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Reappraising prognosis in chronic lymphocytic leukemia

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2016

ISSN 1651-6206
ISBN 978-91-554-9519-0
urn:nbn:se:uu:diva-280943

Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Dag Hammarskjölds väg 20, Uppsala, Wednesday, 11 May 2016 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Professor Clemens Wendtner (University of Cologne, Department of Medicine).

Abstract
Baliakas, P. 2016. Reappraising prognosis in chronic lymphocytic leukemia. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1199. 59 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9519-0.

Chronic lymphocytic leukemia (CLL) exhibits remarkable clinical heterogeneity likely reflecting the underlying biological heterogeneity. The genetic landscape of CLL has been recently enriched with mutations within a number of genes proposed as novel prognostic markers. Mounting evidence also supports the pivotal role of the clonotypic B-cell receptor immunoglobulin (BcR IG) in the natural history of CLL. Interestingly, almost 30% of all CLL patients can be assigned to different patient subsets, each defined by expression of a distinct stereotyped BcR IG. Whether stereotyped subsets exhibit distinct clinical behavior is still an issue of debate. The aim of this thesis was to evaluate the prognostic relevance of recurrent gene mutations and to assess the clinicobiological associations and clinical impact of BcR IG stereotypy in CLL. In a cohort of 3490 patients, *NOTCH1, SF3B1* and *TP53* mutations were enriched within clinically aggressive cases carrying unmutated IGHV genes (U-CLL), frequently co-occurring with trisomy 12, del(11q) and del(17p), respectively. Of note, *SF3B1* mutations increased in parallel with increasing timespan between diagnosis and mutational screening. *NOTCH1* mutations, *SF3B1* mutations and *TP53* abnormalities (*TP53abs*, deletions and/or mutations) correlated with shorter time-to-first-treatment among early stage cases, while in multivariate analysis, only *SF3B1* mutations and *TP53abs* retained independent significance. In a series of 8593 CLL patients, stereotyped subsets showed marked differences in demographics, clinical presentation, cytogenetic aberrations and gene mutational spectrum. Patients within a specific subset generally followed similar clinical courses, whereas patients in different stereotyped subsets—even when displaying similar IG somatic hypermutation status — experienced significantly different clinical outcome. In particular, subset #2 (IGHV3-21/IGLV3-21), the largest overall, was found to exhibit (i) a remarkably high incidence of *SF3B1* mutations (44%), alluding to subset-biased acquisition of genomic aberrations, in the context of particular antigenic stimulation; and, (ii) a dismal clinical outcome, distinct from the remaining IGHV3-21 CLL. Our findings strongly support the adverse clinical impact of *SF3B1* mutations in CLL in addition to *TP53abs*. BcR IG stereotypy also emerges as prognostically relevant, further highlighting that an immunogenetic sub-classification of CLL based on BcR IG configuration could refine patient risk stratification.

Keywords: CLL, prognosis

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ISSN 1651-6206
ISBN 978-91-554-9519-0
urn:nbn:se:uu:diva-280943 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-280943)

“Knowledge, like air, is vital to life. Like air, no one should be denied it.”

Alan Moore, V for Vendetta

List of Papers

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

I. **Baliakas P**, Hadzidimitriou A, Sutton LA, Rossi D, Minga E, Villamor N, Larrayoz M, Kminkova J, Agathangelidis A, Davis Z, Tausch E, Stalika E, Kantorova B, Mansouri L, Scarfo L, Cortese D, Navrkalova V, Rose-Zerilli MJ, Smedby KE, Juliusson G, Anagnostopoulos A, Makris AM, Navarro A, Delgado J, Oscier D, Belessi C, Stilgenbauer S, Ghia P, Pospisilova S, Gaidano G, Campo E, Strefford JC**, Stamatopoulos K**, Rosenquist R**. Recurrent mutations refine prognosis in chronic lymphocytic leukemia. Leukemia. 2015;29(2):329-36.

II. Strefford JC*, Sutton LA*, **Baliakas P***, Agathangelidis A, Malcikova J, Plevova K, Scarfo L, Davis Z, Stalika E, Cortese D, Cahill N, Pedersen LB, Di Celle PF, Tzenou T, Geisler C, Panagiotidis P, Langerak AW, Chiorazzi N, Pospisilova S, Oscier D, Davi F, Belessi C, Mansouri L, Ghia P**, Stamatopoulos K**, Rosenquist R**. Distinct patterns of novel gene mutations in poor-prognostic stereotyped subsets of chronic lymphocytic leukemia: the case of SF3B1 and subset #2. Leukemia. 2013;27(11):2196-9.

III. **Baliakas P**, Agathangelidis A, Hadzidimitriou A, Sutton LA, Minga E, Tsanousa A, Scarfo, Davis Z, Yan XJ, Shanafelt T, Plevova K, Sandberg Y, Vojdeman FJ, Boudjogra M, Tzenou T, Chatzouli M, Chu CC, Veronese S, Gardiner A, Mansouri L, Smedby KE, Pedersen LB, Moreno D, Van Lom K, Giudicelli V, Francova HS, Nguyen-Khac F, Panagiotidis P, Juliusson G, Angelis L, Anagnostopoulos A, Lefran, MP, Facco M, Trentin L, Catherwood M, Montillo M, Geisler CH, Langerak AW, Pospisilova S, Chiorazzi N, Oscier D, Jelinek DF, Darzentas N, Belessi C, Davi F, Ghia P**, Rosenquist R**, Stamatopoulos K**. Not all IGHV3-21 chronic lymphocytic leukemias are equal: prognostic considerations. Blood. 2015;125(5):856-9.

IV. **Baliakas P**, Hadzidimitriou A, Sutton LA, Minga E, Agathangelidis A, Tsanousa A, Scarfo L, Davis Z, Yan X, Shanafelt T, Plevova K, Sandberg Y, Vojdeman F, Boudjogra M, Tzenou T, Chatzouli M, Chu C, Veronese S, Gardiner A, Mansouri L, Smedby K, Pedersen L, Moreno D, Van Lom K, Giudicelli V, Francova H, Nguyen-Khac F, Panagiotidis P, Juliusson G, Angelis L, Anagnostopoulos A, Lefranc M, Facco M, Trentin L, Catherwood M, Montillo M, Geisler C, Langerak A, Pospisilova S, Chiorazzi N, Oscier D, Jelinek D, Darzentas N, Belessi C, Davi F, Rosenquist R, Ghia P**, Stamatopoulos K**. Clinical impact of stereotyped receptors in chronic lymphocytic leukemia. Lancet Haematology. 2014;1(2):74-84.

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List of related papers published during the PhD period

1. **Baliakas P**, Iskas M, Gardiner A, Davis Z, Plevova K, Nguyen-Khac F, Malcikova J, Anagnostopoulos A, Glide S, Mould S, Stepanovska K, Brejcha M, Belessi C, Davi F, Pospisilova S, Athanasiadou A, Stamatopoulos K, Oscier D. Chromosomal translocations and karyotype complexity in chronic lymphocytic leukemia: a systematic reappraisal of classic cytogenetic data. Am J Hematol. 2014;89(3):249-55.

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3. Malcikova J, Stalika E, Davis Z, Plevova K, Trbusek M, Mansouri L, Scarfo L, **Baliakas P**, Gardiner A, Sutton LA, Francova HS, Agathangelidis A, Anagnostopoulos A, Tracy I, Makris A, Smardova J, Ghia P, Belessi C, Gonzalez D, Rosenquist R, Oscier D, Pospisilova S, Stamatopoulos K. The frequency of TP53 gene defects differs between chronic lymphocytic leukaemia subgroups harbouring distinct antigen receptors. Br J Haematol. 2014;166(4):621-5.

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5. Bystry V, Agathangelidis A, Bikos V, Sutton LA, **Baliakas P**, Hadzidimitriou A, Stamatopoulos K, Darzentas N; also on behalf of ERIC, the European Research Initiative on CLL. ARResT/AssignSubsets: a novel application for robust subclassification of chronic lymphocytic leukemia based on B cell receptor IG stereotypy. Bioinformatics. 2015;31(23):3844-6.

6. **Baliakas P**, Hadzidimitriou A, Agathangelidis A, Rossi D, Sutton LA, Kminkova J, Scarfo L, Pospisilova S, Gaidano G, Stamatopoulos K, Ghia P, Rosenquist R. Prognostic relevance of MYD88 mutations in CLL: the jury is still out. Blood. 2015;126(8):1043-4.

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8. Ljungstom V, Cortese D, Young E, Pandzic T, Mansouri L, Plevova K, Ntoufa S, **Baliakas P**, Clifford R, Sutton LA, Blakemore S, 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

9. **Baliakas P**, Mattsson M, Stamatopoulos K, Rosenquist R. Prognostic indices in chronic lymphocytic leukaemia: where do we stand how do we proceed? J Intern Med. 2015.

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Abbreviations

ALC:	Absolute lymphocyte count
Allo-HCT	Allogeneic hematopoietic cell transplantation
AID	Activation induced cytidine deaminase
AS-PCR:	Allele specific PCR
BcR:	B cell receptor
BIRC3:	Baculoviral IAP repeat containing 3
Bp:	Base-pair
BR	Bendamustine plus Rituximab
C:	Constant
CD:	Cluster of differentiation
CDR:	Complementarity determining region
CI:	Confidence interval
CLL:	Chronic lymphocytic leukemia
CSR:	Class switch recombination
CT:	Clinical trial
D:	Diversity
DAPI:	4,6-diamidino-phenylindole
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
DNA:	Deoxyribonucleic acid
ERIC:	European Research Initiative on CLL
F:	Fludarabine
FASAY:	Functional analysis of separated allele in yeast

FC:	Fludarabine-cyclophosphamide
FCR:	Fludarabine-cyclophosphamide-rituximab
FISH:	Fluorescence in situ hybridization
GP:	General practice
HR:	Hazard ratio
HPLC:	High performance liquid chromatography
HRM:	High resolution melting
IG:	Immunoglobulin
IGH:	Immunoglobulin heavy
IGL:	Immunoglobulin lambda
IGHV:	Immunoglobulin heavy variable
iwCLL:	International Workshop on Chronic Lymphocytic Leukemia
J:	Joining
MBL:	Monoclonal B-cell lymphocytosis
M-CLL:	CLL with mutated IGHV genes
MDS:	Myelodysplastic syndrome
MYD88:	Myeloid differentiation primary response 88
NGS:	Next-generation sequencing
OS:	Overall survival
PCR:	Polymerase chain reaction
PFS:	Progression-free survival
RAG:	Recombination activating gene
R	Rituximab
RNA:	Ribonucleic acid
RS:	Richter syndrome
SCT:	Stem cell transplantation
SF3B1:	Splicing factor 3b subunit 1
SHM:	Somatic hypermutation
SS:	Sanger sequencing

TdT:	Terminal deoxynucleotidyl transferase
TLR:	Toll-like receptor
TP53:	Tumor protein p53
TP53abs:	Deletion of the short arm of chromosome 17 and/or *TP53* mutation
TTFT:	Time-to-first-treatment
U-CLL:	CLL with unmutated IGHV genes
V:	Variable

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the *in vivo* accumulation of $CD5^+$ monoclonal B cells in secondary lymphoid organs, bone marrow and blood[1,2]. It is the most common malignancy among the elderly in the western world with a median age at diagnosis of 72 years and a male predominance[3,4]. Mounting evidence suggests that it may be preceded by a pre-leukemic condition, defined as monoclonal B-cell lymphocytosis (MBL), that evolves into CLL requiring treatment at a rate of 1-2%/year[5,6]. Finally, a small proportion of CLL (~5%) may progress to high-grade lymphoma (Richter syndrome – RS)[7,8]. Despite significant progress regarding therapeutic options, CLL continues to be incurable.

The diagnosis of CLL is considered relatively straightforward and is based on the presence of $\geq 5x10^9$ clonal B lymphocytes/L (5000/μL) in peripheral blood expressing CD5, CD19 and CD23; surface immunoglobulin, CD20, and CD79b are expressed at lower levels on CLL versus normal B cells[9]. In general, CLL is considered an indolent malignancy with a median survival of almost 10 years. However, it exhibits remarkable clinical heterogeneity ranging from extremely indolent with no treatment requirement and a life expectancy similar to that of an aged-matched healthy population to an extremely aggressive disease, characterized by refractoriness to standard treatment and reduced life expectancy[10].

Treatment algorithm of chronic lymphocytic leukemia

Despite being diagnosed with a malignancy, most patients with CLL (~85-90%) are not treated at diagnosis, being mostly asymptomatic[11]. Instead, treatment is initiated only in cases with "active disease" as defined by the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) criteria[9] (Table 1).

Table 1. *Criteria regarding treatment initiation in CLL according to the iwCLL.*

Progressive Bone Marrow failure	Development/worsening of anemia and/or thrombocytopenia
Bulky Disease	Massive (≥6 cm below the left costal margin) or progressive/symptomatic splenomegaly Massive nodes (≥10 cm in diameter) or progressive/symptomatic lymphadenopathy
Refractory AIHA/ITP	AIHA and/or ITP poorly responsive to standard therapy
Constitutional symptoms	Weight loss of ≥10% within the previous 6 months Significant fatigue Fever ≥38.0°C for ≥ 2 weeks with no evidence of infection Night sweats ≥1 month with no evidence of infection
Progressive lymphocytosis*	Increase >50% over a 2-month period Doubling time (LDT) of <6 months

*should not be used as a single parameter to define a treatment indication
AIHA: Autoimmune anemia, ITP: autoimmune thrombocytopenia, LDT: lymphocyte doubling time

Once the criteria for treatment initiation are met, the main parameters currently influencing the choice of treatment are the physical condition of the patient and the genetic background of the malignant clone[10] (Table 2). Patients can be classified as follows: (i) those with a good physical condition ("go go") and thus more capable of tolerating toxic regimens; (ii) those with borderline impaired physical conditions ("slow go") where the main goal is to control the symptoms, and: (iii) those with significantly impaired physical conditions ("no go") where a high comorbidity index restricts the treatment choices to a minimum (palliative approach).

For the "go go" group, combined chemo-immunotherapy with purine analogues, alkylating agents and anti-CD20 antibodies is recommended with a fludarabine-cyclophosphamide-rituximab combination (FCR) being the golden standard[12-15], achieving an overall response rate of >90%. More recently, the combination of bendamustine with rituximab (BR) has proven quite effective, with limited toxicity among elderly patients[16-18]. For patients carrying *TP53* aberrations (deletion of chromosome 17p (del(17p) and/or mutation within the *TP53* gene, *TP53*abs), depending on drug availability, the optimal treatment choices are either novel agents which target critical kinases of the B-cell receptor (BcR) pathway (BcR inhibitors, see below) or anti-CD52 (alemtuzumab) based regimens followed, in certain cases, by allogeneic hematopoietic cell transplantation (allo-HCT)[10,19-21]. In the "slow go" group the treatment choice is again influenced by the presence of *TP53*abs; however, in such cases allo-HCT is not an option.

Table 2. *CLL treatment algorithm*

Physical condition	*TP53* aberration	Treatment
”Go go”	Absent	FCR (BR in patients >65)
	Present	BcR inhibitors/BcR inhibitors & anti-CD20 antibodies Alemtuzumab based regimens Allo-HCT?
”Slow go”	Absent	Chlorambucil & anti-CD20 antibodies
	Present	BcR inhibitors/ BcR inhibitors & anti-CD20 antibodies High dose Rituximab Alemtuzumab

Taking into consideration the remarkable clinical heterogeneity of CLL, numerous efforts have been made to classify patients into subgroups with distinct clinical behavior. In every day practice, the Binet[22] and Rai[23] clinical staging systems, easily elaborated with minimum cost and based on physical examination and a blood count (platelets and hemoglobin) are still widely used, more than 30 years after their development. However, they both fail to discriminate between patients with low tumor burden who will experience an indolent disease from those who will follow an aggressive disease course, hence limiting their usefulness. This is especially relevant today when the great majority of CLL patients (~85%) are diagnosed at early clinical stages, with no disease-related symptoms (Figure 1).

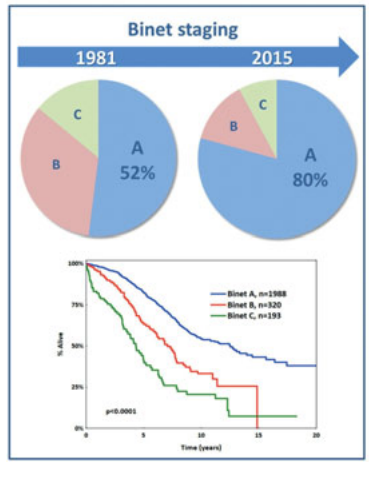

Figure 1. Overtime changes in the proportion of CLL patients assigned to the different Binet clinical stages. Figure adapted from Baliakas et al. J Intern Med. 2015.

The clinical heterogeneity of CLL most likely reflects the underlying biological heterogeneity, where a large number of interacting genetic events (cell-intrinsic aberrations) and microenvironmental stimuli (cell-extrinsic triggers) have been implicated in disease ontogeny and evolution[24-27]. Patient and/or disease related features, e.g. age, gender, performance status, comorbidities and tumor burden, all have an established prognostic value[28-34]. More recently, great progress has been achieved in identifying biomarkers linked to both cell-extrinsic and cell-intrinsic mechanisms[35-38]. Several of these markers are capable of predicting the likelihood of disease progression at the time of diagnosis and, thus, could assist in both risk stratification and the design of follow-up and treatment strategies (Figure 2). Amongst numerous biomarkers with prognostic and predictive relevance in CLL, perhaps the most powerful and widely accepted are related to the clone's genomic profile[39-43] and immunogenetic signature[44,45].

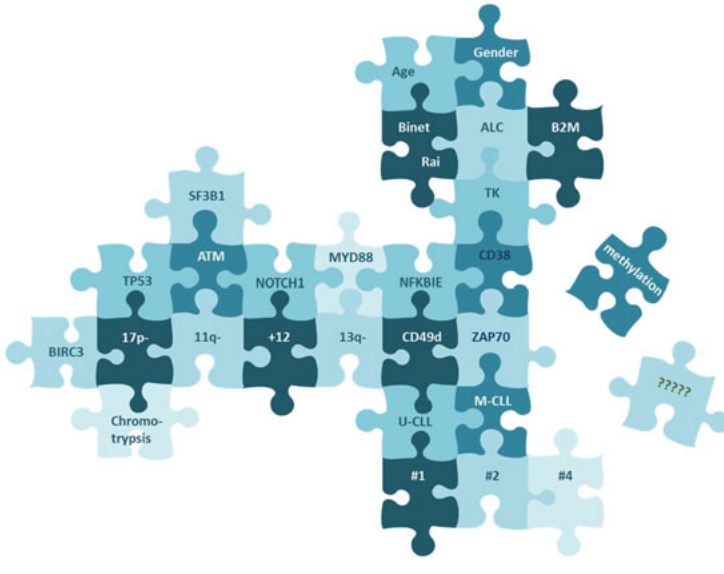

Figure 2. Prognostic markers in CLL. Figure adapted from Baliakas et al. J Intern Med. 2015.

The genomic background of CLL

Recurrent cytogenetic aberrations in CLL

In contrast to other hematological malignancies, CLL lacks disease-specific genomic abnormalities. In the past, due to difficulties in obtaining an adequate number of metaphases, the majority of studies investigating the genetic landscape of CLL have been based on fluorescence *in situ* hybridization (FISH). A seminal finding in the late 1990s revealed that almost 80% of CLL cases carry recurrent aberrations affecting four chromosomes, namely partial deletions of chromosomes 13q, 11q and 17p [del(13q), del(11q), del(17p), respectively] and trisomy of chromosome 12 (+12)[40]. Interestingly, each of these lesions is also associated with a distinct prognosis, and collectively they have formed the basis for the well-established Döhner hierarchical cytogenetic aberrations model (Figure 3). In brief, patients with del(17p) as well as those with del(11q) exhibit significantly worse outcomes compared to patients with isolated del(13q) or patients negative for any of these 4 abnormalities (normal FISH), while cases harboring +12 have an intermediate prognosis, albeit with extreme heterogeneity[46].

FISH-detected abnormalities also influence treatment decisions. *TP53*abs negatively impact on patient outcome and are associated with treatment refractoriness[40,47]. For this reason, screening for del(17p) and *TP53* gene mutations is strongly recommended before treatment initiation (initial as well as subsequent lines of treatment), not only in the context of clinical trials but also in standard clinical practice[9]. Furthermore, in the CLL8 trial, undertaken by the German CLL Study Group (GCLLSG), when comparing FCR vs FC, patients harboring del(11q) displayed a markedly superior outcome after receiving FCR compared to FC alone[12]. However, the favorable impact of the addition of Rituximab to FC for patients carrying del(11q) has not yet been confirmed outside the context of clinical trials[48].

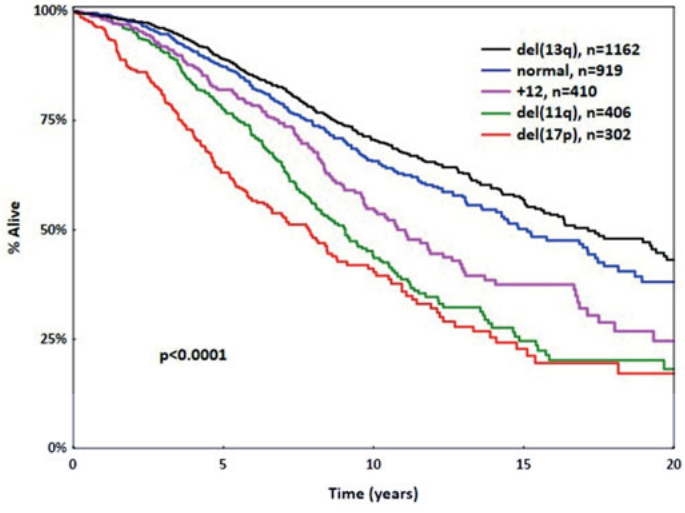

Figure 3. Genomic aberrations and survival in CLL according to the Döhner hierarchical model based on the cohort included in paper IV.

With the advent of novel mitogens, classic cytogenetic analysis was considerably simplified. Consequently, classic cytogenetic studies of large patient cohorts allowed the global evaluation of the karyotype of the malignant clone, thus superseding FISH, which can detect only selected chromosomal abnormalities[39,42,49]. More powerful techniques such as micro-array-based methodologies and, especially, next-generation sequencing (NGS), including targeted, exome and whole-genome sequencing, highlighted further the significance of genomic aberrations in driving the clinical and biological heterogeneity of CLL[50-55]. Besides identifying additional recurrent abnormalities of potential clinical significance, these studies have also confirmed preliminary evidence regarding the clinical consequence of genomic complexity in CLL[41,56,57]. A complex karyotype (≥3 aberrations) has been reported to associate with an unfavorable prognosis and dismal response even within subgroups of patients that would have been classified as having a favorable prognosis in case FISH was the only applied technique[58]. For example, almost 20% of cases with normal FISH according to the Döhner hierarchical model were reported to carry complex karyotypes[39]. More importantly, genomic complexity has been associated with a poor response to treatment even with the advent of novel targeted therapies e.g. BcR inhibitors[59,60].

Novel recurrent mutations in CLL

In addition to chromosomal abnormalities, the genetic landscape of CLL is characterized by recurrent genetic lesions. Until recent years, *TP53* and *ATM* gene mutations were considered the most clinically relevant[47,61-64]. With the application of high-resolution molecular techniques a number of recurrent mutations affecting several genes involved in various biological processes and pathways, such as *NOTCH1* signaling, RNA splicing, DNA damage repair and cell cycle control was revealed[50-53]. Amongst them the *NOTCH1, SF3B1, BIRC3* and *MYD88* genes have attracted great attention and already been proposed as markers that may refine prognostication and empower treatment decision making in CLL.

Independent studies have reported that mutations within the *NOTCH1* gene and the splicing factor *SF3B1* gene are the most frequent and probably the most clinically relevant of all the novel mutations detected in CLL[38,65-67]. Additional lesions of potential importance concern mutations within the anti-apoptotic gene *BIRC3* as well as the *MYD88* gene. Mutations in these latter genes occur at lower frequencies; however, they are attracting interest due to mounting evidence that they may be linked to distinct clinicobiological profiles[51,68,69].

NOTCH1 encodes a class I transmembrane protein that functions as a ligand-activated transcription factor implicated in cell differentiation, proliferation, and apoptosis. The most frequent mutation, accounting for up to 90% of all *NOTCH1* mutations in CLL, concerns a 2-bp frameshift deletion within the PEST domain[66]. Even though the precise oncogenetic role of *NOTCH1* mutations in CLL has not been fully elucidated, it is believed that they activate downstream pathways, thus offering a proliferative advantage to the mutant clone[70]. The frequency of *NOTCH1* mutations in various studies ranges from 5-15%, depending on the composition of the respective cohorts[36,38,65,71-74]. *NOTCH1* mutations are strongly associated with features of aggressive disease, such as advanced clinical stage, RS and U-CLL (U-CLL: limited or no mutations within the IGHV genes, further details provided below), with several studies noting an enrichment of trisomy 12 amongst *NOTCH1* mutated cases. The majority of studies report shorter overall survival (OS) and progression-free survival (PFS) for *NOTCH1* mutated cases; however, it is still unclear whether the prognostic value of *NOTCH1* mutations is independent of other parameters, especially IGHV gene mutational status (discussed in detail below).

SF3B1 is a component of the spliceosome that regulates the splicing machinery, which is of vital significance for cell function. *SF3B1* gene mutations are enriched within a specific subtype of myelodysplastic syndrome (MDS), namely MDS with refractory anemia and ring sideroblasts[75]. In CLL, *SF3B1* mutations have been reported to occur in up to 20% of patients and cluster in specific HEAT repeat regions of the protein, with codon 700

being identified as a mutational "hot spot", harboring almost 50% of all mutations found within this gene[65,76]. *SF3B1* mutations have been associated with U-CLL, advanced clinical stage and del(11q), and significantly correlate with a poor clinical outcome. Interestingly, mutations within this gene are enriched among chemorefractory cases, thereby highlighting their potential relevance for disease evolution.

BIRC3 is an inhibitor of the non-canonical NF-κB pathway and has been found to be disrupted mainly in heavily treated CLL patients. Small frameshift deletions are the most common form of aberrations found within *BIRC3*-mutated cases. The frequency of *BIRC3* mutations is <5% at the time of diagnosis, however amongst fludarabine-refractory cases the frequency may rise to as to as high as 24%[68]. *BIRC3* mutations are strongly associated with U-CLL and del(11q). Interestingly, they are mutually exclusive to *TP53* abnormalities, and show a similarly poor prognosis.

MYD88 plays a pivotal role in B-cell homeostasis and innate immune response where it serves as an adaptor protein for the interleukin-1 receptor/toll-like receptor (TLR) signaling pathways. The predominant *MYD88* mutation concerns a p.L265P substitution within exon 5, which leads to constitutive NF-κB stimulation, thus conferring a survival advantage to the mutant cells. *MYD88* mutations have been identified at relatively low frequencies in CLL, 2-5%[36,65,76]. In contrast to *NOTCH1, SF3B1* and *BIRC3* gene mutations, *MYD88* mutations are almost exclusive to M-CLL (heavily mutated IGHV genes, further details provided below). Their prognostic relevance remains unclear.

Novel mutations and risk-stratification in CLL

Taking into consideration the aforementioned observations between novel recurrent mutations and clinical outcome, the idea to implement novel mutations in the risk-stratification of CLL seems quite reasonable. Incorporation of novel gene mutations into a genetically-orientated prognostic model, i.e. the Döhner hierarchical model[40], could arguably improve prognostication and, thus, more accurately identify patients with distinct clinical outcomes.

To this end, Rossi et al. have published an integrated prognostic model where genetic abnormalities detected by FISH were combined with *TP53, NOTCH1, SF3B1* and *BIRC3* mutations leading to the identification of 4 groups with different OS[76]; namely: (i) a high-risk group, including cases carrying *TP53* and/or *BIRC3* abnormalities; (ii) an intermediate-risk group concerning cases harboring *NOTCH1* and/or *SF3B1* mutations and/or del(11q); (iii) a low-risk group comprising cases with +12 or normal CLL FISH; and (iv) a very low-risk group with cases harboring isolated del(13q). Similarly, Truger et al.[77] reported a model where IGHV gene mutational status was also incorporated; however they failed to reach statistical signifi-

cance amongst all four proposed risk-groups. More recently, Bahlo et al attempted to develop a new prognostic model, however they found that only *TP53*abs and not *NOTCH1* nor *SF3B1* mutations, retained independent significance[78]. These conflicting results may relate to differences between the respective cohorts and/or different analytical methods used, further highlighting the need for more focused approaches within this extremely heterogeneous disease.

Novel mutations in CLL: what is their predictive value?

One of the major questions regarding novel recurrent mutations concerns their potential predictive value and whether they could influence the choice of treatment, especially given the plethora of available therapeutic options. To gain insight into this matter, prospective clinical trials are imperative.

Along these lines, it was recently reported that, in addition to *TP53* mutations, *SF3B1* and *NOTCH1* mutations may also be important when it comes to treatment decisions. In particular, in the UK LRF CCL4 trial both *SF3B1* and *NOTCH1* mutations associated independently with a dismal response[79], while in the CLL8 trial of the GCLLSG, only *SF3B1* mutations retained independent significance and correlated with a worse clinical outcome[80]. Interestingly, in the latter trial, *NOTCH1* mutations were noted as a predictive marker for decreased benefit from the addition of Rituximab to FC.

Immunogenetics in CLL

B-cell receptor

In developing B cells, the B-cell receptor (BcR) is created by a complex combinatorial process resulting in an antibody repertoire that provides the immune system with the ability to recognize and activate immune responses against, in principle, a limitless number of antigens. Briefly, BcR immunoglobulin (IG) diversity lies initially in the random recombination of variable (V), diversity (D; for heavy chains only), and joining (J) genes (*combinatorial diversity*) mediated by the recombination activating genes (RAG)1 and RAG2; followed by the terminal deoxynucleotidyl transferase (TdT) (*junctional diversity*) activity, which trims nucleotides from the recombining genes and/or adds random nucleotides at the junctions, leading to further variability of the IG antigen-binding site, i.e the complementarity determining region 3 (CDR3)[81]. Recombination of IG heavy and light chain genes leads to the formation of a functional BcR IG with unmutated variable domain sequences.

Upon antigen stimulation the IG molecule is further modified and differentiated by the somatic hypermutation (SHM) and class-switch recombination (CSR) processes which take place within secondary lymphoid organs and are mainly orchestrated by the action of the activation induced cytidine deaminase (AID) enzyme[82,83]. SHM is responsible for the introduction of mutations within rearranged genes, while CSR leads to replacement of the constant (IGHC) gene from IGHM to IGHG, IGHE or IGHA. Both mechanisms increase antibody diversity and specificity. The above procedures result in trillions of possible combinations, thus the possibility that two independent B cell clones carry exactly the same BcR ($1:10^{12}$) is minimal if not negligible (Figure 4).

B-cell receptor: the molecular signature of the CLL clone

Clonal B cells are characterized by the BcR IG expressed on their surface and this feature is present from the onset of the clone and remains stable throughout disease evolution. Therefore, analyzing the clonal BcR is considered crucial in understanding the disease history. Consequently, immunoge-

netic studies have been of great interest for all B-cell malignancies, especially CLL.

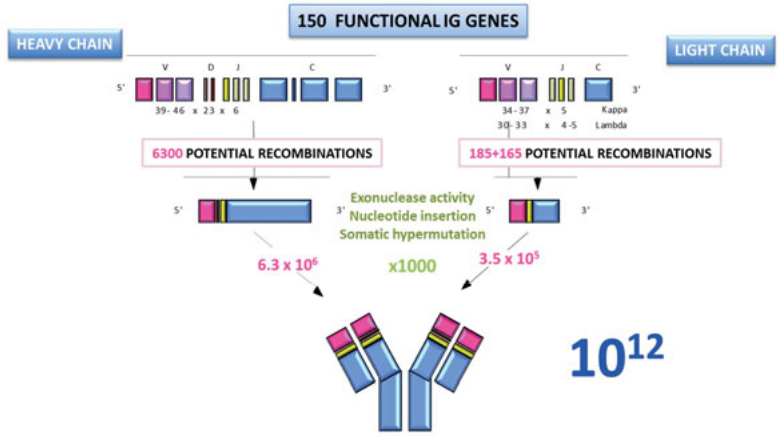

Figure 4. Creation of an IG molecule. Almost 10^{12} different possible combinations. Adapted from the IMGT web resources (http://www.imgt.org/IMGTindex/IGsynthesis.html).

A turning point in the understanding of CLL came when the previous concept that CLL clones consist of resting, inactive and probably naïve, antigen-inexperienced B cells was replaced by the belief that CLL cells are dynamic and continuously interact with the microenvironment to which they are exposed[84] (Figure 5). The BcR is of crucial significance in this crosstalk, highlighting the role of antigen stimulation in CLL onset and evolution[85].

One of the first indications suggesting a role for antigens in the pathogenesis of CLL arose during the early 1990s with the observation that the CLL IGHV gene repertoire was extremely restricted with certain genes, such as IGHV1-69, IGHV4-34, and IGHV3-7, being overrepresented[86,87]. Interestingly, SHM, within CLL patients, is not uniform amongst the aforementioned IGHV genes with some IG genes e.g. IGHV1-69 carrying limited or no SHM, contrasting IG genes such as IGHV3-23 or IGHV4-34 which carry a heavy mutational load. Biased IGHV/IGHD/IGHJ combinations, preferential pairings of specific IG heavy/light chain genes and isotype class switching, provided further evidence in support of the interplay between CLL clones and antigens[88].

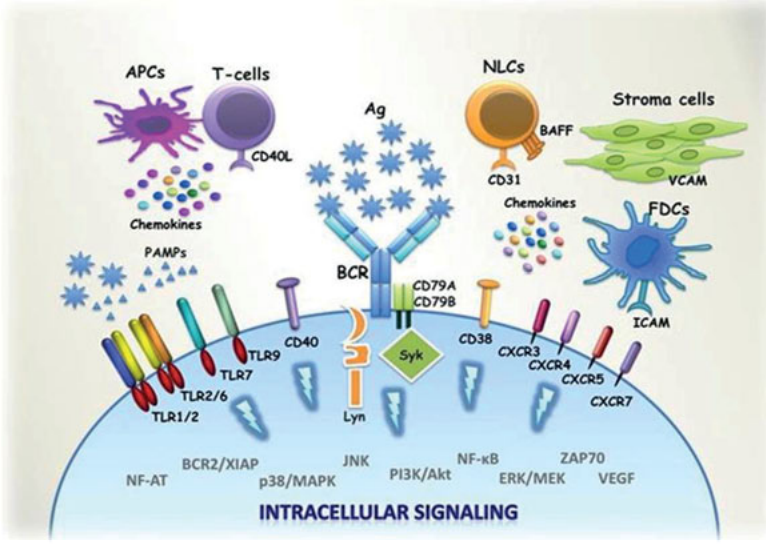

Figure 5. The pivotal role of the B-cell receptor in the interaction of the malignant CLL clone with the microenvironment. Figure adapted by Kostarelli et al. Mediterr J Hematol Infect Dis. 2012.

The critical role of BcR IG in CLL became even more relevant when it was proven that groups with different IGHV mutational load display distinct clinical outcomes. In particular, in 1999 two independent groups reported that cases with no or limited SHM within their IGHV genes [≥98% germline identity, (GI)] experienced aggressive clinical courses and shorter OS compared to cases with mutated IGHV genes (GI<98%) who followed more indolent clinical courses[44,45]. The first group was defined as unmutated CLL (U-CLL) whereas the second group was referred to as mutated CLL (M-CLL). The IGHV gene SHM status has proven to be one of the most robust prognostic markers in CLL, identifiable at diagnosis and independent of clinical stage or other biomarkers, remaining stable throughout disease evolution[29,89]. Moreover, it has a strong predictive value regarding response to treatment, e.g. M-CLL cases display a longer PFS compared to U-CLL after chemo-immunotherapy with FCR, the current gold standard for the treatment of CLL[90,91]. A propos of this, recently IGHV mutational status has been incorporated into a predictive model based on the response to FCR, with M-CLL being associated with long lasting remissions[48].

However, stratification of CLL based on the 98% cut off should not be unconditional. There are studies highlighting the fact that cases with borderline IGHV gene SHM status (GI: 97-97.99%) should be evaluated with extra caution since they may constitute a distinct group[92]. Moreover, cases ex-

pressing specific IGHV genes, such as IGHV3-21, are reported to display unfavorable clinical outcome independently of IGHV gene SHM status[93-95]. Therefore, interpretation of the IGHV SHM status in the clinical setting should always be performed according to well-established guidelines, such as those proposed by the European initiative in CLL (ERIC), thereby ensuring harmonization and standardization of the analysis and interpretation of IGHV gene SHM status[96].

Stereotyped subsets in CLL

Considering the above, it is no surprise that immunogenetic analysis became of prime interest for both biological and clinical/prognostic purposes. As the cumulative number of tested cases increased over the years, it soon became apparent that unrelated and geographically distant CLL patients could carry quasi-similar if not identical BcRs IGs[35,97-99]. Since the probability of identifying identical BcR IGs within different B-cell clones by chance alone approximates to $1:10^{12}$, the observed phenomenon is considered as the strongest molecular evidence for antigen selection in CLL and is aptly termed "stereotypy"[100].

The quest towards the identification of subsets of CLL patients expressing common BcR features was initiated in the mid-1990s, after it was first reported that unrelated CLL cases may carry highly similar, if not identical VH CDR3s, characterized by common amino acid motifs[101]. This observation prompted speculations supporting the involvement of a similar antigenic drive in clonal selection. For instance, a proportion of U-CLL cases expressing the IGHV1-69 gene utilized specific IGHD and IGHJ genes and carried VH CDR3 sequences which were similar and unique to CLL, being absent from that of the normal healthy elderly population[102,103]. A seminal report came in 2003 when it was demonstrated that approximately 50% of IGHV3-21 CLL cases carried quasi-identical, short VH CDR3 sequences with a striking bias towards the usage of the IGLV3-21 light chain gene[93]. This group of patients now constitutes stereotyped subset #2, the largest stereotyped subset, accounting for approximately 3% of all CLL cases[35].

Today, it is widely accepted that 30% of all CLL patients can be assigned to stereotyped subsets, each characterized by a common BcR configuration. Stereotyped subsets vary in size and are present in both U-CLL and M-CLL, though more frequent in the former. According to a recent large-scale study, 19 "major" subsets represent 40% of all stereotyped cases and 12% of all CLL. As mentioned above, stereotyped subset #2 is the largest subset, comprising both M- and U-CLL, and accounts for almost 5-6% of CLL cases requiring treatment. The largest U-CLL stereotyped subset is subset #1, rep-

resenting approximately 5% of all U-CLL; within M-CLL, the most populated subset is stereotyped subset #4 accounting for 2% of all M-CLL cases[35].

One of the primary challenges regarding stereotyped subsets concerns their identification mainly due to the large amounts of IG sequence data that needs to be processed in order to reach robust conclusions[104]. To address this issue, a sophisticated bioinformatics algorithm has been developed which accurately and efficiently identifies stereotypy amongst IGHV sequences. Briefly, three main criteria should be met in order for two IG sequences to be considered sufficiently similar so as to be assigned to the same stereotyped subset: (i) 50% amino acid identity (ii) 70% similarity within the respective VH CDR3 sequences and (iii) usage of IGHV genes belonging to the same phylogenetic clan[35] (Figure 6).

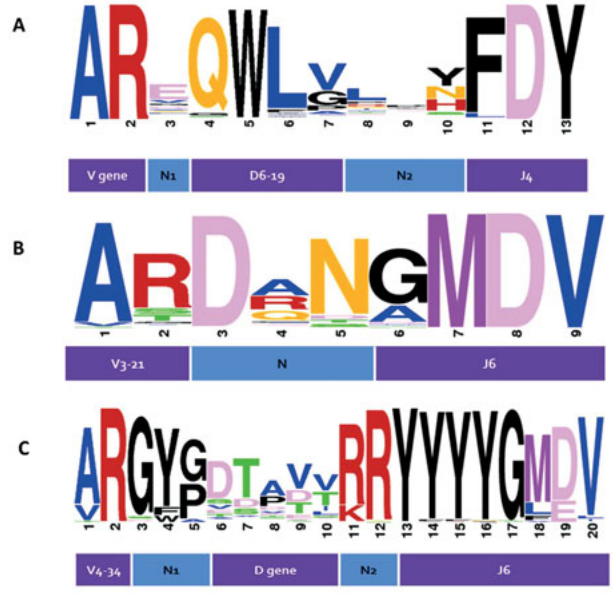

Figure 6. Sequence logos of stereotyped subsets. The height of symbols within the stack indicates the relative frequency of each amino acid at that position. Amino acid position is according to the IMGT numbering for the V domain. A) Stereotyped subset #1: The stereotyped BcR IG of subset #1 combines a heavy chain IGHV1-5-7/IGHD6-19/IGHJ4 gene rearrangement with a kappa light chain IGKV1-39/IGKJ1-2 gene rearrangement. The VH CDR3 length is 13 amino acids and the IGHV gene bears little or no somatic hypermutations. B) Stereotyped subset #2: The subset #2 stereotyped BcR IG is formed by the combination of IGHV3-21/IGHJ6 genes and IGLV3-21/IGLJ3 light chain genes with a 9-aa long VH CDR3 with an acidic residue (aspartic acid D) at position 107. The IGHD gene cannot be reliable assigned. SHM status is variable. C) Stereotyped subset #4: The subset #4 BcR IG consists of a heavy chain IGHV4-34/IGHD5-18/IGHJ6 rearrangement and a light chain IGKV2-30/IGKJ1-2 rearrangement with a VH CDR3 length of 20 amino acids. The pattern defining subset #4 consists of the junctional N2 amino acids [KR]R and the IGHJ6-encoded motif YYYYG. Stereotyped subset #4 cases carry a heavy SHM load.

Preliminary evidence suggests that similarities between different patients extend from BcR IG features to shared genomic aberrations, gene expression, DNA methylation and miRNA profiles as well as similar responses to immune triggering through the BcR IG and/or other receptors[105-108]. These findings allude to distinct interactions between cell-intrinsic and cell-extrinsic mechanisms that may underlie the ontogeny and evolution of different subsets. For example, subsets #2 and #4 are enriched with del(11q) and del(13q), respectively, while subset #1 has been associated with the down-regulation of miR-101[109,110]. Recently, stereotyped subset #8, which exhibits a high risk for transformation to RS, has been linked to excessive antigen polyreactivity[111]; a feature potentially contributing to the clinical aggressiveness observed in this subset.

Based on the above, several reports have favored the concept that stereotyped subsets might represent distinct clinicobiological entities, each tightly linked to the respective shared BcR configuration. However, definitive conclusions could not be drawn due to relatively small patient numbers. This is not unexpected when dealing with stereotyped subsets due to the fact that even the largest subset accounts for less than 5% of all CLL cases, clearly indicating that for meaningful conclusions to be reached, large patient series are imperative.

The B-cell receptor as a treatment target

From a clinical perspective, the most striking evidence supporting the importance of BcR IG in CLL is the remarkable therapeutic efficacy of BcR signaling inhibitors, even among heavily pretreated, relapsed/refractory patients[112-114]. Despite being introduced to the clinical arena less than 5 years ago, these novel agents have changed the scenery of CLL treatment and are considered as the targeted non-chemo-based therapies of tomorrow. Importantly, these new therapeutic options are also considered to be safe in terms of side-effects, which are manageable for the majority of patients. They are also efficacious in cases harboring unfavorable genomic background such as *TP53*abs[115], leading to the recent approval of BcR inhibitors as a first-line treatment choice for these cases.

The BcR inhibitors approved for routine clinical use are ibrutinib, a selective inhibitor of Bruton's tyrosine kinase (BTK), and idelalisib, a selective inhibitor of the delta isoform of phosphatidylinositol 3-kinase (PI3Kδ). These novel drugs target the BcR cascade, thus depriving the malignant clone of critical microenvironmental signals that affect their homeostasis, including survival, proliferation, homing to tissues etc. The observed redistribution of malignant cells from the lymphoid organs to the circulation highlights the dependence of the CLL cells on microenvironmental cues delivered in certain niches. Interestingly, M-CLL patients display a greater rela-

tive increase in the absolute lymphocyte count (ALC) and slower resolution of lymphocytosis compared to U-CLL. This difference in the kinetics of the malignant clone has not yet been fully elucidated[116,117]; however, no difference in the overall response rate between M- and U-CLL has been reported. Of note, BcR signaling inhibitors have demonstrated therapeutic efficacy both as monotherapy as well as in combination with anti-CD20 antibodies e.g. Rituximab. Whether combination with standard chemotherapy regimens will improve the already remarkable therapeutic results observed will be answered by ongoing phase III trials[118,119].

Thesis Aims

The main aim of this thesis was to evaluate the prognostic significance of novel and "traditional" biological markers in CLL, focusing on the genomic and the immunogenetic background of the malignant clones. In particular:

Paper I., To evaluate the prognostic significance of novel recurrent mutations (*TP53, SF3B1, NOTCH1, MYD88* and *BIRC3*) in a series of 3490 patients, within a multicenter collaboration within ERIC. We searched for associations with other features of CLL, aiming to define the clinicobiological profile of each mutated gene and also attempted to address the issue of clonal evolution.

Paper II. To investigate the frequency of *SF3B1, NOTCH1* and *BIRC3* mutations within clinically aggressive subsets #1, #2 and #8, all (cases tested: #1 n=82, #2: n=66 and #8: n=22) and assess the impact of novel mutations on clinical outcome.

Paper III. Almost 50% of all IGHV3-21 cases in CLL can be assigned to stereotyped subset #2 which is associated with unfavorable prognosis. Whether this aggressive clinical behavior results from subset #2 assignment or just from the usage of IGHV3-21 is still a matter of debate. Interestingly, within ongoing clinical trials, IGHV3-21 usage *per se* is considered a high-risk feature. To address this issue, we evaluated the clinicobiological profile of 437 IGHV3-21 cases with 254 (58%) assigned to stereotyped subset #2.

Paper IV. Within a multicenter study including 8593 patients with CLL, we evaluated the clinical implications of stereotypy focusing on 14 major subsets. We extended our analysis from parameters such as gender, age, clinical stage and cytogenetic features to clinical outcome, aiming at addressing whether stereotyped subsets could be recognized as distinct subgroups defined by their immunogenetic identity. We further attempted to incorporate the 3 largest subsets namely subsets #1, #2 and #4 which comprise almost 7% of all CLL, in the well-established Döhner hierarchical model.

Patients and methods

Patients

All patients included in the four studies were diagnosed with CLL according to the 2008 iwCLL diagnostic criteria. Ethical approval was granted by local review committees and informed consent was collected according to the Helsinki Declaration. In particular:

Paper I: 3490 patients from 9 institutions were included and the number of cases tested for each gene are as follows: *NOTCH1*: n=3334, *SF3B1*: n=2322, *TP53*: n=2309, *MYD88*: n=1080 and *BIRC3*: n=919. Of the 3490 cases analyzed, 2813 (81%) were general practice (GP) patients, while 677 patients (19%) were enrolled in clinical trials (UK LRF CLL4: n=493, CLL2H: n=103 and CLL3X: n=81).

Paper II: 170 patients assigned to stereotyped subsets #1 (n=82), #2 (n=66) and #8 (n=22) were included.

Papers III-IV: 8593 patients from 15 institutions were included.

Methods

Analysis of gene mutations

Mutational screening was performed for the following genes: *NOTCH1*: entire exon 34 or targeted analysis for del7544-45/p.P2514Rfs*4; *TP53*: exons 4-8 but also exons 9-10 for some centers; *SF3B1*: exons 14-16; *BIRC3*: exons 6-9 and, *MYD88*: exons 3 and 5 or targeted analysis for p.L265P. Information regarding the timespan between the time of diagnosis and the time-point of the gene analysis is provided in Table 3.

Table 3. *Timespan between the time of diagnosis as well as the time of treatment and the time of the gene analysis.*

Mutation	Tested cases
NOTCH1	Total: 3334
≤12m from diagnosis	1900/2995 (63%)
Before first treatment	2326/2552(91%)
SF3B1	Total: 2322
≤12m from diagnosis	1421/2221 (64%)
Before first treatment	1931/2051 (94%)
TP53	Total: 2309
≤12m from diagnosis	1387/2309 (66%)
Before first treatment	1782/1923 (77%)
MYD88	Total: 1080
≤12m from diagnosis	843/1063 (79%)
Before first treatment	951/1023 (93%)
BIRC3	Total: 919
≤12m from diagnosis	813/915 (89%)
Before first treatment	863/882 (98%)

An overview of the methodologies used for the detection of gene mutations in each institution in paper I, is provided in Table 4.

Table 4. *Overview of the applied methodology regarding analysis of gene mutations in paper I.*

	FASAY/SS	SS	NGS	AS-PCR/SS	HRM/SS	HPLC/SS
NOTCH1		161/1740		45/900	60/694	
TP53	90/615	78/1380	7/196			62/180
SF3B1		98/1306	17/184		111/661	35/171
MYD88		24/1070				
BIRC3		23/942				

FASAY: functional analysis of separated allele in yeast; SS: Sanger sequencing; NGS: Next generation sequencing: AS-PCR: Allele specific PCR: HRM: High resolution melting; HPLC: High performance liquid chromatography.

PCR amplification of IGHV-IGHD-IGHJ rearrangements - Sequence analysis, including stereotyped subset assignment

PCR amplification and sequence analysis of IGHV-IGHD-IGHJ rearrangements were performed on either genomic DNA (gDNA) or complementary DNA (cDNA), as previously reported[35]. PCR amplicons were subjected to direct sequencing on both strands. Sequence data were analyzed using the IMGT® databases and the IMGT/V-QUEST tool (http://www.imgt.org). Only productive rearrangements were evaluated. Output data from IMGT/V-QUEST for all productive IGHV-IGHD-IGHJ rearrangements were parsed, reorganized, and exported to a spreadsheet through the use of computer programming. Information was extracted regarding IG gene repertoires, VH CDR3 length and amino acid sequence and SHM.

To identify and cluster stereotyped rearrangements, we used a purpose-built bioinformatics method, as previously described[35]. This method places VH CDR3 sequences into subsets based on a series of parameters and criteria that guide the process to reflect meaningful sequence relationships. The first criterion concerns the amino acid composition of the VH CDR3, an important determinant of antigen recognition, with cases initially clustered together only when they share at least 50% amino acid identity and 70% similarity. Furthermore, given that VH CDR3 length also affects antigen interactions, clustered sequences must have identical VH CDR3 lengths and identical locations of shared patterns. The final criterion concerns the IGHV gene that accompanies the VH CDR3, with only sequences carrying IGHV genes of the same phylogenetic clan placed in the same cluster. Iterative clustering ultimately leads to higher levels of hierarchy describing more distant, and thus relaxed, sequence relationships with more widely shared sequence patterns (affecting only the number - and rarely the location - of these patterns, but neither the VH CDR3 length nor the phylogenetic makeup of the cluster) in progressively larger clusters, which eventually form the collection of subsets.

Cytogenetic analysis

Preparations for FISH analysis were counterstained with 4,6-diamidino-phenyl-indole (DAPI) and a minimum of 200 interphase nuclei were examined using commercially available probes for chromosomal bands 13q14-34, 11q22, 17p13 and chromosome 12. For 369 cases included in paper I, data regarding these recurrent genomic aberrations was obtained following analysis on the Affymetrix 250K SNP-array. FISH data was available for 2772 and 3662 in paper I and III-IV, respectively. Median time from diagnosis to FISH analysis was 2 and 17 months in papers I and III-IV, respectively. Amongst those patients who were treated before the completion of the stud-

ies, FISH analysis was conducted before treatment administration for 85% and 70% of the cases in papers I and III-IV, respectively.

CD38 and ZAP70 expression

CD 38 and ZAP70 expression was assessed with flow-cytometry (threshold for positivity: 30% and 20% respectively). In papers III-IV, CD38 and ZAP70 data were available for 3928 (46%) and 1926 (22%) of the patients.

Statistical analysis

Descriptive analysis included frequency distributions for all the categorical variables. Quantitative variables were dichotomized using published thresholds, laboratory norms and quartiles. The main endpoint of the statistical analysis was TTFT, defined as the time between diagnosis and date of first treatment. Subjects without a documented event were censored at the time of last follow-up. OS was measured from the date of diagnosis until the date of last follow-up or death. Cases with loss of follow-up were censored at the time of follow-up loss. Survival rates and standard errors were determined using the Kaplan-Meier method and survival curves were compared using log-rank tests. The prognostic relevance of each factor was evaluated by applying Kaplan-Meier methodology and Cox proportional-hazards regression analyses. All variables that showed significant association with TTFT on univariable analysis were consequently included in multivariable analysis. Multivariable Cox regression models were used to test the simultaneous effect of factors on outcome taking into account the relative effect of remaining parameters. Robustness of the multivariable Cox model was verified by a cross-validation test obtained using the crossfold Stata procedure. The proportional Hazards assumption was tested by running the respective plots and results revealed that the assumption was not violated. *C*-statistics were calculated to further evaluate the discriminatory value of multivariable analysis (c=1 indicates perfect discrimination; c=0.5 equates to chance). All tests were two-sided and significance was defined as a p-value of <0.05. Statistical analyses were performed using IBM SPSS Statistics 20.0 (IBM Corp. Armonk, NY), the Statistica Software 10.0 (StatSoftInc, Tulsa, OK) and STATA 13 (STATA, College Station, TX), the latter was also used for generating the figures.

Results-Discussion

Paper I: Recurrent mutations refine prognosis in chronic lymphocytic leukemia

Main findings and conclusions

The prognostic significance of the novel recurrent mutations regarding TTFT was the primary endpoint of the study. We report that amongst early stage Binet A patients, in addition to *TP53*, *SF3B1* mutations emerge as an independent prognostic marker, thus contrasting mutations within the *NOTCH1* gene that failed to retain significance in multivariate analysis (Table 5).

Table 5. *Univariate and multivariate analysis for time-to-first-treatment (TTFT).*

	Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
NOTCH1	2.633	1.928-3.598	<0.0001	1.305	0.923-1.847	0.128
SF3B1	2.635	1.855-3.745	<0.0001	1.644	1.134-2383	0.008
TP53abs	2.266	1.580-3.252	<0.0001	2.081	1.431-3.021	0.0001
U-CLL	4.840	3.860-6.069	<0.0001	3.701	2.833-4.434	<0.0001
idel(13q)	0.524	0.413-0.663	<0.0001	0.98	0.740-1.209	0.889
del(11q)	2.924	2.229-3.849	<0.0001	1.421	1.031-1.970	0.03
Trisomy 12	1.997	1.520-2.623	<0.0001	1.338	0.972-1.841	0.07

HR: hazard ratio; 95% CI: 95% confidence interval; idel(13q):isolated deletion of chromosome 13q; *TP53*abs: *TP53* mutation and/or deletion of chromosome 17p; U-CLL: CLL carrying unmutated IGHVgenes; del(11q): deletion of chromosome 11q

Moreover, *SF3B1* mutations were associated with dismal outcome even among U-CLL, as well as among cases carrying del(11q) or del(13q). On the contrary, *NOTCH1* mutations had no impact amongst cases harboring trisomy 12 (Figure 7). Notably, when we evaluated OS, results were similar. Regarding *MYD88* and *BIRC3* mutations, the limited number of mutated cases precluded definitive conclusions.

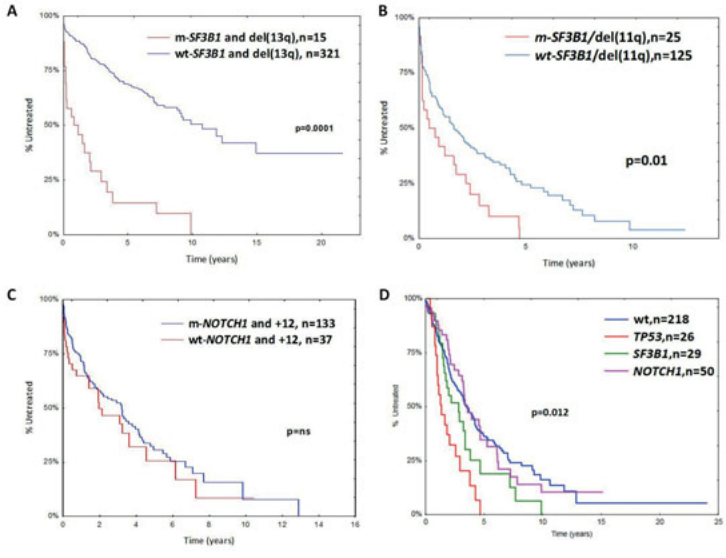

Figure 7. Kaplan Meier curves for time-to-first-treatment (TTFT) in Binet A patients. (A) Impact of *SF3B1* mutations within cases carrying del(13q). (B) Impact of *SF3B1* mutations within cases carrying del(11q). (C) Impact of *NOTCH1* mutations within cases carrying trisomy 12. (D) Impact of novel recurrent mutation within U-CLL.

Figure 8. *TP53*, *SF3B1* and *NOTCH1* gene mutations, IGHV mutational status and cytogenetic abnormalities; each column represents an individual patient and each row corresponds to the indicated lesion. Black: positive, grey: negative, white: no data.

Focusing on the clinicobiological profiles of novel recurrent mutations, we verify and further extend previous findings about the association of *SF3B1, NOTCH1* and *BIRC3* mutations with U-CLL and advanced clinical stage, contrasting *MYD88* mutations that were exclusive to M-CLL. Furthermore, *SF3B1, NOTCH1* and *MYD88* mutations were enriched for specific FISH detected aberrations, namely del(11q), trisomy 12 and del(13q), respectively (Figure 8). *BIRC3* mutations correlated not only with not only del(11q), as earlier reported, but also with trisomy 12, while, interestingly, both *TP53*

and *BIRC3* mutations frequently co-existed frequently (up to 30% and 25%, respectively) with other mutations.
We next attempted to validate the integrated prognostic index proposed by Rossi et al, however risk-groups could not be clearly separated. In particular, no difference was observed between the high- and intermediate-risk groups or between the low- and very-low-risk groups. Taking a step further, through integrating IGHV gene SHM status, we could identify a distinct prognosis for low-risk and very-low-risk groups, highlighting the importance of BcR IG features in the prognostication of CLL (Figure 9).

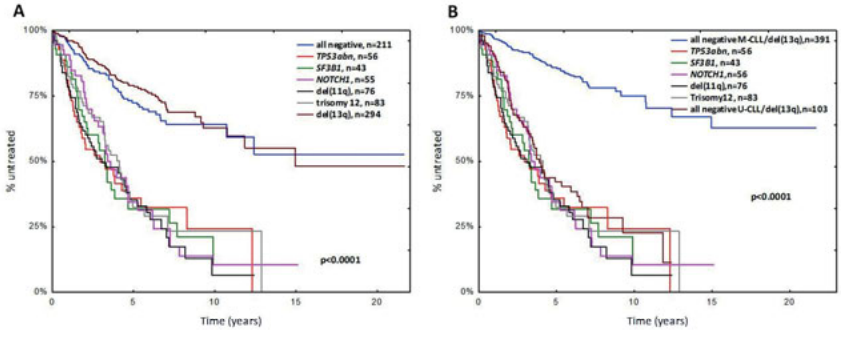

Figure 9. Integration of IGHV gene SHM status and genomic aberrations for assessing the prognosis of Binet stage A patients. Kaplan-Meier curves for time-to-first-treatment. (A) Patients carrying isolated del(13q) (brown line) or lacking any recurrent aberration/mutation (blue line) had a superior outcome, thus contrasting patients with any other recurrent aberration. (B) Patients carrying isolated del(13q) or negative for any recurrent aberration but harboring unmutated IGHV genes (brown line) exhibited similar clinical outcome with patients carrying unfavorable genomic aberrations (n=103/494, 21%) .

Finally, we report that *SF3B1* mutations increase in frequency as the interval between initial diagnosis and mutational screening increases, in contrast to *NOTCH1* mutations which remained stable. This observation indicates that spliceosome dysfunction may be crucial for CLL evolution (Figure 10).

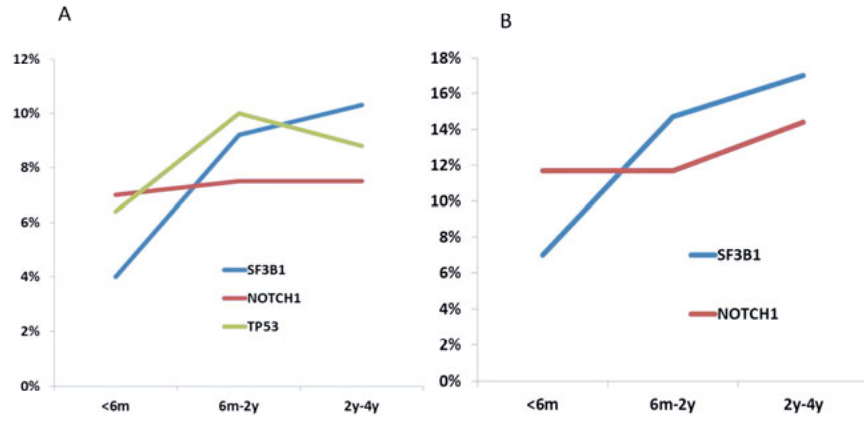

Figure 10. Fluctuation of the incidence of *SF3B1* and *NOTCH1* mutations over time**.** Binet A patients were grouped according to the time elapsed between diagnosis and the time of mutational screening. (A) all cases, (B) cases who have progressed and have since required treatment. *SF3B1* mutations exhibit increased frequency overtime compared to *NOTCH1* mutations.

Limitations

The main limitation of the present study concerns its retrospective nature as well as the fact that the cohort consists of heterogeneous populations of patients who received varying treatments (Table 6). Moreover, the methodology regarding the mutational screening was not uniform in all participating centers, while not all cases were tested for all 5 genes. Finally, the ideal approach to address the issue of clonal evolution would be to analyze longitudinal samples.

Table 6. *Significant differences regarding the spectrum of novel recurrent mutations in CLL depending on the type of the evaluated cohort.*

	Early vs advanced clinical stage			**Untreated vs treated cases**		
	Binet A: n, %	**Binet B/C: n, %**	**p-value**	**Untreated: n (%)**	**Treated: n, %**	**p-value**
NOTCH1	124/1951, 6%	71/535, 13%	<0.0001	33/953, 3.5%	211/1793, 12%	<0.0001
SF3B1	79/1346, 6%	70/387, 18%	<0.0001	23/823, 2.8%	227/1406, 16%	<0.0001
TP53	104/1237, 8%	71/428, 17%	<0.0001	25/646, 3.8%	198/1532, 13%	<0.0001
BIRC3	13/665, 1.9%	9/204, 4.5%	0.05	3/431, 0.7%	20/454, 4%	0.0005
MYD88	16/765, 2%	6/235, 2.5%	ns	11/487, 2.2%	13/540, 2.4%	ns

Paper II: Distinct patterns of novel gene mutations in poor-prognostic stereotyped subsets of chronic lymphocytic leukemia: the case of *SF3B1* and subset #2.

Main findings and conclusions

The main finding of this study is the remarkable enrichment of *SF3B1* and *NOTCH1* mutations within different major, poor-prognostic stereotyped subsets, indicating the acquisition of certain genomic lesions under selective antigen stimulation. In particular, almost 50% of subset #2 cases carried *SF3B1* mutations, whereas *NOTCH1* mutations reached up to 19% and 14% within subsets #1 and #8, respectively (Figure 11). Of note, *SF3B1* mutations were almost absent within subsets #1 and #8, while subset #2 exhibited a very low frequency of *NOTCH1* mutations.

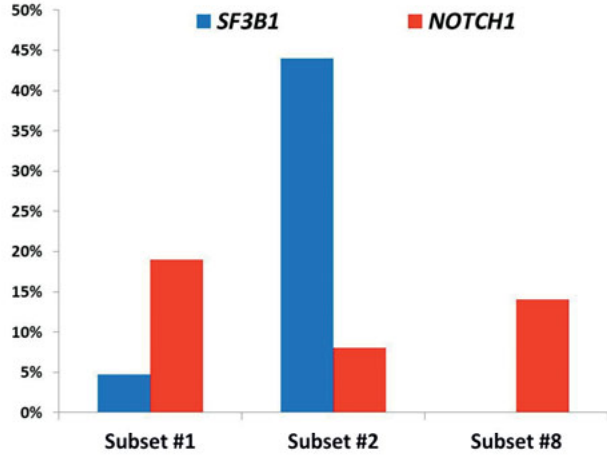

Figure 11. Different distribution of *SF3B1* and *NOTCH1* mutations in clinically aggressive stereotyped subsets.

Regarding their clinical significance, *SF3B1* mutations had no impact on outcome within subset #2, raising the possibility that additional lesions may contribute to the dismal prognosis experienced by patients assigned to this subset. The prognostic significance of *NOTCH1* mutations within subsets #1 and #8 could not be reliably evaluated due to the limited number of cases. Finally, *BIRC3* mutations were extremely rare with no bias to any of the evaluated stereotyped subsets.

Limitations

The main limitation of the present study concerned the low number of tested cases, especially regarding stereotyped subset #8. Moreover, even though the included subsets were selected due to their clinical aggressiveness, they represent only a proportion of the CLL fraction assigned to major stereotyped subsets for which data regarding novel recurrent mutations is still lacking.

Paper III: Not all IGHV3-21 chronic lymphocytic leukemias are equal: prognostic considerations.

Main findings and conclusions

In accordance to previous reports, we verified that >50% of all IGHV3-21 CLL can be assigned to stereotyped subset #2 (Table 7).

Table 7. *Main clinicobiological features of subset #2 vs non-subset #2 IGHV3-21 CLL.*

	#2 (n=212)	non-subset#2/IGHV3-21, (n=143)	p-value
Male	132/254, 62%	87/183, 61%	ns
Age (median)	63.5 years	62.2 years	ns
Binet A	72/156, 46%	50/81, 61%	0.023
U-CLL	86/212, 40%	84/143, 59%	0.0007
GI:97-97.99%	83, 33%	19, 10%	<0.0001
CD38 expression	35/92, 38%	31/73, 42%	ns
ZAP70 expression	23/54, 43%	16/33, 48%	ns
del(13q)	50/81, 61%	27/51, 53%	ns
Trisomy 12	5/103, 5%	5/65, 8%	ns
del(11q)	23/109, 21%	10/70, 14%	ns
del(17p)	4/103, 4%	3/69, 4%	ns
TTFT	22 months	60 months	0.001

#2: assignment to stereotyped subset #2; U-CLL: CLL carrying unmutated IGHV genes; GI: Germline identity; del(13q) deletion of chromosome 13q; del(11q):deletion of chromosome 11q; del(17p): deletion of chromosome 17p; TTFT: time-to-first-treatment.

Interestingly, while both groups (i.e. subset #2 and non-subset #2/IGHV3-21) exhibited mixed SHM status, within subset #2 M-CLL, cases with borderline germline identity (GI: 97-97.99%) predominated contrasting non-subset #2/IGHV3-21 M-CLL, which exhibited heavier SHM loads.

Focusing on clinical behavior, subset #2 exhibited significantly shorter TTFT and OS in comparison to non-subset #2/IGHV3-21 CLL, a difference which remained even when the analysis was restricted to cases with similar SHM status or among cases with early clinical stage at diagnosis (Figure 13). Subset #2 cases displayed a similar clinical behavior to non-IGHV3-21 U-CLL, independently of IGHV gene SHM status. In contrast, amongst non-subset #2/IGHV3-21 cases, the clinical outcome was mainly dictated by IGHV gene SHM status, similar to the remaining CLL cases. Interestingly, within M-CLL subset #2, del(11q) was associated with shorter TTFT, while no such difference was observed among U-CLL subset #2 (Figure 12).

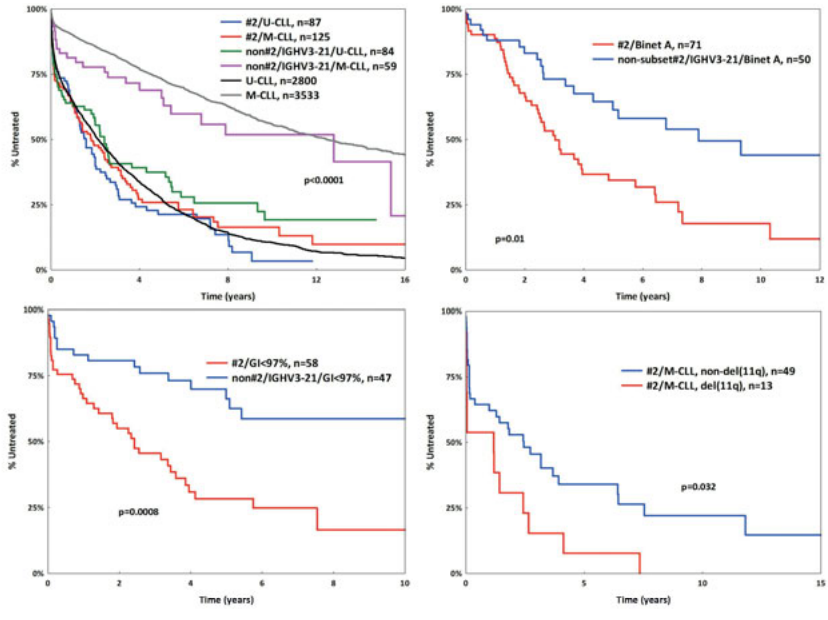

Figure 12. Kaplan Meier curves. A) Time-to-first-treatment (TTFT) for stereotyped subset #2, non-subset #2/IGHV3-21 CLL and non IGHV3-21 CLL in relation to IGHV mutational status. B) TTFT for Binet A subset #2 and non-subset #2/IGHV3-21 CLL cases. C) TTFT for borderline M-CLL non-subset#2/IGHV3-21 CLL cases. D) TTFT for U-CLL subset #2 cases with and without del(11q).

Based on the above, IGHV3-21 gene usage in CLL should not be considered *per se* as an unfavorable prognostic marker. On the contrary, assignment to stereotyped subset #2, which emerges as uniformly aggressive, is associated with dismal prognosis independently of SHM status. Therefore, knowledge regarding subset #2 membership is of primary clinical importance, especially in the context of clinical trials.

Limitations

The main limitation of paper III is the retrospective nature of the study. As a result, even though the question regarding TTFT was adequately evaluated, the lack of data regarding the type of administered treatment limited our ability to address the issue of possible differences regarding treatment response between subset #2 and non-subset #2/IGHV3-21 CLL.

Paper IV: Clinical impact of stereotyped receptors in chronic lymphocytic leukemia.

Main findings and conclusions

Overall, 2878/8593 cases (33.5%) were assigned to stereotyped subsets, with 1122 (13% of the cohort) belonging to one of 14 subsets with at least 20 cases for whom clinicobiological information was available, thus enabling meaningful comparisons (Figure 13). The largest group was subset #2 which displayed a mixed SHM profile and accounted for almost 3% of the whole cohort, while within U- and M-CLL the most populated subsets were subset #1 and #4, respectively.

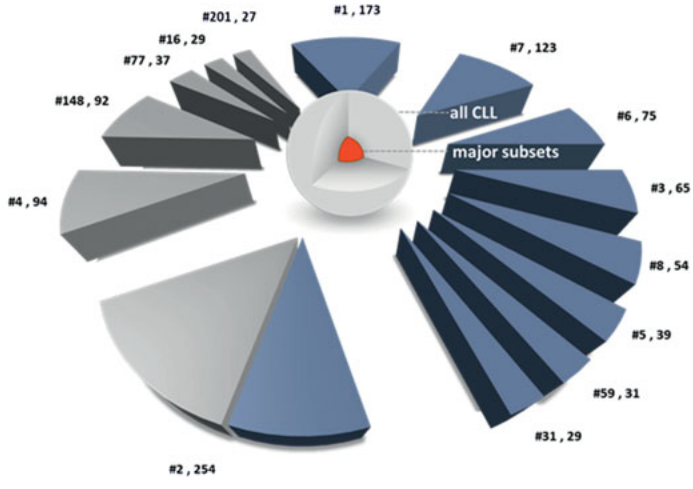

Figure 13. Major stereotyped subsets within the present series. Blue: U-CLL stereotyped subsets; grey: M-CLL stereotyped subsets; stereotyped subset #2 consists of both U- and M-CLL. The numbers of cases in each subset are indicated.

Each stereotyped subset exhibited distinct age and gender distributions as well as distinct profiles regarding clinical stage at diagnosis, CD38 expression and cytogenetic aberrations, differing significantly from remaining CLL cases utilizing the same IGHV gene and with similar SHM status.

Striking examples include: (i) subsets #4 (M-CLL), #31 (U-CLL) and #77 (M-CLL) concerned younger patients (>40% patients younger than 55 years at diagnosis); (ii) subsets #8 (U-CLL) and #201 (M-CLL) displayed a

male/female ratio of 1.0 and 0.85 respectively; (iii) subsets #59 (U-CLL) and #6 (U-CLL) had 31% and 73% of CD38-positive cases, respectively; (iv) certain subsets were enriched for or had a notably absence of certain recurrent cytogenetic aberrations e.g. #77 (M-CLL) and del(13q) (79%), subset #8 and trisomy 12 (60%), subset #8 and trisomy 12 (60%), subset #59 and del(17p) (0%), subset #31 and trisomy 12 (0%), subset #7 and del(11q) (65%).

Interestingly, significant differences were also observed among subsets carrying similar SHM loads and expressing the same IGHV gene e.g. subsets utilizing the IGHV1-69 gene (subsets #3, #5, #6, #7, #59, U-CLL) and those using the IGHV4-34 gene (#4, #16, #201, M-CLL).

In order to evaluate whether the expression of a particular stereotyped IG may be linked to a distinct outcome, beyond IGHV gene SHM status, we assessed TTFT. Focusing on U-CLL, the median TTFT ranged from 1 year in subsets #31 and #59 to 2.7 years in subsets #3 and #7 while within M-CLL median TTFT had not yet been reached for subset #16, whereas it was 11 and 6.7 years for subsets #4 and #201. Again, differences were also significant amongst subsets utilizing the IGHV1-69 or IGHV4-34 gene. Interestingly, in some instances, cases within the same subset experienced a similar clinical course independent of the presence of a particular cytogenetic aberration, i.e., trisomy 12 had no impact on TTFT within subset #8, del(11q) did not correlate with shorter TTFT among subset #3 and #7 cases while, similarly, isolated del(13q) had no impact on TTFT among subset #4 cases. In multivariate analysis regarding TTFT, assignment to subset #2 retained independent significance as an unfavorable parameter not only in the entire cohort but also when the analysis was restricted to early stage patients (Table 8).

Taking a step further, we attempted to integrate BcR IG stereotypy into the well-established Döhner hierarchical model of cytogenetic aberrations. We focused on incorporating subsets #1, #2 and #4 in the model due to their size and also since they represent clear opposites in terms of clinical behavior and are biologically well characterized (Figure 14). These subsets emerged as clinically distinct, even when the analysis was restricted to subset cases negative for aberrations that could influence the results, e.g. including only non-del(11q) subset #2 cases, thus refining prognosis beyond cytogenetic aberrations. Similar results were obtained when the analysis was restricted to early stage patients.

Table 8. *Univariate and multivariate analysis for time-to-first-treatment (TTFT) for the entire cohort and also when restricted to Binet A cases.*

ALL cases (n=1538)	Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
male	1.347	1.243-1.459	<0.0001	1.116	0.970-1.284	0.122
U-CLL	3.742	3.536-4.065	<0.0001	2.898	2.484-3.381	<0.0001
Binet A	0.192	0.176-0.209	<0.0001	0.273	0.237-0.313	<0.0001
#1 assignment	2.121	1.757-2.561	<0.0001	0.984	0.692-1.339	0.937
#2 assignment	2.120	1.814-2.478	<0.0001	1.547	1.155-2.072	0.003
#4 assignment	0.478	0.329-0.694	0.0001	0.769	0.407-1.455	0.42
#148 assignment	0.290	0.177-0.474	<0.0001	0.889	0.417-1.934	0.785
CD38 expression	2.214	2.016-2.433	<0.0001	1.276	1.098-1.483	0.001
del(13q)	0.871	0.767-0.988	0.03	0.976	0.827-1.153	0.782
del(11q)	2.442	2.186-2.728	<0.0001	1.128	0.942-1.355	0.18
Trisomy 12	1.380	1.228-1.551	<0.0001	1.025	0.848-1.238	0.79
del(17p)	1.847	1.605-2.125	<0.0001	1.302	1.048-1.617	0.016
BINET A cases (n=1043)	HR	95% CI	p-value	HR	95% CI	p-value
male	1.202	1.067-1.354	0.002	1.160	0.963-1.395	0.116
U-CLL	4.324	3.853-4.852	<0.0001	4.084	3.321-5.021	<0.0001
#1 assignment	2.434	1.746-3.395	<0.0001	0.846	0.507-1.410	0.521
#2 assignment	2.165	1.616-2.900	<0.0001	3.012	1.865-4.864	<0.0001
#4 assignment	0.564	0.349-0.910	0.01	0.815	0.414-1.603	0.55
#148 assignment	0.317	0.142-0.709	0..005	1.024	0.365-2.875	0.963
CD38 expression	2.593	2.222-3.024	<0.0001	1.552	1.248-1.930	0.0001
del(13q)	0.770	0.653-0.910	0.02	1.144	0.911-1.436	0.224
del(11q)	2.848	2.369-3.425	<0.0001	1.469	1.118-1.930	0.0057
Trisomy 12	1.491	1.241-1.792	<0.0001	0.954	0.722-1.260	0.742
del(17p)	1.666	1.335-2.080	<0.0001	1.206	0.882-1.650	0.24

HR: hazard ratio; 95% CI: 95% confidence interval; del(13q): deletion of chromosome 13q; del(11q): deletion of chromosome 11q; del(17p): deletion of chromosome 17p; U-CLL: CLL carrying unmutated IGHVgenes.

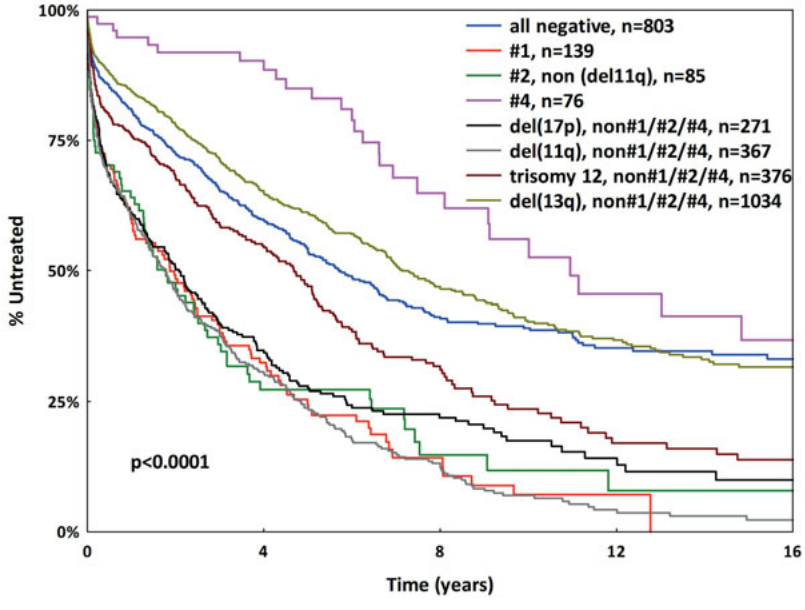

Figure 14. Immunogenetics refines the cytogenetic risk stratification of CLL. Time-to-first-treatment (TTFT) Kaplan-Meier curves for major stereotyped subsets #1, #2 and #4 as well as groups defined according to the Döhner hierarchical aberrations model. Distinct clinical outcomes for major stereotyped subsets were observed, independent of genomic aberrations.

The major finding of the present study is that the immunogenetic sub-classification of CLL based on BcR IG stereotypy is clinically relevant. Indeed, our results demonstrate that stereotypy: (i) defines distinct clinical entities, (ii) refines the Döhner hierarchical model of cytogenetic aberrations (currently the gold standard model for prognostication in CLL), and (iii) supersedes the crude M-CLL versus U-CLL distinction which can seriously underestimate the heterogeneity of CLL for those cases that fall into stereotyped subsets. This compartmentalized approach facilitates the grouping of patients into more homogeneous subsets that exhibit consistent and subset-biased profiles, including age and gender distribution, disease burden at diagnosis, cytogenetic aberrations and timing of clinical progression.

Limitations

Similar to paper III, the main limitation of paper IV is that it is based on retrospective data. Moreover, some of the comparisons concerned relatively small subgroups, despite this being the largest cohort ever evaluated in CLL.

Concluding remarks

Taking into consideration the remarkable clinical heterogeneity of CLL, it is no surprise that numerous efforts have been made towards the identification of prognostic markers that could empower clinicians in everyday clinical practice. Throughout the years, various biological features related to both cell-intrinsic and cell-extrinsic mechanisms have been proposed as prognostically relevant with cytogenetic aberrations and the immunogenetic signature of the clone being the most widely accepted.

Regarding genomic aberrations, the recent identification of novel recurrent mutations has revealed a complex genomic landscape. However, the prognostic relevance of these "new players" is still a matter of debate and thus, these new players have yet to be incorporated into clinical routine. Consequently, *TP53*abs are still the only aberration influencing clinical decisions. While efforts have been undertaken to address this matter, a major caveat or obstacle concerned relatively small cohorts as well as lack of integration with other biological parameters such us IGHV gene mutational status. Therefore, we conducted a multi-institutional study in order to overcome these limitations and report that, in addition to *TP53*abs, mutations within the *SF3B1* gene emerge as a strong prognosticator for fast clinical progression.

Focusing on the immunogenetic signature of BcR, a feature stable over time and unaffected by the disease evolution, we argue that stereotyped subsets are distinct entities with specific clinicobiological features, superseding not only the rather crude discrimination between M-CLL and U-CLL but also genomic abnormalities (as prognosticators) in some cases. Interestingly, the remarkable association of certain genomic aberrations with stereotyped CLL subsets alludes to a subset-biased acquisition of genomic aberrations perhaps in the context of a particular antigenic stimulation.

Overall, we argue that a compartmentalized approach focusing on and comparing different subsets may shed light on CLL biology and clinical behavior and improve our capacity to stratify patients for prognostic purposes. Thus, BcR IG stereotypy could be considered as a companion molecular diagnostic for personalized medicine in CLL, akin to what is already the norm in other hematological malignancies e.g. acute myeloid leukemia. That said, the major difference from molecular stratification schema adopted in other blood cancers is that rather than oncogenetically-oriented, our proposed sub-classification is immunogenetically-oriented: this makes it even

more appealing and relevant, since signaling inhibition has recently emerged as a powerful, non-chemotherapeutic approach towards eventually curing CLL.

Acknowledgments

I am not good at saying “thank you” so I will start with saying “sorry”. “Sorry” to all who I may forget to mention in the following lines.

During the last years I have received enormous support from many people and for that I am grateful. Without their help, completing my PhD studies would not be feasible. Probably I would have “lost myself” somewhere between my moving to Sweden, my decision to obtain a second specialty, my personal worries regarding the impact of my choices on the lives of others, mostly my family. It has been a very exciting and demanding period full of lists with “things to do”, long-lasting discussions, “productive” stress and thankfully many moments of joy.

Richard, I do not know if you remember our first discussion almost 4 years ago. I certainly do. Thank you for being honest from day one. I mostly thank you for allowing me to test myself, to take responsibilities, to make mistakes. Mostly thank you for helping me stay on the right track.

Kostas, thank you for being “here”, even if you were physically some thousands miles away. You were the one who inspired me when I was still a resident in hematology and “taught” me the basic principles of research. I dare to say that you are the person “responsible” for my scientific development.

Lesley, thank you for helping me with all the “details”, which were actually of major importance. Thank you for your patience in trying to understand what I really want to say, your patience in trying to explain what you really mean, mostly for protecting me from the wrong choices. Of course, thank you for tolerating my ignorance on everything that has to do with technology…..

Larry, thank you for being patient enough to teach me the basic on statistical analysis; that had to be challenging…Viktor, thank you for spending hours of your free time to help me prepare figures, thank you for being kind enough trying to understand my Swedish….Sujata, thank you for all your positive thinking…Diego, thank you for our discussions… Emma, thank you for “taking care” of me…. Mattias, thank you for sharing with me the Swedish “clinical reality”.

Aliki, thank you for being next to me, reminding me that friendship can overcome distances.

Great thanks to all my colleagues in the Medical Genetics Department. You all have been so helpful making me feel welcomed from the first day. I

consider myself lucky working with people who really believe in what they are doing. Special thanks to all BMA, Lucia, Marie, Monica, Mattias, Anette, Helen, Cate, Ylva, Christos and Stefanos.

I feel the need to express my deep gratitude to all my friends, who have tolerated me the past three years. Thank you, Katerina and Kostas, and so many others.

Before the end, I would like to thank the family that raised me: my father, mother and brother. Thank you all three, for cleaning up the mess I have been causing since I was a child. Finally, my greatest "thank you" and my most honest "sorry" to Stavroula, Thanasis and Eleni. As long as you are next to me, "there is no mountain high enough"……

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