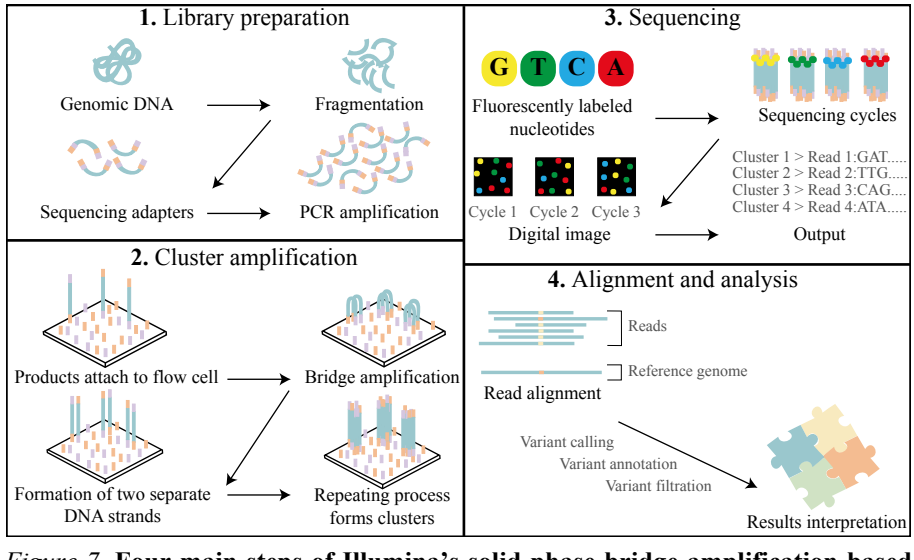


Figure 7. **Four main steps of Illumina's solid-phase bridge amplification based NGS.**

We are now in the era of the $1000 dollar genome. To give perspective on this cost, between 1999-2000 the Human Genome Project estimated its cost of generating just a "draft" of the human genome to be $300 million. So it goes without saying that advancements in whole-genome sequencing (WGS)

have come along way with regards to cost, accessibility and speed. The depth of coverage of WGS is currently at ~30X, giving a comprehensive overview of the genomic landscape. A complication in WGS lies in the complexity of the analysis of results and the logistical issues of storing and processing the data. One whole genome requires approximately 150GB of storage space and 2 full days of computational processing time.

On the other hand, WES targets the coding regions of the genome, increasing the coverage to ~100X, offering a "zoomed in" view of the genomic landscape, while significantly decreasing the amount of data generated. Variants with allelic frequencies of >10% can be confidently called, however, sub-clonal mutations require greater depth of coverage.

Targeted NGS selects specific regions of interest across the genome. Coverage can reach ~1000X, a depth suitable for identifying sub-clonal mutations accurately. Targeted sequencing can be achieved through designing target specific probes, which are included in the library preparation. Using individual "barcode" sequences up to 384 samples can be multiplexed and pooled into one sequencing run, dramatically decreasing time and cost.

NGS has contributed to discoveries of many genetic alterations in lymphoid malignancies, illuminating molecular pathways involved in pathogenesis that were previously unknown. These findings can have a significant influence on how we diagnose, treat and monitor patients, opening up the possibility of patient tailored therapy targeting specific pathways.

Appendix

The following lymphoid malignancies were also investigated in this thesis; classical Hodgkin lymphoma (cHL), mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL), primary central nervous system lymphoma (PCNSL), splenic marginal zone lymphoma (SMZL), follicular lymphoma (FL) and T-cell acute lymphoblastic leukemia (T-ALL). Table 2 and table 3 give brief summaries of the clinical and molecular characteristics of each disease.

Table 2. **Summary of clinical characteristics of other lymphoid malignancies included in this thesis.**

Lymphoid malignancy		Immunophenotype	5 year survival rate	No. of cases diagnosed annually in Sweden	Median age*	Ratio ♂:♀
cHL		CD30^{+}, CD15^{+}, 20% of cases are CD20^{+}	~95%	100-120	39 range: 18-59	1.2:1
MCL		CD20^{+}, CD5^{+}, CD10^{-}, CD23^{-}, Cyclin D1^{-}	50-70%	80-100	73	2.6:1
DLBCL	Activated B-cell like (ABC) subtype	CD20^{+}, CD10^{-}, BCL6^{+}, MUM1^{-}, FOXP1^{-}	55%	500-600	70	1.2:1
	Germinal center B-cell-like (GC) subtype	CD20^{+}, CD10^{-}, BCL6^{-}, MUM1^{+}, FOXP1^{+}	70%			
PCNSL		CD20^{+}, CD3^{-}, MUM1^{+}, BCL6^{+}, BCL2^{+}	~30%	50-60	61	1.3:1
SMZL		CD20^{+} CD5^{-}, CD10^{-}, CD23^{-}	80%	15-20	69	1:1.8
FL		CD20^{+}, CD10^{+}, BCL2^{+}, BCL6^{+}	50-90%	200-250	65	1:1
T-ALL		TdT^{+}, variable expression of CD1a, CD2, CD3, CD4, CD5, CD7, and CD8	45-75%	1-2	29 range: 5-50	2.7:1

*Refers to the median age reported in Sweden. Information for each individual malignancy was ascertained from the following references; cHL[75,224-227], MCL[75,227-231], ABC[75,197,232-235], GC[75,197,232-234], PCNSL[75,203,236,237], SMZL[75,238-240], FL[75,241-244], T-ALL[75,197,245,246].

Table 3. **Summary of immunogenetic and genetic characteristics of other lymphoid malignancies included in this thesis.**

Lymphoid malignancy		Postulated cell of origin	SHM	Preferential IGHV gene usage	Genetic aberrations	
cHL		Antigen experienced B cells from the GC (thymic)	Ongoing SHM	none	*REL/BCLIIA* (45-75%) *SOCS1* (61%) *TNFAIP3* (43%) *NFKBIA* (22%) *PTPN1* (20%)	*TP53* (~12%) *NFKBIE* (16%) *CIITA* (15%) *JAK2* (27%)
MCL		Antigen experienced SOX11- B-cells	SHM present	IGHV3-21*, IGHV4-34, IGHV1-8 IGHV3-23,	t(11;14) (95%) *ATM (41-61%)* *TP53* (14-31%) *KMT2D* (12-23%)	*KMT2C* (5-16%) *CARD11* (3-15%) *NOTCH1* (5-14%) *BIRC3* (6-10%)
		Naïve SOX11+ pre-GC B-cell from mantle zone	SHM absent			
DLBCL	ABC subtype	Post-GC B-cells	Low rate of SHM, not ongoing	IGHV4-34	*BCL6* (24%) *PRDM1* (22%) *TP53* (16%) *MYD88* (14%)	*CARD11* (13%) *CD79B/A* (11-20%) *TNFAIP3* (6%)
	GC subtype	GC experienced B-cells	Ongoing SHM	none	t(14;18) (30-40%) *CREBBP* (39%) *MLL2* (24%)	*EZH2* (20%) *PTEN* (10%)
PCNSL		GC B-cells	SHM with absence of CSR	IGHV4-34	*PIM1* (44-100%) *MYD88* (85.4%) *CD79B* (44%)	*MPEG1* (32%) *PRDM1* (19%)
SMZL		Post follicle marginal zone B-cell	Ongoing SHM	IGHV1-2, IGHV4-34, IGHV3-23	*KLF2* (20-40%) *NOTCH2* (6.5-25%) *NOTCH1* (5%) *CARD11* (~7%) *IKBKB* (7%)	*MYD88* (5-13%) *TNKAIP3* (7-13%) *BIRC3* (6.3%) *TP53* (~12%)
			SHM absent			

FL	GC experienced B-cell	SHM present	IGHV3-23	t(14;18) (>85%) *MLL2* (78%) *CREBBP* (68%) *TNFRSF14* (30%) *STAT6* (21%)	*EZH2* (17%) *HIST1H1E* (11%) *RRAGC* (14%) *EP300* (10%) *CARD11* (10%)
T-ALL	GC B-cells	-	-	*NOTCH* (22%)	*FBXW7* (10%)

* Only seen in MCL with unmutated IGHV genes. Immunogenetic and genetic characteristics for each individual malignancy was ascertained from the following references: cHL[213,219,247-257], MCL[2,258-266], ABC[267-273,274,275], GC[274,276-280], PCNSL[281-284], SMZL[285-296], FL[268,274,297-301], T-ALL[282,302-304]

Thesis aims

The overall aim of this thesis was to investigate recurrent gene mutations in lymphoid malignancies and evaluate their clinical impact, with a particular emphasis on CLL and PMBL. More specifically, we had the following aims:

I To evaluate, on a large scale, the presence of recurrent genetic lesions (*TP53*, *SF3B1*, *NOTCH1*, *MYD88*, *BIRC3* and cytogenetic aberrations) in CLL patients harboring stereotyped B-cell receptors.

II To investigate the frequency of *NFKBIE* deletions in a large collection of lymphoid malignancies in an effort to evaluate the difference in frequencies and assess their potential prognostic relevance.

III To determine the frequency of *EGR2* mutations in a large multi-institutional cohort and explore their clinical correlations and potential use as a prognostic marker in CLL.

IV To further elucidate the genomic landscape of CLL patients assigned to the clinically aggressive subsets #1 and #2 and the more indolent subset #4 using WGS.

Materials and methods

Patient material

All patients included in this thesis were diagnosed according to the WHO criteria for their individual lymphoid malignancy[2,197]. In particular, the CLL patients included were diagnosed according to the 2008 iwCLL criteria[73]. Informed consent was obtained in accordance with the declaration of Helsinki. Local ethical review boards granted ethical approval. Figure 8 details the patients analyzed in **Paper I-IV.**

Paper I: 565 CLL patients assigned to one of 10 major stereotyped subset were included in this study. Pre-treatment samples were available for >80% of patients. Samples were analyzed from research centers in Greece, France, the Czech Republic, the Netherlands, UK, Sweden, USA, Italy, Denmark and Spain.

Paper II: A total of 1460 patients diagnosed with a lymphoid malignancy were analyzed from collaborating institutions in Denmark, Germany, Greece, France, Spain, Sweden and the UK, including patients diagnosed with DLBCL (n=520), FL (n=225), PMBL (n=203), MCL (n=189), SMZL (n=175), T-ALL (n=94), PCNSL (n=34), cHL (n=11), and SLL (n=9). All patients were sampled prior to treatment administration.

Paper III: A large multicenter screening cohort of 1283 CLL patients from the Czech Republic, Denmark, France, Greece, Germany, Italy, Sweden, the UK and USA was first analyzed. Seventy-six percent of these cases were sampled prior to treatment. As a primary validation cohort, 366 CLL patients enrolled in the UK LRF CLL4 trial were included[305]. A secondary validation cohort from the CLL research consortium (CRC) in the US was also included; 81% of the CRC samples were taken prior to treatment initiation. Additionally, 233 patients from a Chinese CLL cohort and 31 Richter transformed CLL patients were screened.

Paper IV: Seventy CLL patients assigned to subset #1 (n=25), #2 (n=26) or #4 (n=19) from collaborating institutes in the Netherlands, the Czech Republic, the UK, Italy, Sweden, Greece and France were included in this study. Normal (non-tumor) samples were obtained for all patients from either of sorted T-cells or buccal swabs.

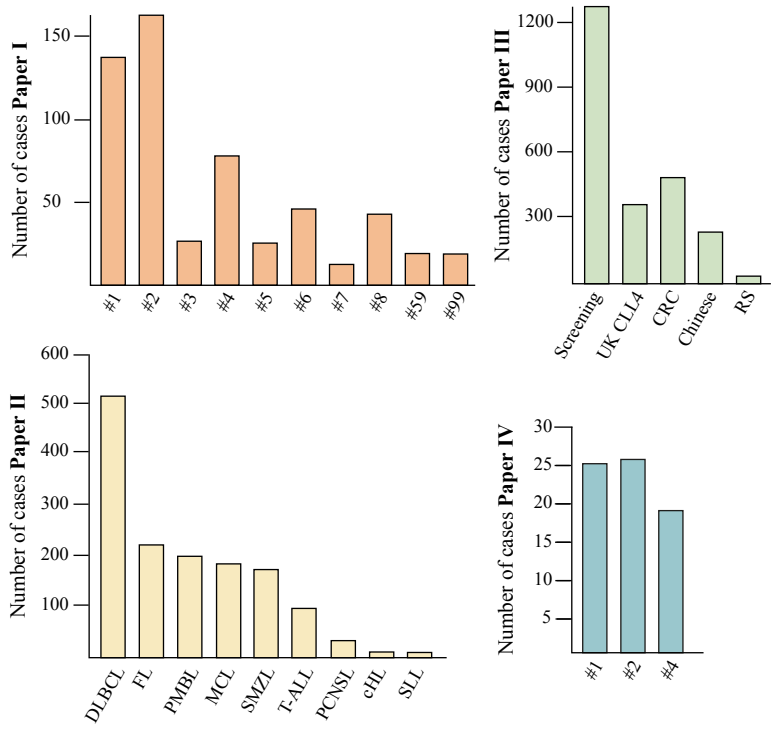


Figure 8. **Number of cases analyzed in Papers I-IV**. **Paper I** and **paper IV** refer to specific stereotyped subsets. In **paper II** the different lymphoid malignancies are indicated and in **paper III** the discrete CLL cohort analyzed is specified.

Methods

Cell sorting and DNA extraction

CLL cells were sorted through either fluorescent activated cell sorting (FACS) or negative selection magnetic bead sorting. Vital frozen cells from CLL patients were stained for both CD5 and CD19 using MACS antibodies (Milteny Biotec, Bergisch Gladbach, Germany). Double positive cells, representing the CLL population, were sorted by FACS. Double negative cells were sorted and served as normal counterparts. Alternatively, negative depletion magnetic bead sorting was employed using the MACS B-CLL isolation kit. Extraction of DNA from all sorted samples was achieved using the universal AllPrep DNA/RNA/miRNA kit (Qiagen, Venlo, Netherlands) with subsequent quantification of DNA using Qubit fluorometric technology (Life Technologies, Waltham, MA, USA).

Sanger sequencing

A hot-start polymerase chain reaction (PCR) using primers designed to target specific exons or hotspot regions within the gene of interest was preformed (Table 4). Subsequent bi-directional Sanger sequencing of the PCR product using BigDye Terminator v3.1 Cycle Sequencing Kit was completed and the purified sequencing product was run on the ABI3730XL DNA Analyzer (both Life Technologies, Carlsbad, CA). Sequences were analyzed using either CodonCode aligner v6.0.2 (CodonCode Corp., Centerville, MA) or Sequence Scanner v2.0 (Applied Biosystems, Waltham, MA). Sanger sequencing was used to analyze the majority of genes in **paper I** and was also utilized in **papers II, III and IV.**

Fragment analysis

Fragment analysis assays were designed for the analysis of the recurrent deletions in *NOTCH1* in **paper I** and *NFKBIE* in **paper II**. Amplification of the DNA fragment was achieved using a hot-start PCR. Importantly, the forward primer was labeled with a fluorophore. The fluorescent DNA fragments were then analyzed through capillary electrophoresis on the ABI3730XL DNA Analyzer (Life Technologies) with subsequent analysis using Peak Scanner Software v1.0 (Thermo Fischer Scientific, Waltham, MA).

Table 4. **Description of the hot spot regions and most frequently used techniques used in this thesis to detect mutations.**

Gene	Target exons or mutations	Detection method
TP53	Exons 4-9/10/11	Sanger sequencing
SF3B1	Exons 14-16	Sanger sequencing
NOTCH1	p.P2514fs (exon 34)	Fragment analysis
MYD88	p.L265P (exon 5)	Sanger sequencing
BIRC3	Exons 6-9	Sanger sequencing
NFKBIE	p.T253fs (exon 1)	Fragment analysis
EGR2	p.R324 to p.P476 (exon 2)	Sanger sequencing
RPS15	Exon 4	Sanger sequencing

Next-generation sequencing

Targeted deep-sequencing

Using a standard hot-start PCR protocol the hot-spot regions in either the *NFKBIE* (**paper II**) or *EGR2* (**paper III**) genes were amplified, and the PCR product was bead purified. Following that, the Nextera XT kit (Illumina, San Diego, CA) was used to prepare libraries from the respective amplicons. These libraries were sequenced on the MiSeq instrument (Illumina) using paired end sequencing. Sequences were mapped using BWA (v.0.7.12)[306],

variant calling was completed using VarScan 2 (v.2.3.7)[307], and variants were annotated using ANNOVAR[308].

Customized targeted gene panel

In **Paper III**, a Haloplex (Agilent Technologies, Santa Clara, CA) gene panel was designed targeting 27 known or postulated CLL driver genes. Probes were designed using Agilents SureDesign service. Following the manufactures guidelines, pooled libraries were paired-end sequenced using v4-sequencing chemistry across one lane of the HiSeq 2500 (Illumina). TrimGalore (v.0.3.7) was used for Illumina adapter removal; the trimmed reads were aligned to the hg19 human reference genome using BWA (v.0.7.12) and variants called using VarScan 2(v.2.3.7)[307].

Whole-exome sequencing

WES libraries were prepared using the Agilent SureSelect QXT protocol for 7 tumor-normal PMBL pairs. The libraries were paired-end sequenced in high-output mode on the Illumina NextSeq500 instrument. Raw reads were processed using the bcbio-nextgen framework and alignment to the Hg19 reference genome was achieved using BWA (version 0.7.15)[148]. Realignment around indels was performed using GATK (version 3.5)[309]. Sambamba (version 0.6.3) was used to identify PCR duplicates with SNVs and indels detection called using Varscan2 (version 2.3.9)[307] using a variant allele frequency (VAF) cutoff of 10%. Variants were subsequently filtered against a panel of normal samples using GATK Variant Filtration.

Whole-genome sequencing

Libraries for WGS were generated in **paper IV** using the TruSeq Nano Kit (Illumina). Cluster generation and 125 cycle paired-end sequencing was performed on the Illumina HiSeq system, using v4 chemistry. Raw sequencing reads were either processed in-house using Piper, a pipeline built on top of a GATK queue, or by the Beijing Genomics Institute. Reads were aligned to the Grch37 or HG19 reference using BWA (v0.7.12)[306]. Applying a 10% cutoff for variant calling, VarScan 2 (v.2.3.7)[307] was used to detect somatic single nucleotide variations (SNV) and small indels. Copy number aberrations were identified and called using Control-FREEC(v.8.7)[310].

Gene expression profiling

Gene expression of *NFKBIE*-deleted and *NFKBIE*-wildtype cases was analysed using the Nanostring PanCancer Pathway Panel with the addition of 30 custom chosen genes implicated in PMBL and/or the NF-κB pathway (Nanostring Technologies, Seattle, WA). This technology, also referred to as nCounter Analysis System, utilizes target-probe hybridization whereby probes are designed to target a gene of interest; one probe is barcoded with a

fluorophore and another probe acts as an anchor for imaging. RNA from paraffin-embedded PMBL tissue was hybridized overnight at 65°C with the PanCancer Pathway Code Set and custom CodeSet. The hybridized probes were then purified and bound to a cartirage on the nCounter Prep Statio. The cartirage was scanned on the nCounter Digital Analyzer where the probes are imaged and counted. The NanoString RCC files were imported into NanoString nSolver2.6 software form the NanoString Digital Analyzer where quality control checks were completed. Differential expression (DE) and gene set analysis was performed using the PanCancer Pathways Analysis Module (v1.0.48) and DE was presented as log fold-changes of at least 40% in *NFKBIE*-deleted vs. *NFKBIE*-wildtype.

Statistical analysis

Throughout **papers I-IV** various descriptive statistic tests were performed, evaluating mutational frequencies. **Paper I** utilized Monte Carlo simulation to generate *P* values with 10,000 replicates. Additionally, Fisher's exact test was used to compare subsets in a two-sided manner. Within this paper the Bonferroni correction method was also applied to allow for multiple comparisons, with a significance level set at $P<0.001$. The free, publically available software, R (version3.1.2) was used for these calculations. In **paper IV**, using R, two sided Students t-tests were applied in testing the difference between subsets. Kaplan-Meier curves were generated in **papers I**, **II** and **III** in order to assess survival times with log-rank tests used to evaluate statistical differences between groups. OS was calculated using date of diagnosis or time of randomization and date of last follow up or date of death. TTFT was calculated from treatment initiation to date of last follow up or date of death. In order to investigate the prognostic significance of *NFKBIE* and *EGR2* mutations in **papers II** and **III**, respectively, multivariate analysis using a Cox proportional-hazards regression analysis was applied. Throughout **papers I**, **II** and **III**, where applicable, a cut-off of $P<0.05$was considered statistically significant. *P*-values associated with DE fold changes in gene expression analysis were derived using the Benjamin-Yekutieli false discovery rate method. The following softwares were utilized: **paper I** Statistica Sofware 10.0 (Stat Soft Inc., Oklahoma city, OK); **paper II** SPSS Version 23.0 (IBM, Armonk, NY); and **paper III** Statistica Software 13.0 (Dell Inc., Oklahoma city, OK).

Results and discussion

Paper I: Recurrent gene mutations in stereotyped CLL subsets

Limited information regarding the frequencies of recurrent mutations in stereotyped subsets was previously known. Initially smaller scale studies of stereotyped subsets were investigated for *TP53 NOTCH1*, *SF3B1 BIRC3* and *MYD88* mutations, revealing a remarkably high frequencies of *SF3B1* mutations in subsets #2 patients (44-52%) and *NOTCH1* mutations in subset #8 (14-62%)[124,311,312]. Following on from this, we wished to investigate using Sanger sequencing a much larger cohort with additional subsets for mutations within known hot-spot regions of these genes. We not only confirmed but also greatly extended the finding of biased enrichment of recurrent gene mutations in different subsets.

While we reported a complete lack of *MYD88* mutations throughout all subsets analyzed and overall, very few *BIRC3* mutations, mutations within *SF3B1*, *NOTCH1* and *TP53* varied greatly, not only between different subsets, but also amongst immunogenetically related subsets i.e. similar SHM status and IGHV gene usage (Figure 9).

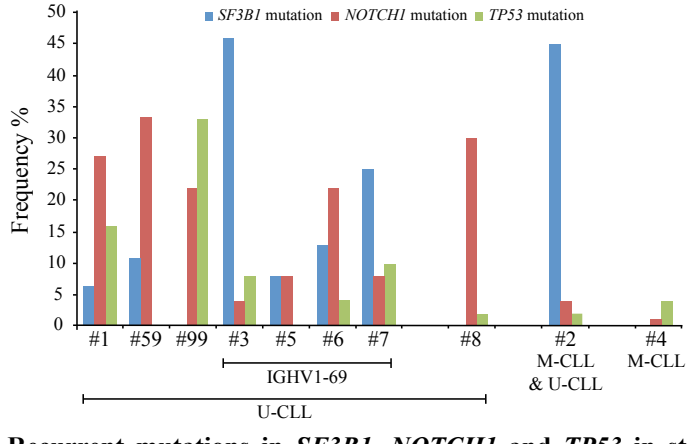


Figure 9. **Recurrent mutations in *SF3B1*, *NOTCH1* and *TP53* in stereotyped subsets.**

Besides confirming the *SF3B1* mutational bias in subset #2 (72/161; 45%), we also reported an unprecedented, marked enrichment of *SF3B1* mutations in subset #3 (12/26; 46%) despite being immunogenetically distinct. In contrast, low frequencies of *TP53* and *NOTCH1* mutations were seen in both subset #2 and #3. This strongly supports the role of *SF3B1* but not *NOTCH1* and *TP53* mutations in the underlying aggressiveness of these subsets.

The U-CLL subsets utilizing Clan I genes (subset #1, #99 and #59) and the clinically aggressive subset #8 were enriched for *NOTCH1* mutations (22%, 30%, 33% and 30%, respectively). The results from subset #1 and subset #8 are in agreement with previously reported studies[124,311]. Using Bonferroni corrections, difference in frequencies of *NOTCH1* mutations between subset #2 and #4 with subset #1, #6, #8 and #59 reached statistical significance. *NOTCH1* mutations thus likely play an important role in the aggressive phenotype the subsets harboring these mutations.

Finally, frequencies of *TP53* mutations were enriched in Clan I gene expressing subsets #1 and #99 (21/135; 16% and 6/18; 33%, respectively), however they were completely absent from subset #59, whom also utilize IGHV Clan 1 genes.

The pathogenic mechanism and evolution of CLL remains puzzling, here we have taken a step closer to elucidating the architecture supporting disease aggressiveness in stereotyped subsets. Within heterogeneous CLL i.e. non-stereotyped patients, overall mutational frequencies are customarily low; while preferential acquisition of mutations in subsets show remarkable skews. This is highly indicative that a selective advantage is afforded to the cells that acquire mutations on the basis of their expressed IG molecule and these mutations drive various distinct pathways ultimately towards clinical aggressiveness.

Although the cohort analyzed represents the largest of its kind studied for this purpose, a limitation within this study remains to be sample size, especially for rarer subsets (e.g. subset #3, #5 and #7). In the majority of cases only hot-spot regions within the genes were targeted, mutations occurring outside of theses regions were therefore missed in this analysis. Sanger sequencing, although still very useful, is now considered a cumbersome technique quickly being surpassed by NGS technologies. Targeted gene panels would make for a better solution in this type of analysis, since recurrently mutated genes can be analyzed simultaneously in a cost effective and high-throughput fashion[313]. It would also be of interest to investigate the presence of sub-clonal mutations within this cohort as they may be of both biological and clinical importance in CLL and have yet to be explored in stereotyped subsets[123,314].

Paper II: *NFKBIE* deletions in PMBL

We recently reported an enrichment of *NFKBIE* truncating mutations in aggressive CLL (7%), particularly within clinically aggressive subset #1 cases (15%). Within the same study *NFKBIE* mutations were also observed in MCL (5%), DLBCL (4.5%) and SMZL (2%)[116]. Since NF-κB is constitutively active in many lymphoid malignancies these recurrent *NFKBIE* mutations may represent a possible common mechanism of NF-κB deregulation[269,315-316]. To study this further we analysed a larger cohort of patients (n=1430) diagnosed with a range of lymphoid malignancies.

We reported the highest frequencies of *NFKBIE* mutations in cHL (27.3%; 3/11), and PMBL (22.7%; 46/203). While frequencies in SMZL, FL and T-ALL were all below 2%, in SLL, MCL, DLBCL and PCNSL frequencies were slightly higher, ranging between 3% and 10% (Figure 10).

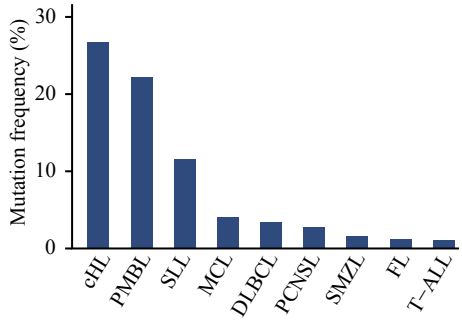


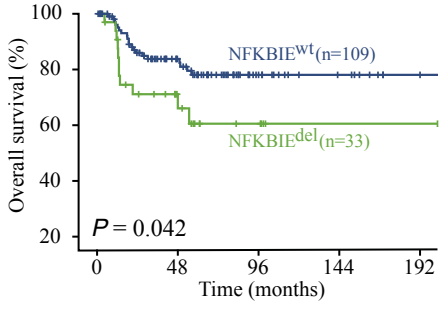
Figure 10. **Frequencies of *NFKBIE* aberrations detected in samples from 9 lymphoid malignancies (n=1460).**

A hallmark of both cHL and PMBL is activation of the NF-κB pathway[206-209]. The markedly high frequencies of *NFKBIE* aberrations detected in these lymphomas is perhaps not unexpected considering previously reported biological similarities between these two malignancies including gene expression profiles and recently reported recurrent mutations in the novel driver gene *PTPN1* and *TNFAIP3* tumor suppressor gene[206,219,223].

Focusing on PMBL, we first aimed to explore the clinical and prognostic implications of *NFKBIE* aberrations in PMBL. *NFKBIE*-deleted PMBL cases had a higher incidence of primary chemorefractoriness in comparison to wildtype cases (25% vs. 6%; *P*=0.022) and a significantly shorter survival (5-year survival, 59% vs. 78%; *P*=0.042) (Figure 11A). In multivariate analysis *NFKBIE*-aberrations remained an independent marker of poor outcome (*P*=0.003) (Figure 11B). Though based on relatively few cases, we present evidence indicating *NFKBIE*-aberrant PMBL patients may benefit from consolidative radiation therapy. Evidently, larger cohort size,

preferably including patients receiving similar treatment regimens is required before we can conclude that the *NFKBIE* mutational status can used as a prognostic and even predictive marker in PMBL.

A



B

Variable	HR	95% CI	*P*-value
Gender (male)	1.59	0.56-4.54	0.387
Ann Arbor stage (low)	0.32	0.12-0.84	0.020
Radiation	0.20	0.06-0.64	0.007
Rituximab	0.16	0.05-0.50	0.002
Intensified CHOP	0.19	0.04-0.94	0.042
***NFKBIE* deletion**	**4.46**	**1.65-12.04**	**0.003**

Figure 11. **Clinical implications of *NFKBIE* aberrations in PMBL**. (A) Kaplan-Meier curve presenting overall survival of 143 PMBL cases (B) Multivariate analysis completed on PMBL (cases: n=111; events: n=19).

To further characterize *NFKBIE*-deleted tumors molecularly, we applied WES to investigate differences in genetic aberrations and gene expression profiling to identify commonly deregulated pathways compared to *NFKBIE* wildtype patients. Overall, PMBL tumors showed a high frequency of exonic mutations (average of 218). Of note, mutations in other genes involved in the NF-κB pathways and JAK-STAT signaling (e.g. *TNFAIP3*, *PTPN1* and *BCL6)* were often observed in *NKFBIE*-deleted patients, hence pointing to complex deregulation of interrelated pathways. Interestingly, *NFKBIE* aberrations and *STAT6* mutations appeared to be mutually exclusive, suggesting that they represent distinct pathogenetic mechanisms, which warrants further investigation. In *NFKBIE*-deleted cases, an increased gene expression in genes associated with the NF-κB pathway e.g. *CD79B*, *CD40* and *BCL2L* was also observed compared to wildtype patients. Taken together, these data clearly suggest that *NFKBIE* deletions causing deregulation of the NF-κB pathway play an important role in disease pathogenesis.

Hence, the *NFKBIE* deletion status appears to represent a novel prognostic marker in PMBL linked with particularly dismal outcome and chemo-refractoriness. Considering that few prognostic markers have thus far been identified in PMBL, this body of work has potential clinical impact, though it has to be verified in independent studies.

Paper III: *EGR2* mutations in CLL

EGR2 mutations were recently indicated as potential early events in CLL pathobiology as they were identified in hematopoietic precursor cells in CLL patients. Enrichment for *EGR2* mutations were also reported in 8.3% of advanced stage CLL patients[115]. To further investigate the presence of *EGR2* mutations in CLL, we screened large, well-characterized cohorts; representing CLL from general practice, referral centers, clinical trials, different geographical locations and Richter transformed patients.

We reported an overall frequency of 3.8% (91/2403) with similar inter-frequencies observed within the screening cohort (3.9%), UK CLL4 cohort (3.7%), CRC cohort (3.7%), Chinese cohort (3.9%) and Richter transformed cohort (6.5%). Figure 12 details the breakdown of the various CLL cohorts analyzed and the frequencies and localization of *EGR2* mutations.

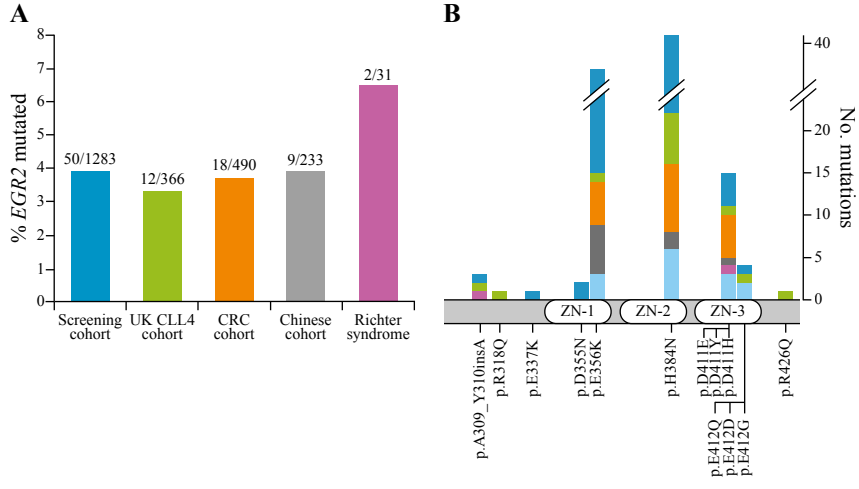


Figure 12. ***EGR2* mutations in CLL.** (A) Distribution of mutations within the separate cohorts analyzed. (B) Genetic localization of *EGR2* mutations.

In the screening cohort (n=1283), *EGR2* mutations were associated with a younger age (57 vs. 62 years: *P*=0.004), and advanced stage of diagnosis (Binet B/C 56 vs. 30%; *P*=0.001), U-CLL genes (81 vs. 61% P=0.004) and del(11q) status (33 vs. 18%; *P*<0.0001).

Table 5. **Multivariate Cox proportional hazard analysis.** Time-to-first treatment (TTFT n=735) and overall survival (OS n=688) in screening cohort.

	TTFT			OS		
Variable	Hazard ratio	95% Confidence interval	*P*-values	Hazard ratio	95% Confidence interval	*P*-values
EGR2	**1.52**	**1.06-2.20**	**0.024**	**1.92**	**1.23-2.97**	**0.003**
Age	1.07	0.88-1.28	0.490	2.48	1.93-3.18	<0.001
Gender	1.11	0.92-1.34	0.264	1.18	0.92-1.52	0.193
Binet stage	NA	NA	NA	1.90	1.47-2.46	<0.001
IGHV	3.77	3.03-4.65	<0.001	3.14	2.36-4.17	<0.001
NOTCH1	1.12	0.85-1.49	0.429	1.30	0.90-1.86	0.159
SF3B1	1.60	1.22-2.06	<0.001	1.47	1.03-2.10	0.035
*TP53*abn	1.35	1.05-1.60	0.019	1.80	1.30-2.48	<0.001

In comparison to *EGR2*-wildtype patients, *EGR2* mutated patients had a significantly poorer TTFT (median, 7.8 vs. 35.5 months; $P<0.001$) and OS (median, 74.7 vs. 127.2 months; $P<0.001$) (Figure 13 A & B). *EGR2* status remained an independent marker of prognosis for both TTFT and OS in multivariate analysis in a model that included known strong prognostic markers, such as age, gender, Binet stage, IGHV mutational status, del(11q) and *TP53*abn (Table 5). Notably, there was no significant difference between *TP53*abn and *EGR2* mutated patients ($P=0.900$); patients double positive for both mutations (n=7) were included in the *TP53* arm (Figure 13 C & D).

In the first validation cohort, UK CLL4 trial cohort (n=366), a similar trend was observed, with a significantly reduced OS from time of randomization (median, 24.6 vs. 75.7 months; $P=0.004$) and *EGR2* mutational status remained an independent marker of prognosis in multivariate analysis (HR: 2.22, 95% CI: 1.03-4.77, $P=0.036$). Within the CRC cohort (n=486), again a shorter TTFT (median, 35.8 vs. 55.7 months; $P=0.007$) and OS (median, 98.3 vs. 152.9 months; $P=0.036$) was observed. Confirmation of *EGR2* as an independent prognostic marker was, however, found only with regards to TTFT in the CRC cohort (HR: 1.92, 95% CI: 1.11-3.32, $P=0.020$). We speculate that this is due to shorter follow-up time and fewer events in this cohort.

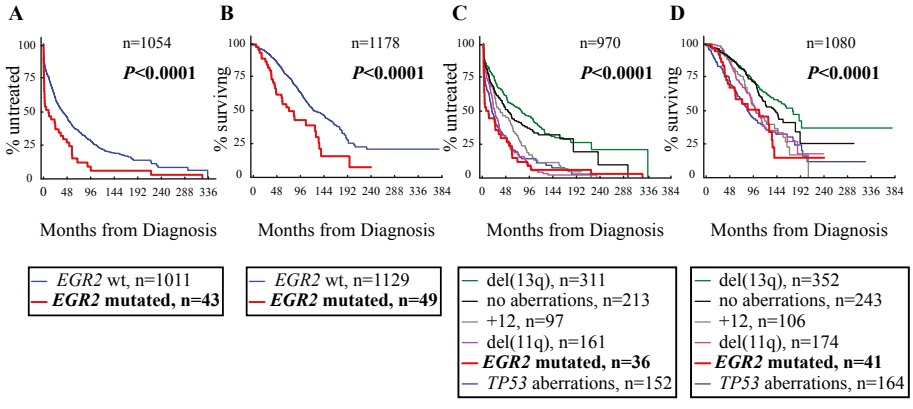


Figure 13. **Kaplan-Meier curves for TTFT and OS in screening cohort**. (A) TTFT in screening cohort according to *EGR2* status. (B) OS in screening cohort relative to *EGR2* status. (C) TTFT and (D) OS according to *EGR2* status and the Döhner hierarchal classification[94]. The *EGR2* arm is indicated by the red arm in all curves.

Another element we wished to address in this paper was the aspect of possible co-occurring mutations within *EGR2*-mutated patients. Results from the targeted Haloplex panel revealed several concomitant mutations within *EGR2* mutated patients (Figure 14). The most noteworthy include *ATM* (12/38; 31.6%), *TP53* (7/38; 18.4%) and *SF3B1* (4/38; 10.5%). Furthermore, 7 *EGR2* mutated patients presented with co-occurring mutations in genes affecting the Notch signaling pathway, *NOTCH1* (3/38), *FBXW7* (3/38) and *SPEN* (1/38).

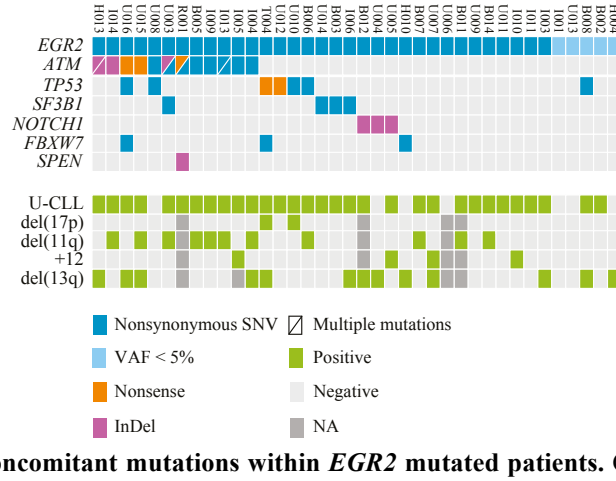


Figure 14. **Concomitant mutations within *EGR2* mutated patients.** Columns and rows represent individual patients and genes investigated, respectively.

Taken together, the data presented here is compelling in the favor of *EGR2* as a novel prognostic marker of very aggressive disease, similar to *TP53*

aberrations, and should be considered for inclusion in the diagnostic work-up of CLL. That said, it will be important to verify the prognostic relevance prospectively and in the context of novel targeted therapy. Since CLL is continuously being diagnosed in younger people (<55 years) the correlations between *EGR2* mutational status and age at diagnosis may also suggest its use as a molecular marker of aggressive disease in younger patients.

Paper IV: WGS in stereotyped CLL subsets

Frequencies of recurrent genetic mutations in stereotyped subsets of CLL have previously been reported in small cohorts and in a larger cohort in **Paper I** of this thesis[124,311,312]. These studies gave the first glimpse of the potential molecular pathways involved in the evolution of specific subsets. However, there is thus far no comprehensive analysis pertaining to both the coding and non-coding parts of the genomes of stereotyped CLL patients.

Comparing CLL to other cancers, there is a huge disparity between total numbers of mutations in the coding region of their respective genomes. CLL has a median of 0.9 mutations per Mb in comparison to cancer types characterized by chronic mutagenic exposure such as melanoma caused by UV exposure (median of 18.54 mutations per Mb) or lung cancer caused by tobacco smoke (median of 10.5 mutations per Mb)[108,317]. Pediatric hematological malignancies can have as low as 0.1 mutations per Mb and this can be accounted for the short time period the leukemic clone has to acquire genetic lesions. However, as CLL is a chronic malignancy occurring in the latter decades of life, a higher mutational burden would be expected. It is therefore speculated that the non-coding regions of CLL could harbor important driver mutations.

In 2015, Puente *et al.* examined WGS in 150 CLL and MBL cases, identifying novel recurrent non-coding mutations located in the 3' promoter region of *NOTCH1,* causing mRNA splicing errors and associated with adverse prognosis, and in an enhancer region of *PAX5*, a B-cell specific transcription factor. This study italicizes the need to look deeper into the so-called "dark side of the genome"[318].

Approximately 50-60% of general practice CLL patients are M-CLL, meaning they have undergone SHM of the BcR. WGS results from CLL patients have an added level of complexity as the cells not only will harbor cancer associated somatic mutations but also somatic alterations derived from SHM. As expected, at the whole-genome level, the mutation burden in the indolent subset #4 (M-CLL; average ≈ 2400 mutations per sample) was significantly higher than that of aggressive subset #1 (U-CLL; average ≈ 1800 mutations per sample) (P<0.005) and the aggressive subset #2 (both M-CLL and U-CLL) had an intermediate mutational burden (average ≈ 2100 mutations per sample), a direct consequence of the different levels of SHM (Figure 15A). However, when we looked into the numbers of non-synonymous exonic mutations (excluding IG genes) within all three subsets the mean number was similar, with no statistically significant differences (subset #1 14.3, subset #2 15.1 and subset #4 11.9) (Figure 15B).

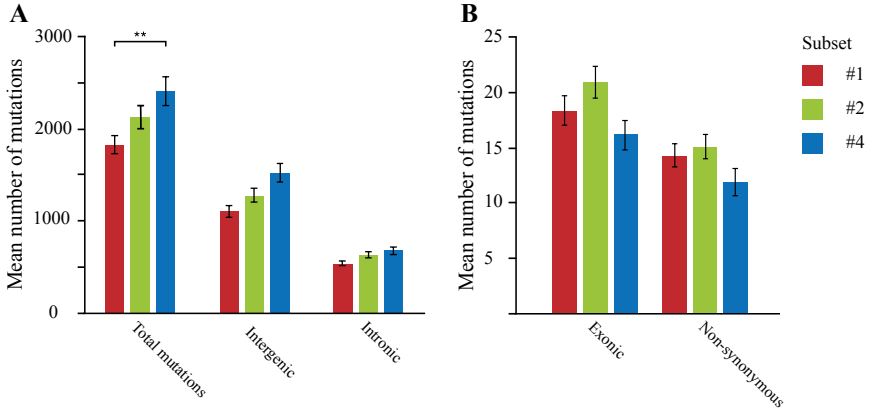


Figure 15. **Frequencies of mutations in stereotyped subset #1, #2 and #4.** (A) Frequencies of mutations relative to genomic location. (B) Frequencies of mutations relative to mutation type.

Two distinct mutational signatures were detected through the analysis of SNVs and were similar to that reported by Puente *et al*. signature 1, an age related signature characterized by C-to-T transitions at CpG sites; and signature 2, involving T:A-to-G:C transversions, specific for M-CLL patients[318]. Signature 1 concerned subset #1 (U-CLL) cases, not surprisingly signature 2 was seen in subset #4 (M-CLL) cases, while subset #2 had aspects of both signatures 1 and 2.

Results from the coding regions of these patients mirrored that of previous findings by us and others, including a skewed distribution of *SF3B1* mutations in subset #2 (7/26; 27%) and *NOTCH1* mutations which were almost exclusively seen in subset #1 (5/25; 20%) (Figure 16)[124,311]. As a novel finding, mutations in the recently reported driver gene *RPS15*, encoding a component of the 40S ribosomal subunit, were observed soley in subset #1 (3/70; 4.5%). This finding was validated in a further 82 subset #1 cases (11% (9/82)), pointing to a role for ribosomal mutations in this particular subset. Furthermore, novel *CTCFL* mutations were observed in subset #2 (3/26; 12%). *CTCFL* encodes for a chromatin regulator protein and has recently been implicated in the upregulation of expression of *NOTCH3* in T-ALL[319].

In the non-coding regions, we identified several candidate intronic mutations that represent possible novel mechanisms of disease pathobiology. Intronic mutations in *MSI2* (encoding for an RNA binding protein) were observed in ~15% of patients with bias in subset #2 patients. Using RNAseq, Mansouri *et al* identified a novel-splice isoform of *MSI2* unique to subset #1[320]. A bias was observed towards subset #1 patients for intronic mutations in *CTBP2* (transcriptional co-repressor). These two intronic mutations warrant further

functional validation in order to understand their potential role in disease pathogenesis.

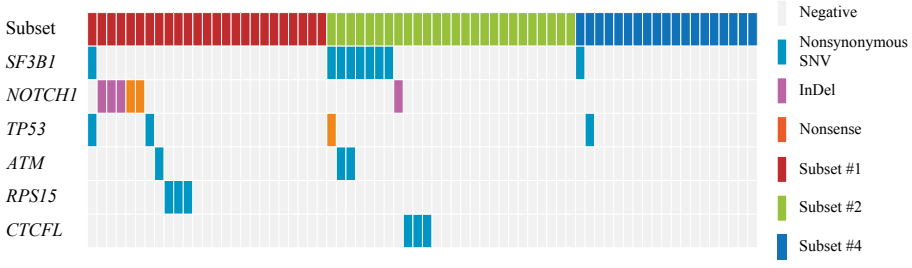


Figure 16. **Recurrently mutated genes in stereotyped subsets from WGS.** Each column represents one patients and each row represents a gene with the exception of the top row, which denotes stereotyped subset of patients (subset #1, #2 and #4).

Within the coding and non-coding regions of the genome, we presented candidate mutations that were found enriched in specific stereotyped subsets and deemed suitable for further investigation. However, the next immediate steps are to extend the number of cases analyzed and expand the scope of subsets included. In addition, specialized filtering strategies must be devised in order to delineate which of the very high number of the non-coding variations are relevant to the disease.

Concluding remarks

Advancements in biological and clinical research within lymphoid malignancies has advanced rapidly in the last few years resulting in the fourth edition of the World Health Organizations (WHO) classification of hematopoietic and lymphoid tumors undergoing a major revision in 2016[2,197]. The findings in this thesis have extended our understanding of both biological and clinical factors of lymphoid malignancies, in particular in CLL and PMBL.

CLL patients harboring stereotyped BcRs represent an intriguing branch of research within the disease. A compartmentalized view of this otherwise heterogeneous disease is afforded in the guise of immunogenetically and clinically well-defined subgroups. The identification of clear selective pressure in mutation acquisition relative to a specific BcR indicates the involvement of different intracellular pathways and processes determining the mechanisms of clinical aggressiveness. Complementary results from the WGS performed in this thesis illuminate novel pathways and cellular processes, such as RNA metabolism and chromatin regulation within stereotyped subsets. The non-coding regions of stereotyped subsets could also hold more answers, however, more samples and enhanced bioinformatics analyses and filtering are required to pinpoint the most relevant non-coding mutations. While investigating the mutational profile of stereotyped subsets may have important prognostic and even predictive impact, taking a look at the bigger CLL-picture, novel findings within the realm of stereotyped subsets may also have applicability in "heterogeneous" or non-subset CLL.

In the era of NGS, we are seeing more and more clinics implementing targeted gene panels. Recently, we assessed the accuracy and reproducibility of a targeted CLL panel using Haloplex technology for routine clinical use. We demonstrated that it is not only feasible in a clinical setting, but also meets the stringent and accurate standards of a diagnostic laboratory. Compared to traditional Sanger sequencing, targeted NGS panels are both labor and time effective. Possibly in the next decade, all cancer diagnoses will be followed up with a disease specific NGS panel. Evidence from this thesis strongly suggests the addition of *EGR2* for CLL and *NFKBIE* for PMBL to the diagnostic framework.

Cancer research is often referred to as a puzzle, with each publication adding a new piece. Although research in the field has advanced expeditiously, especially in the last decade the "puzzle" is not nearly complete. This is owing to its multi-system, ever-changing progressive nature. The findings in this thesis along with other studies in the field bespeak a change in not only prognostication of CLL patients but also how we approach treatment. Mutations within certain pathways could lead to the development of mutation-specific small molecule inhibitors. Advancements in these areas of research could eventually one day lead to patient tailored monitoring and therapy programs based on mutational profiles as the treatment touchstone.

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