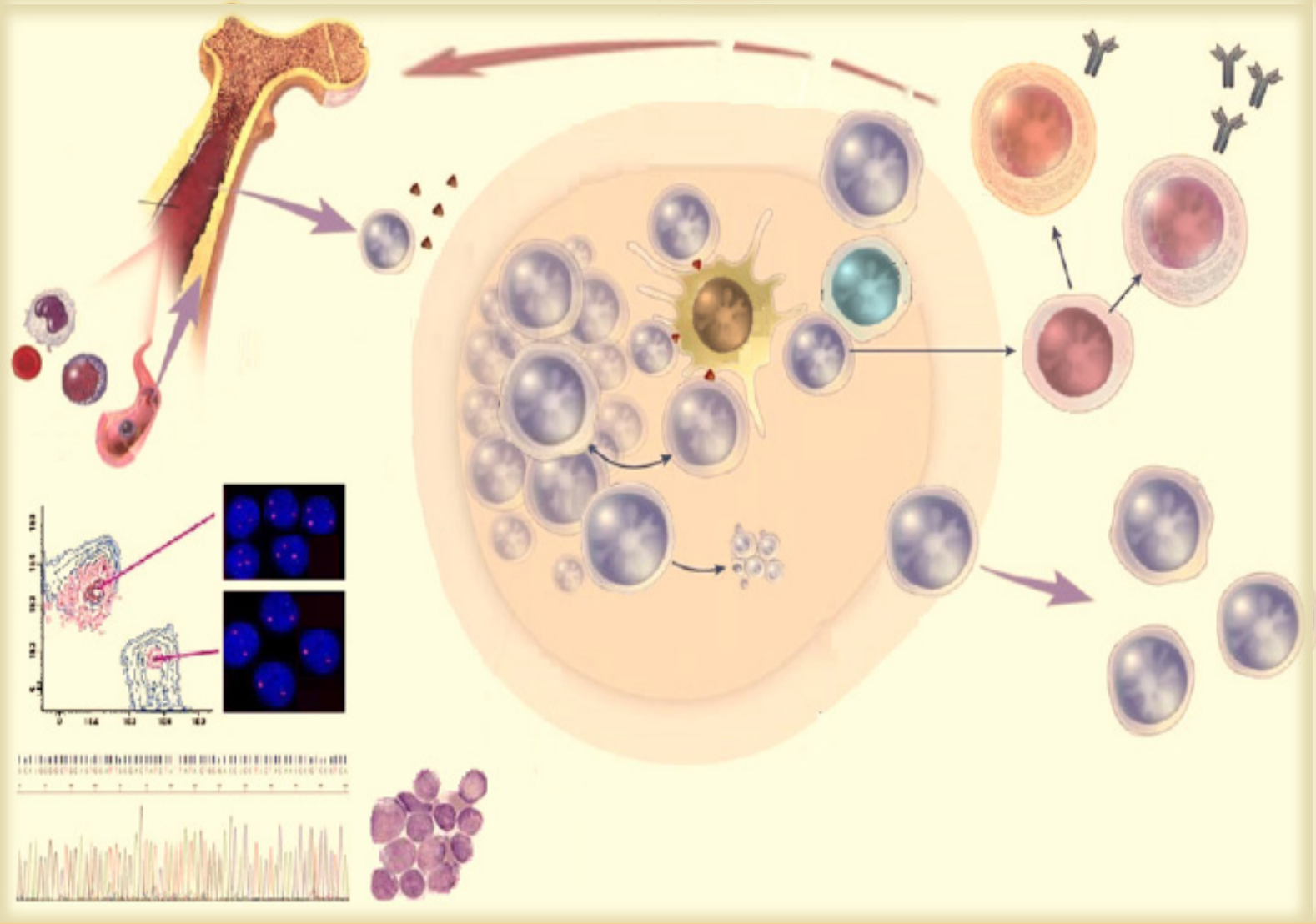

Ana Filipa Parreira Carvalheira dos Santos Henriques

Immunophenotypic, genetic and molecular characterization of B-cell chronic lymphoproliferative disorders: multiclonal versus monoclonal nature

Tese de Doutoramento em Biociências, ramo de especialização em Biologia Celular e Molecular, orientada pelo Professor Doutor Alberto Órfão e pelo Professor Doutor Carlos Faro e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Julho 2014

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Aos meus avós,
Aos meus pais,
À minha irmã

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"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis."

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TABLE OF CONTENTS

Abbreviations

A

A: Adenine
aa: Amino acid
ADCC: Antibody-dependent cell-mediated cytotoxicity
AF700: AlexaFluor 700
Ag: Antigen
AID: Activation-induced cytidine deaminase
ALK: Anaplastic lymphoma kinase
AML: Acute myeloid leukemia
APC: Allophycocyanin
APRIL: Proliferation-inducing ligand
APS: Automated population separator
ATM: Ataxia telangiectasia mutated

B

BAFF: B-cell activating factor
BCL2: B-cell lymphoma/leukemia 2
B-CLPD: B-cell chronic lymphoproliferative disorders
BCR: B-cell receptor
BDB: Becton/Dickinson Biosciences
BM: Bone marrow
B-NHL: Non-Hodgkin B-cell lymphoma
BSAP: B-cell-specific activator protein

C

C: Cytosine
CDK4: Cyclin-dependent kinase 4
CDR: Complementary-determining region
CLL: Chronic lymphocytic leukemia
CMV: Cytomegalovirus
CSR: Class switch recombination
CTLA-4: Cytotoxic T-lymphocyte antigen 4
Cy: Intracellular

D

D: Diversity
DLBCL: Diffuse large B-cell lymphoma
DNA: Deoxyribonucleic acid
ds: Double stranded

E

EBF: Early B-cell factor
EBV: Epstein–Barr vírus

F

FBXW7: F-box and WD repeat domain containing 7
FITC: Fluorescein isothiocyanate
FL: Follicular lymphoma
FR: Framework region
FSC: Forward light scatter

G

G: Guanine
GC: Germinal center
GEP: Gene expression profile

H

H: Heavy chain
HC: Hairy cell
HCDR3: Heavy chain complementary-determining region 3
HCL: Hairy cell leukemia
HCLv: Hairy cell leukemia variant
HCV: Hepatitis C virus
HHV8: Human herpes virus 8
HIV: Human immunodeficiency virus
HL: Hodgkin lymphoma
HLA: Human leukocyte antigen
HS1: Specific Lyn substrate 1
HSA: Heat stable antigen
HSC: Hematopoietic stem cell

I

iFISH: Interphase fluorescence *in situ* hybridization
Ig: Immunoglobulin
IGHV: Immunoglobulin heavy chain variable region
IGHC: Immunoglobulin heavy chain constant region
IL: Interleukin
IMGT: ImMunoGeneTics (data base)

J

J: Joining

K

KLHL6: Kelch-like family member 6

L

L: Light chains
LDL: low-density lipoprotein
LN: Lymph nodes
LPL: Lymphoplasmacytic lymphoma

M

mAb: Monoclonal antibody
MALT: Mucosa-associated lymphoid tissue
MBL: Monoclonal B-cell lymphocytosis
MBL^{high}: MBL with absolute B-lymphocytosis or high count MBL
MBL^{low}: MBL without absolute B-lymphocytosis or low count MBL
MCL: Mantle cell lymphoma
MDM2: E3 ubiquitin-protein ligase
MDS: Myelodysplastic syndrome
MEAC: Myosin-exposed apoptotic cell
MFC: Multiparameter flow cytometry

MGUS: Monoclonal gammopathy of undetermined significance
miRNA: Micro ribonucleic acid
MM: Multiple myeloma
mRNA: Messenger ribonucleic acid
MYD88: Myeloid differentiation primary response gene 88
MYHIIA: Non-muscle myosin heavy chain IIA
MZL: Marginal zone lymphoma

N

NLR: Nucleotide oligomerization domain-like receptors
NMZL: Nodal marginal zone lymphoma

O

ORF: Open reading frame

P

PacB: Pacific blue
PacO: Pacific orange
PB: Peripheral blood
PBM-MZL: Primary bone marrow marginal zone lymphoma
PC: Principal component
PCA: Principal component analysis
PCR: Polymerase chain reaction
PE: Phycoerythrin
PE-Cy7: PE–cyanin 7
PerCPCy5.5: Peridinin chlorophyll protein-cyanin 5.5
PLL: B-cell prolymphocytic leukemia

R

R: Replacement mutations
RAG: Recombinase activating gene protein
RB: Retinoblastoma gene
RF: Reading frame
RSS: Recombination signal sequence

S

S: Silent mutation
SF3B1: Splicing factor 3b subunit 1
SHM: Somatic hypermutation
SLL: Small lymphocytic lymphoma
Sm: Surface membrane
SMZL: Splenic marginal zone lymphoma
SO: Spectrum orange
SpA: *Staphylococcus aureus* protein A
ss: Single-stranded
SSC: Sideward light scatter
SSCb: Sodium chloride citrate buffer

T

T: Thymine
TD: T cell-dependent
TdT: Terminal deoxynucleotidyl transferase
TI: T cell-independent
TIR8: Toll IL-1R 8
TLR: Toll-like receptor
TP53: tumor protein p53

U

U: Uracil

V

V: Variable

W

WBC: White blood cell
WHO: World Health Organization
WGS: Whole-genome sequencing
WM: Waldenström macroglobulinemia

X

XPO1: Exportin 1

Abstract / Resumo

Abstract

Increasing knowledge exists about the mechanisms involved in the pathogenesis of B-cell chronic lymphoproliferative disorders (B-CLPD). Generally, tumor cell survival and/or proliferation depend both on the genetic abnormalities of neoplastic cells and the tumor microenvironment. Therefore, the development and widespread of molecular techniques for the characterization of both tumor cell genetic alterations and B-cell receptor (BCR) features, have been pivotal in the understanding of B-CLPD. As a consequence, chronic lymphocytic leukemia (CLL) is now considered as the prototype for several B-cell diseases where microenvironmental interactions, rather than a specific genetic abnormality, are critical in the onset, expansion and even progression of the disease, in at least a fraction of cases. Thus, a biased repertoire of the immunoglobulin heavy chain variable region (*IGHV)* genes with a particular mutational status, or even closely homologous antigen (Ag) binding sites among otherwise unrelated cases ("stereotyped" BCR), is generally considered as evidence for the involvement of a limited set of Ags, superantigens or both, in the development of CLL, fostering research about the early phases of the disease, e.g. monoclonal B-cell lymphocytosis (MBL). In this regard, flow cytometry has facilitated the identification of MBL cases with (MBL^{high}) or without (MBL^{low}) absolute B-lymphocytosis which precedes most CLL cases, allowing the investigation of potential mechanisms involved in the transition from such MBL precursor states to overt CLL. Since tumorigenesis is a multi-step process, the first transforming events may occur at earlier stages, either directly in the normal counterpart of a CLL cell or perhaps, even in the hematopoietic stem cell compartment of CLL patients.

In order to address this issue, in the present doctoral thesis we investigated multiple phenotypic and BCR features of clonal B-cells and their microenvironment in a relatively large series of MBL, CLL/B-CLPD clones, from both monoclonal and multiclonal cases. In order to explore whether particular Ag could be involved in specific cytogenetic pathways during early oncogenesis, we first investigated the potential association between unique cytogenetic profiles and specific *IGHV* repertoires. In a second step, we compared the BCR and cytogenetic features of B-cell clones from monoclonal vs. multiclonal cases to determine whether or not the latter were associated with a higher BCR homology, potentially reflecting occurrence of B-cell mediated immune responses. Finally, we compared the features of stereotyped vs. non-stereotyped MBL and CLL cases.

Overall, we detected three major groups of clones with distinct but partially overlapping patterns of *IGHV* gene usage, mutational status and cytogenetic alterations: 1) a group enriched in MBL^{low} clones expressing specific *IGHV* genes (e.g. *VH3-23*) with no or isolated good-prognosis cytogenetic alterations; 2) a group which mainly consisted of MBL^{high} and advanced stage CLL with a skewed, but different, *IGHV* gene repertoire (e.g. *VH1-69*),

often associated with complex karyotypes and poor-prognosis cytogenetic alterations, and; 3) a group with intermediate features, prevalence of mutated *IGHV* genes and higher numbers of del(13q)$^{+}$ clonal B-cells. Altogether, these results suggest that BCR features of CLL-like B-cell clones may modulate the type of cytogenetic alterations acquired by the transformed cell, their rate of acquisition, and potentially also, their clinical consequences.

As referred above, recent findings support the existence of underlying chronic B-cell stimulation by a restricted set of epitopes in CLL. In line with this, expansion of ≥2 B-cell clones has been frequently reported in B-CLPD, mainly in MBL, which could be an epiphenomenon of a chronic and persistent antigenic stimulation. Thus, we hypothesized that multiclonality could be associated with particular BCR features indicating a greater probability of interaction with shared immunological determinants. Comparative analysis of CLL-like and non-CLL-like B-cell clones from multiclonal vs. monoclonal MBL, CLL/B-CLPD cases showed clonotypic BCR of multiclonal cases have a slightly higher degree of HCDR3 homology, together with unique hematological and cytogenetic features, which are typically associated with earlier disease stages. Among these cases a subgroup of phylogenetically related (coexisting) B-cell clones which displayed unique molecular and cytogenetic features, was identified. Altogether, these results would support the Ag-driven nature of such multiclonal B-cell expansions and the potential involvement of multiple epitopes in promoting the development of MBL and favor their progression into full disease (e.g. CLL). However, the scenario in which these events occur, remains unknown.

In order to gain insight into the above scenario in the last part of our work we further investigated the potential relationship between an altered/clonal hematopoiesis and antigenic driving forces, during the expansion of stereotyped vs. non-stereotyped CLL and CLL-like MBL clones. Overall, former cases more frequently used *IGHV1* rather than *IGHV3* genes, together with longer HCDR3 and unmutated *IGHV* sequences. The overall size of the stereotyped B-cell clones in peripheral blood (PB) did not appear to be associated with their cytogenetic profile but it was more closely related to presence of myelodysplasia-associated immunophenotypes on PB myeloid cells. Such unique association suggests that the emergence and/or expansion of CLL-like B-cell clones in these stereotyped cases could be favored by an underlying altered hematopoiesis.

In conclusion, our results highlight the potential involvement of different Ag-driven pathways in the early stages of development of MBL and transformation to CLL, where BCR recognition of multiple epitopes together with the co-existence or not of an underlying altered hematopoiesis, would modulate further patterns of acquisition of cytogenetic alterations in the pathway to CLL, through different transitional stages from multiclonal MBL to monoclonal CLL clones carrying more complex cytogenetic profiles.

Resumo

Hoje o conhecimento dos mecanismos envolvidos na patogenia das doenças linfoproliferativas crónicas de célula-B (B-CLPD) assume uma importância crescente. De forma geral, a sobrevivência e/ou proliferação da célula tumoral depende tanto das anomalias genéticas das células neoplásicas como do microambiente tumoral. Neste sentido, o desenvolvimento generalizado de técnicas moleculares para a caracterização quer das alterações genéticas presentes nas células tumorais, quer das características do recetor das células B (BCR), mostrou-se fundamental. Como consequência, a leucemia linfocítica crónica (CLL) é hoje considerada como o protótipo para várias doenças de células B em que as interações com o microambiente, mais que a presença de uma anomalia genética específica, são cruciais no surgimento, expansão ou mesmo na progressão da doença, em pelo menos uma fração dos casos. Neste sentido, a existência de um repertório de genes da região variável da cadeia pesada da imunoglobulina (*IGHV*) tendencioso juntamente com um estado mutacional particular e a recente identificação em casos não relacionados de locais de ligação ao antigénio (Ag) praticamente homólogos (BCR "estereotipados") é, regra geral, indicativo do envolvimento de um conjunto limitado de Ags, superantigénios ou ambos, no desenvolvimento da doença, fomentando a investigação das fases iniciais da mesma, p.e., através do estudo da linfocitose monoclonal de células B (MBL). Por isso, a citometria de fluxo veio facilitar a identificação de casos de MBL com (MBL^{high}) ou sem (MBL^{low}) linfocitose B absoluta, a qual precede a maioria dos casos de CLL, permitindo assim a investigação de potenciais mecanismos envolvidos na transição de tais estados precursores tipo MBL, para CLL. Uma vez que a tumorigénese consiste num processo em várias etapas, os primeiros eventos transformantes podem ainda ocorrer em etapas mais precoces, quer diretamente na contrapartida normal da célula de CLL ou talvez, mesmo no compartimento de células estaminais hematopoiéticas de doentes com CLL.

Para resolver esta questão, na presente tese de doutoramento investigámos múltiplas características fenotípicas e do BCR de células B clonais assim como do seu microambiente, numa série relativamente ampla de clones MBL, CLL/B-CLPD, tanto de casos monoclonais como multiclonais. De forma a explorar se determinados Ags poderão estar envolvidos em vias citogenéticas específicas durante as fases inicias do processo oncogénico, na primeira parte do estudo, focámos o nosso interesse na potencial associação entre determinados perfis citogenéticos e repertórios *IGHV* específicos. Num segundo passo, foram comparadas as características do BCR e as alterações citogenéticas dos clones de células B de casos monoclonais vs. casos multiclonais para determinar neste último grupo de doentes, a possível existência de uma maior homologia nos BCR que fosse potencialmente indicadora da

ocorrência de respostas imunes mediadas por células B. Por fim, comparámos as características dos casos com clones MBL e CLL estereotipados vs. não estereotipados.

De uma forma geral, foram detetados três grupos principais de clones com padrões distintos, mas parcialmente sobrepostos, relativamente ao uso dos genes *IGHV*, ao estado mutacional desses genes e às alterações citogenéticas: 1) um grupo enriquecido em clones MBL^{low} expressando genes *IGHV* específicos (p.e. *VH3-23*) sem alterações citogenéticas ou com alterações isoladas de bom prognóstico; 2) um grupo principalmente constituído por clones MBL^{high} e estágios avançados de CLL com um repertório *IGHV* restrito, mas diferente (p.e., *VH1-69*), muitas vezes associado com cariótipos complexos e alterações citogenéticas de mau prognóstico, e; 3) um grupo com características intermédias, com prevalência de genes *IGHV* mutados e com números mais elevados de células clonais B $del(13q)^{+}$. Estes resultados sugerem que as características do BCR de clones de células B com fenótipo de CLL podem modular o tipo de alterações citogenéticas adquiridas pela célula transformada, a sua taxa de aquisição, e eventualmente também, as suas consequências clínicas.

Tal como referido anteriormente, os resultados recentes apoiam a existência em doentes com CLL, de uma estimulação crónica subjacente das células B por um conjunto restrito de epítopos. Neste sentido, expansões de ≥ 2 clones de células B têm sido frequentemente relatadas em B-CLPD, principalmente na MBL, a qual parece constituir um epifenómeno de estimulação antigénica crónica e persistente. Assim, foi colocada a hipótese de a multiclonalidade se encontrar associada com características particulares do BCR indicando uma maior probabilidade de interação com determinantes imunológicos partilhados. A análise comparativa de clones de células B com fenótipo de CLL e com fenótipo não-CLL de casos de MBL, CLL/B-CLPD multiclonais vs. monoclonais mostrou que, nos casos multiclonais o BCR clonotípico apresenta um grau ligeiramente maior de homologia de HCDR3, juntamente com características hematológicas e citogenéticas únicas, que estão tipicamente associadas com os estágios iniciais da doença. De entre estes casos foi ainda identificado um subgrupo de clones de células B (coexistentes) filogeneticamente relacionados que exibiam características moleculares e citogenéticas únicas. No seu conjunto, esses resultados apoiariam a natureza de tais expansões de células B multiclonais associada ao Ag e o potencial envolvimento de múltiplos epítopos em promover o desenvolvimento da MBL e favorecer a sua progressão para doença (p.e., LLC). No entanto, o cenário no qual podem ocorrer esses eventos permanece desconhecido.

De forma a ganhar um maior conhecimento acerca deste cenário, na última parte do nosso trabalho, investigamos ainda a potencial relação entre uma hematopoiese alterada/ clonal e o estímulo antigénico durante a expansão dos clones de CLL e MBL estereotipados vs. não estereotipados. No geral, os casos estereotipados exibiam mais frequentemente genes *IGHV1* em vez de *IGHV3*, juntamente com sequências HCDR3 mais longas e genes *IGHV* não mutados. O tamanho dos clones de células B estereotipados no sangue periférico (PB) não

mostrou estar relacionado com o seu perfil citogenético, mas sim com a presença de imunofenótipos associados com mielodisplasia em células mielóides do PB. Tal associação particular sugere que o surgimento e/ou expansão de clones de células B de CLL nestes casos estereotipados pode ser favorecido por uma hematopoiese alterada subjacente.

Em conclusão, os nossos resultados destacam o potencial envolvimento de diferentes vias induzidas pelo Ag nos estágios iniciais de desenvolvimento da MBL e de transformação para CLL, onde o reconhecimento de múltiplos epítopos pelo BCR, juntamente com a coexistência ou não de uma hematopoiese alterada subjacente, poderão modular os padrões de aquisição de alterações citogenéticas na patogénese da CLL, através de diferentes vias de transição desde os estágios de MBL multiclonal até aos clones de CLL monoclonal com perfis citogenéticos mais complexos.

Key-words / Palavras-chave

Key-words: B-cells, B-cell chronic lymphoproliferative disorders, monoclonal B-cell lymphocytosis, chronic lymphocytic leukemia, multiclonality, immunogenetics, cytogenetics, immunophenotyping.

Palavras-chave: células B, doenças linfoproliferativas crónicas de célula B, linfocitose monoclonal de células B, leucemia linfocítica crónica, multiclonalidade, imunogenética, citogenética, imunofenotipagem.

Chapter 1 | GENERAL INTRODUCTION

B-cell chronic lymphoproliferative disorders (B-CLPD) are a heterogeneous group of diseases with a highly variable clinical course.[1] Despite the well-defined clinical, biological and histopathological features of the distinct World Health Organization (WHO) clinical entities, the specific factors associated with the ontogeny of these disorders still remain largely elusive.

As in other tumors, chromosomal and molecular/genetic alterations, particularly those genetic mutations and chromosomal translocations involving the immunoglobulin (Ig) heavy chain genes and to a lower extent also the light chain gene loci, and their distinct partnering proto-oncogenes, are a hallmark of many types of B-cell lymphoma.[2] In recent years, important progress has been made as regards the identification of oncogenic mutations – e.g. *BRAF* and *MyD88* gene mutations in hairy cell leukemia (HCL)[3] and lymphoplasmacytic lymphoma (LPL),[4] respectively – and chromosomal translocations – e.g. t(11;14) in mantle cell lymphoma (MCL)[5] –. However, for other B-CLPD such as B-cell chronic lymphocytic leukemia (CLL), despite extensive research has been done, no universal oncogenic alteration has been identified thus far.[6] In this regard, several factors other than genetic/chromosomal alterations have also been associated with the ontogenesis of specific subtypes of B-CLPD. Concerning this, tumor cells from most chronic B-cell leukemias and non-Hodgkin B-cell lymphomas (B-NHL) express a unique B-cell receptor (BCR) molecule and in several B-cell malignancies, antigen (Ag) activation of tumor cells through BCR signaling seems to be an important factor in the pathogenesis of the disease.[7] For example, It has been hypothesized that chronic antigenic stimulation could drive CLL development,[8,9] as it has also been proposed for indolent B-cell lymphomas that are supposed to derive from the marginal zone – e.g. gastric lymphomas of mucosa-associated lymphoid tissue (MALT) –. The latter lymphomas are commonly associated with chronic antigenic stimulation either as a result of infection (e.g. *Helicobacter pylori* in the stomach) or autoimmune responses/disease in other MALT lymphomas (e.g. Sjögren syndrome and salivary glands lymphoma).[10]

Immunogenetic analyses of the tumor cell BCR have provided new insights into the ontogenic relationship between B cell malignancies and Ags that they might interact with within their tissue of origin.[11,12] Thus, a biased *IG* gene repertoire is seen as evidence for an underlying selection of progenitor cells by Ag in diseases such as CLL or marginal zone lymphoma (MZL). Additional evidence is provided by the differential prognosis of cases with distinct mutational status of the clonotypic BCR in CLL, and the existence of subsets of patients with highly-selected or even quasi-identical (e.g stereotyped) BCR, which account for up to around one-third of all CLL cases.[13] These observations have been instrumental in shaping the notion that the ontogeny and progression of CLL are functionally driven and dynamic, rather than a simple stochastic process.

Interestingly, a precursor condition for B-CLPD, particularly for CLL, has been identified as a premalignant state: monoclonal B-cell lymphocytosis (MBL) with (MBL^{high}; high count MBL) or without (MBL^{low}; low count MBL) absolute B-lymphocytosis in PB.[14] Extensive research performed in recent years in MBL has also contributed to a better understanding of the mechanisms involved in the genesis of lymphoma/leukemia and the identification of those factors involved in the transition from a B-cell lymphoma/leukemia precursor state to an overt lymphoproliferative disorder. Thus, most MBL cases are characterized by the presence of circulating monoclonal B-cells, which have an immunophenotypic profile that fully overlaps with that of CLL (CLL-like MBL).[15,16] At present, the precise factors and the likelihood of MBL to progress to CLL over time are still largely unknown. In this regard, the overall prevalence of MBL, which is significantly greater than that of CLL, is consistent with the expectation that most MBL will not progress to CLL; even more, for MBL^{low}, progression appears very unlikely,[17] while for MBL^{high} the risk of progression to CLL requiring therapy is of approximately 1% per year.[16,18] In turn, it has also been shown that many MBL are oligoclonal based on interphase fluorescence in situ hybridization (iFISH) but also on single cell Ig sequence analyses;[15,19] in contrast, only around 5% of the B-CLPD display two phenotypically distinct populations of clonally unrelated B lymphocytes coexisting in the same patient, either simultaneously or at different time points during follow-up.[20,21] Such particularly high prevalence of multiclonality at the earliest stages of MBL (≥20% vs. 5%), would further support the potential reactive nature of MBL among individuals with normal lymphocyte counts, prior to stepwise acquisition of genetic alterations and progression to MBL^{high} and CLL;[22-24] such a model could be similar to that occurring in other cancers, indicating that development of CLL might be initiated at a polyclonal B-cell population, one clone progressively taking over. Consequently, the evolution from a reactive to a neoplastic expansion of MBL clones, and their transformation to CLL, might provide a model for the development of CLL, where analyses of *IG* genes and the associated cytogenetic profiles can assist in better understanding the precise mechanisms leading to the genesis of the tumor and its malignant transformation.

In this section, we will first review the BCR structure and repertoire, along the B-cell differentiation; afterward, we will focus on the major features of distinct WHO subtypes of B-CLPD, particularly of CLL and MBL, and their monoclonal vs. multiclonal nature.

1. B-CELL ANTIGEN RECEPTOR

1.1. Basic structure of the B-cell receptor

The BCR for Ags consists of two monomeric molecules: the Ig responsible for Ag binding and CD79 which delivers intracellular signals for B-cell activation. Igs are heterodimer molecules composed of two heavy (H) and two light (L) chains (Figure 1).[25,26] Each H and L chain consists of a variable (V) domain, which binds to the Ag, and between one and four constant (C) domains, which carry out the effector function of that chain. Diversity is asymmetrically distributed within the V domain, each V domain containing three segments of higher variability which form those loops recognizing the Ag termed complementarity determining regions (CDR); CDR are separate one from each other and from the external sequences by four conserved sequences, known as the framework regions (FR) (Figure 1).[27] Two of the CDR loops are encoded by the *V* genes (CDR1 and CDR2) whereas the third, and most polymorphic one, is encoded by the junction between the rearranged *V*, (*D*) and *J* genes. The four FR of both the H and L Ig chains fold to form the scaffold that brings together the three H chain and the three L chain CDRs to create the Ag binding site in the 3-dimensional structure.[26]

Through the transmembrane and intracellular domains of their H chains, Igs are linked in the B-cell membrane to CD79a and CD79b, to form the functional BCR. CD79 is a disulphide-linked transmembrane heterodimer which belongs to the immunoglobulin superfamily and that is responsible for the transduction of BCR signals, upon Ag recognition by B cells.

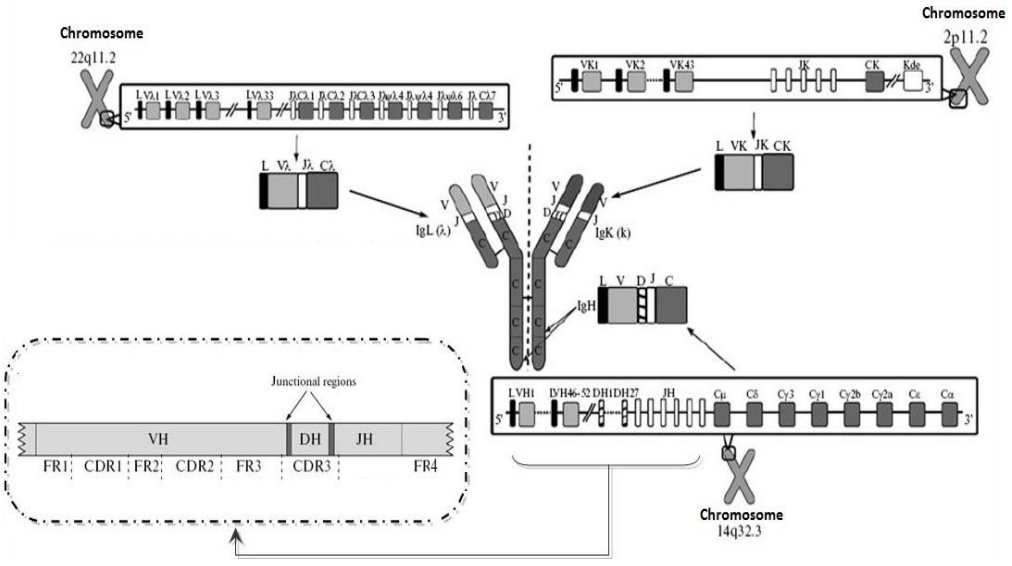

Figure 1. Schematic diagram of the structure of the genetic loci of the immunoglobulin heavy and light (λ and κ) chain genes [adapted from Zakharova *et al.*[28]].

1.2. The B-cell receptor repertoire

Signaling through the BCR is required throughout B cell development, as well as during peripheral B-cell maturation and for the selection of the B-cell repertoire. Additionally, the avidity and the context in which Ag is encountered will determine both cell fate and differentiation in the periphery, once the Ig genes are further diversified during immune responses.[29,30] Consequently, the study of the BCR repertoire by sequence analysis of Ig heavy and light chain gene transcripts can refine the categorization of B cell subpopulations and can shed light on the selective forces that act during aging, immune responses (e.g. infections) or immune dysregulation that result from B-CLPD.[31,32] In contrast to nearly all other proteins, the components of Ig molecules are not encoded by germline DNA. The genetic elements in the Ig loci, the Variable (*V*), Diversity (*D*) and Joining (*J*) genes, need to be rearranged to encode a functional protein essentially through processes of *V(D)J* recombination, exonuclease trimming of germline genes, and the random addition of nucleotides that are not encoded in a DNA template. In the *IGH* locus, one of each *V*, *D* and *J* genes are randomly coupled to form a functional exon while similar rearrangements are initiated between one *V* and one *J* gene segment in the *IGK* and *IGL* loci.[33,34]

While somatic point mutations have given B cell studies a major focus on variable (*IGHV*, *IGLV*, and *IGKV*) genes, *D* genes identified in BCR *VDJ* rearrangements allowed the processes and elements that contribute to the incredible diversity of the Ig heavy chain CDR3 (HCDR3) to be analyzed in detail. Such diversity is in contrast with that of the light chain where a small number of polypeptide sequences dominate the repertoire.[35]

1.2.1. Germline immunoglobulin genes and lymphocyte diversity

The variable locus of the *IG* gene consists of multiple genes which have evolved through gene duplication in order to generate a diverse germline repertoire.[36] Analysis of homology among the *V* gene segments has revealed that these can be grouped in discrete *V* gene families,[37,38] which can further be regrouped in clans[39] which reflect the earliest events of gene duplication in the evolution of the *IG* locus.[40,41] The organization in multiple copies of variable genes, plus the somatic processes of recombination and hypermutation, allow the immune system to generate an antibody repertoire of great diversity. Moreover, selective pressures have shaped the evolution of the germline genes of the Ig. The nature of these selective forces is still a matter of controversy.[42]

Analysis of nucleotide and amino acid (aa) substitutions at the coding region of the *V* genes has shown that the regions involved in the interaction with the Ag present high variability, in contrast to the remaining relatively conserved FRs, pointing out that different selective forces act over these two regions.[36,42,43] Evaluation of other aspects like polymorphisms,[36,44,45] sequence variability[46,47] and phylogeny[39,40,48] has provided additional evidence of selective forces acting over *V* genes in order to shape their variability. In fact, current knowledge about the germline genes is far from being complete; this is due in part, to the complexity of the loci, where numerous highly similar genes are thought to have evolved via gene conversion,[49] duplication and divergence,[50] and further interspersed with many pseudogenes and repetitive elements.

IGHV genes are by far the longest of the recombining *IGH* genes, and they are the main targets of the mutational machinery.[51,52] As it is necessary to be certain of the germline origin of mutated sequences, the complete and accurate definition of the set of germline *IGHV* genes and allelic variants should remain clear. The official human *IGHV* germline gene dataset, created by the ImMunoGeneTics (IMGT) group (www.imgt.org), includes 129 functional genes, open reading frames (ORF), and pseudogenes, as well as over 200 allelic variants which have increased in recent years, as 40 new allelic variants have been reported since 2005.[53-56] In turn, the 27 human *IGHD* genes include 25 functional genes, 23 of which are unique.[57] The *IGHJ* locus includes 6 functional genes, which are all found downstream of the *IGHD* locus in a single cluster.

1.2.2. Biases in combinatorial and junctional diversity and shaping of the BCR repertoire

Many BCR repertoire studies which have utilized different sets of primers, and amplified different source materials, are surprisingly consistent with the occurrence of strong gene utilization biases. Some data show that segments in the *V3* family are most frequently used (namely the *IGHV3-23* gene), followed in descending order by *V4*, *V1*, *V5*, *V2*, *V6*, and lastly *V7*.[58] Different *IGHV* genes are used at frequencies that range from 0.1% to more than 10% of all rearrangements in an individual's naïve B-cell repertoire, their relatively frequency also varying between alleles.[59,60]

Frequencies of usage of some *IGHV* families are surprisingly constant among different individuals (e.g. *IGHV1-46*, *IGHV3-21* and *IGHV3-49*),[61] while *IGHV1-69* varies at frequencies that range from 3.1 to 9.1% (average 6.2%).[59] Biased gene usage is not confined to the *IGHV* genes since *IGHD* gene usage also varies from < 1% (e.g. *IGHD4-4/11*) to > 15% (e.g. *IGHD3-22*)

of total rearrangements.[62] For each *D* segment, there is one reading frame (RF) encoding predominantly hydrophilic aa residues (specially tyrosine and serine; RF1), followed by a hydrophobic RF (RF2), and lastly, a third RF that often encodes a stop codon (RF3). Thus, the RF3 can be used only if either somatic mutations or nucleotide losses during *VDJ* recombination delete the germline stop codon. Finally, there is also considerable variation between the frequencies of usage of *IGHJ* genes (e.g. *IGHJ4* gene is present in approximately 45–50% of rearrangements, *IGHJ6* accounts for 20–25% of *VDJ* rearrangements[63,64] and *IGHJ1* is only used by 1% of all rearrangements).[65]

In a similar way, analysis of *IGK* rearrangements from sequence databases also showed a preferential gene usage with under- and over-utilization of the different *JK* gene segments,[66] while the *IGLV* usage is strongly skewed toward a limited number of the functional *V* segments with 3 of the 30 *IGLV* accounting for > 50% of the expressed rearrangements.[67] Only four of the seven *IGLJ* are considered functional[68] and their frequencies range from almost 55% of the expressed B-cell repertoire for *IGLJ7*, to just 5.5% for *IGLJ1.*[69]

On the other hand, variations in the recombination signal sequences (RSS) also influence the frequencies of BCR gene usage, while they cannot explain all differences in allele utilization.[70-72] In addition to the underlying biases in utilization of germline genes, a final bias has been identified that affects the contribution of recombination frequencies to repertoire diversity. For reasons that still remain unclear, the analysis of 6,500 *IGH VDJ* sequences collected from public databases appears to confirm pairing preferences for some *IGHD* and *IGHJ* genes that increase the frequency of particular *IGHD-IGHJ* pairs within the repertoire (i.e. *IGHD2-2* and *IGHD3-3* with *IGHJ6*, and of *IGHD3-22* with *IGHJ3*).[64,73] In addition, biases in the pairing of germline heavy and light chain genes have been also described in early studies;[74] however, such germline heavy and light chain gene pairing preferences were not supported by later studies,[75,76] including a recent study that applied high-throughput sequencing to generate thousands of linked heavy and light chain gene sequences.[77]

On top of all the above, at present it is also well established that N (non-germline encoded) nucleotides contribute significantly to the diversity of the BCR repertoire.[78] Non-template encoded N-additions are intrinsically biased owing to the nucleotide preferences of the terminal deoxynucleotidyl transferase (TdT) enzyme toward the incorporation of guanine (G) nucleotides; such TdT preference by G nucleotides ensures that the germline gene-encoded regions of the CDR3 are frequently flanked by small aa encoded by G-rich codons such as glycine, that promote flexibility of the CDR3 loop.[79] Exonuclease trimming which results in the loss of nucleotides from the coding ends of the genes during rearrangement is perhaps the least understood process that contributes to the BCR repertoire, but a number of

features of the process have been described, and intrinsic biases have been identified.[80] In this regard, it seems that sequences enriched in adenine (A)/thymine (T) might be more susceptible to nucleotide loss, while G/cytosine (C) enriched sequences would be more resistant to processing.[81-84] The gene sequence ends that remain after exonuclease processing provide a final bias that shapes the repertoire.

Without the added diversity that comes from *D* genes, the kappa and lambda repertoires would be strongly shaped by biased gene usage and minimal processing giving rise to repertoires with a surprisingly limited diversity.

2. B-CELL ONTOGENY

B cells are generated throughout life from long-lived and self-renewing hematopoietic stem cells (HSC) in the bone marrow (BM). B-cell maturation occurs in two clearly defined stages which are localized in different tissues: Ag‑independent precursor B‑cell differentiation from an HSC to naïve mature B-lymphocytes occurs in the BM,[85] whereas Ag-dependent B-cell maturation to memory B-cells and effector plasma cells takes place mostly in secondary lymphoid tissues, e.g. lymph nodes (LN), MALT, BM and spleen (Figure2).[86]

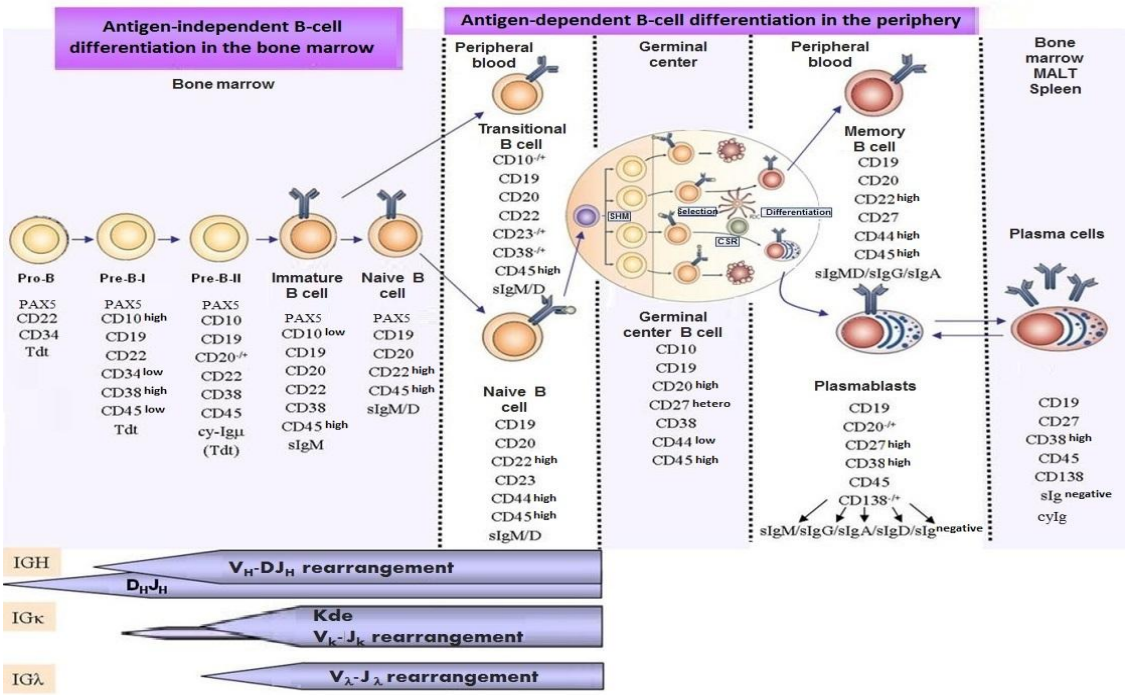

Figure 2. Antigen-independent B-cell differentiation occurs in the bone marrow, whereas Ag-dependent B-cell differentiation occurs in the periphery. The immunophenotypic profile of the distinct human B-cell differentiation stages including *V(D)J* recombination bars are shown for both the BM and peripheral B-cell differentiation pathways [adapted from Vinuesa, *et al*.[87] and Perez-Andres, *et al*.[88]].

2.1. Antigen independent B-cell differentiation in the bone marrow

Differentiation of B cells from early committed progenitors to mature B-lymphocytes is a multistep maturation process that can be monitored by the coordinated acquisition and loss of leukocyte differentiation Ags and the status of rearrangement of the *IGH* and *IGL* genes (Figure 2). The major goal of precursor B-cell differentiation to mature B-lymphocytes is to generate a functional Ig receptor via an ordered *V(D)J* recombination of the genes encoding the Ig heavy (IgH) and the Ig light (Igk or Igʎ) chains. Double stranded (ds)DNA breaks at the *V*, *D* and *J* gene segments are induced by the recombinase activating gene proteins products 1 and 2 (RAG1 and RAG2) that specifically recognize short conserved DNA sequences termed RSS.[89] The first gene rearrangements that occur during precursor B-cell differentiation involve *D* to *J* rearrangements in the *IGH* locus.[90,91] These rearrangements are generally initiated in parallel on both *IGH* alleles.[92] Subsequently, only one of the alleles starts complete *V* to *DJ* rearrangements, whereas the second one only rearranges *V* to *DJ* when the first allele is not successful, e.g. if there is no functional IgH protein. In the majority of precursor B cells, *V* to *J* gene rearrangements in the *IGK* and *IGL* loci are initiated only after a functional IgH protein is formed. Still, it has been demonstrated that a minor fraction of pro-B cells can rearrange *IGL* genes before the assembly of a productive *IGH.*[93,94]

Based on the order of *IG* gene rearrangements, precursor B-cells are classified into distinct stages of maturity (Figure 2). Thus, pro-B-cells represent the first committed B-cell precursors,[95,96] which can be distinguished from pre-pro-B-cells by surface expression of CD19, upon expression of Pax5.[97] In these cells, the Igα–Igβ heterodimer (CD79a/CD79b) is expressed on the cell surface in association with calnexin and potentially also other chaperone molecules.[98]

D to *J* rearrangement in the *IGH* locus is initiated in the pre-pro-B-cells and continues with *V* to *DJ* rearrangement at the pro-B cell stage. The pre-BCR is not required for lineage commitment and the initiation of recombination but, this is rather dependent upon the intrinsic expression of two main transcription factors, E12 and E47,[99] and the transcription factor EBF (early B-cell factor),[100] which have been shown to up-regulate expression of the B-cell-specific genes *λ5,* VpreB, Igα/CD79a and Igβ/CD79b, as well as of the lymphoid-specific RAG-1 and RAG-2, and the B-cell-specific transcription factor Pax5 or BSAP (B-cell-specific activator protein).[101-103] Lineage commitment is enforced at the pro-B-cell stage by Pax5, which both activates B-cell-specific genes (including BLNK, CD19 and Igα/CD79a) and represses the expression of other non-B-lineage genes (including Notch1).[104,105]

Early B-cell development is not entirely intrinsically regulated by the future B-cell precursor, as signalling through the interleukin-7 (IL-7) receptor is required to generate pro-B-cells.[106] IL-7 signalling also induces pro-B-cells to proliferate and expand, and it has been shown to up-regulate expression of CD19 and Pax5.[107,108]

Surface expression of a signalling-competent pre-BCR, containing an in-frame *V(D)J* rearrangement of the Ig heavy chain, allows progression from the pro-B-cell to the pre-B-cell stage; the pre-B cell stage is the first stage at which BCR signalling becomes required. Appropriate pre-BCR signalling results in allelic exclusion at the heavy-chain locus, at the same time it leads to parallel changes in the phenotype of developing B-cells;[109] cells become larger as they undergo a proliferative burst of two to five cycles and become more responsive to IL-7.[110,111] After proliferation, cells enter the small pre-B stage, where they down-regulate HSA (heat stable Ag), CD43 and IL-7R, becoming IL-7 unresponsive. Then, they begin the process of light chain rearrangement, first at the kappa locus and then at the lambda locus.[112]

Upon light-chain rearrangement, heavy and light chains are co-expressed on the cell surface, in association with Igα/CD79a and Igβ/CD79b, to form a functional Ig receptor; subsequently, the new B cell will be positively selected and will become an immature B-lymphocyte. The new IgM^+ IgD^- immature B-lymphocytes frequently carry autoreactive or polyreactive receptors, which need to be removed from the immune repertoire through a BCR receptor-mediated negative selection process. These cells are assumed to either undergo apoptosis/deletion in response to high-avidity ligands, to become anergic if they encounter lower-avidity ligands and unresponsive to Ig receptor crosslinking, or to modify the reactivity of the Ig receptor by initiation of a secondary Ig gene rearrangement (receptor editing).[113-115] Of note, short-lived anergic cells down-regulate surface IgM expression and exhibit a characteristic intracellular signalling signature in association with a unique gene expression profile[116] that appears to be maintained throughout chronic engagement of the BCR with low-avidity ligands. Which of these three tolerance mechanisms is invoked depends on many different factors, including receptor affinity, receptor expression levels, developmental stage and site of encounter (e.g. ligation of the immature BCR in a BM environment results in receptor editing, whereas ligation in a splenic environment induces B-cell deletion).[117,118]

Negative selection of cells with polyreactive and autoreactive BCR takes place during two checkpoints. Thus, a central checkpoint occurs in the BM and results in the removal of cells with both autoreactive and polyreactive BCR. Consequently, the frequencies of autoreactive (≈75%) and polyreactive (≈55%) BCR in early immature B-cells decrease to ≈45% and <10% among immature B-cells, respectively.[119] In turn, a second peripheral checkpoint occurs upon B cell migration from the BM to the periphery, and it is mainly directed against

the remaining autoreactive BCR which often display long (>20 aa) and positively charged IGH-CDR3 regions,[119] reducing their frequency to ≈20% among naïve mature B-cells.

2.2. Antigen dependent B-cell maturation in the periphery

Following successful Ag-independent differentiation in the BM, B cells migrate to peripheral lymphoid organs and recirculate in blood. The cells require external signals for survival, which thereby ensure stable homeostasis of the total B-cell pool.[86,120] Only those cells that recognize their cognate Ag initiate further differentiation and generate memory B-cells and antibody-producing plasma cells.[86] The maturation pathways will differ depending on the anatomic localization of the response (e.g. LN vs. gut, lung or splenic marginal zone) and the type of Ag (e.g. protein vs. polysaccharide).

2.2.1. Peripheral distribution and maturation of immature to naïve B cells

Recent BM emigrants are functionally immature, i.e. they do not respond to BCR stimulation. Immature B-lymphocytes, also referred as transitional B cells, represent ≈5–10% of all B cells in blood of healthy adults and have a characteristic phenotype which includes expression of surface membrane (Sm)IgM and SmIgD, CD21, CD22, CD5 and high expression levels of CD24 and CD38.[121-123] Of note, B lymphocytes leaving the BM consist of cells at different maturation stages between the immature and naïve mature B-cell compartments; therefore, they typically show heterogeneous features; these cells have unmutated *IGHV* genes, express phenotypic features of immature B-cells, show a lower ability to proliferate and differentiate to Ab-secreting cells after *in vitro* stimulation when compared to naïve mature B-cells together with a higher κ/λ ratio vs. other PB B-cell subsets.[121-124] Of note, the frequency of these immature B-lymphocytes in PB seems to increase in autoimmune diseases and other immunological diseases (e.g. systemic lupus erythematosus, common variable immunodeficiency, X-linked lymphoproliferative disease), as well as during BM regeneration after transplantation,[125] in parallel to decreased numbers of memory B-cells.[121-123]

Maturation into pre-naïve B-cells is accompanied by downregulation of CD38 and CD24 which makes them partially responsive to BCR stimulation and CD40 ligation. Upon subsequent downregulation of CD5, pre-naïve B cells finally become naïve B-cells, which are fully responsive to Ag. Naïve B-cells are a relatively frequent B cell compartment in the PB and comprise about 60–70% of circulating B-cells; they simultaneously co-express IgM and IgD and display unmutated *IGV* sequences.

2.2.2. T-cell dependent and T-cell independent B-cell responses to antigen

B cells respond to Ags which are specifically recognized by their BCR. Upon binding to its cognate Ag, the BCR induces downstream signaling through the same pathways as the pre-BCR, to initiate target gene transcription. The CD19-complex, consisting of CD19, CD21, CD81 and CD225, is necessary for sufficiently strong signaling.[126,127]

In addition to Ag recognition via the BCR and CD19 signaling, B cells require a second signal to become activated. Activated T cells can provide such a signal via CD40L that interacts with CD40 on B cells. T cell-dependent (TD) B-cell responses are characterized by germinal center (GC) formation. In the GC, B lymphocytes undergo extensive proliferation, affinity maturation and Ig class switch recombination (CSR).[128] Thus, after the GC reaction, high-affinity memory B-cells and Ig-producing plasma cells are formed.

Alternatively, B cells can respond to T cell-independent (TI) Ags that either activate them via the BCR and another (innate) receptor (TI type 1 response) or via extensive cross-linking of the BCR due to the repetitive nature of the Ag (TI type 2 response). The Ags triggering TI B-cell responses can be both lipid and carbohydrate structures;[129] similarly, the co-stimulatory receptors include various types of receptors particularly pattern recognition receptors, such as Toll-like receptors (TLR) and nucleotide oligomerization domain-like receptors (NLR) that have been implicated in TI responses.[130,131] Usually, TI responses are directed against blood-borne pathogens in the splenic marginal zone and in mucosal tissues (reviewed in [132,133]). Among other proteins and molecules, the B-cell activating factor (BAFF) and the proliferation-inducing ligand (APRIL) protein must likely support TD and TI, as well as induction of affinity maturation and Ig CSR.[134,135]

2.2.3. Somatic hypermutation and Ig class-switch recombination

The Ig variable regions of activated B cells are targets for somatic hypermutation (SHM). In this process of SHM, the activation-induced cytidine deaminase (AID) enzyme is a key player. AID initiates deamination of cytidine to uracil (U) on single-stranded (ss)DNA through preferentially targeting of RGYW and WRCY DNA motifs where R are purine nucleotides, Y are pyrimidines, and W is either A or T.[136,137] Although SHM can be introduced through the entire Ig variable regions, mutations in post-GC cells are preferentially found in the CDR sequences. In part, this is due to overrepresentation of AID-targeted RGYW and WRCY DNA motifs in the Ig CDR[138,139] vs. the FR and other Ig regions,[140,141] and results on selection of GC B-cells with higher affinity for the target Ag, which will therefore have preferentially mutated CDR3. AID-

associated mutations in the DNA sequence of the *IG* gene can either be silent (S) mutations with no effect on the aa composition, or replacement (R) mutations which lead to aa substitutions. R mutations in the FR regions are likely to impair the Ig structure, and cells that acquired these mutations are most frequently removed from the repertoire. In turn, R mutations in the CDR regions can have either positive or negative effects on the recognition and affinity of the BCR for the Ag. Hypermutated B cells that cannot recognize the Ag undergo apoptosis, while those that carry mutations which increase their affinity for the Ag will survive and proliferate. In general, a high ratio of R vs. S mutations (R/S ratio) in the *IGHV* CDR is regarded as a molecular sign of an underlying affinity maturation.[142]

AID does not only play a crucial role in the generation of SHM, but it is also involved in the process of CSR, which leads to changes in the Ig receptor effector functions.[136] In this regard, it should be noted that the *IGH* locus contains multiple constant region-encoding genes downstream of *IGHM*. In precursor and naïve mature B-cells, these regions are not used, and rearranged *VDJ* exons are spliced to the *IGHM* and *IGHD* exons. During the GC response, the B cell is capable of rearranging the Ig switch region upstream of *IGHM* with one of the switch regions upstream, resulting in the deletion of the intervening DNA and splicing of *VDJ* exons to the exons of an *IGHC* other than *IGHM*.

The process of CSR does not affect the Ag specificity and/or the affinity of the BCR, but it influences the effector functions of the antibodies the cell will eventually produce, due to differential recognition of Ig subclasses (e.g. isotypes) by Fc receptors on immune cells and by soluble proteins (e.g. complement proteins). Also the type of Ig subclasses has an impact on the avidity of the Ig, since the ability of IgM and IgA antibodies to form polymers also increases their avidity.[143,144]

IgG is the predominant Ig class in human serum, and can act locally in the tissues. All IgG subclasses are involved in neutralization of pathogens, but only IgG1 and IgG3 are potent activators of the complement system and inducers of antibody-dependent cell-mediated cytotoxicity (ADCC).[145] Complement activation is also the predominant function of IgM, while the two IgA subclasses act as neutralizing antibodies with different susceptibility to digestion by bacterial proteases.[146,147] Finally, IgE is involved in mast cell and basophil sensitization and it is the mediator of allergic responses and of responses to parasitic infections.[148]

2.2.4. Circulating human memory B cells and their diversity

A substantial fraction of B cells in adults (≈20–30% of all PB B-cells) are Ag-experienced and shows hallmarks of memory B-cell. One of these hallmarks is an increased responsiveness

which results from upregulation of co-stimulatory and activation molecules (i.e. CD80, CD86, CD180, TACI), and downregulation of Ig signaling inhibitors (i.e. CD72, LAIR1).[149-151] Moreover, these Ag-experienced cells may display SHM within their *IGHV* and *IGLV* regions and around half of them have also undergone Ig CSR,[152,153] as reflected by surface membrane expression of a switched IgH (e.g. to SmIgG or SmIgA); (23% ± 10% and 21% ± 9% of adult PB memory B-cells express SmIgG and SmIgA, respectively). Meanwhile, the other half of memory B-cells still coexpress SmIgM and SmIgD (52% ± 15% of memory B-cells), or potentially SmIgM or SmIgD only. Even considering that Ig class switching specializes the future effector function of the antibodies that will be produced by Ag-specific B-cells, through replacement of the IgM and IgD gene exons (Cμ and Cδ) by the IgG (Cγ), IgA (Cα), or IgE (Cε) exons via genetic recombination, it should be noted that a small percentage of B-cells (1%–3%) actually class switch from Cμ to Cδ at the genetic level using cryptic switch regions between the Cμ and Cδ exons; this results in an $SmIgM^{-}SmIgD^{+}$ memory B-cell phenotype. Recently the presence of very low numbers of $SmIgE^{+}$ memory B-cells has also been described in PB;[154] murine studies suggest that IgE-secreting plasma cells could be generated both indirectly via CSR to memory $SmIgE^{+}$ B-cells and directly from IgG_1 memory B-cells,[155] although the latter possibility remains controversial.[156]

Until recently, human memory B-cells have been defined based on the expression of the CD27 protein on their surface membrane.[157] However, recent studies have demonstrated that memory B-cells are a more complex and heterogeneous group of B cells than originally thought, and that they can also be $CD27^{-}$; such heterogeneity of memory B-cells probably reflects the fact that they consist of multiple different and diverse subsets originated from functionally distinct types of immune responses.[154,158]

The majority of circulating memory B-cells in healthy adult PB derives from TD responses in the GC. Thus, $CD27^{+}SmIgG^{+}$ and $CD27^{+}SmIgA^{+}$ GC-derived memory B-cells have typically undergone the highest rate of proliferation and SHM; this supports the notion that at least part of the $CD27^{+}SmIgG^{+}$ and $CD27^{+}SmIgA^{+}$ B-cell subsets in healthy adults, occur later in the course of an immune response and/or have undergone multiple immune responses.[128,159] Interestingly, despite these two memory B-cell subsets share selection mechanisms, $CD27^{+}SmIgA^{+}$ B-cells display a clearly higher frequency of *IGHV* gene mutation vs. $CD27^{+}SmIgG^{+}$ B-cells. A potential explanation for such difference might be the different localization of the immune responses which generate most of the $CD27^{+}SmIgA^{+}$ vs. $CD27^{+}SmIgG^{+}$ memory B-cells, since IgA class switching mostly occurs in MALT, while IgG is typically predominant in other lymphoid tissues such as the LN.[160] Compared to $CD27^{+}SmIgA^{+}$ and $CD27^{+}SmIgG^{+}$ memory B-cells, $CD27^{+}SmIgM^{+}$ memory B-cells contain less SHM but show molecular footprints of (early)

GC emigrants that did not undergo CSR[161] and participate in IgM responses initiated early in primary infection. Interestingly, in contrast to these $CD27^+$($SmIgM^+SmIgD^-$) "IgM-only" cells, $CD27^+SmIgM^+SmIgD^+$ "natural effector" B-cells are present in patients with CD40 or CD40L deficiency, indicating that at least part of this subset can be generated independently of T cell help outside of the GC.[131,159] Furthermore, natural effector B-cells resemble prediversified marginal zone populations that can be generated independently of functional GC and that have a limited replication history compared to GC B-cells (both centroblasts and centrocytes) and $CD27^+SmIgD^-$ memory B-cells.[128,159] Thus, $CD27^+SmIgM^+SmIgD^+$ natural effector B-cells probably consist of a mixed population of GC-derived and splenic marginal zone-derived memory B-cells. Moreover, these cells more frequently use a subset of Ig variable region genes which have long been associated with autoreactivity, at the same time, they also show evidences of receptor editing during B-cell development,[162-164] which suggests that these memory B-cells are either generated by a mechanism of immune tolerance or that they evade immune tolerance.

Regarding IgD-only B-cells, at present it is known that these cells have undergone a Cμ deletion due to a non-canonical CSR event, they typically express Igλ, contain extremely high levels of SHM and show a strongly biased *IGHV3-30* gene usage that can be also seen in some malignant B-cell disorders.[165]

In addition to all above subsets of memory B-cells, there are three other minor populations of IgG, IgA and IgE class-switched B-cells which lack CD27 expression (21% ± 10%, 9% ± 6% and <1% of all memory B-cells, respectively), which are present in the PB of healthy individuals.[166] The specific origin of these cells and their relationship to the $CD27^+$ memory B-cell subsets remain currently unknown; overall, these $CD27^-$ memory B-cells show a lower frequency of SHM.[154,158] In addition, $CD27^-SmIgG^+$ B-cells are derived from primary GC-dependent responses and compared to their $CD27^+$ counterparts, they show dominant usage (> 90%) of the *IGHM*-proximal *IGHG1* and *IGHG3* genes, which are potent activators of the complement system and inducers of ADCC, revealing their potential role in autoimmunity.[167] The $CD27^-SmIgA^+$ memory B-cell subset is a smaller population and can be derived independently from T cell help, through TI IgA responses in the splenic marginal zone and locally in the gastrointestinal system.[135] The nature of $CD27^-SmIgE^+$ memory B-cells still remains to be elucidated.[154]

Taking all these findings in consideration, 8 different subsets of antigen-experienced B cells have been described. They all exhibit an activated phenotype but different molecular signs of Ag experience (i.e. levels of SHM of rearranged Ig genes and participation in primary vs. secondary phases of GC responses).

2.2.5. Terminal B-cell differentiation to plasmablasts and plasma cells

Very low numbers of CD20$^{-/+}$ SmIg^{+} CD19^{+} CD27^{high} CD38^{high} CD43^{+} CD138^{-} CD45^{+} HLA-class II^{+} plasmablasts/plasma cells newly generated in the LN and which are derived from activated B cells following a different transcriptional program than memory B-cells, are found in steady state PB of healthy adults.[168] These circulating plasmablasts/plasma cells are induced to circulate for a short period until they reach a niche in the BM, spleen, MALT, LN or chronically inflamed tissues. They ensure regulation of normal Ig production in view of the competition of newborn plasmablasts generated after Ag immunization with older plasma cells for binding to a niche, inducing the old plasma cells to recirculate.[168] In the plasma cells niches, early plasma cells encounter all factors they require to survive and further differentiate into long-living mature (CD20^{-} SmIg^{-} CD138^{+}) plasma cells. Overall, circulating plasmablasts/plasma cells only represent about 1–3% (1–5 cells/μL) of all PB B-cells in healthy adults under steady state conditions, although they can be found at higher frequencies of all circulating B-cells in specific disease conditions associated with active immune responses (e.g. acute infection).[88] Since plasma cells progressively loose membrane BCR expression while maturing, they depend on other mechanisms for long-term antibody production and survival in the BM.[169]

In contrast to memory B-cells, the most represented subset of plasmablasts/plasma cells in PB is that of circulating SmIgA^{+} plasmablasts/plasma cells (49% ± 12% of all PB plasmablasts/plasma cells); SmIgM-only plasmablasts/plasma cells represent around 18% ± 12% and SmIgG^{+} cells are about 13% ± 11% of all PB plasmablasts/plasma cells. The remaining 14% ± 12% of circulating plasmablasts/plasma cells do not express any SmIg.[168,170] Interestingly, presence of circulating IgD^{+}IgM^{-} plasmablasts/plasma cells (<5% of plasmablasts/plasma cells) has been recently reported in the PB of healthy adults,[168] being specifically associated with immune responses involving the upper respiratory tract.[171,172]

3. B-CELL CHRONIC LYMPHOPROLIFERATIVE DISORDERS

B-CLPD consist of a heterogeneous group of clonal/neoplastic conditions associated with the accumulation of variable numbers of mature-appearing clonal B-lymphocytes blocked at distinct stages of B-cell differentiation from transitional/immature B-lymphocytes to plasma cells. The expanded neoplastic B-cells typically combine features of their normal B-cell counterpart, with aberrant characteristics. Whereas the former features reflect the maturation stage and tissue homming profile of equivalent normal B-cells, the latter are frequently related

to primary genetic and molecular alterations occurring during either the early stages of malignant B-cell transformation (e.g. derailment and oncogenic *V(D)J* gene rearrangements) or during antigen-driven GC reactions in lymphoid tissues,[173-175] and/or to secondary genetic events associated with disease progression.

Since expanded B-CLPD lymphocytes are clonal and (frequently) aberrant, at the same time they accumulate in one or more "easily" accessible tissues, such as PB, BM and lymphoid tissues, morphologic and multiparameter flow cytometry (MFC) analyses of B-CLPD have emerged with the years as the most well-suited approaches for the specific identification, enumeration and phenotypic characterization of the expanded tumor cells. In this regard, in recent years, MFC has become particularly relevant because it has proven to be a highly sensitive and specific method for discriminating between normal and leukemic B-cells and the recognition of B-CLPD-phenotypes associated with distinct disease categories, even when the neoplastic B-cells are present at very low frequencies among a major population of normal hematopoietic cells and therefore, may go undetected with conventional microscopic-based morphologic and histologic techniques. Furthermore, MFC requires single cell suspensions, which can be easily obtained from PB samples, LN biopsies and fine needle aspirates, as well as BM aspirated samples.

In contrast to most normal and reactive B-cell populations, expanded mature B-cells from B-CLPD typically display (mono)clonal features including identical *V(D)J* gene rearrangements and Ig light chain expression restricted to Igk or Igʎ.[176] In addition, these cells also display aberrant cell Sm and/or intracellular (Cy) protein expression profiles.[177] Altogether, these features allow for a highly specific and sensitive identification of mature neoplastic B-cells and their distinction from normal cells by MFC.

In the above sections of this introduction chapter we have already described in detail the normal B-cell phenotypes. In turn, in B-CLPD four major types of aberrant phenotypes have been described so far, which include: i) crosslineage Ag expression (e.g. expression of T-/NK-cell-associated markers such as CD2 on neoplastic B-cells); ii) asynchronous expression of markers associated with distinct maturation stages (e.g. Bcl2 high expression on $CD10^+$ GC-like B-cells); and expression of either iii) abnormally high amounts (e.g overexpression of CD305 on hairy cells) or; iv) abnormally low levels – e.g. decreased intensity of staining for CD19 and CD20 in follicular lymphoma (FL) and CLL B-cells, respectively – of B-lineage associated markers.[178] In practice, detailed characterization of mature B-cell-associated aberrant phenotypes, contributes both to the discrimination between normal and neoplastic B-cells, as well as to a more accurate classification of the disease, including the screening for specific genetic alterations for fast subsequent molecular studies.[179]

The current WHO classification of B-CLPD is based on combined assessment of cytomorphological/histopathological, immunophenotypic, and genetic/molecular features of tumor B-cells, together with the clinical characteristics of the disease.[180,181] Thus, in the WHO 2008 classification of hematopoietic and lymphoid neoplasias, B-CLPD are identified as neoplastic conditions of mature lymphoid B-cells, and they are stratified into three major groups of diseases which are mostly related to the pattern of tissue infiltration, including: i) peripheral blood involvement (peripheral/mature chronic B-cell leukemias); ii) infiltration of secondary lymphoid tissues (peripheral/mature B-cell lymphomas) and; iii) BM (and also other non-lymphoid tissues) involvement (neoplastic plasma cell disorders) (Table 1).[181] Of note, within each multiple diagnostic disease categories of these three groups of B-CLPD, those showing common genetic events – e.g. t(11;14) in MCL or CMYC gene rearrangements in Burkitt lymphoma – are included together with other genetically heterogeneous disorders that display either a uniform immunophenotype (e.g. CLL) or a similar pattern of tissue involvement (e.g. MZL). In addition, the WHO 2008 classification has also drawn attention for the first time to pre-malignant B-cell neoplasias related to peripheral/mature lymphoid leukemias/ lymphomas, such as MBL[14,182] and both *in situ* FL[183,184] and *in situ* MCL (Table 1).[5] Although such cases might share many biologic features with their malignant counterparts, they usually display a benign clinical course with relatively low rates of malignant transformation.

Table 1. List of the distinct subgroups of mature B-cell neoplasias and their major features, as defined in the WHO 2008 classification [adapted from Campo *et al*.[181]].

MATURE B-CELL NEOPLASMS
Peripheral / Mature chronic B-cell leukemias
Monoclonal B-cell lymphocytosis (MBL)
Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL)
B-cell prolymphocytic leukemia (PLL)
Hairy cell leukemia (HCL)
Peripheral / Mature B-cell lymphomas
In situ follicular lymphoma
In situ mantle cell lymphoma
Splenic marginal zone lymphoma (SMZL)
*Splenic lymphoma/leukemia, unclassifiable**
Lymphoplasmacytic lymphoma (LPL)
Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Nodal marginal zone B-cell lymphoma (NMZL)
Follicular lymphoma (FL)
Primary cutaneous follicle center lymphoma
Mantle cell lymphoma (MCL)
Diffuse large B-cell lymphoma (DLBCL), not otherwise specified (NOS)
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
Primary cutaneous DLBCL, leg type
ALK+ large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
Burkitt lymphoma (BL)
Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma
Neoplastic plasma cell disorders
Heavy chain disease
Monoclonal gammopathy of undetermined significance (MGUS)
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma

*ALK, anaplastic lymphoma kinase; HHV8, human herpes virus 8.*This histologic type is a provisional entity for which the WHO Working Group felt there was insufficient evidence to recognize as distinct diseases at this time.*

In the following sections of the introduction chapter we will review the most relevant biological as well as clinical features of the major subtypes of B-CLPD, particularly focusing on CLL-like MBL and CLL.

3.1. Monoclonal B-cell lymphocytosis

3.1.1. Diagnostic criteria for MBL and its subtypes

Several hospital-based and population-based flow cytometry immunophenotypic studies have been reported, in which the presence of relatively small (usually CLL-like) B-cell clones has been demonstrated in PB from a substantial fraction of otherwise healthy adults classified as with or without increased B-cell counts; MBL^{low} or MBL^{high}, respectively.[15,16,185]

In most MBL cases (85%), clonal B-cells display an immunophenotype which is similar to that of CLL cells (CLL-like MBL) consisting of $CD5^+$, $CD19^+$, $CD20^{low}$, $CD23^+$, $SmIg^{low}$ and $CD79b^{low}$ clonal B-lymphocytes (Table 2).[14,182,186] In turn, in a small fraction of $CD5^+$ MBL cases (atypical-CLL-like MBL) and in a minor proportion of cases corresponding to $CD5^-$ MBL (non-CLL-like MBL), clonal B-cells display variable phenotypes which are distinct from those typically found on CLL cells (e.g. $CD23^+$, $CD20^{low}$, $CD79b^{low}$ and $SmIg^{low}$); such phenotypes frequently overlap with those of MZL cells (Table 2).[14] Since clonal B cells of individuals with CLL-like MBL^{low}, CLL-like MBL^{high}, and CLL share an identical immunophenotypic profile and overlapping cytogenetic alterations, at present the distinction between the two MBL subgroups and CLL is mostly based on the absolute count of B-lymphocytes in PB. Therefore, while diagnosis of CLL requires the presence of ≥ 5,000 B-cells/μl, MBL includes only those cases with < 5,000 B-cells/μl in the absence of organ infiltration (Table 2).[14] Among MBL cases, there is consensus as regards the usage of a threshold of 500 B-cells/μl to distinguish between MBL^{low} (< 500 B-cells/μl) and MBL^{high} (≥ 500 B-cells/μl) cases. However, it should be noted that such cutoff for the definition of MBL^{low} vs. MBL^{high} still deserves confirmatory approval in the coming WHO classifications of mature B lymphoid neoplasias. Despite this, in all cases it is mandatory to actively exclude an underlying (e.g. BM, LN, splenic) mature-B-cell neoplasm other than MBL, particularly among non-CLL-like subjects and in atypical CLL-like MBL cases with circulating $CD5^-$ or $CD5^+CD23^-$ monoclonal B-cells at levels below $5x10^9$/L clonal B-cells, through e.g. BM cytomorphological/histopathological and imaging techniques (e.g. computerized tomography and/or magnetic resonance imaging).

At present, the precise significance of MBL B-cell clones, particularly of the MBL^{low} ones, still remains controversial. Thus, it has been hypothesized that expanded clonal B-cells

circulating in the PB of MBL subjects might either result from a transient and/or chronic activation of the immune system during e.g. infection,[187] or they may represent premalignant states of CLL and NHL.[5,184] So far, most reported data has focused on CLL-like MBL, while there is very limited information concerning individuals with $CD5^-$ non-CLL-like MBL.[188]

Table 2. Diagnostic criteria and nomenclature for MBL [adapted from Shanafelt *et al*.[185]]

Diagnostic criteria for MBL
1. Documentation of one or more clonal B-cell population[1] by ≥1 of the following criteria: a) Light chain restriction: kappa: lambda ratio >3:1 or <0.3:1, or > 25% B-cells lacking SmIg or expressing $SmIg^{low}$ b) Monoclonal *IGHV* gene rearrangement
2. Presence of a B-CLPD disease-specific immunophenotype[2]
3. Absolute B-cell lymphocyte count $<5x10^9$/L
4. No other clinical features compatible with a lymphoproliferative disorder: a) absence of B symptoms, b) normal physical examination (no lymphadenopathy or organomegaly), c) no autoimmune/infectious disease
Subclassification of MBL:
CLL-like MBL
● CLL phenotype: $CD5^+CD19^+CD20^{low}CD23^+sIg^{low}CD79b^{low}$
● Light chain restriction with $SmIg^{low}$ [3]
Atypical CLL-like MBL
● $CD5^+CD19^+$, but $CD23^{-\text{ or low}}$ and/or $CD20^{high}$, and/or $SmIg^{high}$ and/or $CD79b^{high}$
● Light chain restriction with moderate to bright SmIg expression[3]
● Exclude t(11;14) to rule out mantle cell lymphoma
Non-CLL-like MBL
● $CD5^-CD19^+CD20^+$ B-cells
● Light chain restriction with moderate to bright SmIg expression[3]

[1] *Where possible, repeated assessment(s) should also demonstrate that the monoclonal B-cell population is stable over a 3-month period.*

[2] *In the absence of a disease-specific immunophenotype, a highly skewed kappa:lambda ratio may result from a reactive process/immune response.*

[3] *Very small MBL clones may be oligoclonal and thus not light chain restricted.*

3.1.2. Prevalence of MBL

The prevalence of MBL in the general population varies substantially (from 0.6% to up to 20% of cases) depending on the population-cohorts investigated and the sensitivity of the MFC immunophenotypic approach used.[15,16,185] Thus, in the general population, MBL is more commonly detected among males, its frequency increasing with age, similarly to what is observed in CLL.[15,189] Reports on more limited series of first degree relatives of CLL patients also suggest familial aggregation and consequently, a genetic predisposition/ inheritance.[190,191]

The prevalence of MBL in the general population appears to be very rare under the age of 40.[16,186] Thereafter, it increases from 5.1% of adults with 40–49 years and 5.3% of adults aged 50 to 59 years, to much higher rates: 17.5%, 21.7%, 27.3% and 75% among the 60–69, 70–79, 80–89 years and >89 years age groups, respectively.[15] In turn, the risk for MBL appears to be increased by 4-fold in first-degree relatives of CLL patients.[190,191] Conversely, there is almost no data about the prevalence of MBL among first-degree relatives of individuals with MBL.

Regarding non-CLL-like MBL, controversial results have been reported in the literature with respect to the age at onset of the underlying B-cell clones. Ghia *et al.*[186] suggested that non-CLL-like MBL clones are already detectable in the general population in sizeable amounts among individuals younger than 40 years and that their frequency is only marginally affected by age. Thus, based on the observations of Ghia *et al.*[186] it could be hypothesized that while CLL-like MBL could be related to physiologic immune senescence-associated mechanisms, this would not explain the observation of other non-CLL-like MBL cases. However, it should be noted that, in contrast to what occurs in CLL-like MBL, currently there are no highly sensitive assays for the identification of non-CLL-like B-cells in PB, which weaknesses the potential conclusions about the exact prevalence (and even phenotypes) of non-CLL MBL. In addition, Nieto *et al*.[192] found a progressively higher frequency of non-CLL-like MBL cases in the general population with increasing age, the frequencies observed ranging from 0.4% among subjects aged 40–59 years to 5.4% among individuals over 80 years; such findings, suggest a similar behavior in the general population for non-CLL-like and CLL-like MBL, as regards its prevalence and distribution per age.

3.1.3. Risk factors for progression from CLL-like monoclonal B-cell lymphocytosis to chronic lymphocytic leukemia

The strong association reported between CLL-like MBL and increasing age, has promoted the hypothesis that MBL could be one of many signs of "immunosenescence".[193] Immunosenescence is a physiological process which involves an impaired function of immune cells. Among B-cells, immunosenescence is associated with accumulation of B cell populations producing polyreactive and autoreactive antibodies, lower incidence of SHM, and a more limited *IGHV* gene usage together with the emergence of oligo and even (mono)clonality, including the presence of detectable (mono)clonal component peaks in the serum.[194-196] Actually, presence of tiny numbers of clinically indolent (mono)clonal B cells with a CLL or other B-CLPD associated phenotype, is a rather common finding at the very early phases of

most mature B-cell neoplasms. The most well-recognized example of such early events is monoclonal gammopathy of undetermined significance (MGUS), which shows an incidence of around 1% in the general population older than 50 years, such frequency increasing thereafter to up to 10% among subjects older than 75 years.[197] Of note independent studies[198,199] have demonstrated that in most multiple myeloma (MM) patients, the disease is preceded by MGUS with a malignant transformation rate of MGUS to MM of between 1% and 2% cases per year.[200] More recently, a similar behavior and relationship has been identified for CLL-like MBL and CLL[5,184] as discussed below.

Population-based screening for CLL-like MBL has shown that MBL^{low} is more commonly associated with oligoclonality than CLL (20% of MBL vs. 5% of CLL) and MBL^{low} is enriched for lower risk CLL markers, such as mutated *IGHV* sequences (around 87% of MBL^{low} clones have mutated Ig genes vs. around 50% of CLL cases).[20] These findings suggest that the acquisition of a CLL-like cell surface immunophenotype does not necessarily go along with the emergence of a single clone, but it might more likely reflect a functional state potentially associated with prolonged/chronic B-cell stimulation, activation and/or immunesenescence, similarly to what has been previously described for T cells in the aging population, where chronic and persistent viral infections induce the emergence of oligoclonal and even monoclonal expansions of $CD4^+CD8^+$ T lymphocytes, under the influence of a specific genetic background (e.g. HLA-class II haplotypes).[201,202] In this regard, it should be noted that preliminary investigations of *IGHV* gene usage in CLL-like MBL^{low} based on highly-sensitive single cell purification techniques, have shown that clonal B-cells from MBL^{low} cases less frequently use VH CDR3 stereotypes (see definition in section 3.2.3.2.), their restricted *IGHV* repertoire (e.g. the *IGHV4–59* and I*GHV4–61* families are more frequently used than others) being also distinct from that detected in both mutated and unmutated CLL cases.[15,20,203] Overall, the above biological differences observed between expanded clonal B-cells in CLL-like MBL^{low} vs. CLL suggest that detection of CLL-like MBL in an otherwise healthy subject is not always equivalent to a preleukemic state, because specific BCR configurations are more prone than others for disease transformation. In line with this hypothesis, it has been recently shown in a cross-sectional epidemiological study that in the general population, MBL^{low} is significantly associated with a personal history of pneumonia and meningitis and infectious diseases among their children, while it was less commonly observed among subjects vaccinated against pneumococcus and influenza; these results suggest that exposure to infectious agents leads to serious clinical manifestations in the patients or their relatives and that they may more frequently trigger immune events leading to MBL.[204]

In contrast to what has been described for MBL^{low}, in MBL^{high} cases the biology of the expanded CLL-like B-cell clone more closely mimicks what is also seen in good-risk CLL: it shows molecular features similar to good-prognosis CLL (e.g. a bias toward mutated and clinically favorable BCR) different from those that are more frequently found in MBL^{low}.[16] For example, recent investigations[203,205] indicate that Ig genes commonly expressed in CLL (*IGVH1–69* and *IGVH4–34*) are also frequently used in CLL-like MBL^{high} (*IGVH4–34* and *IGVH3–23*), but rarely observed in MBL^{low}.

In line with what has been described above for the *IGHV* repertoire of MBL, cytogenetic analysis of clonal B-cells from CLL-like MBL subjects has also demonstrated alterations – typically restricted to del(13q) and trisomy 12 – in about 40% of cases vs. >50% of CLL patients.[16,206] These abnormalities are usually seen in only a fraction of all abnormal cells, such fraction increasing from MBL^{low} cases to CLL.[20] Despite MBL^{low} does not show poor-prognosis cytogenetic/molecular alterations – e.g. del(17p), del(11q) or *NOTCH1* mutations – currently there is no cytogenetic marker which identifies MBL individuals who are likely to develop progressive disease.[203,206] Of note, in some MBL^{low} cases, cytogenetic alterations are observed even when the number of circulating MBL cells is extremely small, once again, in the absence of any evidence of progression to MBL^{high} and CLL.[187] Overall, these findings mimick what has been previously reported also for most MGUS cases, where the (mono)clonal plasma cells frequently bear overlapping chromosomal abnormalities and cytogenetic profiles with symptomatic MM;[207,208] at the same time, it may also contribute to explain why cells carrying the t(14;18) translocation (the cytogenetic hallmark of FL) can be found in the PB of approximately 50% of healthy persons in the absence of evidence for disease progression.[209] In more detail, del(13q14) can be detected in approximately an equal proportion of CLL-like MBL and CLL, particularly among *IGHV* mutated clones, independent of the absolute number of circulating CLL cells.[210,211] Once again, these observations highlight the fact that development of very small CLL clones with "good risk" cytogenetic and biological features is a common finding in the elderly and it may be a consequence of the ageing of immune system more than an actual oncogenic event; at the same time, they indicate that additional factors are required to drive small CLL clones to expand. In contrast to del(13q) cytogenetic abnormalities which are associated with poor prognosis CLL, such as del(17p13), del(11q22) and *NOTCH1* mutations, have not been reported in MBL^{low} and they are only occasionally seen in CLL-like MBL^{high}.[15,16,18] These observations, together with the restricted but different, *IGHV* repertoire detected in MBL^{low} vs. MBL^{high}, suggest that in addition to the occurrence of specific genetic alterations, specific BCR signaling would be also required for the expansion of CLL-like MBL cells and for the transformation of MBL to symptomatic CLL, at least in a fraction of the cases.

In this regard, it has been recently shown that CLL is always preceded by an MBL state,[212] similarly to what occurs with MM and MGUS. In turn, this is also consistent with the fact that most MBL cases will not progress to CLL, particularly among MBL^{low} subjects, where progression appears to be very unlikely.[213] Due to the arbitrary cutoffs used for the distinction between MBL and CLL Rai stage 0, at present it is well-accepted that it is more appropriate to define the risk of MBL progression as the risk for developing CLL requiring treatment, than just the risk to develop CLL. Based on such criteria, follow-up studies of large series of MBL^{high} cases have found an annual risk of progression of MBL^{high} to CLL requiring treatment of between 1 and 2% vs. 5 to 7% for CLL Rai stage 0 patients.[16,18,214] Today, the only prognostic factor known to predict progression of MBL to CLL is the actual number of clonal B lymphocytes in PB.[16,18] However, other prognostic markers that are informative within CLL might also be applied to CLL-like MBL, to predict which cases might progress to CLL and eventually require treatment. Therefore, established risk factors for CLL, such as the *IGHV* mutational status, cytogenetic/molecular aberrations, expression of specific phenotypic markers (e.g. ZAP-70, CD38 and CD49d), are potentially also adverse prognostic factors among CLL-like MBL subjects, their precise value deserving further investigations.

Other factors that might contribute to understand the pathogenesis of MBL and predict transformation of MBL into CLL include an altered homeostasis and functionality of both NK- and/or T-cells, which have been suggested to influence survival of CLL B-cells and progression of the disease.[19,215] In this regard, recent studies have shown that in MBL subjects, $CD4^{+}CD8^{+}$ double-positive T-cells are significantly reduced in absolute numbers while $CD8^{+}CD4^{-}$ T-cells are increased, supporting the notion that an impaired immunosurveillance function may favor the emergence of MBL clones.[216] Moreover, presence of MBL clones in healthy subjects is associated with reduced counts of normal circulating PB B-cells, mainly at the expense of immature and naive B-cells, such decrease being more pronounced as the number of MBL cells increases.[216] Based on these observations, it could be hypothesized that the reduced PB counts of normal B-cells at the expenses of recently produced B-cell subsets in MBL^{low}, could depend on the size of the B-cell clone in the BM, suggesting a potential suppressive effect of the MBL clone on normal B-lymphopoiesis. Whether these abnormalities occur before or after the emergence of the MBL clone(s), and whether this reduction derives from immune-suppression or it may reflect a decrease in B-cell production at BM niches due to MBL-cell competition, requires further investigations.

Altogether, the above observations raise the question about whether there is a time-dependent pipeline of unavoidable events leading from MBL^{low} to MBL^{high} and also to CLL, or MBL^{low} simply represents one of many features of immunesenescence, while (clinical) MBL^{high}

is already a pre-malignant CLL state. At present, further research is still required to elucidate this question. However it should be noted that the understanding of the molecular and biological features underlying the risk of progression of MBL to CLL may significantly modify the currently used strategies for the follow-up of MBL, leading to a more refined management of CLL premalignant states and a better follow-up of MBL cases at risk of progression, for early adoption of measures that would potentially block or delay malignant transformation.

3.2. Chronic lymphocytic leukemia

3.2.1. Definition and diagnostic criteria for CLL

CLL is the most common leukemia in adults in the Western world.[217,218] It is a chronic incurable disease which is characterized by progressive accumulation of B cells in the PB, BM, and/or lymphoid tissues.[219] When the disease mostly involves the PB and BM, it is called CLL, while when LN or other tissues are preferentially infiltrated by tumor cells with identical morphologic and immunophenotypic features to CLL, in the absence of the typical leukemic manifestations of the disease, it is called small lymphocytic lymphoma (SLL). In the WHO 2008 classification,[180,181] these two entities (CLL and SLL) are simply considered as different clinical manifestations of the same disease. Current diagnostic criteria for typical CLL requires the presence of at least 5,000 B lymphocytes/µl of PB with a CLL immunophenotype, or LN involvement by CLL cells in case of SLL.[220] BM involvement is typically present, infiltrating CLL cells usually represent more than 30% of all nucleated cells in the aspirated BM sample.

Despite a remarkable phenotypic homogeneity, CLL is characterized by an extremely variable clinical course with very heterogeneous responses to treatment; thus, while some patients do not require therapy for rather long periods of time after diagnosis or they reach complete and prolonged remissions after treatment, others relapse early and need several lines of treatment and frequently die from the disease.[221] Such clinical heterogeneity most likely reflects the underlying molecular and cellular heterogeneity of the disease.[22] In fact, several lines of research have demonstrated that CLL can be subdivided into multiple subgroups with distinct biological features, based on underlying genomic features, cytogenetic/molecular aberrations and/or the immune signaling pathways that can be activated via cell surface and/or intracellular receptor molecules of both the innate (e.g. TLRs) and adaptive (e.g. BCR) immune system.[22,222,223]

3.2.2. Immunophenotypic features of CLL cells

The typical immunophenotypic profile of CLL small lymphocytes includes coexpression of CD5 and CD23 in the absence of FMC7 and low expression levels of SmIg (often IgM with or without IgD);[224] in addition, the levels of expression of several other B-cell-associated cell surface membrane molecules like CD20, CD22, CD79b and CD81 are also significantly decreased compared to those found on normal mature B-lymphocytes.[225] CLL cells also coexpress CD43 and CD200 which may provide further information in differentiating CLL from other B-cell NHL and chronic lymphoid leukemias such as FL (e.g. CD43 is positive in CLL but usually negative in FL) and MCL (e.g. CD200 is consistently expressed in CLL whilst it is negative or very weakly expressed in MCL).[226] Moreover, CLL cells are usually positive for CD21, CD24, CD25, CD27, CD39, CD40, CD45RA, CD62L and CXCR5 (CD185). In turn, CD11c, CD38, CD45RO, CD49d, CD80, CD95, CD124, CD126, CD130, ZAP-70 and other markers that recognize adhesion molecules, are expressed at variable levels in a fraction of all CLL/SLL cases.

3.2.3. Molecular features of CLL cells

3.2.3.1. Immunoglobulin heavy chain variable region gene usage in CLL

In recent years, several studies have confirmed that the *IGHV* gene repertoire of CLL is restricted and different from that of normal IgM^+ B-cells;[227-229] thus, several *IGHV* genes are clearly over-represented in CLL (e.g. the *IGHV1-69*, *IGHV4-34*, and *IGHV3-7* genes).[227] Similarly, a restricted usage of specific *IGHD* and *IGHJ* genes has also been described in CLL.[13] In this regard, only five *IGHD* genes are used by almost half of all CLL cases, the *IGHD3-3* gene being the most frequently selected; in turn, the *IGHJ* gene repertoire of CLL cells is characterized by a preferential usage of the *IGHJ4* and *IGHJ6* genes.[230,231] Such restricted *IGH* gene repertoire leads to the predominant usage of specific *IGHV*, *IGHD* and *IGHJ* gene combinations. For example, *IGHV1-69* gene rearrangements are strongly biased toward the usage of the *IGHD3-3* and the *IGHJ6* genes; in contrast, no significant (or minor) biases are noted for cases expressing other *IGHV* genes, particularly *IGHV4-34*, *IGHV3-7* and *IGHV3-23.*[230,232] Furthermore, the imprint of SHM is not uniform across different *IGHV* genes in CLL; thus, the *IGHV1-69* gene most frequently carries very few or no mutations, as opposed to the *IGHV3-7*, *IGHV3-23* and *IGHV4-34* genes, which are mutated in a relatively significant proportion of cases.[227]

3.2.3.2. Stereotyped B-cell receptors in CLL

Immunogenetic studies of the BCR repertoire of CLL cells has revealed the presence of homologous BCR in a significant proportion of cases, suggesting that recognition of common epitopes or classes of structurally similar epitopes is likely involved in the selection of CLL clones.[233-235] Overall, different types of homologous BCR have been defined, enabling different cases to be grouped into subsets based on common sequence features of the BCR, particularly the homology of their VH CDR3 sequences. These highly similar BCR have been referred as "stereotyped" BCR.[233] So far, more than 200 different subsets of cases carrying stereotyped BCR have been defined, 8 of such subsets accounting for ≈30% of all stereotyped CLL cases.[236]

In 2007, a set of criteria was proposed for the definition of stereotyped BCR based on the specific underlying *IGH V(D)J* gene rearrangements;[237] these criteria include: 1) a VH CDR3 aa identity ≥60%, in line with established bioinformatic concepts for evaluating sequence conservation in protein sequences; 2) usage of the same *IGHV/IGHD/IGHJ* germline genes or different *IGHV* genes, as long as the above criterion for VH CDR3 sequence conservation is met; 3) usage of the same *IGHD* gene RF.

Based on this clustering approach for stereotyped BCR in CLL, it has been shown that the frequency of BCR Ig stereotypes in CLL can exceed 25% of the entire CLL patient cohort;[13] although BCR Ig stereotypes exist among both mutated and unmutated CLL, they are significantly more frequently observed in the latter group.[233,238] Of note, different versions of BCR Ig stereotypes can be defined based on shared VH CDR3 aa sequence patterns, which are distinct for each subset.[239] Interestingly, the relative size of each subset differs markedly, from just two to large numbers of cases with homologous BCR, since individual genes show a markedly different susceptibility to be used in stereotyped rearrangements in CLL. Hence, while the frequency of stereotyped rearrangements has been shown to exceed 30% of the cases for some *IGHV* genes (e.g. *IGHV3-21, IGHV1-69, IGHV1-2, IGHV1-3, IGHV4-39, IGHV3-48*), it is rather low (<5%) for other *IGHV* genes (e.g. *IGHV3-7*, *IGHV3-74*).[237] In addition, individual subsets of cases with stereotyped VH CDR3 are often characterized by restricted Ig light chain gene usage and CDR3 features.[237,240,241] On top of all the above, specific subsets of stereotyped CLL cases may be also associated with distinct clinical/phenotypic features including a different outcome (see section 3.2.5.2. below), raising the possibility that a particular Ag binding site can be critical in determining clinical presentation and potentially also even disease prognosis.[237,242] Finally, although BCR stereotypes have also been found in other B-CLPD, those observed in CLL are frequently different from the ones reported in other B-cell malignancies

such as splenic MZL (SMZL) and MCL, alluding to distinct disease-biased, selective and ontogenetic processes.[243,244]

Based on the above observations it might be concluded that depending on the BCR repertoire, CLL actually consists of two major different categories which show important biological and ontogenetic differences;[13] the first includes cases with heterogeneous BCR (non-clustered cases), while the second is characterized by a remarkably high frequency of BCR stereotypy (clustered cases). The biological significance of BCR stereotypy still remains to be fully understood.

3.2.3.3. Recognition of conserved epitopes by CLL cells

Detailed dissection of the potentially relevant antigenic epitopes recognized by the BCR in CLL, indicates that these might probably correspond to molecular structures usually involved in eliminating cellular debris, scavenging apoptotic cells and apoptotic bodies, and/or providing a first line of defense against pathogenic bacteria.[245-247] For instance, non-muscle myosin heavy chain IIA (MYHIIA), which is expressed on a subpopulation of apoptotic cells (called myosin-exposed apoptotic cells or MEAC), emerged as the antigenic target of CLL monoclonal antibodies (mAbs) from a subset of cases expressing *IGHV1-69*/*IGHD3-16*/*IGHJ3* stereotyped BCR, as well as of several other CLL mAbs.[246] Other Ags recognized by CLL cells have been functionally associated with specific bacterial infections – e.g. molecular mimicry driven by *Streptococcus pneumoniae* capsular polysaccharides, Gram-positive, Gram-negative bacterial strains and oxidized low-density lipoprotein (LDL) –.[248] Viral infections have also been suggested to drive subgroups of BCR stereotyped CLL cases; accordingly, persistent infection by Epstein–Barr virus (EBV) and cytomegalovirus (CMV) has been correlated with a stereotyped *IGHV4-34* subset[249] and hepatitis C virus (HCV) has been recently associated with stereotyped *IGHV4-59/IGKV3-20* CLL mAbs, albeit indirectly, since such CLL mAbs can exhibit rheumatoid factor activity.[250] Interestingly, the existence of rheumatoid factor with restricted Ig gene sequences in various conditions (e.g. CLL, SMZL, myoepithelial sialadenitis in primary Sjogren's syndrome, mixed cryoglobulinemia type II) may allude to cross-reactivity or molecular mimicry of the antigenic elements which are involved in the selection of the clonogenic B-cell progenitors involved in distinct pathologic conditions.[250]

3.2.4. Genomic aberrations in CLL

Genomic aberrations can be identified in around 50-80% of CLL cases by iFISH analysis of CLL cell nuclei with a disease-specific probe set.[251,252] The most common chromosomal abnormalities in CLL are del(13)(q14), trisomy 12, del(11)(q22–23), del(17)(p13) and del(6)(q21).[222] Del(13q14) followed by trisomy 12 are the two most frequent cytogenetic alterations in CLL, being present in between 8% and 60% and between 10% and 25% of all cases, respectively.[222,252,253] In turn, del(17p13) and del(11q23.3-q23.1) are detected in a significantly lower proportion of cases, ranging between 5% and 16% and between 5% and 15% of all CLL cases, respectively.[222,252,253] In common, these cytogenetic abnormalities, and a number of other chromosomal defects, are known to target genes that play a key role in the regulation of cell proliferation, survival, and/or DNA repair, such as the tumor protein p53 (*TP53*), retinoblastoma (*RB*), ataxia telangiectasia mutated (*ATM*)*, CYCLIN D2,* cyclin-dependent kinase 4 (*CDK4*), and E3 ubiquitin-protein ligase (*MDM2*) genes. Despite the high frequency of both del(13q14) and trisomy 12 and the fact that they have been suggested to play a primary and central role in the transformation process in CLL,[254] the pathogenic role of del(13q) and trisomy 12 in CLL is not fully understood. In this regard, at present it is known that the deleted chromosome 13q region, always comprises the locus coding for two microRNA (miRNA), miRNA-16-1 and miRNA-15a, which have been suggested to act as tumor suppressor genes in the pathogenesis of CLL via down-regulation of expression of the antiapoptotic Bcl2 protein;[255,256] in turn, in a fraction of cases carrying del(13q), larger deletions that include the region coding for the *RB1* gene occur, such larger deletions being associated with a poorer clinical outcome, potentially due to an altered control of cell proliferation and survival.[257-259]

Besides its relevance in the pathogenesis of CLL, miRNA expression profiles may also be relevant prognostic markers in CLL. In this regard, it should be noted that critical processes of the B-cell physiology, including immune signaling through the BCR and/or TLR, are targeted by miRNA and, changes on the expression of specific miRNA (e.g. upregulation of miRNA-150, miRNA-29c, miRNA-143 and miRNA-223 and downregulation of miRNA-15a) which are differentially expressed among mutated vs. unmutated CLL cases and in certain CLL stereotypes (e.g downregulation of miRNA-101 in subset 1), may also modulate the biological and clinical behavior of CLL clones.[260] As an example, miRNA-101 regulates the expression levels of the enhancer of zeste homolog 2 (EZH2) protein; consequently, its overexpression is associated with more aggressive CLL.[260]

Recent studies devoted to whole CLL genome sequencing have shown the presence of more than one thousand distinct mutations across CLL patients. However, the frequency at

which such mutations were found in CLL was relatively low, except for a few genes.[6,261,262] The recently identified genes, included the Notch 1 (*NOTCH1,* 12.3%), splicing factor 3b subunit 1 (*SF3B1,* 9.0%), *TP53* (7.1%), exportin 1 (*XPO1,* 3.4%), F-box and WD repeat domain containing 7 (*FBXW7,* 2.5%), myeloid differentiation primary response gene 88 (*MyD88*, 1.5%) and the kelch-like family member 6 (*KLHL6,* 1.8%) genes, apart from those that had been previously described.[6,263] Since, some of these mutations can also emerge during the clinical course of CLL,[264] future studies are required to clarify how this might influence treatment strategies, as data suggest that therapy may disrupt the interclonal equilibrium and lead to evolution of subclones with driver mutations such as *SF3B1*, *NOTCH1* or *TP53* mutations.[265] Furthermore, a recent cohort study has shown that different subgroups of CLL can be defined according to co-occurrence of distinct patterns of genetic alterations for some of these mutations: 1) mutations in *SF3B1* gene are frequently associated with del(11q); 2) mutations in the *NOTCH1* and the *FBXW7* genes are associated with trisomy 12, whereas these two mutations and *SF3B1* mutations were nearly mutually exclusive, and; 3) mutations in the *MYD88* gene are mainly found in combination with isolated del(13q), while *NOTCH1*, *FBXW7* and *SF3B1* mutations are rarely found in this cytogenetic subgroup of CLL.[263] Thus, the combination of molecular markers with discrete cytogenetic subgroups may hint at distinct ways of CLL pathogenesis and may explain the clinical heterogeneity of the disease.

3.2.5. Outcome and prognosis of CLL patients

As discussed above, CLL is a clinically and biologically heterogeneous disease, with a highly variable outcome. During the last decades, several clinical and biological variables, including the immunogenetic profiles of CLL cells, have been related with the prognosis of the disease.

3.2.5.1. Clinical and biological prognostic factors

The standard clinical procedures to estimate prognosis in CLL are based on the staging systems developed by Rai *et al*.[266] and reformulated latter on by Binet *et al*..[267] These systems (Table 3) define early (Rai 0, Binet A), intermediate (Rai I/II, Binet B) and advanced (Rai III/IV, Binet C) stage disease with, median estimated overall survival rates of >10 years, 5-7 years, and only 1-3 years, respectively. Because most patients present early or intermediate stage disease and a heterogeneous course of disease occurs for individual patients within the same

disease stage, additional markers are needed to stratify patients who are at increased risk of disease progression with a potentially decreased survival, within individual disease stages.

A relatively high number of prognostically relevant markers have long been identified in CLL. Among other, these include clinical characteristics (e.g. patient age, gender, and performance status) in addition to the stage of the disease, as well as laboratory parameters reflecting the tumor burden and/or activity of the disease, (e.g. lymphocyte count, BM infiltration pattern or lymphocyte doubling time) (Table 4).[268] More recently, prognostic markers related to the biology of the tumor have been also identified, including serum parameters, immunophenotypic markers and cytogenetic/molecular characteristics of CLL cells, such as the mutational status of the *IGHV* genes (Table 4) that will be specifically discussed in the following section of this chapter.[222,269-271]

Table 3. Rai *et al*. and Binet *et al*. staging systems for the prognostic classification of CLL.

Staging System	Stage	Definition	Median survival
Rai *et al.* staging system	0 (low risk)	Lymphocytosis only	11.5 years
	I (intermediate risk)	Lymphocytosis and lymphadenopathy	11.0 years
	II (intermediate risk)	Lymphocytosis in blood and marrow with splenomegaly and/or hepatomegaly (with or without lymphadenopathies)	7.8 years
	III (high risk)	Lymphocytosis and anemia (hemoglobin <110g/L or a hematocrit <33%)	5.3 years
	IV (high risk)	Lymphocytosis and thrombocytopenia (platelet count <100x10^9/L)	7.0 years
Binet *et al.* staging	A	Enlargement of <3 lymphoid areas (cervical, axillary, inguinal, spleen, liver); no anemia or thrombocytopenia	11.5 years
	B	Enlargement of ≥3 lymphoid areas	8.6 years
	C	Anemia (hemoglobin <100 g/L) or thrombocytopenia (platelet count <100x10^9/L), or both	7.0 years

At present, it is widely accepted that chromosomal aberrations are one of the most relevant prognostic factors in CLL, being associated with different clinical and prognostic features of the disease. Thus, in general a normal karyotype and presence of isolated del(13q) are both associated with a significantly better outcome, whereas trisomy 12 confers an intermediate prognosis associated with atypical CLL, and both del(11q) and del(17p) are associated with a particularly poorer outcome.[222,252,272] Despite this, recent studies have shown that there is a significant heterogeneity in the outcome of patients with isolated del(13q),[273-276]

the clinical impact of biallelic vs. monoallelic del(13q) still remaning controversial. In this regard, it should be noted that while biallelic loss of chromosome 13q is frequently seen as a secondary cytogenetic event with a limited impact on prognosis, the progressive accumulation of high percentages of cells (>90%) carrying del(13q) has been described as an independent prognostic factor for risk stratification of patients with del(13q).[277]

TP53 and *SF3B1* mutations emerge among the strongest biological markers with an independent prognostic value in CLL patients receiving current standard first-line therapy.[278] CLL patients with *TP53* mutation have a poor prognosis when treated with fludarabine (F)-based chemotherapeutic regimens regardless of the presence of del(17p) and should be considered for alternative treatment approaches.[279] In turn, *SF3B1* mutations have a lower incidence in early CLL stages, and they are more commonly observed in advanced disease, tending to be associated with a poorer prognosis.[16,280] *NOTCH1*, *XPO1* and *MYD88* are other three genes that are recurrently mutated in CLL, being associated with a better (e.g. *MYD88*) or a poorer outcome (*NOTCH1*, *XPO1*).

In addition to tumor cytogenetics, several immunophenotypic markers of CLL cells, have also been associated with the prognosis of the disease. Thus, both CD38 and ZAP70 expression have been shown to be associated with a poorer outcome in CLL.[281,282] Similarly, increased expression of the CD49d integrin has consistently been identified as an independent negative prognosticator for CLL, particularly among *IGHV* unmutated cases, defining a subset of CLL patients characterized by an aggressive and accelerated clinical course;[271,283,284] in contrast, preliminary data also suggests that CD305 (LAIR1), an inhibitory B-cell signaling molecule, appears to confer a better prognosis in CLL.[285] Other markers that have been also associated with adverse prognostic features of CLL include: higher expression of CD74[286] and absence of CTLA4 expression.[287] Of note, specific polymorphic variants of CTLA4 have been associated with advanced Rai stages and an increased risk of progression of CLL.[288]

Table 4. Outcome of CLL patients distributed according to selected prognostic factors [Adapted from Shanafelt *et al.*[268]].

		Clinical Risk	
		Low	**High**
Major	*Sex*	Female	Male
	Clinical stage	Binet A	Binet B or C
		Rai 0, I	Rai II, III, IV
	BM infiltration pattern	Non-diffuse	Diffuse
	Morphology of lymphocytes	Typical	Atypical
	Lymphocyte doubling time	> 12 months	< 12 months
	% of CD38$^+$CLL cells	<20-30%	>20-30%
	Interphase FISH cytogenetics/	Normal; del13q (sole)/	del(11q); del(17p)
	Whole-genome sequencing mutated	*MYD88*	*TP53*
	genes		*SF3B1*
			NOTCH1
			XPO1
	IGHV mutational status	Mutated (≥2%)	Unmutated (<2%)
	Cellular expression of ZAP-70	Low	High
	Cellular expression of CD49d	Low	High
	Cellular expression of CD74	Low	High
	Cellular expression of CD305	High	Low
	Cellular expression of CTLA4	High	Low
	Serum thymidine kinase activity	Low or Normal	Elevated
Other	*Beta2-microglobulin (serum levels)*	Low (<2.5 mg/L)	High (>2.5 mg/L)
	Soluble CD23 (serum levels)	Low (<574 U/mL)	High (>574 U/mL)

3.2.5.2. Immunogenetic parameters and profiles with prognostic impact in CLL

The load of somatic mutations across the sequence of the rearranged *IGHV* genes was the first highly accurate molecular marker for prognostication of CLL and it still remains one of the strongest prognostic markers of the disease, which is independent from the Binet/Rai staging (Table 4). Thus, mutated (<98% homology with germline sequences) CLL is usually associated with early stage and more stable forms of the disease, whereas unmutated (≥98% homology) CLL typically shows more aggressive features and a poorer outcome.[278,289] Most interestingly, the mutational status of the *IGHV* genes also appears to be associated with acquisition of specific genetic alterations. Thus, mutations of the *MYD88* gene are predominant in CLL with mutated *IGHV* and define a good-prognosis subgroup of young adults;[290] in contrast, *NOTCH1*, *SF3B1* and *XPO1* mutations are mainly detected in patients with

unmutated Igs.[263] These patterns of association between the *IGHV* somatic mutation profile and specific genetic changes, support the notion that recurrent mutations of the *NOTCH1*, *MYD88, SF3B1* and *XPO1* genes are oncogenic changes that contribute to the clinical evolution of specific subsets of CLL patients defined by their *IGHV* gene status.[6]

In addition to the *IGHV* mutational status, specific *IGHV* genes have been also associated with the prognosis of CLL. The first hints suggesting that not only the mutational status of the *IGHV* genes, but also other BCR features could be prognostically relevant in CLL, came more than 10 years ago, in 2002. At that time, it was already found that usage of the *IGHV3-21* gene was associated with an adverse prognosis, regardless of the SHM load.[291] Afterward, other reports confirmed the association between specific *IGHV* genes and prognosis of CLL; while some *IGHV* genes confer an adverse prognosis (e.g. *IGHV3-23*)[292] others are associated with a more favorable outcome (e.g. *IGHV3-30* and *IGHV3-72*) of CLL.[293] However, except for the data on the prognostic impact of the *IGHV3-21* gene, for which there is strong evidence,[294,295] the prognostic association of other *IGHV* gene usage profiles is based on retrospective analyses of rather small patient series, hence requiring definitive confirmation in prospective studies including larger numbers of patients.

In parallel, specific associations have also been reported in CLL between subsets of cases with (quasi-identical) 'stereotyped' BCR and several clinical features of the disease.[238,296] As an example, CLL cases expressing stereotyped *IGHV4-39*/*IGHD6-13*/*IGHJ5* BCR more frequently experience aggressive disease complicated by severe recurrent infections, Richter's transformation and/or the occurrence of secondary neoplasias (e.g. solid tumors).[238] Another clinically relevant example of such association, relies on cases showing expression of stereotyped *IGHV4-34*/*IGKV2-30* BCR of the IgG isotype together with unique patterns of SHM, occurring in younger CLL patients[237,297,298] presenting a more indolent disease.[237,297] Also, the cooperative association between *IGHV4-39* gene usage and specific BCR stereotypes, *NOTCH1* mutation and trisomy 12, more frequently leads to CLL transformation into Richter syndrome;[236] similarly, coexistence of *SF3B1* mutations and specific BCR stereotypes associated to the *IGHV3-21* gene, invariably leads to disease progression among such CLL cases.[236]

3.2.6. The cell of origin of CLL-like MBL and CLL

In human hematopoietic cells, genetic alterations which are responsible for oncogenic transformation should in principle accumulate in cells that already have (or will acquire) self-renewing proprieties, such as HSCs. Such cells can continuously produce a number of

progenitors which carry the same genetic alteration and that are potential targets for additional mutations,[299] which might become leukemia stem cells with self-renewal capacity and a limited ability to differentiate normally.[300,301]

While myeloproliferative neoplasms and myelodysplastic syndromes are known to arise at the HSC stage in hematopoiesis, many other hematologic malignancies have been linked to later stages in the hematopoietic hierarchy.[302-304] For example, aggressive acute leukemias are generally believed to arise from early progenitors, with some initial leukemogenic events occurring in the HSC themselves.[303,304] In turn, most mature B cell lymphomas and leukemias are classified according to their presumed cell of origin along the continuum of B cell development and differentiation, early oncogenic events potentially targeting long living pre-GC B-cells, memory B-cells and/or plasma cells.[7]

In recent years, the focus of research in CLL has moved towards the molecular genetic level. This is due to the fact that there is a general believe that the identification and the understanding of those genetic and molecular mechanisms directly responsible for the course of the disease, such as clinical progression, response to treatment and overall survival, will contribute to accelerate the cure of the disease, at the same time they will provide insight into those events leading to the development and malignant transformation, in CLL. Consequently, an increasing number of studies have been reported which provide useful information to delineate part of the history of the malignant CLL clone, particularly during the period that precedes malignant transformation, e.g. at the stage of MBL.

3.2.6.1. Multistep models for human CLL development

The precise B-cell subset targeted at the initial stages of CLL, still remains a matter of debate, and current knowledge about the history of the cell of origin of CLL, prior to leukemic transformation, is incomplete. Consistent expression of CD5 on CLL cells led to initial speculations that CLL might be a malignancy of $CD5^+$ B-cells,[305,306] a subset of B-lymphocytes that represent a distinct B cell lineage (B1 B-cells) in mice where they are capable of self-renewing, producing natural/polyspecific antibodies and responding to T-independent Ags; based on this hypothesis, CLL cells were claimed to be potentially stimulated *in vivo* in a continued way by self-antigens.[307,308] Recently, human PB $CD5^+$ B cells have been shown to correspond to the so called immature/transitional B cells,[121] as confirmed by unsupervised analysis of gene expression profile (GEP) for >10,000 transcripts of CLL cells vs. distinct subsets of normal mature B-lymphocytes.[309,310] However, despite most $CD5^+$ B-lymphocytes are regarded as pre-GC B-lymphocytes with unmutated *IGHV* genes, around half of all CLL cases

harbor mutated *IGHV* genes.[270,311,312] In line with these findings, a small fraction of $CD5^+$ B-cells (4–17%) that coexpress the memory B-cell associated CD27 marker has also been described in healthy subsets.[152] Moreover, a small subpopulation of all $CD5^+$ B-cells (between 0.5 and 2% of all $CD5^+$ PB B cells), appears to be class-switched to IgG or IgA.[309]

In fact, not only unmutated CLL but also mutated CLL appears to be highly similar to normal $CD5^+$ B-lymphocytes.[311-313] Thus, transcriptome analyses revealed that *IGHV* gene unmutated CLL might derive from unmutated mature $CD5^+$ B lymphocytes, while mutated CLL would most likely derive from a distinct $CD5^+CD27^+$ post-GC B cell subset.[309] Notably, these $CD5^+$ B cell populations appear to be enriched in stereotyped *IGHV* gene rearrangements, at the same time they also include oligoclonal expansions already among young healthy adults. Therefore, these $CD5^+$ PB B-cells may potentially already represent an early phase in CLL development, prior even to the CLL-like MBL precursor cell. Alternatively, it has also been proposed that CLL cells could derive from splenic marginal zone B-cells, based on the functional similarities between CLL and this subset of normal B-lymphocytes (Figure 3A).[22] In this regard, it should be noted that several studies which have focused on *IGHV/IGLV* gene usage by CLL cells, including analysis of the *IGHV* mutational status and the *IGHV* repertoire, gene expression profiling and cellular phenotypes (e.g. CD27, ZAP-70 or CD38 expression) have found that CLL cells are more similar to memory than to naïve B-lymphocytes; consequently, CLL cells appear to potentially have a past history that would include Ag stimulation.[227,228,237,292,314-316]

Interestingly, the Ag reactivity profile of the BCR of CLL cells appears to overlap with that of natural antibodies produced in the absence of exogenous Ag stimulation, and that play a crucial role in immediate host defense against a wide range of pathogens.[247,317] Further studies have demonstrated that both self-reactive and polyreactive BCR profiles are inversely related to the mutational load of the BCR.[318] These apparently discrepant findings could be related to the fact that BCR-dependent transductional signaling pathways are still efficient in unmutated CLL, while deficient in mutated CLL cells.[319] Since unmutated *IGVH/IGVL* genes generally encode for antibodies with a natural/polyspecific profile, it has been proposed that the neoplastic B-cells using these genes would be continuously stimulated *in vivo* by self-antigens that have the capacity to react with their SmIg.[320] Thus, neoplastic transformation might potentially occur when these B cells are expanding in response to T-independent Ags, and they may continue to do so, also after neoplastic transformation. This would explain, at least in part, the expression of activation markers like ZAP-70 and/or CD38 on such stimulated and transformed cells, which occurs less frequently among those cases where the neoplastic cells used mutated *IGHV* genes.[321] These latter cases typically express surface Ig which do not

show a natural/polyspecific reactivity profile.[322] Although such *IGHV* mutated CLL cells may have expanded in response to antigenic stimuli acting before (or during) transformation they do not appear to be affected by antigenic stimulation thereafter, once the transformation process had been completed.[323]

Based on all the above, it could be hypothesized that unmutated CLL would derive from marginal zone B-cells, while mutated CLL cases would have their origin on memory B-cells that have exited the GCs at the end of their selection process.[8] However, several features of these two groups of CLL (unmutated and mutated CLL) do not seem to confirm such hypothesis. Firstly, most memory B-cells that transit through the GC and undergo isotype switch, express surface IgG or IgA, rather than IgM;[324] secondly, CLL cells from both unmutated and mutated cases present rather similar gene expression profiles as assessed by high-density microarrays.[315,325,326] An alternative possibility would be that both subsets of CLL originate from the same type of cell (e.g. from marginal zone B-cells), *IGHV* mutations potentially occurring in mutated CLL cases, while the cells respond to a particular TI Ag, either prior to or during neoplastic transformation in the marginal zone (Figure 3A).[327] As a result of these mutations, the polyspecificity of the antibody itself is lost and the cells are no longer susceptible to *in vivo* stimulation by self-antigens. Hence, without this important promoting factor in leukemogenesis, B-cells which had undergone SHM would expand less rapidly and would exhibit a more benign clinical course than the unmutated CLL cases. In line with this latter hypothesis, site-directed mutagenesis tests on the *IGHV/IGLV* genes have demonstrated that elimination of the mutated spots and reversion to the unmutated *IGVH/IGVL* gene configuration, results in the synthesis of antibodies with natural/polyspecific activity;[318] these observations reinforce the concept that the same cells that initially had natural/polyspecific activity had given origin to both the mutated and the unmutated CLL cases.[318] If this hypothesis holds true, then, it would also contribute to explain their similar surface phenotype and GEP, as well as the shared biological features of CLL cells from these two groups of CLL cases.

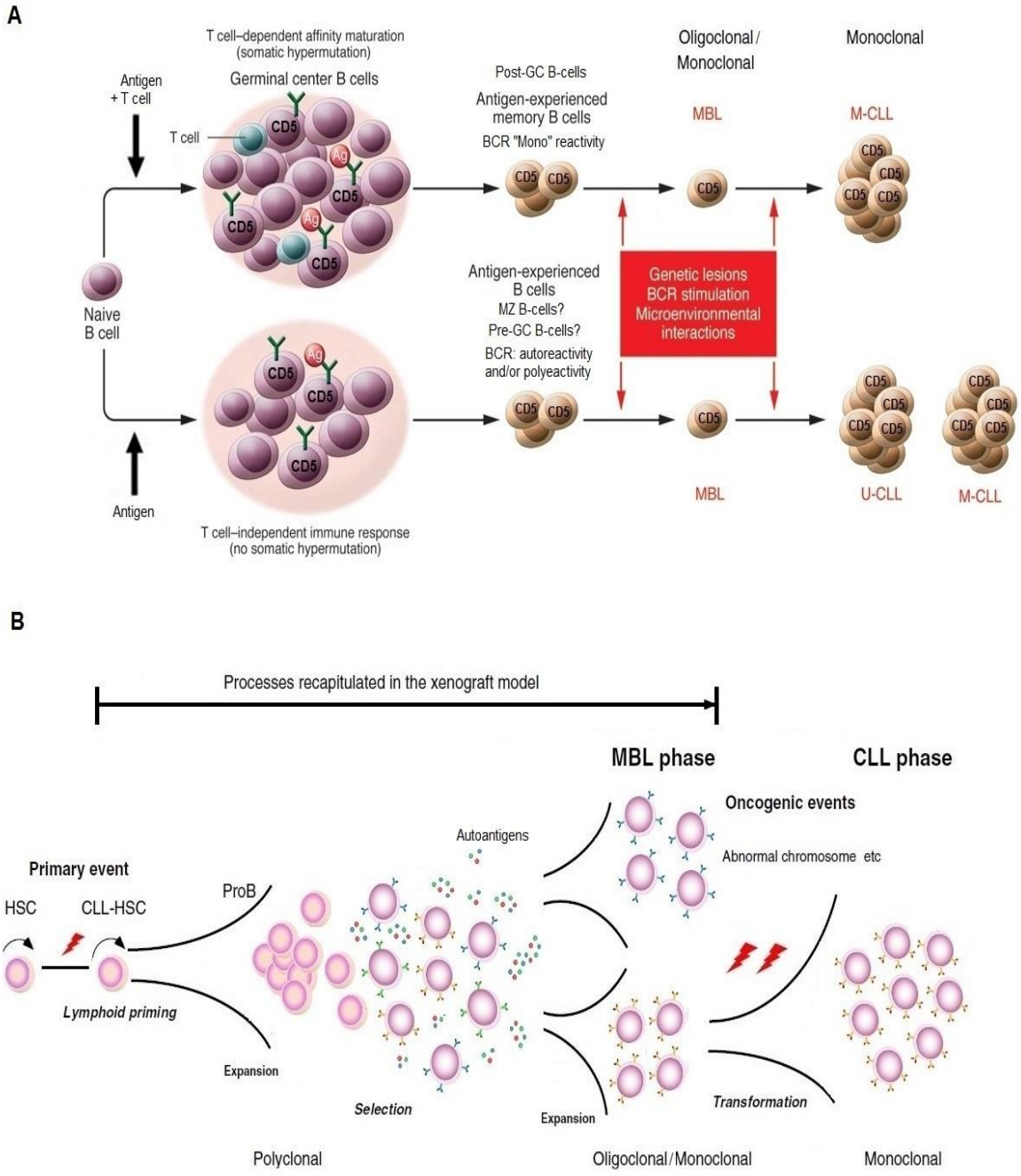

Figure 3. Schematic representation of "multistep" models for human CLL development. Panel A: Encounter of naive B-cells with antigen may proceed either through a T cell–dependent reaction occurring in the germinal center (GC), leading to the generation of memory B-cells that have undergone somatic hypermutation of the *IGHV* genes, or in T cell–independent immune responses, it may lead to the formation of antigen-experienced B cells harboring unmutated *IGHV* genes. CLL, and the preceding MBL phase, may originate from either of these subsets of antigen-experienced B cells. Two distinct $CD5^+$ B cell subsets (unmutated mature $CD5^+$ B cells and $CD5^+CD27^+$ post–GC B cells) and marginal zone (MZ) B cells have been identified as the potential cell compartment in which CLL originates. Panel B: alternatively, based on a xenogeneic transplantation model, it has been shown that genetic abnormalities can accumulate on CLL hematopoietic stem cells (HSC) that might amplify B cell differentiation and produce a high number of polyclonal B cells carrying the same genetic aberrations. B cell clones would be then selected and they would expand in response to BCR signaling, presumably driven by autoantigens, simulating progression of MBL. Additional abnormalities such as aberrant karyotypes might play a role in progression from MBL into CLL in humans [Adapted from: Gaidano, G *et al*. [328] and Kikushige, Y *et al*.[329]].

3.2.6.2. Stepwise model of development of MBL into CLL from early HSC

In order to trace the origin of CLL B cells, it should be noted that CLL cells from individual patients are not always monoclonal, and that two or more coexisting CLL clones are found in up to ≈5% of CLL patients.[21] Furthermore, a recent cohort study has shown that 44/45 patients with CLL had a precursor MBL state which had been identified between 6 months and 7 years in advance.[212] Of note, MBL is more frequently composed of two or more coexisting B cell clones (20%–70% of total cases) than CLL, this being a particularly frequent finding among MBL^{low} and in familial CLL cases.[15,20,203] These findings strongly support a model for stepwise development of CLL from an MBL oligoclonal precursor state, similarly to what has been described for other human cancers. Thus, stepwise development of MBL into CLL could have its onset in a mixture of polyclonal cells with one single dominant clone progressively taking over, such expanded B-cell clone either obscuring or eliminating other MBL clones. Recent evidences suggest that in such model, the first oncogenic event could be traced back at least up to the progenitor or HSC that has not rearranged the *IGHV* genes (Figure 3B).[329] In parallel, signaling through receptors on the expanded cells, particularly BCR signaling, would play a critical role in the pathogenesis of CLL, through its contribution to further oligoclonal and/or monoclonal expansion of B cells from the initial pool of (precursor) polyclonal B-cells.

In line with this hypothesis, recent studies by Kikushige *et al.*[329] in a xenogeneic mouse model, reported that HSC from CLL patients have an increased susceptibility to generate expansions of (oligo-mono)clonal B cells carrying *V(D)J* gene rearrangements which are always unrelated to those of the original CLL cells. These findings, suggest that aberrant CLL-HSC have already intrinsic abnormalities that cause their skewing towards the B-cell lineage, supporting early involvement of HSC in the pathogenesis of the disease, as aberrant preleukemic cells that produce an increased number of polyclonal (altered) pro-B cells (Figure 3B). The resulting mature B cells could be then selected most likely via the recognition of autoantigen(s), resulting in expanded mono- and/or oligoclonal B cell populations. Later on, along the B-cell differentiation pathway, additional genomic abnormalities would develop, eventually resulting in a typical clonal MBL and/or CLL disorder. Despite all the above, it should be noted that the role of such genetic abnormalities of HSC may be very limited (or even nonexisting) in the early phases of development of MBL, whenever the microenvironmental stimuli would play a more prominent role. Subsequently, genetic abnormalities disrupting the control of cell growth and survival may cooperate with microenvironment triggered events, mainly represented by antigen-mediated BCR and coreceptor stimulation, to trigger and fuel clonal B-cell expansion. In line with this, whole-genome sequencing (WGS) approaches have contributed to the

identification of multiple recurrent somatic mutations (e.g. *NOTCH1*, *MYD88* and *XPO1*), which appear to contribute to determine or modulate the clinical evolution of CLL.[6] In this regard, integrated mutational and cytogenetic analyses, have shown that clonal evolution from lower to higher risk CLL is associated with the emergence of molecular alterations (e.g. mutations) of the *NOTCH1*, *SF3B1*, and *BIRC3* genes in addition to those of the *TP53* and *ATM* genes;[264] moreover, *SF3B1* mutations primarily occur in CLL cases with del(11q)[330] and *NOTCH1* mutations are frequently associated with trisomy 12.[331] In addition, other altered genes such as *BAFF,* specific Lyn substrate 1 (*HS1*) and Toll IL-1R 8 (*TIR8*) appear to influence the natural history of the disease.[332] Most interestingly, previous studies indicate that virtually all recurrent mutations described so far in CLL appear to emerge and act only at relatively late stages of MBL and/or CLL; some of them may even underlie CLL transformation to Richter syndrome.[333] Therefore, such molecular/genetic alterations appear to contribute more to elucidate the genetic basis of CLL progression, rather than of MBL evolution, the potential initiating genetic alterations still remaining to be identified. The only exception relies on a recent report which is based on a model where deletion of the entire minimal deleted region at 13q14 chromosome, encoding for the *DLEU2/miR-15a/16-1 gene* cluster, which suggests that del(13q) could lead to the development of low penetrance indolent B-cell clonal lymphoproliferative disorders that appear to recapitulate the whole spectrum of human CLL-associated phenotypes from MBL to Richter syndrome.[334] This approach underlines the critical importance of the deleted region, which appears to harbor gene(s) involved also in the first steps of leukemogenesis in CLL, but it would not explain the origin of cases which have unaltered chromosome 13q14 sequences.

3.3. B-cell lymphoproliferative disorders (B-CLPD) other than chronic lymphocytic leukemia (CLL)

Classification of B-CLPD other than CLL relies to a significant extent on the relationship between the expanded B-cells and the GC reaction within secondary lymphoid organs. Thus, B-CLPD other than CLL are currently classified as pre-GC, GC and post-GC neoplasms since these WHO 2008 categories of ‘‘mature’’ B-cell tumors are considered to arise from B cells, that have already undergone *V(D)J* recombination at the *IGH* and *IGL* loci and that are independent of early hematopoiesis and HSC.[181] However, it should be noted that most evidences supporting a model of cell-of-origin that links these mature B cell tumors to post-*VDJ* recombination stages of the B-lymphoid ontogeny and the GC reaction, are indirect evidences, based on shared phenotypes by normal and tumor B-cells such as the pattern of expression of cell surface

markers and the capacity for ongoing SHM of the tumor cells and their "normal" counterparts (Figure 4).[7]

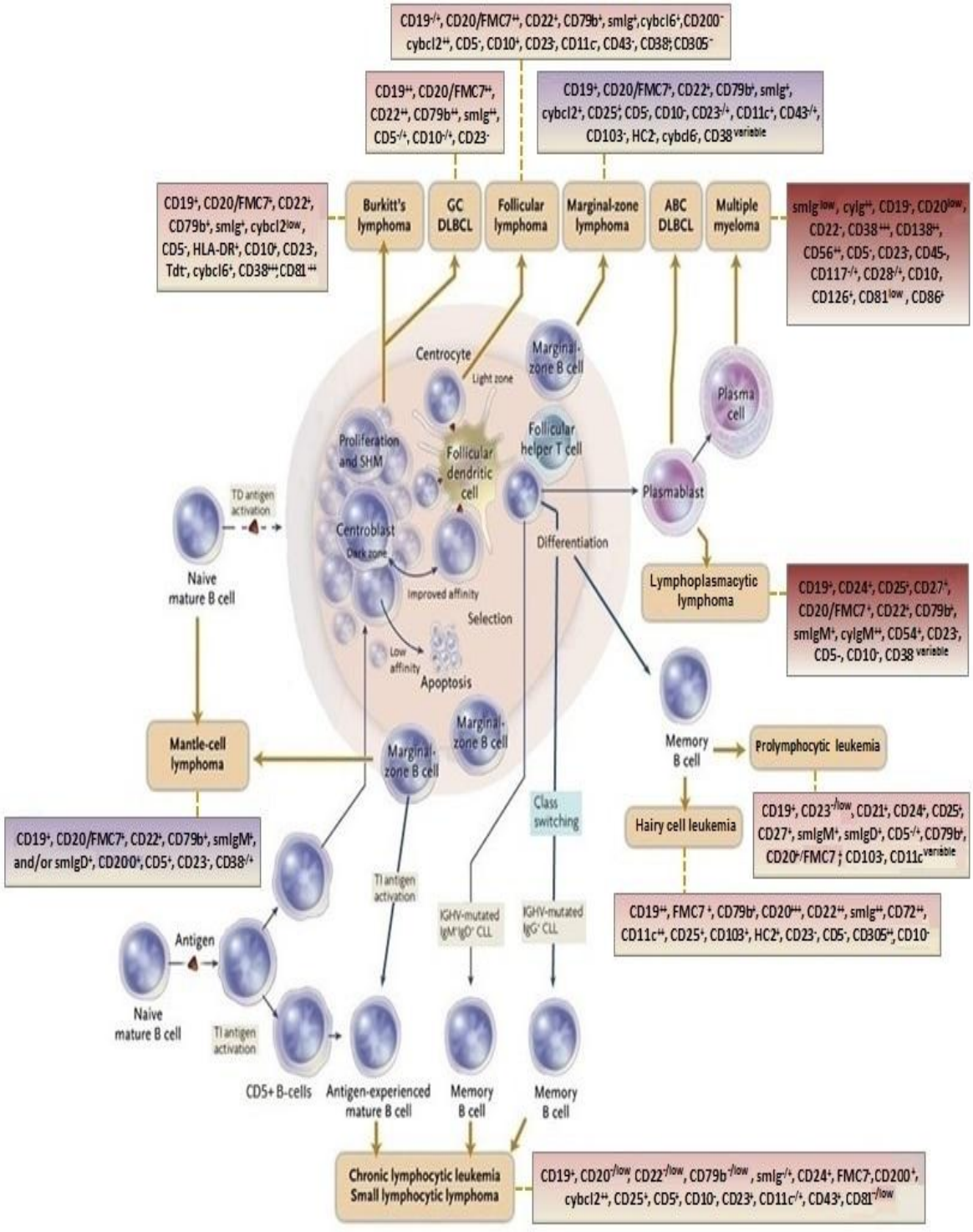

Figure 4. Cellular origin and immunophenotypic profile of human B-cell lymphoproliferative disorders (B-CLPD). Human B-CLPD are assigned to their potential normal B-cell counterpart, despite they also display aberrant patterns of protein expression which are presumably related to the cytogenetic abnormalities carried by the tumor cells and/or the altered microenvironment. ABC DLBCL, activated B-cell diffuse large B-cell lymphoma; GC DLBCL, germinal center derived DLBCL [adapted from Sagaert, *et al.*[324] and Zenz, *et al.*[335]].

3.3.1. Peripheral/mature B-cell chronic lymphoid leukemias other than CLL

Peripheral/mature B-cell chronic lymphoid leukemias other than CLL, traditionally include two major WHO 2008 disease categories: B-cell prolymphocytic leukemia (B-PLL) and hairy cell leukemia (HCL).

3.3.1.1. B-cell prolymphocytic leukemia

B-PLL is a rare and aggressive mature/peripheral lymphoid malignancy. For its diagnosis, the presence of prolymphocytes which account for more than 55% of all PB lymphoid cells on morphological grounds (usually > 90%), is required.[181] Of note, mature B-cell leukemias, such as CLL (particularly CLL/PLL), MCL or SMZL, and HCL variant (HCLv) share similar features at presentation to those of PLL, and recent studies indicate that most PLL cases could in fact represent a unique subgroup of MCL.[336] Despite its similarity to both SMZL and HCLv in terms of its clinical presentation, no cytoplasmic hairy projections or "villi " are seen in PLL, and the presence of B symptoms, a very high white blood cell (WBC) count (> 100×10^9/L), and an aggressive clinical course, are much more characteristic of PLL than of SMZL or HCLv.[337,338] In comparison to CLL/SLL, PLL has been described as having strong Ig, CD20/FMC7 and CD79b expression, in association with $CD23^{low/-}$ and decreased staining for CD5 in a fraction of the patients (Figure 4).

The most common cytogenetic abnormality seen in PLL is del(17p) involving loss of the *TP53* gene[339] and t(11;14) associated with expression of cyclin D1 and/or SOX11, the latter supporting the close relationship between PLL and MCL with a leukemic presentation.[340,341] Regarding their molecular features, PLL leukemic cells express a skewed repertoire characterized by predominant usage of distinct members of the *IGHV3* gene family (73%), preferentially of the *IGHV3–23* gene (50% of the *IGHV3* genes).[342] The type and distribution of *IGHV* mutations clearly indicates that in some cases the tumor cells have undergone through an Ag selection process.

3.3.1.2. Hairy cell leukemia

HCL is a rare type of leukemia characterized by the presence of large mature-appearing B cells, with an abundant cytoplasm and characteristic micro-filamentous ("hairy") projections – hairy cells (HC) –.[343] HC typically infiltrate the BM, the spleen, and to a lesser extent also the liver, LN and the skin.

Studies about the GEP of B cells from discrete subsets of normal B-cells vs. HCL cells have shown that HC are related to memory B-cells, although with an altered pattern of expression of genes controlling cell adhesion and expression of chemokine receptors.[344] Moreover, several phenotypic features of HC can be explained in the context of a highly activated B-cell that appears to have undergone the sequence of reactions that during normal immune responses occur upon stimulation by Ag, accessory cells and cytokines. Therefore, HCL is typically associated with an increased expression of activation-associated markers at intermediate to bright intensity, such as CD25, CD11c, FMC7, and CD103.[345] Other unique immunophenotypic features of HC that contribute to the diagnosis of the disease include: overexpression of CD20, CD22, CD19, CD72 and CD305 (LAIR1); intermediate intensity of expression of CD79b, CD123, T-bet, anexin A1 and HC2; intermediate to bright positivity for SmIg (generally SmIgM or SmIgD but also SmIgG or SmIgA), and; lack of expression of CD23 and CD24.[346-350] In addition, CD5 and CD10 are expressed on HC from a minority of cases (Figure 4).[177,345] Although, AID is expressed in HCL, HC fail to express GC-associated markers such as CD38, CD10 and Bcl6, at the same time they lack the CD27 memory B-cell marker in the great majority of cases.[351]

For many years, no recurrent chromosomal translocations and molecular alterations had been identified in HCL. More recently, the BRAF V600E mutation has been reported in the classic forms of HCL only, as the first genetic alteration recurrently associated with this disease.[352] Some cytogenetic alterations involving monoallelic deletions of *TP53* and *BCL6* have also been reported in a subset of cases.[353]

Due to the rarity of the disease, analysis of the *IGHV* gene has been only performed in small series of cases.[354] Despite this, results show that most HCL carry mutated *IGHV* genes with low levels of intraclonal heterogeneity, unmutated *IGHV* genes occurring only in a minor subset of patients who are refractory to (single-agent) cladribine and have a more aggressive behavior.[351,355] In turn, HCL is characterized by biased usage of the *IGHV3-21*, *IGHV3-30* and *IGHV3-33* genes which are highly homologous gene segments; thus, the BCR of HCL patients may share the ability to bind identical or highly similar Ags, and potentially react with common bacterial superantigens, such as modified *staphylococcus aureus* protein A or the natural *staphylococcus aureus* enterotoxin A.[356] In line with this hypothesis, *IGHV3-30* has been reportedly involved in the immune response against *Toxoplasma.*[356] Clearly, the pressure on *IGHV* selection in HCL appears to be different from that potentially occurring in other B-cell neoplasms, particularly in CLL. For example, *IGHV4-34* is used predominantly in a mutated conformation by CLL cells, while it is preferentially unmutated in HCL cases.[357,358] Interestingly,

the apparent lack of HCDR3 stereotypy in HCL is another distinctive feature of HCL vs. CLL and suggests that Ag driving in HCL may not rely on HCDR3-mediated interactions.

3.3.2. Peripheral/mature B-cell lymphomas other than SLL

3.3.2.1. Marginal zone lymphoma

MZL represents about 8-10% of all lymphomas and the third most frequent type of lymphoma, just after diffuse large B-cell lymphoma (DLBCL) and FL. At present it is widely accepted that MZL comprises distinct lymphoma types, which are considered to originate from marginal zone B cells. Depending (mainly) on the organ in which the lymphoma arises, the current WHO 2008 classification recognizes three distinct types of MZL: nodal (NMZL), extranodal MZL of MALT type (or MALT lymphoma) and splenic marginal zone lymphoma (SMZL), depending on whether the disease arises in the LN, mucosal sites and the spleen, respectively.[180] MZL shares some common immunophenotypic features, but the clinical course and molecular characteristics are different for each of the distinct subtypes of the disease. More recently, a fourth subtype of MZL named "primary BM MZL" (PBM-MZL) has also been described as a provisional entity that still deserves full recognition prior to inclusion into the new WHO classification.[359]

3.3.2.1.1. Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue or MALT lymphoma

MALT lymphoma is the most common type of MZL. It is a clinically indolent disease which preferentially arises in the stomach and small intestine, but that can occur in almost all mucosal tissues and in the skin. MALT lymphoma is therefore thought to arise from peripheral lymphoid tissues by long-standing (auto)antigenic stimulation, such as that observed during chronic infection and inflammatory disorders.[360,361] Several infectious agents and inflammatory disorders have been associated with MALT lymphomas, among which the association between gastric MALT lymphoma and *Helicobacter pylori* infection (up to 90% of cases) is the best characterized.[361]

MALT lymphoma cells express CD19, CD20, CD22, CD25, CD79b and CXCR3, typically in the absence of CD5, CD10 and CD45RO, and they show variable patterns of expression of CD23 and CD27 (Figure 4).[362] From the genetic/molecular point of view, it presents with several recurrent genomic lesions, including chromosomal translocations and unbalanced

alterations.[10] Thus, MALT lymphomas frequently have translocations involving the *API2*, *MALT1*, *BCL10*, and *FOXP1* genes.[363] T(11;18)(q21;q21) involving the *API2* gene at chromosome 11q21 and the *MALT1* gene at 18q21 chromosome, is the most common structural chromosomal abnormality in MALT lymphoma and its presence is usually correlated with the lack of any further genetic instability or chromosomal imbalances; in turn, t(3;14) and t(14;18) fuse the *BCL10* and *MALT1* genes to the heavy chain of the Ig locus, respectively.[364] Of note, the above mentioned chromosomal translocations are mutually exclusive and they typically show a different anatomic distribution.[365]

3.3.2.1.2. Splenic marginal zone lymphoma

SMZL is a relatively rare disease comprising 1-2% of all NHL and roughly 20% of MZL. It is mostly, but not always, a clinically indolent lymphoma.[180] The lymphoma originates in the spleen, and spreads in most cases to the BM; in addition, it is also detectable in the PB in a variable number of cases. SMZL is assumed to arise from the splenic marginal zone B-cells and it has a concordant $CD20^{high}$, $CD19^{+}$, $CD79b^{high}$, $CD20/FMC7^{+}$, $CD22^{+}$, $CD43^{+/-}$, $CD11c^{+}$, $SmIgM^{high}$, $SmIgD^{low}$, $CD27^{+}$, $CD5^{-/+}$, $CD103^{-}$, $HC2^{-}$, $CD25^{-}$, $CD10^{-}$ and $CD23^{-}$ immunophenotype (Figure 4).[366]

Skewed *VH* gene usage is pronounced in SMZL, with about 30% of cases using the *IGHV1-2* genes.[244] Also highly homologous CDR3 regions are observed among the latter cases, which may indicate Ag stimulation as part of SMZL lymphomagenesis.[367,368] In geographic areas where HCV and malaria infections are endemic, SMZL cases associated with these infections are frequently observed.[369]

In the largest series of SMZL (n=330 cases) in which cytogenetic analyses have been performed, it has been shown that 72% of cases exhibit chromosomal aberrations, of which 53% include complex genetic changes.[370] However, none of these cytogenetic abnormalities is considered to be characteristic of SMZL, with the exception of del(7q32) that occurs in 39% of the cases.[370] Other cytogenetic alterations involving chromosomes 8, 9p34, 12q23-24, 18q, and 17p have been reported.[370] In contrast to what occurs in MALT lymphoma, chromosomal translocations involving the Ig loci are rarely found in SMZL.

3.3.2.1.3. Nodal marginal zone lymphoma

NMZL, also called monocytoid B-cell lymphoma, is a primary nodal B-cell MZL in the absence of evidence for splenic or extra-nodal involvement; it represents 1.5–1.8% of all

lymphoid neoplasms.[181,371,372] Most NMZL cells express pan-B-cell markers including CD20, CD79a and PAX5, together with CD43 in around half of the cases.[373] NMZL cells typically lack expression of CD5, CD23, CD10, Bcl6, anexin A1, and cyclin D1, whereas Bcl2 is positive in most cases.[181,367,373]

As for other types of MZL, NMZL cases have been reported in association with infections (e.g. HCV)[374,375] and autoimmune disorders (e.g. rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome).[376] However, this evidence remains far from sufficient to establish a definitive role for these stimuli in lymphomagenesis. In turn, multiple studies have shown that in the majority of NMZL patients, SHM of rearranged Ig genes and preferential usage of *IGHV3* and *IGHV4* (particularly of *IGHV4-34*) occurs, further suggesting that antigenic stimulation is involved in the ontogenesis of this type of MZL.[377] Despite preferential usage of *IGHV4-34*, no association with EBV and CMV has been established, as for CLL.[249] However, in NMZL patients with HCV, *IGHV1-69* seems to be preferentially used.[378,379]

Finally, no unique and/or characteristic cytogenetic abnormality of NMZL has been reported so far. The cytogenetic alterations reported in this subtype of MZL include trisomy 3 (present in about 20-25% of cases),[380] 7, 12, and trisomy 18, plus structural rearrangements of chromosome 1 with breakpoints at the 1q21 or 1p34 chromosomal regions;[381,382] in contrast, SMZL-related 7q losses have not been reported among NMZL cases.[383]

3.3.2.1.4. Primary bone marrow marginal zone lymphoma

Among MBL cases with <5,000 B-cells/μl of PB and a $CD5^-$ non-CLL-like immunophenotype in the absence of LN, spleen and extranodal involvement, a new B-CLPD entity showing clonal B-cell lymphocytosis with marginal zone features, has been identified.[359] These cases usually correspond to adults between the 4^{th} and 8^{th} decades of life with a similar distribution per sex, and variable levels of PB infiltration by small B-lymphocytes with a monocytoid-like morphology, mixed with villous lymphocytes and lymphocytes with plasmacytoid features and a MZL phenotype; presence of serum paraproteinemia is observed in one third of the cases[359] and BM infiltration with a mixed nodular-interstitial pattern that varies from 10% to 70% of the whole BM cellularity, is also usually present. From the cytogenetic point of view, most cases show a normal karyotype (≈70% of cases) while others have complex cytogenetic profiles, where chromosomes 3, 12, 17 and 7 are those chromosomes more frequently involved, with e.g. del(7q) and iso17q in 13% and 17% of the cases, respectively. Most PBM-MZL patients show mutated *IGHV* genes with a clear predominance of the *IGHV4-34* gene.

From the clinical point of view, most PBM-MZL display an indolent and stable (clinical) course of the disease, although in some cases (particularly those with complex karyotypes) progression to symptomatic MZL, usually to a splenic MZL, is observed.[384]

3.3.2.2. Follicular lymphoma

FL accounts for 20%–30% of all lymphoid tumors, with the highest incidence in the Western countries.[385] From a biological point of view, FL represents the neoplastic equivalent of the reactive GC B-cell, presenting with varying and abnormal ratios of centroblasts and centrocytes. Tumor cells may leave and (re)enter the GC; consequently, they may periodically acquire novel SHM in their *IGH* and *IGL* genes. Indeed, either analysis of individual tumor cells or molecular cloning of *IGH* gene rearrangements from a pool of tumor cells, have both demonstrated that not all tumor cells share the same SHM within each individual lymphoma, as a result of subclonal evolution associated with distinct patterns of ongoing SHM of the *IG* loci.[386,387]

The t(14;18)(q32;q21) chromosomal translocation represents the cytogenetic hallmark of FL and it is detected in 80%–90% of cases. Its molecular consequence is the juxtaposition of the *BCL2* protooncogene close to the enhancer sequences of the *IGH* promoter region;[388,389] this leads to deregulated expression of Bcl2, resulting in overexpression of the Bcl2 protein in the neoplastic follicles.[390] Less frequently, variant translocations of t(14;18) also occur, such as t(2;18) or t(18;22), in which the *BCL2* gene is juxtaposed to the loci of the Ig light chains genes (κ or λ, respectively). All these events lead to accumulation of inappropriately rescued GC B-cells with a prolonged life span, allowing for the occurrence of additional genetic hits that are required for the establishment of an overt FL. Consequently, a relatively high number of secondary chromosomal alterations have been described in FL including structural and numerical changes. Of note, it has long been recognized that these alterations occur in a non-random fashion, and a temporal order in the emergence of these aberrations has been described, some occurring early in the course of the disease, whereas others usually represent late genetic events.[391-394] In addition, some of the reported alterations are mutually exclusive, while others frequently cluster together, potentially leading to coordinated deregulation of specific genetic pathways.[395] From the clinical point of view, such secondary genetic hits are crucial to distinguish different subgroups of FL with distinct biological behavior, risk of transformation to aggressive lymphoma, prognosis and overall survival (e.g. *BCL2* plus *CMYC* double hit transformed FL).[396]

Phenotypically, tumor cells from FL are most frequently characterized by overexpression of Bcl2, strong reactivity for SmIgM, CD20/FMC7, CD22 and CD79b. As normal GC B-cells, FL cells also express intermediate intensity CD10, Bcl6 and CD38 (Figure 4).[397,398] Conversely, these cells usually lack CD5, CD11c, CD23, CD43, CD103, CD200, CD305 and HC2 expression. CD19 is frequently underexpressed, or even negative in some FL cases (Figure 4).[397,398] Ki-67 is typically recommended for the assessment of the proliferation index of FL which is highly variable.

3.3.2.3. Mantle cell lymphoma

MCL is a relatively rare subtype of B-cell lymphoma, comprising 5%–7% of all lymphomas.[399] Typically, MCL is a very aggressive disease with a poor outcome,[400] that has specific genetic lesions and a characteristic $CD5^+$ immunophenotype. This phenotype differs from that of typical CLL/SLL in that MCL cells express CD20, CD22, CD79b and SmIg at moderate to bright intensity, and they are usually CD23 and CD200 negative while $FMC7^+$ (Figure 4).[397,401] Both CD38 and the proliferation marker Ki-67 show variable levels of expression.[402] Recently, an indolent variant of MCL termed asymptomatic B cell lymphocytosis with cyclin D1 expression has been described with a unique $CD38^-$ $CD200^{low}$ vs. $CD38^{low/+}$ $CD200^-$ immunophenotype.[403]

The genetic lesion typical of MCL is the t(11;14)(q13;q32) translocation which juxtaposes the *CCND-1* gene (also known as *BCL-1* or *PRAD-1* gene) encoded at chromosome 11q13 with an enhancer of the *IGH* gene at chromosome 14q32.[404] As a consequence, this translocation leads to an enhanced/continuous cell proliferation due to constitutive activation of the *CCND1* proto-oncogene and overexpression of cyclin D1 mRNA and protein.[404] In rare cases, MCL is cyclin $D1^-$; when this happens, cyclin D2 or cyclin D3 can be overexpressed.[405]

The t(11;14)(q13;q32) translocation most likely occurs as an initial oncogenic event at the pre–B-cell stage of differentiation in the BM, during recombination of the *V(D)J* segments of the *IGHV*. However, the tumor is composed of a unique population of mature-appearing B lymphocytes, indicating that the full neoplastic phenotype is acquired at later stages of the B-cell differentiation process.[406] Based on the pattern of expression of SmIgM/SmIgD and CD5 by the tumor cells, their topographic distribution in the mantle zones, and the predominant usage of unmutated *IGHV* genes, naive B-cells have been generally considered the normal counterpart of MCL tumor cells.[407] However, analysis of BCR diversity in MCL has shifted this view to a more complex ontogenetic model, in which Ag selection would play also an important role in the pathogenesis of the disease, at least in a subset of the cases. For

example, some studies have shown that 15%–40% of MCL carry *IGHV* hypermutation, with around 10% of MCL showing stereotyped Ig heavy chain CDR3 sequences and a restricted VH gene repertoire, associated with predominant usage of the *IGHV3-21*, *IGHV4-34*, and *IGHV5-51* genes.[408] Of note, the two former *VH* genes have a strong association with autoimmunity and the *VH3-21* gene seems to be also overrepresented in CLL with mutated *IGHV* genes, and associated with a significantly poorer overall survival than that found for CLL cases with other mutated *IGHV* genes.[357,409] Moreover, *IGHV3-21*$^{+}$ MCL patients appear to almost exclusively show usage of the *IGVλ3-19* gene.[357] In contrast to CLL, the *IGHV* gene mutation status is not prognostically informative among MCL patients.

3.3.2.4. Diffuse large B-cell lymphoma

DLBCL is the most common subtype of NHL, accounting for approximately one-third of all adult lymphomas.[410] It is characterized by a diffuse proliferation of large mature B-cells that mainly occurs in LN, although involvement of extranodal sites is observed in up to 40% of the cases.[362] BM involvement is found in 11% to 27% of all cases, whereas PB is rarely (5%-10%) infiltrated.[180] DLBCL is a heterogeneous lymphoma which may arise either as primary (*de novo)*, or may result from a transformation of an indolent lymphoma.

The great morphological, clinical and biological heterogeneity of DLBCL confirms the existence of several subtypes of the disease with different clinical outcomes. According to the WHO 2008 classification, DLBCL may be subdivided into several morphological variants (Table 1).[180,411,412] Similarly, GEP studies grouped DLBCL into three subtypes which are morphologically indistinguishable; these three GEP subgroups of DLBCL are associated with different pathogenetic mechanisms and they include activated B-cell DLBCL, germinal center DLBCL and primary mediastinal DLBCL (Table 1).[413] Consistent with this heterogeneity, the genetic lesions associated with DLBCL are also diverse and include balanced reciprocal translocations deregulating the expression of *BCL6*, *BCL2* and *MYC*, gene amplifications, nonrandom chromosomal deletions (e.g. 6q) and aberrant somatic hypermutation.[414-418] Nonetheless, in a significant fraction of DLBCL, no specific genetic changes have been identified which could potentially contribute to the pathogenesis of the disease.

The *BCL6* gene is one of the most frequently affected genes in DLBCL. *BCL6* promotes a higher cell survival and genetic instability, which both contribute to malignant transformation.[419] In turn, t(14;18) results in overexpression of the Bcl2 protein in an identical way as in FL, this cytogenetic alteration being found in 15% to 30% of all DLBCL cases and virtually all GC-associated DLBCL patients.[419,420] Genetic recombination of the *MYC* gene can be

found in 2% to 16% of DLBCL cases being specially frequent among extranodal DLBCL cases; *MYC* gene rearrangements are associated with a great rate of central nervous system involvement, lower complete remission rates and a poorer overall survival.[421]

There is no specific DLBCL immunophenotype. Large neoplastic B-cells have strikingly higher forward light scatter (FSC) and sideward light scatter (SSC), once compared to normal B lymphocytes, and they express variable patterns of pan-B-cell markers (CD19, CD20, CD22 and CD79b) (Figure 4); despite a mature B-cell phenotype, a subset of these lymphomas does not demonstrate SmIg or CyIg expression.[422] A proportion of cases express CD5 and/or CD10, the former marker being associated with an unfavorable clinical course.[423] Indeed, $CD5^+$ DLBCL may consist of a large cell lymphoma transformed from a lower-grade $CD5^+$ B-cell neoplasm such as CLL/SLL (Richter syndrome) or *de novo* $CD5^+$ DLBCL, including extremely rare cases of $CD5^+$ intravascular large B-cell lymphoma (Table 1).[424]

3.3.2.5. Burkitt lymphoma

Three different clinical variants of BL have been described so far: EBV-associated endemic and sporadic BL, and immunodeficiency (HIV)-associated BL.[180,181] Although all three variants share many morphologic and immunophenotypic features, they have distinct clinical and geographical presentations.[181] Regarding cytogenetics, there is considerable overlap among the three subtypes of BL, but unique genetic features have also been described among these variants, potentially reflecting distinct pathogenetic mechanisms.[425]

BL cells express SmIgM, Bcl6, CD19, CD20, CD22, and CD79a, and they are negative for CD5, CD23, and TdT, in association with a characteristic $CD38^{high}$, $CD81^{high}$, $CD10^+$ GC-phenotypic profile (Figure 4). Since CD10 is positive in nearly all cases, the typical absence of Bcl2 expression in association with very high levels of CD81 is helpful in ruling out “double-hit” lymphomas with dual translocations affecting *MYC* and *BCL2*.[426,427] Translocations involving the *CMYC* oncogene are the molecular hallmark of BL, and they are seen in virtually all cases in association with a high proliferation rate. In most cases, *CMYC* gene rearrangements involve the *IGHV* gene on chromosome 14; less commonly, the light chain genes on chromosomes 2 and 22 are involved in the *CMYC* translocation.[426,427] Sometimes, *CMYC* translocations may also involve a non-*IG* partner gene.[428,429]

3.3.2.6. Lymphoplasmacytic lymphoma / Waldenström macroglobulinemia

LPL/WM is a B-cell lymphoproliferative disorder characterized by IgM paraproteinemia and the accumulation of clonally related lymphocytes, lymphoplasmacytic cells and plasma cells in the BM.[362,430] Some patients suffer from hyperviscosity caused by exceeding levels of the IgM paraprotein, which may be useful in differentiating LPL/WM from other neoplasms with plasmacytoid differentiation.[362,431]

Morphologically, LPL/WM is characterized by proliferation of small B-cells, plasmacytoid cells and also plasma cells. The plasma cell component expresses the same Ig light chain as the lymphocytic component, it is positive for CD138 and shows low expression of B cell–associated Ags such as CD20, Pax5 and SmIg.[432] The lymphoma cells typically express CD19, CD20, CD22 and CD79b, CD27 and Bcl2, while they usually lack CD5, CD10 and CD23, which helps to discriminate LPL/WM from FL, CLL and MCL (Figure 4).[432,433] However, expression of these latter markers may be found in 10–20% of cases, often at weak and variable levels.[434] The distinction between LPL/WM and other $CD5^-$ small B-cell lymphomas is more difficult, and it often requires detailed evaluation of a combination of immunophenotypic, genetic, morphologic and clinical features.

At the molecular level, the neoplastic clone from most LPL/WM cases has undergone *IGHV* somatic hypermutation, but not isotype class switching, retaining the capability for plasma cell differentiation.[431] The lack of a unique molecular/genetic hallmark of the disease has complicated the distinction between LPL/WM and other B-cell lymphomas presenting a monoclonal serum component.[435] Recently, the *MYD88 L265P* mutation has been found in >90% of cases of LPL/WM, pointing out the utility of this molecular marker to distinguish LPL/WM from other entities,[431] and the potential involvement of e.g. TLR-signaling in the pathogenesis of this disease.

In the WHO 2008 classification it is stated that t(9;14) would be present in at least 50% of cases. However, translocations involving the *IGH* genes are actually rare in LPL/WM.[436] The t(9;14)(p13;q32) translocation results in up-regulation of *PAX5*, implicating this gene as a putative oncogene in the pathogenesis of a subset of LPL.[437,438] Overexpression of *PAX5* seems to prevent expression of high levels of Ig that result in paraproteinemia, supporting the absence of t(9;14)(p13;q32) in typical LPL/WM cases associated with a serum monoclonal component.[439] In turn, there are sporadic reports of WM tumors with IGH gene rearrangement, t(11;18)(q21;q21) and chromosome 6q abnormalities, which may also occur in other B-cell lymphomas types.[439]

On immunophenotypic grounds, both the lymphoid and the plasma cell compartments of LPL/WM neoplastic cells, show an immunophenotype that largely overlaps with their normal counterparts with expression of CD25 on the B lymphocyte compartment and positivity for immature markers (e.g. $CD20^{+}$ and $SmIg^{+}$) on plasma cells, usually associated with expression of SmIgK light chains (≈80% of cases).

3.3.3. Multiclonal lymphoproliferative disorders

Generally, neoplastic B-cells from B-CLPD are believed to result from the expansion of a single B-cell clone within which all cells share an original transforming mutation, identical *V(D)J* gene segments of the *IGH* gene, and a restricted expression of the rearranged Ig light chain (e.g. kappa or lambda). Additional genetic alterations in association also with different phenotypic features, may be gained during the evolution of the disease. However, presence of two or more morphologically and/or phenotypically different populations of neoplastic lymphocytes in the same patient, detected either simultaneously or at different time points during follow-up, has been reported in the literature[440-443] at an overall frequency of around 5% of all B-CLPD.[21] Actually, once the prevalence of B-cell neoplasias in elderly patients is considered, simultaneous emergence of two distinct clones turns out to be not that uncommon. Usually the presence of such coexisting ≥ 2 B-cell populations is interpreted as a result of either two completely different (apparently) unrelated B-cell populations – multiclonality – or subclone formation within the original malignant tumor stem cell line – intraclonal evolution/heterogeneity –.

3.3.3.1. Composite lymphomas

Composite lymphomas are defined by the coexistence of two or rarely, more than two morphologically and/or immunophenotypically different types of lymphoid neoplasms in the same patient, which arise synchronously or metachronously within the same anatomic site.[444] Although these lymphomas can be clonally related or unrelated, and they may comprise many different combinations of mature B-cell subtypes,[445] they are broadly categorized as follows: 1) cases where distinct types of NHL of the same lineage, mostly B-cell lymphomas, coexist; 2) coexistence of a B-NHL and a Hodgkin lymphoma (HL);[446] 3) distinct types of NHL of different lineages, e.g. B- and T-NHL,[447] and; 4) colocalization of a T-NHL and a HL.[448] Most cases in the first category represent tumor progression and transformation from an indolent B-cell lymphoma, with two or more abnormal B-cell populations usually showing distinct

morphological features, light scatter characteristics and/or DNA cell contents, but an identical phenotype; for example, Richter transformation of CLL/SLL or transformation of a FL to DLBCL.[449] In contrast, in a few cases, simultaneous occurrence of two distinct small B-cell lymphomas is demonstrated which may involve any specific B-NHL subtype, e.g. MCL and/or SMZL. A recent report, about composite lymphoma cases included patients diagnosed with MCL and FL, MCL with Hodgkin lymphoma (HL), and MCL with plasmacytoma or SLL, together with a case of composite FL with SLL; of note, in none of these patients, the two neoplastic B-cell compartments were found to be clonally related, based on molecular analysis of the Ig gene rearrangement profile.[450] Rare cases of composite lymphomas involving SMZL include cases associated with HL,[451] DLBCL,[452] BL,[453] or CLL,[454] and also, cases of T-cell large granular lymphocyte leukemia associated with SMZL.[455,456]

3.3.3.2. Intraclonal evolution versus multiclonality in B-cell chronic lymphoproliferative disorders

Coexistence of two apparently distinct hematological malignancies always raises the question about whether or not they share a common cell of origin. As mentioned above, the detection of two different, apparently unrelated B-cell clones – already at diagnosis or during follow up –, is usually interpreted as the emergence of two clonal populations arising from a single primary tumor (intraclonal evolution),[457,458] rather than coexistence of two distinct lymphoid tumors (true multiclonality).[459] Intraclonal evolution and conversion between two histological subtypes of B-cell lymphoma is commonly interpreted as being associated with differentiation of neoplastic B-cells towards more aggressive disease leading to a shortened survival. Conversely, multiclonality appears to be rare. However, multiclonality might have been underestimated, since the precise diagnosis of these cases usually requires a multidisciplinary approach encompassing histopathology, cytomorphology, immunophenotypic, cytogenetic and molecular analyses in each of the cell populations, and such studies have frequently not been performed in a substantial fraction of the composite lymphomas reported in literature, the potential clonal relationship between the two tumoral components, being therefore disregarded.

3.3.3.3. Criteria for multiclonality

Documentation of multiclonality greatly depends on the type of diagnostic methods used for the characterization of the co-existing tumor cell populations. Histopathological and

immunophenotypic studies, usually reveal this situation – e.g. coexistence of two or more distinct and expanded abnormal lymphoid populations –, but neither of the two approaches guarantees the nature of the clonal relationship that exists among the coexisting tumor cell populations from most cases. Only the genetic rearrangements of the *IGHV* and/or the cytogenetic abnormalities of the coexisting B-cell populations may provide definitive evidence to distinguish between a potentially common vs. distinct origin of the involved tumor cell populations.[460] Hence, multiclonality can only be clearly demonstrated through the combination of these techniques (i.e., histopathology and/or flow cytometry plus molecular and cytogenetic characterization of each individual population) (Figure 5).

Regarding MFC immunophenotyping, usage of multicolor gating strategies, involving the combined evaluation of the patterns of expression of both Ig heavy and light chains, together with maturation-associated markers, is mandatory for precise identification and definition of such coexisting cell populations and the identification of distinctive features that might be used for their purification, for subsequent molecular/cytogenetic analyses. Physical isolation of each individual pathological cell populations through high-throughput cell sorting procedures or laser microdissection techniques, allows for a better characterization of the coexisting B-CLPD (sub)clones by other methods, such as PCR analysis of *IGH* gene rearrangements and/or the assessment of different genetic abnormalities by iFISH procedures.

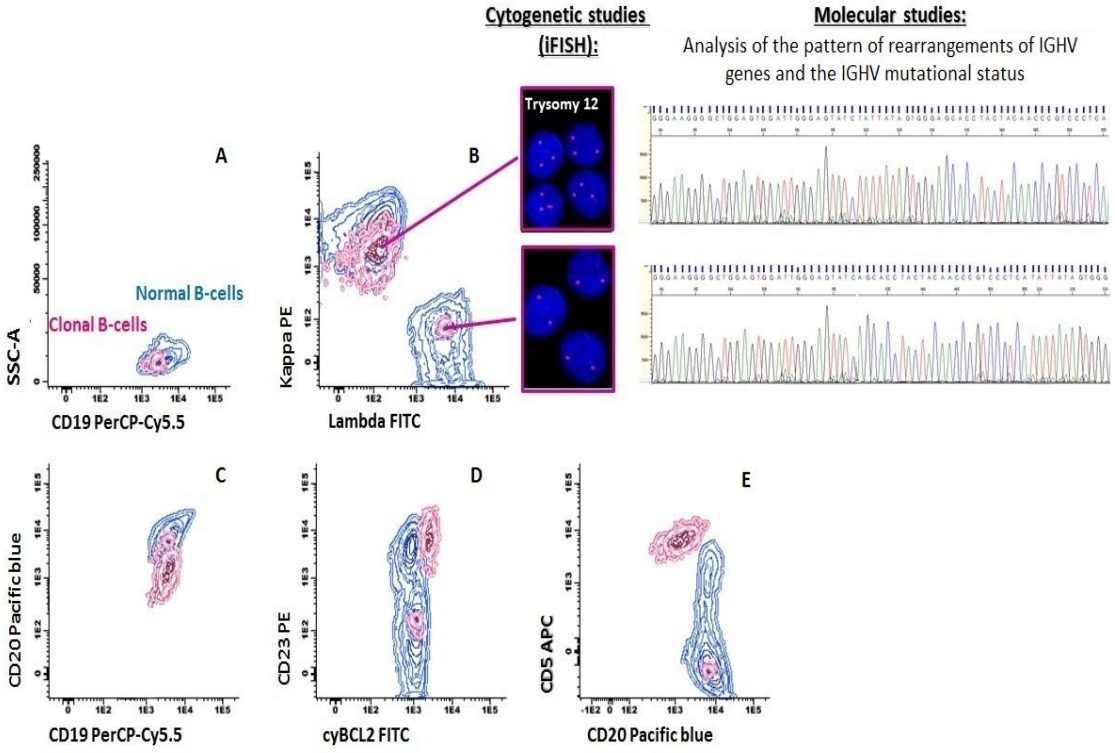

Figure 5. Illustrating example of a gating strategy based on the definition of $CD19^{+}$ cells (Panel A) for the immunophenotypic identification and characterization of multiclonal B-CLPD using multiparameter flow cytometry. Panels B to E show the gating strategy used for the identification of the two coexisting pathologic B-cell populations

(pink dots) and their phenotypic discrimination from the remaining normal B-cells (blue dots) coexisting in the same peripheral blood sample. After cell sorting the purified B-cell populations can be evaluated by e.g. PCR analysis of *IGH* gene rearrangements and iFISH (upper right panels) which demonstrated the presence of different *IGHV* gene rearrangements in the two B-cell clones and trisomy 12 retricted to only one of them. SSC, sideward light scatter; APC, allophycocyanin; Cy, cytoplasmic; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCPCy5.5, peridinin chlorophyll protein-cyanin 5.5.

3.3.3.4. Multiclonality in chronic lymphocytic leukemia

So far, multiclonal CLL has not been fully characterized since only rare cases of CLL with >2 CLL-like or non-CLL-like clones have been reported in the literature.[21,441,459,461,462] Reported cases include multiclonal CLL associated with MCL,[443] HCL,[463] FL,[464] and another CLL.[462] Therefore, the occurrence of multiclonal CLL is currently viewed as unusual and raises questions about whether these patients are particularly susceptible to develop B-CLPD in general, particularly CLL, and their actual clinical significance. Despite all the above, preliminary studies indicate that the frequency of multiclonality among CLL ranges between 5% and 10% of cases, and it may appear already at diagnosis (≈ 5% of cases) or during follow-up, sometimes even more than ten years after the initial diagnosis.[465] From the clinical point of view, CLL patients who present ≥2 B-cell clones, particularly when the CLL clone coexists with a non-CLL B-cell clone, seem to have a poorer outcome, as regards the requirement for early treatment (treatment-free survival).[21] In turn, no data about the impact of multiclonality on the final outcome (overall survival) of CLL patients followed for long periods of time (≥ 5-10 years), is currently available.

4. HYPOTHESIS AND OBJECTIVES

B-CLPD are a heterogeneous group of diseases that usually show (mono)clonal expansion of a single mature-appearing and aberrant population of B-lymphocytes, arrested at a given stage of differentiation. Despite this, patients diagnosed with composite lymphomas and other B-cell chronic lymphocytic leukemias – e.g. CLL – have been occasionally reported in the literature for decades, particularly among immunocompromised subjects.[21,441,461] Although early reports considered this phenomenon to be a rare event, current data suggests that it might have been underestimated due to the need for sophisticated multidisciplinary approaches encompassing combined histopathology, cytomorphology, immunophenotypic and cytogenetic techniques, and/or molecular analyses of purified cell populations. Actually, B-CLPD consisting of two or more phenotypically distinct populations which are clonally

unrelated, but that coexist in the same patient (detected either simultaneously or at different time points during follow-up), have been reported in the literature (e.g. multiclonal B-CLPD) at an overall incidence of around 5% of all B-CLPD.[21] Interestingly, presence of different unrelated B-cell clones in individual subjects has been shown to be particularly higher (>20%) in premalignant B-cell states such as MBL.[20,22] In this regard, preliminary data based on a relatively small series of subjects also demonstrated that the frequency of multiclonality could be particularly high among CLL-like MBL cases from CLL relatives.[20] Altogether, these findings suggest that, similarly to what occurs in other types of cancer, the development of CLL and potentially also of other B-CLPD, at the earliest steps, may involve a mixture of polyclonal cells, with one to a few clones progressively taking over. However, the immunobiology of such abnormally expanded B cell clones in multiclonal MBL and CLL, remains largely unknown.

In parallel to the above findings, it has been demonstrated that the SHM status of the *IGHV* genes can divide CLL into two subgroups and that these subgroups experience markedly distinct clinical outcomes; at the same time, the identification of these *IGHV* subgroups of CLL also represented an important advance in the understanding of the disease and its pathogenesis. Thus, at present it is well-established that CLL B cells, as well as tumor B-cells from other B-CLPD, express a distinct restricted BCR repertoire, which suggests a selection process driven by specific Ags. Moreover, recent identification of quasi-identical (stereotyped) BCR expressed by different CLL patients, reinforces the notion that BCR reactivity may play an important role in the B-cell transformation process leading to CLL, although the specific Ags recognized by CLL antibodies still remain largely elusive.

Altogether, these observations indicate that CLL and potentially also other B-CLPD, could represent antigen-driven expansions of specific subsets of normal B cells which have undergone neoplastic transformation from a multiclonal phase to a monoclonal state, in association with stepwise acquisition of genetic alterations and potentially also BCR-independent growth stimulation, MBL representing an intermediate stage in this oncogenic process. Despite this and the already recognized risk factors, it should be noted that at present, very limited knowledge exists about the transformation events that select individual normal B-cells to become first CLL-like MBL and then, CLL B cells.

Based on the above background, the **general objective** of this study aimed at gaining insight into the early stages of development of B-CLPD through detailed analysis of the immunophenotypic, genetic and molecular characteristics of clonal B-cells, paying special attention to the role and features of the BCR in both CLL-like MBL and CLL, and their subsets of

monoclonal vs. multiclonal cases, as different stages of a putative model of disease progression and malignant transformation.

In order to accomplish this goal, we addressed the following three **specific objectives**:

- To investigate whether specific *IGHV* repertoires are associated with unique cytogenetic profiles, as the basis for stepwise transformation pathways from MBL to CLL;

- To analyze the molecular and cytogenetic features of the expanded B-cell clones from multiclonal vs. monoclonal B-cell lymphoproliferative disorders (particularly CLL-like MBL and CLL cells) in order to determine whether multiclonality is associated with molecular features indicating a greater probability of interaction with shared/common immunological determinants, and;

- To investigate the potential relationship between an altered/clonal hematopoiesis and antigenic driving forces acting during the expansion of stereotyped vs non-stereotyped CLL and CLL-like MBL clones.

In the following sections of this doctoral thesis, a detailed description of the patients from whom samples were obtained and studied, the materials and methods used for such studies, as well as the results obtained are presented and discussed separately for each of the proposed three specific objectives.

Chapter 2 | Combined patterns of *IGHV* repertoire and cytogenetic/molecular alterations in monoclonal B lymphocytosis versus chronic lymphocytic leukemia

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2.1. Abstract

CLL-like MBL with (MBL^{high}) or without (MBL^{low}) absolute B-lymphocytosis precedes most CLL cases, the specific determinants for malignant progression remaining unknown. For this purpose, simultaneous iFISH and molecular analysis of well-established cytogenetic alterations of chromosomes 11, 12, 13, 14 and 17 together with the pattern of rearrangement of the *IGHV* genes were performed in CLL-like cells from MBL and CLL cases. Our results based on 78 CLL-like MBL and 117 CLL clones from 166 subjects living in the same geographical area, show the existence of three major groups of clones with distinct but partially overlapping patterns of *IGHV* gene usage, *IGHV* mutational status and cytogenetic alterations. These included a group enriched in MBL^{low} clones expressing specific *IGHV* genes (e.g. *VH3-23*) with no or isolated good-prognosis cytogenetic alterations, a second group which mainly consisted of clinical MBL^{high} and advanced stage CLL with a skewed but different CLL-associated *IGHV* gene repertoire (e.g. *VH1-69*), frequently associated with complex karyotypes and poor-prognosis cytogenetic alterations, and a third group of clones with intermediate features, with prevalence of mutated *IGHV* genes, and higher numbers of $del(13q)^{+}$ clonal B-cells. These findings suggest that the specific *IGHV* repertoire and *IGHV* mutational status of CLL-like B-cell clones may modulate the type of cytogenetic alterations acquired, their rate of acquisition and/or potentially also their clinical consequences. Further long-term follow-up studies investigating the *IGHV* gene repertoire of MBL^{low} clones in distinct geographic areas and microenvironments are required to confirm our findings and shed light on the potential role of some antigen-binding BCR specificities contributing to clonal evolution.

2.2. Materials and Methods

2.2.1. Patients and samples

A total of 166 subjects presenting one or more CLL-like MBL and/or CLL clonal B-cell populations were included in this study: 15 cases (9%) corresponded to healthy individuals with MBL^{low} – <200 clonal B-cells/µL in peripheral blood (PB); 5 males and 10 females; mean age of 68±13 years; range: 49-84 years, –33 (20%) were MBL^{high} – ≥200 and <5,000 clonal CLL-like B-cells/µL of PB – (20 males and 13 females; mean age of 72±12 years; range: 37-89 years), –114 (69%) had newly-diagnosed untreated CLL (66 males and 48 females; mean age of 70±13 years; range: 35-89 years) and 4 (2%) had other B-cell lymphoproliferative disorders (B-CLPD) with coexistence of one or two CLL-like MBL B-cell population(s) (Table 5). From the 33 MBL^{high}

cases, 20 (61%) showed clinical MBL (>2,000 clonal B-cells/µl of PB). Individuals corresponded to consecutive MBL and CLL subjects from Salamanca (Spain) and Coimbra (Portugal) in the western area of the Iberian Peninsula.

PB samples were obtained from each subject after written informed consent was given, and the study was approved by the local ethics committees of the two participating centres (University Hospital of Salamanca and Histocompatibility Centre of Coimbra). Diagnosis of MBL and CLL was based on the WHO 2008 criteria.[466] Clinical staging of CLL subjects according to Binet classification[466] was collected retrospectively; 46/77 (60%) CLL cases were diagnosed as stage A and the remaining cases (31/77, 40%) as stage B/C. Overall, 37/166 subjects (22%) showed co-existence of two or three phenotypically different aberrant B-cell populations (multiclonal cases; 25 males and 12 females with a mean age of 76±8 years; range: 57-89 years), while the remaining 129 individuals showed one single phenotypically aberrant monoclonal B-cell population (monoclonal cases; 69 males and 60 females with a mean age of 68±12 years; range: 35-89 years). In 26/37 multiclonal cases, all different B-cell populations showed a typical CLL-like phenotype, while in the remaining 11 cases only one B-cell population displayed a typical CLL-like phenotype co-existing with population(s) phenotypically compatible with other B-CLPD.[192] For this study, analysis was focused only on those aberrant B-cell populations displaying a typical CLL-like and CLL phenotype (n=195 B-cell clones). The distribution of all CLL-like and CLL clonal populations analyzed in the distinct diagnostic categories was as follows: 27 corresponded to CLL-like MBL^{low}, 51 to CLL-like MBL^{high} and 117 to CLL (Table 5).

Table 5. Distribution of subjects included in the study and the corresponding CLL and CLL-like MBL clones, according to diagnosis.

			Diagnostic subgroups			
		No. of cases	**MBL^{low}**	**MBL^{high}**	**CLL**	**Other B-CLPD**
Subjects	Monoclonal	129	13 (87%)	25 (76%)	91 (80%)	-
	Multiclonal	37[†]	2 (13%)*	8 (23%)*	23 (20%)*	4 (100%)*
	Total	**166**	**15**	**33**	**114**	**4**
B-cell clones	From monoclonal cases	129	13 (48%)	25 (49%)	91 (78%)	-
	From multiclonal cases	66	14 (52%)	26 (51%)	26 (22%)	-
	Total	**195**	**27**	**51**	**117**	**-**

**For multiclonal CLL and CLL-like MBL cases as well as for other B-CLPD cases other than CLL, only CLL-like clones were considered; the later B-CLPD cases included the following diagnoses: HCL, hairy cell leukemia; SMZL/MALT, splenic marginal zone B-cell lymphoma/ extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue lymphoma. CLL, chronic lymphocytic leukemia; MBL, monoclonal B-cell lymphocytosis; B-CLPD, B-cell chronic lymphoproliferative disorders. [†]The number of clones per multiclonal case was of two in all diagnostic subgroups, except in three tri-clonal subjects corresponding to one CLL patient, one MBL^{high} case and one patient with a B-CLPD other than CLL.*

2.2.2. Immunophenotypic analyses

Immunophenotypic studies to screen for the presence and full characterization of clonal B-cell populations were performed on erythrocyte-lysed PB samples according to procedures which have been previously described in detail.[192,467,468] PB white blood cells (WBC) were systematically stained with the following monoclonal antibody (mAb) combinations following the EuroFlow recommendations:[469,470] 1) CD20-pacific blue (PacB)/ CD45-pacific orange (PacO)/ CD8-fluorescein isothiocyanate (FITC)+ anti-SmIgλ-FITC / CD56-phycoerythrin (PE) +anti-SmIgκ-PE/ CD4-peridinin chlorophyll protein-cyanin 5.5 (PerCPCy5.5)/ CD19-PE–cyanin 7 (PE-Cy7)/ CD3-allophycocyanin (APC)/ CD38-AlexaFluor 700 (AF700); 2) CD20-PacB/ CD45-PacO/ Cybcl2-FITC/ CD23-PE/ CD19-PerCPCy5.5/ CD10/-PE-Cy7 CD5-APC/ CD38-AF700 and 3) CD20-PB/ anti-sIgλ-FITC/ anti-SmIgκ-PE/ CD19-PerCPCy5.5/ CD10-PE-Cy7/ CD5-APC. All cases showed a clonal (imbalanced SmIgκ:SmIgλ ratio of >3:1 or <1:3) and/or an aberrant $CD5^+$ B-cell population for the above mAb combinations, as reported elsewhere;[15] in every case, the phenotypic study was extended with the following additional 5- and 6-color stainings (PacB/FITC/PE/PerCPCy5.5/PECy7/APC): 1) CD20/ CD22/ CCR6/ -/ CD19/ CD5; 2) CD20/ CD103/ CD25/ CD5/ CD19/ CD11c; 3) CD20/ CD43/ CD79b/ CD5/ CD19/ CD49d; 4) CD20/ SmIgM/ CD27/ -/ CD19/ CD5; 5) CD20/ FMC7/ CD24/ - / CD19/ CD5; and 6) CD20/ CD3/ Cyzap70/ -/ CD19/ CD5. For the staining of Cybcl2 and Cyzap70, the Fix & Perm™ reagent kit (Invitrogen, Carlsbad, CA, USA) was used, following the recommendations of the manufacturer. All reagents were purchased from Becton/Dickinson Biosciences (BDB; San Jose, CA, USA), except for CD19-PECy7 (Beckman/Coulter, Miami, FL), CD20-PacB (e-Biosciences, San Diego, CA, USA), CD38-AF700 (Exbio, Prague, Czech Republic), CD45-PacO (Invitrogen, Carlsbad, CA, USA), CD79bPE, CD24PE and CD43FITC (Immunotech, Marseille, France), and bcl2-FITC, anti-IgM-FITC, anti-SmIgλ-FITC and anti-SmIgκ-PE (DAKO, Glostrup, Denmark).

Data acquisition was performed on a FACSCanto II flow cytometer (BDB) using the FACSDiva software (v6.1; BDB); in MBL^{low} cases, acquisition was done using a double-step procedure: firstly, information on $1x10^5$ events corresponding to the whole sample cellularity was stored; in the second step, information was stored on $CD19^+$ and/or $CD20^+$ gated events, containing a minimum of $5x10^6$ leucocytes/tube.

Instrument setup and calibration were performed according to well-established protocols, and a daily quality control program was followed, using the Cytometer Setup and Tracking (CST)™ Beads and CST Module (BDB).

Data analysis was performed using the INFINICITY™ software program (Cytognos SL, Salamanca, Spain). B lymphocytes were identified according to their SSC/$CD19^+$ distribution

and their numbers were calculated after excluding cell debris and platelets. The definition of a CLL-like/CLL phenotype was based on the presence of a $CD19^{+}$, $CD5^{+}$, $CD20^{low}$, $CD23^{+}$, $CD79b^{low}$, $FMC7^{-/low}$, $Cybcl2^{high}$ and $SmIg\kappa^{low}$ or $SmIg\lambda^{low}$, in the absence of CD10 expression. The minimum number of cellular events required to define the presence of a CLL-like/CLL B-cell cluster was of 50 cells.

The frequency of distribution of $sIg\kappa^{+}$ vs. $sIg\lambda^{+}$ populations within the CLL-like/CLL B cells were visually analyzed by superimposing the two fluorescence profiles in a double exposed picture (κ/λ distribution). An imbalanced sIgκ/sIgλ ratio of >3:1 or <1:3 was considered abnormal.

2.2.3. Fluorescence-activated cell sorting (FACS) purification of B-cell populations (FACSorting)

For all individuals studied, each CLL-like $CD5^{+}$ B-cell population identified was purified in a FACSAria III flow cytometer (BDB). In those samples with more than one aberrant B-cell population (n=37), discrimination among them was based on their distinct patterns of expression for ≥1 of the B-cell markers analyzed, as described elsewhere.[467] The clonal nature of each FACS-purified B-cell population (purity: 98%±0.8%) was assessed by both cytogenetic and molecular techniques, as described below.

2.2.4. Cytogenetic and molecular studies

The presence of the most common cytogenetic abnormalities associated with CLL was investigated by multicolour iFISH on slides containing FACS-purified and fixed CLL-like and CLL cells, as previously described.[253] Analysis of trisomy 12, del(11q23), del(17p13.1), and del(13q14), as well as structural abnormalities involving the IgH gene were systematically investigated using the following DNA probes purchased from Vysis (Downers Grove, IL): CEP12 DNA probe conjugated with spectrum orange (SO), LSI ATM (11q22.3), LSI MLL (11q23.3) dual-color probe, LSI p53 (17p13.1) conjugated with SO, LSI13/RB1 gene (13q14) conjugated with SO, LSI D13S25 (13q14.3) conjugated with SO, and LSI IgH/bcl2 t(14;18)(q32;q21) dual color probe, respectively. FISH studies were then performed on purified CLL-like/CLL B-cells fixed in 3/1 (vol/vol) methanol/acetic and hybridized. Briefly, pepsin-digested slides containing both the cells´ DNA and the probes´ DNA were denatured at 75°C for 1 min and immediately hybridized (overnight at 37°C), in a Hybrite thermocycler (Vysis). Then, slides were sequentially washed (5 min at 46°C) in 50% formamide/2X saline sodium chloride citrate buffer (SSCb) and

PBS with 1% Tween-20 (vol/vol), and counterstained with 35 µl of a mounting medium containing 75 ng/ml of DAPI (Sigma, St Louis, MO); Vectashield (Vector Laboratories, Burlingame, CA) was used as antifading agent.

Analysis of the patterns of rearrangement of the *IGHV* genes was performed for each FACS-purified CLL and CLL-like B-cell population. Genomic DNA preparation, PCR amplification, sequencing and analysis of *V, (D), J* genes were performed following well-established protocols.[471,472]

High molecular weight DNA from sorted CLL-like/CLL B-cells was isolated by standard proteinase K digestion, and isopropanol precipitation in the presence of glycogen to increase the DNA yield; final washing of the DNA pellet was performed in ice cold 70% ethanol. For amplification of complete *IGH VDJ* rearrangements, six different family-specific VH primers and one JH consensus primer were used in one multiplex PCR reaction covering framework region (FR) 1. The primers were produced in scale (0.02 µM) and they were purified by standard HPLC (InvivoScribe Technologies, La Ciotat, France). The BIOMED-2 consortium has developed and clinically validated these primers for immune receptor amplification and their sequences were as follows: Primer name VH1/7-FR1 (5´ GGCCTCAGTGAAGGTCTCCTGCAAG-3´), Primer name VH2-FR1 (5´ GTCTGGTCCTACGCTGGTGAAACCC-3´), Primer name VH3-FR1 (5´ CTGGGGGGTCCCTGAGACTCTCCTG-3´), Primer name VH4-FR1 (5´ CTTCGGAGACCCTGTCCCTCACCTG-3´), Primer name VH5-FR1 (5´ CGGGGAGTCTCTGAAGATCTCCTGT-3´), Primer name VH6-FR1 (5´ TCGCAGACCCTCTCACTCACCTGTG-3´) and Primer name JH consensus-FR1 (5´ CTTACCTGAGGAGACGGTGACC-3´). PCR amplifications were performed using 50 or 100 ng of template genomic DNA, 10 pmol of each primer, and 0.2 µl of AmpliTaq Gold enzyme (Applied Biosystems, Foster City, CA) per 35 µl reaction. The PCR amplification used was as follows: denaturation at 95°C for 7 min; 40 cycles at (94°C for 30s, 59.8 °C for 45s, 72°C for 90s); and a final extension at 72°C for 10 min. To obtain high-quality sequencing results, efficient post-PCR amplicon purification using ExoSAP-IT reagent (USB products, Affymetrix, Santa Clara, CA) was carried out. After ExoSAP-IT PCR clean up, the DNA was sequenced in an Applied Biosystems 3130xl Genetic Analyzer.

The IgBLAST algorithm (National Center for Biotechnology Information) was used to localize those sequences which show a perfect match between forward (F) and reverse (R) reads. For each B-cell clone, we generated a consensus *IGHV-D-J* sequence with the matched F-R region and the correct base reads found before and after such region in the original chromatograms. Alignment of rearranged *IGHV-D-J* sequences to germ line *V*, *D* and *J* segments and determination of *V-D* and *D-J* junctions were performed using the IMGT

database and tools (http://imgt.org). For MBL^{low} clones, whole genomic amplification (WGA) was performed prior to analysis, using the Replig^{R}UltraFast Mini kit (Qiagen, Valencia, CA) as per the recommendations of the manufacturer. Those MBL^{low} cases showing more than one productive rearrangement corresponding to different *IGHV* genes within each purified CLL-like B-cell population were excluded from this study, because in such cases we could not establish the precise association between each IGHV gene and the underlying cytogenetic alterations detected. For each FACS-sorted B-cell population, only in-frame rearrangements were evaluated. Sequences containing >2% deviation from the germline sequence were considered as being somatically mutated.

Analysis of CLL-associated *NOTCH1* mutations[331] was performed via PCR of previously amplified genomic DNA from each FACS-purified CLL-like B-cell population for a total of 70 clones (5 MBL^{low}, 14 MBL^{high} and 51 CLL clones).

2.2.5. Statistical methods

Conventional descriptive and comparative statistics – the nonparametric Kruskal-Wallis and Mann-Whitney U tests (for continuous variables), or the Pearson's $\chi 2$ and Fisher exact tests (for categorical variables) – were performed using the SPSS software program (SPSS 15.0 Inc. Chicago, IL). *P* values <0.05 were considered to be associated with statistical significance.

For multivariate comparisons among MBL^{low}, MBL^{high} and CLL clones, based on the count of clonal B cells/μL and the percentage of aberrant/clonal cells carrying the different cytogenetic profiles, principal component analysis (PCA) was applied, and graphically visualized with the 3D Automated Population Separator (APS) view – Principal Component 1 (PC1) vs PC2 vs PC3 – of the Infinicyt^{TM} software (Cytognos SL, Salamanca, Spain). As previously described in detail, in this APS view, each axis of a plot is represented by a different PC as a linear combination of parameters with distinct statistical weights.[473]

For the assignment of MBL^{low}, MBL^{high} and CLL clones to different groups, the size of the clone and the percentage of altered cells for each cytogenetic abnormality were the continuous variables included in the PCA-based assay performed with the Infinicyt software^{TM}, while *IGHV* gene usage, *IGHV* mutational status and clinical staging of CLL subjects according to the Binet classification were treated as categorical variables, used only for labelling the different clones within each group, after applying the PCA.

2.3. Results

2.3.1. Overall size and BCR features of CLL-like MBL and CLL B-cell clones

The median relative percentage and absolute count of CLL-like and CLL B-cells progressively increased from MBL^{low} (0.6% and 20 cells/µl), to MBL^{high} (14% and 2,000 cells/µl) and CLL clones (57% and 17,400 cells/µl) (P <0.0001) (Table 6).

Of note, around half of all MBL^{low} and MBL^{high} cell populations (52% and 51%, respectively) derived from multiclonal cases, whereas only 22% of CLL clones were identified in multiclonal cases (P ≤0.03; Table 6). In addition, CLL clones less frequently showed mutated IGHV genes (53%) compared to both MBL^{high} (73%) and MBL^{low} (67%) clones (P ≤0.02) (Table 6).

Table 6. Peripheral blood B-cell counts and BCR features of clonal MBL^{low}, MBL^{high} and CLL B cells.

	MBL^{low} n= 27	**MBL^{high} n=51**	**CLL n=117**	***P* value**
No. of PB clonal B cells ($x10^6$/L)*	20 (0. 09-200)	2,000 (350-4,900)	17,400 (1,300[†]-369,000)	$P < 0.0001^{a,b,c,d,e}$
% of PB clonal B cells from WBC*	0.6% (0.001%-7.5%)	14% (0.7%-45%)	57% (17%-97%)	$P < 0.0001^{a,b,c,d,e}$
No. of B-cell clones from multiclonal cases	14/27 (52%)	26/51 (51%)	26/117(22%)	$P \leq 0.002^{a,b,d}$; $P = 0.03^{e}$
No. of *IGHV* mutated clones	18/27 (67%)	37/51 (73%)	60/113 (53%)	$P \leq 0.02^{a,d}$

*Results expressed as number of B-cell clones and percentage between brackets or as *median value (range). Statistically significant differences (P< 0.05) found between [a]MBL^{high} vs CLL, [b]MBL^{low} vs CLL, [c]MBL^{low} vs MBL^{high}, [d]MBL^{low} plus MBL^{high} vs CLL and [e]MBL^{low} vs MBL^{high} plus CLL. BCR, B-cell receptor; CLL, chronic lymphocytic leukemia; MBL, monoclonal B-cell lymphocytosis; SmIg, surface membrane immunoglobulin; IGHV, immunoglobulin heavy chain variable region genes.[†]Includes 6/117 cases with <5,000 clonal CLL B-cells/µL of PB, diagnosed with small lymphocytic lymphoma (SLL).*

2.3.2. Cytogenetic features and NOTCH1 mutation in CLL-like MBL and CLL B-cell clones

Overall, MBL^{low} B-cell clones showed a significantly lower frequency of genetic alterations associated with CLL (33%) than MBL^{high} (51%) and CLL (62%) B-cells (P ≤0.02) (Table 7). Furthermore, only a small proportion of MBL^{low} (7%) and MBL^{high} clones (14%) showed coexistence of ≥ 2 cytogenetic alterations, while this was found in 33% of all CLL clones (P ≤0.04).

Table 7. Cytogenetic and molecular features of MBL^{low}, MBL^{high} and CLL B-cell clones.

Cytogenetic/molecular alterations	MBL^{low}	MBL^{high}	CLL	*P* value
No. of genetically altered CLL-like/ CLL clones	9/27 (33%)	26/51 (51%)	72/117 (62%)	$P \leq 0.02^{b,e}$
No. of clones with ≥2 genetic alterations	2/27 (7%)	7/51 (14%)	38/117 (33%)[†]	$P \leq 0.04^{a,b,d}$
Type of cytogenetic/molecular changes				
No. of del(13q)$^+$ clones (%)	7/27 (26%)	16/51 (31%)	46/117 (39%)	NS
% del(13q)$^+$cells *	73% (19%-96%)	56% (15%-99%)	87% (10%-99%)	NS
No. of del(13q14.3)$^+$ clones (%)	5/27 (19%)	16/51 (31%)	45/117 (39%)	NS
% del(13q14.3)$^+$cells *	70% (19%-96%)	46% (15%-99%)	75% (5%-99%)	NS
No. of del(13q14)$^+$ clones (%)	1/26 (4%)	3/51 (6%)	18/117 (15%)	$P = 0.04^{d}$
% del(13q14)$^+$cells *	86% (86%-86%)	96% (15%-98%)	79% (10%-99%)	NS
No. of trisomy 12$^+$ clones (%)	2/27 (7%)	10/51 (20%)	20/117 (17%)	NS
% trisomy 12$^+$ cells *	50% (41%-59%)	87% (66%-95%)	77% (33%-97%)	$P \leq 0.04^{b,e}$
No. of t(14q32)$^+$ clones (%)	0/17 (0%)	2/51 (4%)	12/116 (10%)	NS
% t(14q32)$^+$ cells *	-	42% (31%-52%)	82% (18%-98%)	NS
No. of del(11q)$^+$ clones (%)	0/23 (0%)	2/51 (4%)	9/116 (8%)	NS
% del(11q)$^+$cells *	-	57% (20%-93%)	58% (21%-98%)	NS
No. of del(11q22.3)$^+$ clones (%)	0/23 (0%)	2/51 (4%)	7/116 (6%)	NS
% del(11q22.3)$^+$cells *	-	57% (20%-93%)	71% (24%-98%)	NS
No. of del(11q23)$^+$ clones (%)	0/15 (0%)	0/51 (0%)	4/116 (3%)	NS
% del(11q23)$^+$cells *	-	-	32% (21%-64%)	-
No. of del(17p13.1)$^+$ clones (%)	0/24 (0%)	0/51 (0%)	5/117 (4%)	NS
% del(17p13.1)$^+$cells *	-	-	44% (33%-88%)	-
No. of *NOTCH1* mutated clones (%)	0/5 (0%)	0/14 (0%)	5/52 (10%)	NS

*Results expressed as number of B-cell clones with cytogenetic abnormalities from all clones in the corresponding group (percentage) or as *median values of altered cells/clone (range). In 9 clones (1 MBL^{low},1 MBL^{high} and 7 CLL) biallelic del(13q14.3) was detected and hyperdiploidy was found in one MBL^{low} clone. [a]MBL^{high} vs CLL, [b]MBL^{low} vs CLL, [d]MBL^{low} plus MBL^{high} vs CLL and [e]MBL^{low} vs MBL^{high} plus CLL. NS, no statistically significant differences observed ($P \geq 0.05$); CLL, chronic lymphocytic leukemia; MBL, monoclonal B-cell lymphocytosis. [†]Includes the 5/66 cases with NOTCH1 mutation associated to trisomy 12 in 3 cases, to biallelic del(13q14.3) in one and to both mono allelic del(13q14.3) and del(17p) in the remaining cases.*

Regarding each specific cytogenetic/molecular alteration, a tendency towards a greater frequency of del(13q) and trisomy 12 was observed from MBL^{low} to MBL^{high} and CLL clones, although differences only reached statistical significance for the frequency of del(13q14) involving the *RB1* gene (*P* =0.04). In addition, presence of t(14q32) and

del(11q22.3) were exclusively found among MBL^{high} and CLL, while del(17p), del(11q23) and *NOTCH1* mutations were only present in CLL clones (Table 7). Of note, among the cytogenetically altered clones, no significant differences were observed in the percentage of altered cells, except for a greater proportion of B-cells with trisomy 12 among both MBL^{high} and CLL vs MBL^{low} B-cell clones ($P \leq 0.04$) (Table 7).

NOTCH1 mutations occurred in 5/52 CLL cases (10%), in which a preferential association with IGHV unmutated clones (80%, $P = 0.02$) and a high frequency of cases (3/5, 60%) harbouring trisomy 12 as an additional isolated chromosomal abnormality ($P = 0.007$) was observed; in the remaining two CLL cases, the presence of *NOTCH1* mutation was associated with del(13q14) involving the *RB1* gene and to both del(13q14.3) and del(17p), respectively. None of the 19 MBL cases investigated showed *NOTCH1* mutations (Table 7).

2.3.3. Molecular characteristics of CLL-like MBL and CLL B-cell clones

Analysis of the *IGHV* gene revealed single in frame gene rearrangements for each clonal B-cell population analyzed. Interestingly, shorter CDR3 sequences of the *IGHV* gene were found among MBL^{low} versus CLL clones. So, the frequency of CLL clones with CDR3 sequences coding for >20 aa became significantly higher than that observed among MBL^{low} and MBL^{high} clones ($P = 0.02$) (Table 8).

Regarding *IGHV*, *DH* and *JH* gene usage, no significant differences were found between the three groups of CLL-like B-cell clones, except for the *VH3* and *DH3 IGHV* genes (Table 8): CLL showed lower frequency of *VH3* usage and a greater proportion of *DH3*$^{+}$clones vs MBL^{low} and MBL^{high} ($P \leq 0.04$) (Table 8).

Table 8. Molecular characteristics of the BCR of CLL-like MBL^{low}, MBL^{high} and CLL B-cell clones.

	MBL^{low} N= 27	MBL^{high} N=51	CLL N=113	*P* value
*HCDR3 length**	13 (6-22)	17 (8-26)	18 (8-32)	$P \leq 0.02^{b,d,e}$
HCDR3> 20 aa	3/27 (11%)	8/51 (16%)	38/113 (34%)	$P = 0.02^{a,b,d}$
VH subgroups				
VH1	4/27 (15%)	7/51 (15%)	28/113 (25%)	NS
VH2	0/27 (0%)	1/51 (2%)	2/113 (2%)	NS
VH3	18/27 (66%)	31/51 (60%)	47/113 (42%)	$P \leq 0.04^{a,b,d}$
VH4	4/27 (15%)	9/51 (17%)	32/113 (28%)	*NS*
VH5	1/27 (4%)	3/51 (6%)	2/113 (2%)	NS
VH6	0/27 (0%)	0/51 (0%)	1/113 (1%)	NS
DH subgroups				
DH1,4,7	5/27 (18.5%)	7/50 (14%)	15/109 (14%)	NS
DH2	5/27 (18.5%)	12/50 (24%)	19/109 (17%)	NS
DH3	5/27 (18.5%)	16/50 (32%)	48/109 (44%)	$P \leq 0.03^{b,d,e}$
DH5	5/27 (18.5%)	8/50 (16%)	12/109 (11%)	NS
DH6	7/27 (26%)	7/50 (14%)	15/109 (14%)	NS
JH genes				
JH1,2,3,5	4/27 (15%)	14/50 (28%)	23/113 (20%)	NS
JH4	13/27 (48%)	21/50 (42%)	45/113 (40%)	NS
JH6	10/27 (37%)	15/50 (30%)	45/113 (40%)	NS

*Results expressed as number of B-cell clones from all clones in the corresponding group (percentage) or or as *median (range). Statistically significant differences were found between [a]MBL^{high} vs. CLL, [b]MBL^{low} vs. CLL, [c]MBL^{low} vs. MBL^{high}, [d] MBL^{low} plus MBL^{high} vs. CLL and [e]MBL^{low} vs. MBL^{high} plus CLL; NS, no statistically significant differences observed (P ≥0.05); BCR, B-cell receptor; CLL, chronic lymphocytic leukemia; MBL, monoclonal B-cell lymphocytosis; aa, amino acids; HCDR3,heavy chain complementarity-determining region 3.*

Of note, a significant percentage of all CLL (72%), MBL^{high} (74%) and MBL^{low} (85%) clones corresponded to only 12 *IGHV* genes, namely *V3-23, V3-11, V5-51, V3-21, V1-2, V1-3, V4-39, V3-7, V3-30, V4-34, V3-48* and *V1-69* (Figure 6). Among these, preferential usage (*P* <0.04) of the VH3-23 gene was observed in both MBL^{low} (7/27, 26%) and MBL^{high} (10/51, 20%) vs. CLL (5/113, 4%) clones (Figure 6).

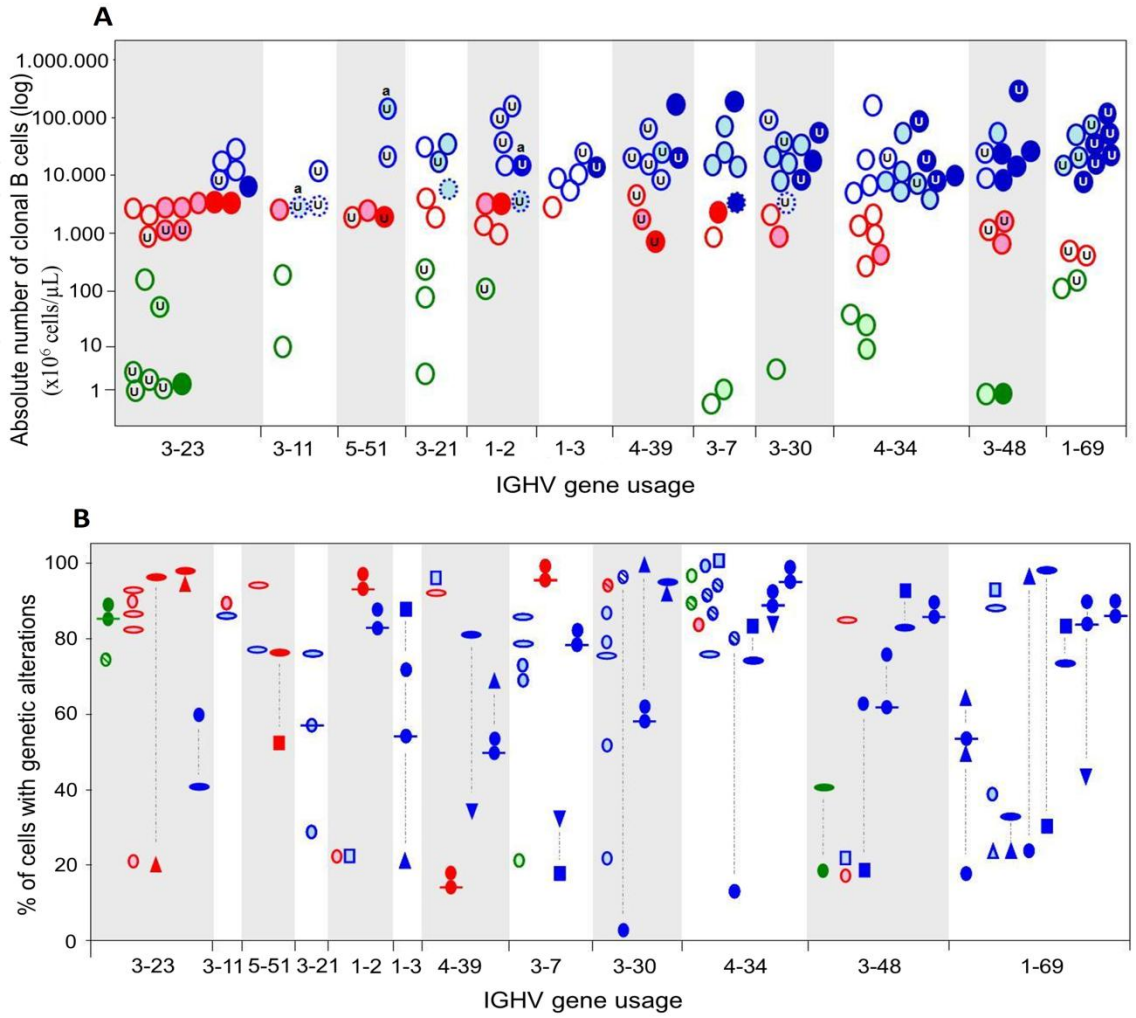


Figure 6. Frequency of CLL-associated cytogenetic alterations (**A**) and the cytogenetic profile (**B**) for those *IGHV* genes most commonly detected in MBL^{low}, MBL^{high} and CLL B-cell clones, as assessed by iFISH. The three diagnostic categories studied are depicted by different colors (green, MBL^{low}; red, MBL^{high}; blue, CLL B-cell clones) and the absence vs. presence of one vs≥2 chromosomal alterations *per* clone, is indicated by empty circles, light colored and dark colored circles, respectively. For each *IGHV* subgroup, the clones are represented in the Y-axis according to the absolute number of clonal B cells per µL of PB (**A**) and the percentage of cells genetically altered, by iFISH (**B**). Different FISH patterns are defined by the following symbols in panel **B**: **0** , del(13q14.3); **0**, biallelic del(13q14.3); **⊖**, del(13q14); **⬭**, trisomy 12; **Δ**, del(11q);**▽**,del(17p) and; **□**, t(14q32); dotted contour lines in panel **A** highlight those clones phenotypically classified as SLL(small lymphocytic lymphoma); dotted blue lines in panel **B** indicate cells from the same B-cell clone showing different cytogenetic abnormalities; U = unmutated clones; a = clones with *NOTCH1* mutation.

2.3.4. Relationship between the most frequently used IGHV genes and the cytogenetic profile of CLL-like MBL and CLL B-cell clones

As mentioned above, preferential usage of the *VH3-23* gene was observed in both MBL^{low} and MBL^{high} vs. CLL clones (Figure 6A). *VH3-23*$^{+}$ MBL clones typically showed no cytogenetic alterations (8/17) or they carried an isolated cytogenetic alteration which corresponded either to trisomy 12 (3/17) or deletion of 13q (3/17) (Figure 6B). Nevertheless,

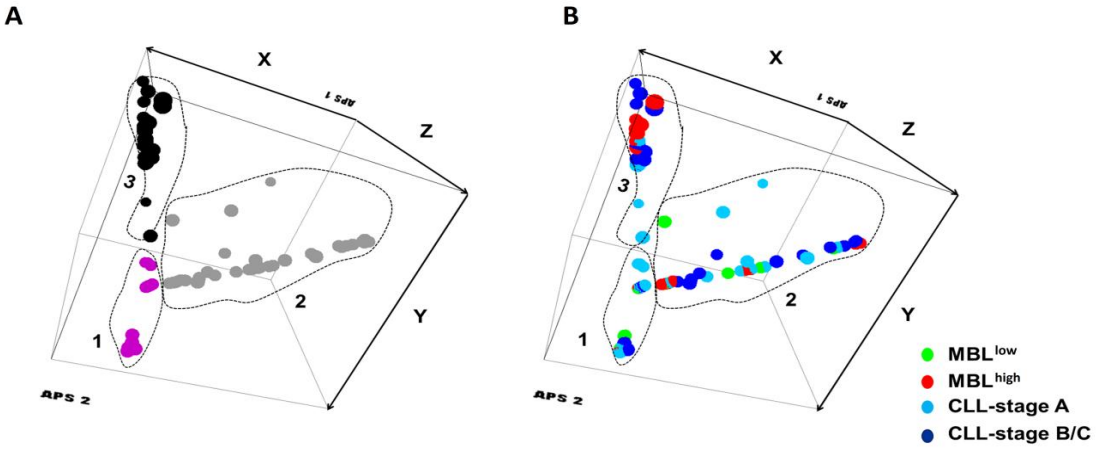
two MBL^{high} clones showed co-existence of trisomy 12 and del(11q22.3) and one MBL^{low} clone showed del(13q) including both the 13q14.3 and 13q14 (*RB1*) chromosomal regions (Figure 6B). From the five *VH3-23*$^{+}$ CLL clones only one carried genetic alterations – trisomy 12 and del(13q) –. Most interestingly, *VH3-23*$^{+}$ MBL^{low} clones frequently showed unmutated IGHV genes, including most unmutated MBL^{low} clones, with <10 CLL-like cells/µl (5/8; 63%), which contrasts to the much lower frequency of unmutated *VH3-23* CLL clones.

A similar frequency of usage of the *VH3-11, VH5-51, VH3-21* and *VH1-2* genes was observed in both MBL^{low} and MBL^{high} vs. CLL (Figure 6). In none of the clones expressing these *IGHV* genes, cytogenetic alterations associated with a poor disease outcome – e.g. del(17p) and/or del(11q) – were found; in addition, most MBL and CLL clones expressing these *IGHV* genes showed no cytogenetic alteration, or they just had a single abnormality. Despite this, *NOTCH1* mutations were more frequently observed among cytogenetically altered, *IGHV* unmutated CLL clones expressing these *IGHV* genes (one *VH3-11*$^{+}$, one *VH3-21*$^{+}$ and one *VH1-2*$^{+}$ clones). Noteworthy, 4/6 CLL cases classified as small lymphocytic lymphoma (SLL) variants were also included among cases with a *VH3-11* (n=2), *VH3-21* (n=1) and *VH1-2* (n=1) repertoire in this group.

Finally, among those clonal B-cell populations which expressed the *VH1-3, VH4-39, VH3-7, VH3-30, VH4-34, VH3-48* and *VH1-69* genes, CLL clones were overrepresented (61/113, 54%) vs. both MBL^{low} (10/27, 37%) and MBL^{high} (18/51, 35%) clones. Notably, a high number of CLL clones carrying these *IGHV* genes in association with one or more cytogenetic alterations, including poor prognosis cytogenetic alterations, was found among these cases (44/61, 72%). In this regard, del(13q) including both the 13q14.3 and 13q14 (*RB1*) chromosomal regions was frequently detected (single or combined lesion) in these CLL and also MBL^{high} clones, particularly among those cases expressing the *VH3-30* and *VH4-34* gene genes; presence of trisomy 12, del(11q) and t(14q32) were also common among these CLL cases (16%, 13% and 18%, respectively) while being infrequent in MBL cases (only 2 MBL^{high} clones showed isolated trisomy 12). Moreover, del(17p) alone and complex karyotypes with ≥3 cytogenetic/molecular alterations were also found in 4 of the CLL clones which expressed the V*H1-3, VH4-39, VH3-30, VH3-48, VH4-34* and *VH1-69 IGH* genes, respectively (Figure 6B). Remarkably, ≥1 genetic alteration was systematically detected in a major fraction of the *VH1-69*$^{+}$ clonal cells (Figure 6A) while being absent in the few MBL clones which expressed this specific *IGHV* gene. Of note, unmutated *IGHV* genes were a hallmark of both *VH1-69*$^{+}$ (12/14 clones; 86%) and *VH4-39*$^{+}$ (9/10 clones; 90%), independently of their MBL vs CLL nature (Figure 6A).

Based on the observation of the above described associations, we performed a multivariate analysis based on PCA, in searching for unique patterns of association between

cytogenetic alterations and *IGHV* repertoires among MBL vs. CLL clones. Three major groups of CLL-like MBL and CLL clones were identified, according to the absolute number of clonal B cells/µL and the percentage of cells carrying cytogenetic alterations (Figure 7) and then labelled according to their pattern of *IGHV* gene usage and the *VH* mutational status. Of note, the most homogeneous group (Group 1) included virtually all MBL^{low} clones (77%) and half of the MBL^{high} clones (54%), but only around one fourth of Binet stage B/C CLL (28%); by contrast, no MBL^{low} clones were included in Group 3 (Figure 7D). Group 2 showed a more heterogeneous distribution with an intermediate pattern. In detail, Group 1 was mainly characterized by cases with a normal karyotype (83%) and lower numbers of cytogenetically altered cells mostly displaying the *VH1-2, VH3-23* and *VH4-34 IGHV* genes (Supplemental Table 1); in turn, Group 2 typically showed a higher number of cases with mutated *IGHV* genes, and higher numbers of del(13q)$^{+}$ clonal B cells, while Group 3 included high numbers of cases with unmutated *IGHV* genes, trisomy 12 and an *IGHV* repertoire enriched in unmutated *VH1-69*$^{+}$ CLL clones (Supplemental Table 1).



C

Axes	Parameters	Contribution to the respective component principal
PC1	Pe. of del(13q14.3)$^{+}$ cells	38%
	Pe. of del(13q14)$^{+}$ cells	27%
	Pe. of trisomy 12^{+} cells	21%
PC2	Pe. of trisomy 12^{+} cells	42%
	Pe. of del(13q14.3)$^{+}$ cells	14%
	Pe. of del(13q14)$^{+}$ cells	13%
PC3	Pe. of t(14q32)$^{+}$ cells	51%
	Pe. of biallelic del(13q14.3)$^{+}$ cells	19%
	Pe. of del(11q22.3)$^{+}$ cells	17%

D

Major Groups	Diagnostic Subgroups			
	MBL^{low}	MBL^{high}	CLL-stage A	CLL-stage B/C
1	17 (77%)	20 (54%)	23 (62%)	8 (28%)
2	5 (23%)	9 (24%)	9 (24%)	10 (36%)
3	0 (0%)	8 (22%)	5 (13%)	10 (36%)

Figure 7. Principal component analysis (3-dimensionalX-Y-Z axis view of PC1 vs. PC2 vs. PC3, respectively) for comparison of MBL^{low}, MBL^{high} and CLL B-cell clones according to the absolute number of clonal B cells/µL and the pattern of cytogenetic alterations (including the percentage of altered cells), using the Infinicyt^{TM}software. Overall, MBL^{low}, MBL^{high} and CLL cases are clustered into groups distinguished by different colors in A: magenta, gray, and black circles (**A**). The distribution of MBL^{low}, MBL^{high}, CLL-stage A and CLL-stage B/C clones are coloured differently in

B: MBL^{low}, green; MBL^{high}, red, CLL stage A and B/C light blue and dark blue, respectively (**B**). The most informative parameters contributing to the best discrimination between 1 x 1 comparisons of the three groups are displayed in a decreasing order of percentage contribution to each of the principal component (**C**); Distribution of MBL^{low}, MBL^{high} and CLL clones among the three major groups defined in panel A by principal component analysis (**D**); CLL, chronic lymphocytic leukemia; MBL, monoclonal B lymphocytosis; PC: principal component.

2.4. Discussion

It is now well established that emergence of CLL is typically preceded by MBL.[212] However, only a fraction of all MBL^{high} will evolve to CLL, at a rate of 1.1% persons/year,[474] while the outcome of MBL^{low} remains unknown. Despite this, general consensus exists in that stepwise acquisition of specific genetic alterations may determine the rate of progression, not only from MBL^{high} to CLL, but potentially also from MBL^{low} to MBL^{high} and eventually to CLL. Concurrence of chronic Ag stimulation through specific BCRs may further support and accelerate the expansion of MBL clones, facilitate acquisition of new genetic alterations and therefore contribute to progression to CLL.[20,218] Although data has accumulated in the last decade about the cytogenetic alterations and the *IGHV* gene repertoire of CLL-like clonal B-cells in both MBL and CLL, to our knowledge, this is the first report about the combined patterns of cytogenetic alterations and *IGHV* gene repertoire in MBL^{low} vs. MBL^{high} vs. CLL clones.

In recent years, more than a thousand different molecular/genetic alterations reflected in multiple distinct and complex cytogenetic/molecular profiles in individual CLL patients, have been described through high-throughput WGS approaches.[475,476] However, only a relatively small number of cytogenetic/molecular alterations recurrently occur at relatively high frequencies (e.g. >5% cases).[333,477] Such alterations include del(13q14), reported in around half of all CLL cases, trisomy 12, present in about one third of the patients and del(11q), del(17p), t(14q32) and *NOTCH1* mutations, which occur in between 5-15% of all CLL cases.[253,333,477] In around half of CLL cases, unmutated *IGH* genes associated with preferential usage of specific IGHV genes (i.e. *VH1-69* and *VH4-34*) and the above described cytogenetic alterations have also been reported in CLL. In turn, MBL^{high} cases share molecular features with good-prognosis CLL in terms of both the *IGHV* gene repertoire and chromosomal alterations,[206,478] with a greater frequency of *IGHV* mutated cases. By contrast, preliminary data indicates that the *IGHV* repertoire expressed by MBL^{low} could be strikingly different from that of both typical CLL and MBL^{high} cases;[479] in addition, such MBL^{low} clones appear to display a much lower frequency of chromosomal alterations, restricted to del(13q14.3) and trisomy 12, with a high prevalence of *IGHV* mutated cases (similar to that of MBL^{high} cases),[20] and no poor-prognosis cytogenetic alterations.[203,206]

In line with such observations, we also found a lower frequency of both cytogenetically altered and *IGHV* unmutated CLL-like clones in MBL^{low} vs. both MBL^{high} and CLL and vs. CLL clones, respectively. Interestingly, the proportion of B-cell clones carrying ≥2 alterations significantly increased from MBL^{high} to CLL. On top of the progressively higher number of cytogenetic/molecular alterations found in MBL^{low} vs. MBL^{high} and CLL, the cytogenetic profile of clonal B-cells also became significantly more heterogeneous among the latter two groups. Accordingly, while del(13q14.3) and to a much lesser extent, del(13q14) involving the *RB1* gene and trisomy 12, were already detected in a small fraction of MBL^{low} clones, del(11q) and t(14q32) emerged at an MBL^{high} stage, whereas del(17p), del(11q23) and *NOTCH1* mutations were only found in CLL. These latter three alterations typically involved CLL clones that already had other cytogenetic alterations and therefore, had more complex cytogenetic/molecular profiles. In line with these findings, the altered CLL-like MBL and CLL clones showed progressively increasing percentages of cells carrying del(13q14.3), del(13q14), trisomy 12, t(14q32),del(11q)and del(17p13.1),respectively. In accordance with previous observations,[480] *NOTCH1* mutations were exclusively detected in CLL (preferentially among unmutated CLL clones) which also had other cytogenetic alterations – e.g., trisomy 12, del(13q14) and/or del(17p).

The overall increased frequency of all cytogenetic alterations, together with the more complex cytogenetic/molecular profiles, observed from MBL^{low} to MBL^{high} and CLL would support the notion that evolution from MBL^{low} to MBL^{high} and CLL is paralleled by progressive acquisition of recurrent cytogenetic alterations, each of which appears to emerge at specific MBL and CLL stages, in line with previous data from our and other groups.[15,481] Accordingly, del(13q), and to a lesser extent trisomy 12, are relatively early cytogenetic events which may frequently occur at an MBL^{low} stage, whereas del(17p), *NOTCH1* mutations, and to a lesser extent also del(11q) and t(14q32), would typically arise later, as secondary cytogenetic events occurring at an MBL^{high} or CLL stage. Acquisition of these and other genetic changes may potentially be associated with an increased proliferation and/or survival of the altered CLL-like cells. At the earliest stages of development of MBL, proliferation and/or survival signals could be provided to the MBL clone by chronically sustained BCR stimulation. If this holds true, the BCR features could also play a critical role in determining the probability of cytogenetic progression. Unfortunately, our series of MBL – particularly of MBL^{low} – is quite short at this time to further confirm this hypothesis, due to the difficulty in collecting cases with enough number of CLL-like B-cells, to perform in parallel reliable iFISH and molecular analyses. In this regard, the limited number of MBL^{low} cases included in our series may predominantly present with the genetic/molecular patterns of a low risk MBL cohort, which may not be related to CLL

progression. Despite this, in accordance with other recent reports,[16,185,482] non-random usage of *IGHV* genes with clearly different *IGHV* gene repertoires was found in our series in MBL vs. CLL. As expected, the most frequently used *IGHV* genes in CLL were the *VH4-34, VH3-30, VH1-69, VH3-48, VH4-39, VH1-2* and *VH3-7* genes, accounting for around half of the CLL clones. Interestingly, also half of the CLL clones showed unmutated *IGHV* genes, strikingly high frequencies of unmutated clones being detected among cells expressing *VH1-69, VH4-39* and *VH1-2*. By contrast, *VH3-23*$^{+}$ B-cells predominated among the MBL^{low} and MBL^{high} clones, most *VH3-23*$^{+}$ MBL^{low} cases showing very low counts of *IGHV* unmutated clonal B-cells. Of note, the *IGHV* genes used by the MBL^{high} clones were commonly observed in either CLL (e.g. *VH4–34, VH1-2, VH3–48* and *VH4–39*) or MBL^{low} (e.g. *VH3-23* and *VH4-34*), but usually at lower frequencies. The fact that these particular *IGHV* genes have been associated with previously reported stereotypic B-cell receptors in CLL clones,[13] together with our own results which show that the CDR3 of the *IGHV* genes are highly homologous in around one fifth of the B-cell clones from our short cohort (Supplemental Table 2), would reinforce the role of some antigen-binding BCR specificities in clonal evolution.

Based on the overall patterns of cytogenetic alterations and *IGHV* gene usage together with the BCR mutational status, it could be concluded that while some unmutated *IGHV* genes appear to be associated with the acquisition of complex cytogenetic profiles, rapid expansion of clonal CLL-like B-cells and progression to CLL (e.g. *IGHV1-69*), others would not (e.g. *IGHV3-23*); the latter clones would show a more benign behaviour. This could potentially be due to a lower binding affinity of the unmutated BCR for the antigen, the recognition of specific subtypes of low concentrated antigens and/or unique immune response profiles. In line with this hypothesis, *IGHV* genes over-represented among CLL clones (e.g.*VH4-39* and *VH1-69*) frequently corresponded to *IGHV* genes enriched in genes encoding for antibodies that recognise a broad variety of relatively common and abundant (auto)antigens, including low-affinity BCR, e.g. myoglobulin, thyroglobulin, actin, and ssDNA[296,483] associated with T-independent, type II autoimmune responses.[235] In contrast, the unmutated *IGHV3-23* BCR was over-represented among our MBL^{low} cases, normal peripheral blood *IGHV3-23*$^{+}$ IgM^{+} B-cells being associated with recognition of superantigens.[24,484,485] Thus, the association between MBL^{low} and unmutated *IGHV3-23* could be potentially due to a low affinity of this particular BCR for low concentrated/prevalent (super)antigens, which would limit the development of repetitive immune responses associated with the expansion of MBL clones and/or their cytogenetic progression. A recent study[203] also reported MBL^{low} cases to display an *IGHV* gene repertoire different from that of CLL patients (e.g. absence of *IGHV1-69*$^{+}$ MBL^{low} clones, together with a low frequency of the *IGHV4-34* gene and overrepresentation of the *IGHV4-*

59/61 genes); however, no preferential usage of the *IGHV3-23* gene was found among MBL^{low} cells in this series. Further studies investigating the *IGHV* gene repertoire of MBL^{low} clones in distinct geographic areas and microenvironments, may shed light on those factors accounting for such apparent discrepancies, as an association between MBL^{low} and previous history of infections has been recently reported in this setting.[204]

Taken together, these results would support the notion that antigen-driven BCR-stimulation could be a triggering factor in driving CLL-like B-cells to expand, in line with recent data showing a significant association between MBL in the general population and the individual history of infectious diseases and vaccination,[204] whilst depending on the nature of the antigenic stimuli, distinct patterns of cytogenetic changes might then occur. Thus, the specific combination of cytogenetic alterations acquired by the CLL-like B-cells may determine, for distinct antigenic stimuli, and specific BCR repertoires, the outcome of the genetically-targeted cell. Long-term longitudinal studies, ideally of the same cases at different time-points and at different stages of the disease, would be crucial to definitively confirm these hypotheses, although based on our preliminary follow-up date[19] this may require decades due to the stable nature of most MBL^{low} clones in the short-term.

In summary, MBL and CLL clones appear to display a distinct but partially overlapping pattern of *IGHV* gene usage, *IGHV* mutational status and cytogenetic alterations, which may translate into distinct groups of clones with different genetic/molecular features associated with a distinct clinical behavior. Sequential studies in larger series of cases followed for long periods of time are ongoing to investigate the risk of progression and outcome of MBL clones with specific *IGHV* and iFISH cytogenetic profiles.

Chapter 3 | Molecular and cytogenetic characterization of expanded B-cell clones from multiclonal versus monoclonal B-cell chronic lymphoproliferative disorders

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3.1. Abstract

Chronic antigen-stimulation has been recurrently involved in the earlier stages of MBL, CLL and other B-CLPD. Among these individuals, expansion of ≥2 B-cell clones has been frequently reported; potentially, such coexisting clones have a greater probability of interaction with common immunological determinants. Here, we comparatively analyzed the BCR repertoire and the molecular profile, as well as the phenotypic, cytogenetic and hematological features of 228 CLL-like and non-CLL-like clones between multiclonal (n=85 clones from 41 cases) vs. monoclonal (n=143 clones) MBL, CLL and other B-CLPD. The BCR of B-cell clones from multiclonal cases showed a slightly higher degree of HCDR3 homology than B-cell clones from monoclonal cases, in association with unique hematological (e.g. lower B-lymphocyte counts) and cytogenetic (e.g. lower frequency of cytogenetically altered clones) features usually related to earlier stages of the disease. Moreover, a subgroup of coexisting B-cell clones from individual multiclonal cases which were found to be phylogenetically related, showed unique molecular and cytogenetic features: they more frequently shared *IGHV3* gene usage, shorter HCDR3 sequences with a greater proportion of *IGHV* mutations and del(13q14.3), than other unrelated B-cell clones. These results would support the antigen-driven nature of such multiclonal B-cell expansions, with potential involvement of multiple antigens/epitopes.

3.2. Materials and Methods

3.2.1. Patients and samples

A total of 184 subjects with one (n=143 monoclonal cases) or ≥2 (n=41 multiclonal cases) CLL/non-CLL B-CLPD (n=140) and/or CLL-like/non-CLL-like MBL (n=88) B-cell clones – as defined by the WHO criteria 2008[486] – were included. Binet staging[466] of CLL subjects was retrospectively collected.

From the 41 multiclonal cases, 2 (5%) corresponded to healthy individuals with CLL-like MBL^{low}, 8 (19.5%) were CLL-like MBL^{high} cases, 23 (56%) had CLL and 8 (19.5%) had B-CLPD other than CLL; 4 of these latter cases showed coexistence of either one or two CLL-like MBL B-cell population(s). In 3/41 multiclonal cases, three coexisting B-cell populations were detected. From the 143 monoclonal cases, 13 (9%) corresponded to healthy adults with CLL-like MBL^{low}, 26 (18%) were CLL-like MBL^{high}, 89 (62%) had CLL, 2 (1%) were non-CLL-like MBL^{low}, 2 (1%) non-CLL-like MBL^{high} cases and 11 (8%) had other B-CLPD. MBL was defined by the presence of

small clones of aberrant B-cells in the PB, with a clonal B-cell count below the threshold for diagnosis of CLL (< 5.0×10^9 cells/L).[14] A MBL case was subclassified as MBL^{low} when the absolute number of clonal B-lymphocytes was less than 200 cells/µL in PB and as MBL^{high} when this number ranged between ≥200 and <5,000 clonal CLL-like B-cells/µL of PB.[468] The age/gender distribution for each diagnostic group, is detailed in Table 9.

Peripheral blood samples were obtained from each subject after written informed consent was given, and the study was approved by the local ethics committees of the University Hospital of Salamanca and the Blood and Transplantation Center of Coimbra/Portuguese Institute of Blood and Transplantation, in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Table 9. Age and gender features of subjects included in the study.

		Diagnostic subgroups					
		CLL and CLL-like MBL			**Non-CLL B-CLPD and non-CLL MBL**		
	Age/Gender Features	**MBL^{low}**	**MBL^{high}**	**CLL**	**MBL^{low}**	**MBL^{high}**	**Non-CLL**
Cases	Monoclonal (n=143)	13 (9%)	26 (18%)	89 (62%)	2 (1%)	2 (1%)	11 (8%)
	Age (years)*	66±13 (49-84)	69±13 (37-89)	69±13 (35-89)	65 & 95	79 & 80	71±9 (53-84)
	Male/Female	4/9	15/11	50/39	1/1	0/2	7/4
	Multiclonal (n=41)	2 (5%)	8 (19.5%)	23 (56%)	- (0%)	- (0%)	8 (19.5%)
	Age (years)*	77 & 83	76±6 (65-82)	75±9 (57-89)	-	-	74±9 (56-81)
	Male/Female	1/1	5/3	16/7	-	-	6/2

*Age values expressed as *media ± one standard deviation (range) when n>2. MBL, monoclonal B-cell lymphocytosis; CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; B-CLPD, B-cell chronic lymphoproliferative disorders other than CLL or MBL.*

3.2.2. Immunophenotypic analyses

Immunophenotypic studies to screen for the presence and full characterization of clonal B-cell populations were performed by high-sensitive MFC on erythrocyte-lysed peripheral blood samples, according to previously described procedures (see the Materials and Methods section of Chapter 2 for detailed descriptions). In all cases studied, each SmIg light chain restricted and phenotypically aberrant B-cell population identified was purified in a FACSAria II flow cytometer (BDB). In those samples (n=41) containing ≥2 aberrant B-cell populations, discrimination among them was based on their distinct patterns of expression for ≥1 of the B-cell markers analyzed, as described elsewhere.[467] The clonal nature of each FACS-purified B-cell population (purity: 98%±0.8%) was assessed by both cytogenetic and molecular techniques.

3.2.3. Cytogenetic and molecular studies

Cytogenetic analyses were performed by multicolor iFISH on slides containing FACS-purified and fixed aberrant B-cells, as previously described in detail (see the Materials and Methods section of Chapter 2 for detailed descriptions).[253,468] In parallel, analysis of the patterns of rearrangement of the *IGHV* and *IGKV* and *IGλV* genes was performed for each FACS-purified B-cell clone (see the Materials and Methods section of Chapter 2 for detailed descriptions).[468,471,472] Each deduced "IMGT/V-QUEST aa sequence" corresponding to individual *IGHV* gene sequences from purified B-cell clones from both monoclonal and multiclonal cases was aligned using the bioinformatic tools available at the web services of the European Bioinformatics Institute (EMBL-EBI Cambridge, UK). More than 12,400 alignments of *IGHV* aa sequences, with a coverage ranging from framework region (FR) 1 to the HCDR3 region (both regions included) were obtained for all B-cell clones (a total of 8,891 alignments within the monoclonal and 3,560 alignments within the multiclonal groups of cases). Then, the percentage of alignment of *IGHV* aa sequences obtained after two-by-two comparisons between the distinct B-cell clones, was calculated for every pair of B-cell clones. Finally, each single paired-alignment obtained – 8,891 and 3,560 in monoclonal vs. multiclonal cases, respectively – was included in a final database, to calculate the median and range of the total *IGHV* aa alignment percentages and to calculate the statistical significance of their differences observed between the two groups (*P*-values).

To investigate the level of phylogenetic relationship among *IGHV* aa sequences corresponding to distinct clones from multiclonal cases, as well as monoclonal cases, a sequence distance tree was built using the neighbor-joining method implemented in the freely available Molecular Evolutionary Genetic Analysis (MEGA) software (version 5.2, http://www.megasoftware.net). Examination of the different branches of the sequence distance tree allowed the distinction of multiclonal cases whose clones had *IGHV* aa sequences phylogenetically closer than others. Thus, sequences in the same major branch were guaranteed to exhibit ≥50% aa identity (from FR1 to HCDR3, both regions included).[239] As might be expected, sequences in sub-branches emerging from the same major branch exhibit even more aa identity, ranging from 60% to 99%. In our analysis, those co-existing B-cell clones with *IGHV* aa sequences that belonged to the same major branch with >60% aa identity or belonged to close located sub-branches were assumed to be “phylogenetically” related sequences. This “identity” threshold was based on previously published concepts about the phylogeny of human *IGHV* genes based on their aa sequences,[239] and on the minimum identity

percentage observed in colocalized sub-branches (presumably with the highest evolutionary relationship[239]) of the sequence distance tree built in this study.

HCDR3-alignments were carried out for each multiclonal case whose co-existing B-cell clones showed HCDR3 regions with identical or one aa differing lengths using the bioinformatic tools available at the web services of the European Bioinformatics Institute (EMBL-EBI Cambridge, UK). Through the EMBL-EBI tools, the identical aa or those with analogous side-chain polarity per case-paired HCDR3 alignments were highlighted taking into account their conserved composition in terms of "hydropathy", "volume" and "chemical characteristics" as outlined in the IMGT classification of aa".

3.2.4. Statistical methods

Comparisons between groups were performed with either the nonparametric Kruskal-Wallis and Mann-Whitney U tests (for continuous variables) or the Pearson's $\chi 2$ and Fisher exact tests (for categorical variables) using the SPSS software/version 20.0 (IBM SPSS Statistics, IBM, Armonk, NY, USA). *P* values <0.05 were considered to be associated with statistical significance.

3.3. Results

3.3.1. Distribution and immunophenotypic features of B-cell clones

A total of 228 B-cell clones were identified. These corresponded to 143 B-cell clones (89 CLL, 11 non-CLL, 39 CLL-like MBL and 4 non-CLL-like MBL clones) from monoclonal cases and 85 B-cell clones (26 CLL, 14 non-CLL, 40 CLL-like MBL and 5 non-CLL-like MBL clones) from multiclonal cases (Table 10). The complete immunophenotypic and cytogenetic features of the individual clones of multiclonal cases are summarized in Supplemental Table 3.

In 26/41 multiclonal cases, all coexisting B-cell clones showed a CLL-like phenotype, while in 11 of the remaining 15 cases, at least one CLL-like B-cell population coexisting with another non-CLL aberrant B-cell population was identified. In the remaining 4 cases, two distinct non-CLL-like B-cell clones were found (Supplemental Table 3). The distribution of all CLL/non-CLL and CLL-like MBL/non-CLL-like MBL clones analyzed (from all monoclonal and multiclonal cases considered together) in the distinct diagnostic categories was as follows: 27 B-cell clones corresponded to CLL-like MBL^{low}, 52 to CLL-like MBL^{high}, 115 to CLL, 5 to non-CLL-like MBL^{low}, 4 to non-CLL-like MBL^{high} and 25 to non-CLL B-CLPD (Table 10). The precise

diagnoses of the B-cell clones from B-CLPD patients other than CLL are specified in the footnote of Table 10.

Table 10. Distribution of subjects included in the study and their corresponding CLL and non-CLL like B cell clones, according to diagnosis.

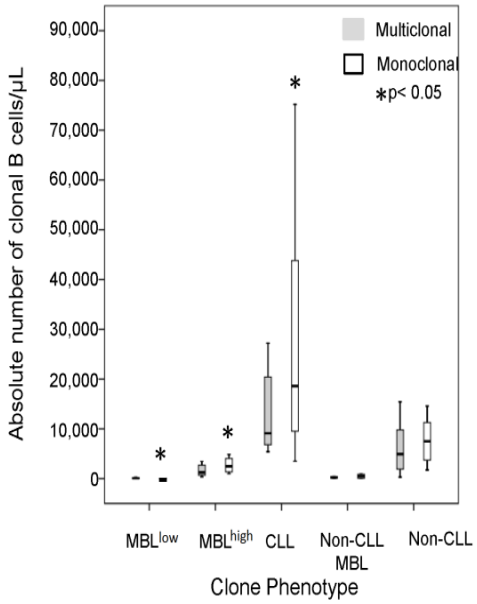
		Diagnostic subgroups					
		CLL and CLL-like MBL			Non-CLL B-CLPD and non-CLL MBL*		
		MBL^{low}	MBL^{high}	CLL	MBL^{low}	MBL^{high}	Non-CLL
Cases	Monoclonal (n=143)	13 (87%)	26 (76.5%)	89 (80%)	2 (100%)	2 (100%)	11 (58%)
	Multiclonal (n=41)	2 (13%)	8 (23.5%)	23 (20%)	- (0%)	- (0%)	8 (42%)
	Total (n=184)	15	34	112	2	2	19
B cell clones	Monoclonal (n=143)	13 (48%)	26 (50%)	89 (77%)	2 (40%)	2 (50%)	11 (44%)
	Multiclonal (n=85)	14 (52%)	26 (50%)	26 (23%)	3 (60%)	2 (50%)	14 (56%)
	Total (n=228)	27	52	115	5	4	25

*CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma (n=115 clones); MBL, monoclonal B-cell lymphocytosis (n=88 clones: 79 CLL-like MBL clones and 9 non-CLL-like MBL clones); B-CLPD, B-cell chronic lymphoproliferative disorders other than CLL (n=25 clones). *Patients other than CLL included the following diagnoses: HCL, hairy cell leukemia (n=1 clone); MZL, marginal zone lymphoma (n=17 clones); MALT, lymphoma of mucosa-associated lymphoid tissue (n=7); MCL, mantle cell lymphoma (n=3 clones); FL, follicular lymphoma (n=4 clones); DLBCL, diffuse large B-cell lymphoma (n=1 clones); LPL, lymphoplasmacytic lymphoma (n=1 clone).*
The precise diagnosis of multiclonal cases (CLL vs. non-CLL) were based on consistent clinic-biological features, according to the WHO 2008 criteria [486].

3.3.2. Overall size and BCR features of B-cell clones from multiclonal versus monoclonal MBL, CLL and other B-CLPD cases

The relative and absolute median number of PB clonal B-cells was significantly lower in multiclonal than in monoclonal cases (13% vs. 45% and 2,692 cells/µL vs. 9,115 cells/µL, respectively; *P* =0.001). Of note, the absolute median number of CLL-like MBL^{high} and CLL B-cell clones were also significantly lower in multiclonal than in monoclonal cases (1,254 vs. 2,464 cells/µL and 9,113 vs. 18,600 cells/µL, respectively; *P* =0.004 and *P* =0.02) (Figure 8). In contrast, the absolute median number of PB CLL-like MBL^{low} B-cell clones was significantly higher in multiclonal than in monoclonal cases (79 vs. 1 cells/ µL, *P* =0.002). No significant differences were found in the clone size between non-CLL like and non-CLL B-cell clones in multiclonal vs. monoclonal cases (Figure 8). In addition, the frequency of CLL-like MBL B-cell clones was significantly higher in multiclonal than in monoclonal cases (47% vs. 27%, respectively; *P* =0.002), whereas the frequency of CLL B-cell clones was higher in monoclonal vs. multiclonal subjects (62% vs. 31%, respectively; *P* =0.001). CLL B-cell clones from multiclonal and monoclonal CLL patients showed a similar distribution in Binet stage A vs.

Binet stages B/C (*P* >0.05). Of note, non-CLL B-cell clones were present at higher frequencies in multiclonal vs. monoclonal cases (17% vs. 8%, respectively; *P* =0.04) (Table 11).



	MBL^{low}		MBL^{high}		CLL		Non-CLL MBL		Non-CLL	
Absolute N. of clonal B-cells/µL	Multiclonal	Monoclonal	Multiclonal	Monoclonal	Multiclonal	Monoclonal	Multiclonal	Monoclonal	Multiclonal	Monoclonal
Median	79	1	1,254	2,464	9,113	18,600	266	465	4,932	7,500
Range (min-max)	(0.6-250)	(0.1-112)	(346-3,458)	(986-4,851)	(5,412-71,485)	(3,507-369,289)	(85-440)	(54-890)	(300-156,168)	(1,728-41,221)
p-value	**.002**		**.004**		**.02**		NS		NS	

Figure 8. Absolute number of CLL-like MBL^{low}, CLL-like MBL^{high}, CLL, non-CLL MBL and non-CLL B-cell clones per µL of peripheral blood in multiclonal vs monoclonal cases distributed according to diagnosis. Boxes extend from the 25th to the 75th percentiles, the lines in the middle represent median values (50th percentile). Vertical lines represent the highest and lowest values that are not outliers or extreme values (being outliers and extreme values those values that lie more than 1.5- and 3- fold the length of the box). The adjacent table compiles the median number and range of each subgroup of CLL-like and non-CLL like clonal B-cells/µL of peripheral blood and the exact P-values obtained after comparing multiclonal vs. monoclonal cases (Mann-Whitney U test) for the MBL^{low}, MBL^{high} and CLL subgroups**.** NS: no statistical significant differences were detected (*P* ≥0.05).

Regarding BCR features, a similar distribution of *IGHV* mutated and *IGHV* unmutated B-cell clones was found in multiclonal vs monoclonal cases − 51/85 (60%) vs. 84/139 (60%) and 34/85 (40%) vs. 55/139 (40%), respectively; Table 11 −. Despite this, the percentage of alignment of *IGHV* aa sequences among B-cell clones from multiclonal cases (n =3,560 two by two comparisons of clonal *IGHV* aa sequence) was slightly higher than that obtained among B-cell clones from monoclonal cases (n =8,891 comparisons): median of 52% vs. 50%, respectively; (*P* =0.001; Table 11).

Table 11. Peripheral blood B-cell counts and BCR features of multiclonal vs. monoclonal B-cell clones from B-CLPD and MBL cases.

	Multiclonal B-cells n=85 clones	**Monoclonal B-cells n=143 clones**	**TOTAL n=228 clones**
N. of PB clonal B cells(x10^6/L)*	**2,692 (0.6-156,168)[a]**	**9,115 (0.1-369,288)**	5,530 (0.1-369,288)
% of PB clonal B cells from WBC*	**13% (0.1%-89%)[a]**	**45% (0.002%-97%)**	35% (0.001%-97%)
CLL like MBL^{low} B-cell clones	14/85 (16%)	13/143 (9%)	27/228 (12%)
CLL like MBL^{high} B-cell clones	**26/85 (31%)[a]**	**26/143 (18%)**	52/228 (38%)
CLL B-cell clones	**26/85 (31%)[a]**	**89/143 (62%)**	113/228 (50%)
CLL-stage A clones	12/20 (60%)	53/89 (60%)	65/109 (60%)
CLL-stage B/C clones	8/20 (40%)	36/89 (40%)	44/109 (40%)
Non-CLL like MBL B-cell clones	5/85 (6%)	4/143 (3%)	9/228 (4%)
Non-CLL B-cell clones	**14/85 (17%)[a]**	**11/143 (8%)**	25/228 (11%)
IGHV mutated CLL-like B-cell clones	40/66 (61%)	76/128 (59%)	116/194 (60%)
IGHV mutated non-CLL-like B-cell clones	11/19 (58%)	8/15 (53%)	19/34 (56%)
% alignment of IGHV aa sequences between coexisting B-cell clones*	51% (38%-79%)	AN	51% (38%-79%)
% alignment of IGHV aa sequences between each B-cell clone and the other clones*	**52% (31%-100%)[a]**	**50% (29%-100%)**	51% (29%-100%)

*Results expressed as number of B-cell clones and percentage between brackets or as *median value (range). PB, peripheral blood; WBC, white blood cells; CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL^{low}, low count monoclonal B-cell lymphocytosis; IGHV, immunoglobulin heavy chain variable region genes; CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL^{high}, clinical monoclonal B-cell lymphocytosis; aa, amino acids. NA, not appropriate. [a]Statistically significant differences (P <0.05) found between clones from multiclonal vs. monoclonal cases. Information about the parameters included in this table is separately displayed for CLL-like vs. non-CLL-like clones in Supplemental Table 4.*

3.3.3. Cytogenetic features of B-cell clones from multiclonal versus monoclonal MBL and B-CLPD cases

The frequency of CLL-like MBL and CLL clones from multiclonal cases that showed cytogenetic alterations was significantly lower than that found among CLL-like MBL and CLL clones from monoclonal cases: 27/66 (41%) vs. 77/128 (60%), respectively (*P* =0.02). Likewise, the proportion of CLL-like B-cell clones showing coexistence of ≥2 cytogenetic alterations was also significantly lower in multiclonal than in monoclonal cases − 8/66 (12%) vs. 32/128 (25%); *P* =0.047 −; this was specially true among B-cell clones from CLL patients − 2/26 (8%) vs. 29/89 (33%), respectively; *P* =0.03 – (Table 12).

Regarding each specific cytogenetic alteration, only a decreased frequency of CLL-like B-cell clones with del(13q14) involving the *RB1* gene and a lower percentage of del(13q14)$^{+}$ cells was found in multiclonal vs monoclonal cases − frequency of del(13q14)$^{+}$ clones − of 5% vs. 15% with a median of del(13q14)$^{+}$ cells of 55% vs. 86%, respectively; *P* =0.01) (Table 12). Of note, these differences were mostly due to the lower frequency of B-cell clones with del(13q14) (4% vs 19%, *P* =0.01) found among CLL clones from multiclonal vs. monoclonal cases (Table 12).

Table 12. Cytogenetic features of CLL-like MBL^{low}, MBL^{high} and CLL B-cell clones from monoclonal versus multiclonal cases.

Cytogenetic alterations	MBL^{low} clones		MBL^{high} clones		CLL clones		TOTAL	
	Multiclonal n=14	**Monoclonal n=13**	**Multiclonal n=26**	**Monoclonal n=26**	**Multiclonal n=26**	**Monoclonal n=89**	**Multiclonal n=66**	**Monoclonal n=128**
No. of cytogenetically altered clones	2/14 (14%)	6/13 (46%)	13/26 (50%)	13/26 (50%)	12/26 (46%)	58/89 (65%)	**27/66 (41%)[b]**	**77/128 (60%)**
No. of clones with ≥2 alterations	1/14 (7%)	1/13 (8%)	5/26 (19%)	2/26 (8%)	**2/26 (8%)[a]**	**29/89 (33%)**	**8/66 (12%)[b]**	**32/128 (25%)**
Type of cytogenetic changes								
No. of del(13q)$^{+}$ clones (%)	2/14 (14%)	5/13 (38%)	7/26 (27%)	8/26 (31%)	8/26 (31%)	36/89 (40%)	17/66 (26%)	49/128 (38%)
*% del(13q)$^{+}$ cells **	46% (19%-73%)	86% (22%-96%)	74% (15%-98%)	38% (21%-99%)	93% (30%-96%)	80% (47%-99%)	84% (10%-98%)	79% (18%-99%)
No. of del(13q14.3)$^{+}$ clones (%)	2/14 (7%)	4/13 (31%)	7/26 (27%)	8/26 (31%)	8/26 (31%)	35/89 (39%)	17/66 (26%)	47/128 (37%)
*% del(13q14.3)$^{+}$ cells **	(19%-73%)	78% (22%-96%)	65% (15%-98%)	38% (21%-99%)	81% (30%-96%)	73% (5%-99%)	80% (15%-98%)	71% (5%-99%)
No. of del(13q14)$^{+}$ clones (%)	0/14 (0%)	1/12 (8%)	2/26 (8%)	1/26 (4%)	**1/26 (4%)[a]**	**17/89 (19%)**	**3/66 (5%)[b]**	**19/127 (15%)**
*% del(13q14)$^{+}$ cells **	-	86% (-)	57% (15%-98%)	96% (-)	**95% (-)**	**79% (47%-99%)**	**55% (10%-98%)[b]**	**86% (47%-99%)**
No. of trisomy 12^{+} clones (%)	0/14 (0%)	1/13 (8%)	6/26 (23%)	5/26 (19%)	2/26 (8%)	17/89 (19%)	8/66 (12%)	23/128 (18%)
*% trisomy 12^{+} cells **	-	59% (-)	87% (19%-95%)	84% (80%-93%)	84% (75%-93%)	76% (33%-97%)	87% (41%-95%)	80% (33%-97%)
No. of t(14q32)$^{+}$ clones (%)	0/12 (0%)	0/10 (0%)	2/26 (8%)	0/26 (0%)	1/26 (4%)	10/89 (11%)	3/64 (5%)	10/125 (8%)
*% t(14q32)$^{+}$ cells **	-	-	42% (31%-52%)	-	98% (-)	82% (18%-94%)	72% (28%-98%)	59% (18%-94%)
No. of del(11q)$^{+}$ clones (%)	0/12 (0%)	0/11 (0%)	1/26 (4%)	1/26 (4%)	1/26 (4%)	7/89 (8%)	2/64 (3%)	8/126 (6%)
*% del(11q)$^{+}$ cells **	-	-	93% (-)	20% (-)	91% (-)	57% (21%-98%)	92% (91%-93%)	57% (20%-98%)
No. of del(11q22.3)$^{+}$ clones (%)	0/12 (0%)	0/11 (0%)	1/26 (4%)	1/26 (4%)	1/26 (4%)	6/89 (7%)	2/64 (3%)	7/126 (6%)
*% del(11q22.3)$^{+}$ cells **	-	-	93% (-)	20% (-)	91% (-)	70% (24%-98%)	92% (91%-93%)	68% (20%-98%)
No. of del(11q23)$^{+}$ clones (%)	0/12 (0%)	0/11 (0%)	0/26 (0%)	0/26 (0%)	0/26 (0%)	3/89 (3%)	0/64 (0%)	3/126 (2%)
*% del(11q23)$^{+}$ cells **	-	-	-	-	-	32% (21%-64%)	-	40% (24%-64%)
No. of del(17p13.1)$^{+}$ clones (%)	0/13 (0%)	0/12 (0%)	0/26 (0%)	0/26 (0%)	0/26 (0%)	5/89 (6%)	0/65 (0%)	5/127 (4%)
*% del(17p13.1)$^{+}$ cells **	-	-	-	-	-	44% (33%-88%)	-	44% (33%-88%)

*Results expressed as number of clones with cytogenetic changes from all clones in the corresponding group (percentage) or as *median values of altered cells/clone (range). In seven clones (1 multiclonal MBL^{low}, 3 monoclonal and 3 multiclonal CLL clones) biallelic del(13q14.3) was detected, and polysomy was found in 1 multiclonal CLL clone. Statistically significant differences found between multiclonal vs. monoclonal B-cell clone groups for [a]CLL clones (P =0.01) and [b]all (total) clones (P =0.01). CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL, monoclonal B-cell lymphocytosis.*

No statistically significant differences were observed in the cytogenetic patterns of non- CLL B-cell clones from multiclonal vs monoclonal cases, which is probably due to the relatively low number of non-CLL clones included in the study; the precise cytogenetic alterations found in non-CLL/non-CLL-like MBL cases are shown in Supplemental Table 3 and Table 4. In turn, the overall cytogenetic features of non-CLL like B-cell clones from multiclonal (n=19; 3 non-CLL MBL^{low}, 2 non-CLL MBL^{high}, 14 non-CLL B-cell clones) vs. monoclonal (n=15; 2 non-CLL MBL^{low}, 2 non-CLL MBL^{high}, 11 non-CLL B-cell clones) subjects were similar, as regards both the frequency of cytogenetically altered clones (6/19, 32% and 6/15, 40%) and the percentage of cases with ≥2 genetic alterations − 2/19 (11%) vs. 2/15 (13%) – (Supplemental Table 3 and Table 5).

3.3.4. Molecular characteristics of the BCR of B-cell clones from multiclonal versus monoclonal MBL and B-CLPD cases

The molecular profile of the BCR of CLL-like MBL^{low}, MBL^{high} and CLL B-cell clones and of B-cell clones other than CLL from multiclonal vs. monoclonal cases was very similar (Table 13 and Table 14).

No statistically significant differences in multiclonal vs monoclonal *VH* gene usage were found for most groups. Despite this general behavior, CLL-like MBL^{high} B-cell clones from multiclonal cases less frequently showed usage of the *DH1*, *DH4* and *DH7* gene families than B-cell clones from monoclonal cases; in addition, JH6 genes were also less frequently used by CLL B-cell clones from multiclonal vs. monoclonal cases (Table 13). Overall, 33 functional *IGHV* gene rearrangements were identified from which 12 (*V4-34, V3-23, V3-48, V3-30, V1-69, V3-21, V4-39, V3-33, V3-11, V3-53, V1-2, V3-7*) were highly represented among the B-cell clones (≥5% of all B-cell clones corresponding to ≥4 and ≥5 B-cell clones sharing the same *IGHV* gene in multiclonal and monoclonal cases, respectively) (Figure 9A). Interestingly, 11 of these *IGHV* genes were found at similar frequencies within the clones of multiclonal vs. monoclonal cases, while the *V3-33* gene was typically associated with multiclonal cases (6% vs. 1%, *P* =0.03). Regarding *IGHD* genes, no significant differences were observed between B-cell clones from multiclonal and monoclonal cases, the *D3-3, D5-12, D3-10, D6-19, D2-15*, and *D2-2* genes being the most frequently used and shared by both groups of B-cell clones (Figure 9B). Among *IGHJ* genes, significant differences were only observed for the *JH6* gene, which was more frequently used in monoclonal cases (40% vs. 26%, *P* = 0.03) (Figure 9C).

Table 13. Molecular characteristics of the BCR of CLL-like MBL^{low}, MBL^{high} and CLL B-cell clones from monoclonal versus multiclonal cases.

	MBL^{low} clones		MBL^{high} clones		CLL clones		TOTAL	
	Multiclonal n= 14	**Monoclonal n= 13**	**Multiclonal n= 26**	**Monoclonal n= 25**	**Multiclonal n= 26**	**Monoclonal n= 87**	**Multiclonal n= 66**	**Monoclonal n= 126**
HCDR3 length (N. of aa)*	15 (6-22)	13 (11-20)	16 (8-23)	17 (9-26)	17 (11-26)	18 (8-32)	17 (6-26)	17 (8-32)
VH families								
VH1	2/14 (14%)	2/13 (15%)	3/26 (12%)	4/25 (16%)	4/26 (15%)	25/87 (29%)	9/66 (14%)	31/125 (25%)
VH3	9/14 (65%)	9/13 (69%)	15/26 (58%)	16/25 (64%)	11/26 (42%)	36/87 (41%)	35/66 (53%)	61/125 (49%)
VH4	2/14 (14%)	2/13 (15%)	6/26 (23%)	3/25 (12%)	9/26 (35%)	23/87 (27%)	17/66 (26%)	28/125 (22%)
VH2, VH5, VH6	1/14 (7%)	0/13 (0%)	2/26 (8%)	2/25(8%)	2/26 (8%)	3/87 (3%)	5/66 (7%)	5/125 (4%)
DH families								
DH1, DH4, DH7	3/14 (21%)	2/13 (15%)	**1/26 (4%)[a]**	**6/24 (25%)**	6/26 (23%)	11/86 (13%)	10/66 (15%)	19/123 (16%)
DH2	2/14 (14%)	3/13 (23%)	5/26 (19%)	7/24 (29%)	4/26 (15%)	15/86 (17%)	11/66 (17%)	25/123 (20%)
DH3	3/14 (21%)	2/13 (15%)	9/26 (35%)	7/24 (29%)	13/26 (50%)	36/86 (42%)	25/66 (38%)	45/123 (37%)
DH5	2/14 (14%)	3/13 (23%)	6/26 (23%)	2/24 (8%)	3/26 (12%)	9/86 (11%)	11/66 (17%)	14/123 (11%)
DH6	4/14 (29%)	3/13 (23%)	5/26 (19%)	2/24 (8%)	0/26 (0%)	15/86 (17%)	9/66 (13%)	20/123 (16%)
JH genes								
JH1, JH2, JH3, JH5	3/14 (21%)	2/13 (15%)	7/26 (27%)	7/24 (29%)	9/26 (35%)	14/86 (16%)	19/66 (29%)	23/123 (19%)
JH4	6/14 (43%)	7/13 (54%)	13/26 (50%)	8/24 (33%)	10/26 (38%)	34/86 (40%)	29/66 (44%)	49/123 (40%)
JH6	5/14 (36%)	4/13 (31%)	6/26 (23%)	9/24 (38%)	**7/26 (27%)[a]**	**38/86 (44%)**	**18/66 (27%)[a]**	**51/123 (41%)**
LCDR3 length (N. of aa)*	9 (8-13)	10 (8-10)	**10 (8-12)[a]**	**9 (7-12)**	10 (8-15)	9 (5-12)	**10 (8-15)[a]**	**9 (5-12)**
VK families								
VK1	0/6 (0%)	1/4 (25%)	6/14 (43%)	3/12 (25%)	6/12 (50%)	23/48 (48%)	12/32 (38%)	27/64 (42%)
VK2, VK5, VK6	1/6 (17%)	0/4 (0%)	1/14 (7%)	3/12 (25%)	2/12 (17%)	8/48 (17%)	4/32 (13%)	11/64 (17%)
VK3, VK4	5/6 (83%)	3/4 (75%)	7/14 (50%)	6/12 (50%)	4/12 (33%)	17/48 (35%)	16/32 (50%)	26/64 (41%)
JK genes								
JK1, JK3, JK5	3/6 (50%)	1/4 (25%)	5/14 (36%)	7/12 (58%)	5/12 (42%)	21/47 (45%)	13/32 (40%)	29/63 (46%)
JK2	2/6 (33%)	2/4 (50%)	8/14 (57%)	3/12 (25%)	4/12 (33%)	10/47 (21%)	14/32 (44%)	15/63 (24%)
JK4	1/6 (17%)	1/4 (25%)	1/14 (7%)	2/12 (17%)	3/12 (25%)	16/47 (34%)	5/32 (16%)	19/63 (30%)
Vλ families								
Vλ3	2/3 (67%)	NA	1/7 (14%)	0/7 (0%)	2/7 (29%)	8/25 (32%)	5/17 (29%)	8/32 (25%)
Other	1/3 (33%)	NA	6/7 (86%)	7/7 (100%)	5/7 (71%)	17/25 (68%)	12/17 (71%)	24/32 (75%)
Jλ genes								
Jλ1	1/3 (33%)	NA	0/7 (0%)	0/7 (0%)	4/7 (57%)	6/21 (71%)	5/17 (29%)	6/28 (21%)
Other	2/3 (67%)	NA	7/7 (100%)	7/7 (100%)	3/7 (43%)	15/21 (29%)	12/17 (71%)	22/28 (79%)
IGHV mutational status								
Mutated IGHV	9/14 (64%)	8/11 (73%)	17/26 (65%)	20/25 (80%)	14/26 (54%)	46/86 (54%)	40/66 (61%)	74/122 (61%)
Unmutated IGHV	5/14 (36%)	3/11 (27%)	9/26 (35%)	5/25 (20%)	12/26 (46%)	40/86 (47%)	26/66 (39%)	48/122 (39%)

*Results expressed as number of B-cell clones from all clones in the corresponding group (percentage) or as *median (range). CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL, monoclonal B-cell lymphocytosis; BCR, B-cell receptor; HCDR3, heavy chain complementarity-determining region 3; LCDR3, light chain complementarity-determining region 3; aa, amino acid. NA, not analyzed; [a] statistically significant differences found between groups of clones from multiclonal vs monoclonal cases (P ≤0.03).*

Table 14. Molecular characteristics of the BCR of non-CLL B-cell clones from both MBL and B-CLPD other than CLL.

	Non-CLL B-cell clones n=34 clones	
	Multiclonal	**Monoclonal**
HCDR3 length (N. of aa)*	14 (8-26)	16 (7-22)
VH families		
VH1	4/19 (21%)	3/15 (20%)
VH3	12/19 (63%)	8/15 (53%)
VH4	3/19 (16%)	2/15 (13%)
VH5	0/19 (0%)	1/15 (7%)
VH6	0/19 (0%)	1/15 (7%)
DH families		
DH1, DH4, DH7	5/19 (26%)	2/14 (14%)
DH2	3/19 (16%)	4/14 (29%)
DH3	4/19 (21%)	2/14 (14%)
DH5	2/19 (11%)	5/14 (36%)
DH6	5/19 (26%)	1/14 (7%)
JH genes		
JH1, JH2, JH3, JH5	7/19 (37%)	3/15 (20%)
JH4	8/19 (42%)	7/15 (47%)
JH6	4/19 (21%)	5/15 (33%)
LCDR3 length (N. of aa)*	9 (9-11)	9 (8-12)
VK families		
VK1	4/11 (36%)	3/12 (25%)
VK2	2/11 (18%)	1/12 (8%)
VK3-4	5/11 (46%)	8/12 (67%)
JK genes		
JK1, JK3, JK5	5/11 (42%)	5/11 (45%)
JK2	2/11 (33%)	2/11 (21%)
JK4	4/11 (25%)	4/11 (34%)
Vλ families		
Vλ3	1/3 (33%)	1/1 (100%)
Other	2/3 (67%)	0/1 (0%)
Jλ genes		
Jλ2	2/3 (67%)	1/1 (100%)
Jλ3	1/3 (33%)	0/1 (0%)

*Results expressed as number of B-cell clones from all clones in the corresponding group (percentage) or as *median (range). Non-CLL, clones mimicking or compatible with B-cell chronic lymphoproliferative disorders other than chronic lymphocytic leukemia; BCR, B-cell receptor; HCDR3, heavy chain complementarity-determining region 3; LCDR3, light chain complementarity-determining region 3; aa, amino acid.*

Except for slightly longer LCDR3 sequences of the *IGKV* and *IGLV* genes found among B-cell clones from multiclonal versus monoclonal cases, specially among CLL-like MBL^{high} clones (Table 13), no other significant differences were found in the molecular characteristics of the

immunoglobulin light chain genes, neither among CLL-like nor non-CLL like B-cell clones from multiclonal vs. monoclonal cases (Table 13 and Table 14). Regarding *IGKV* and *IGLV* genes, only the *VK1-33* gene was associated with multiclonal cases (6% vs. 0%, *P* =0.02) (Figure 9D)

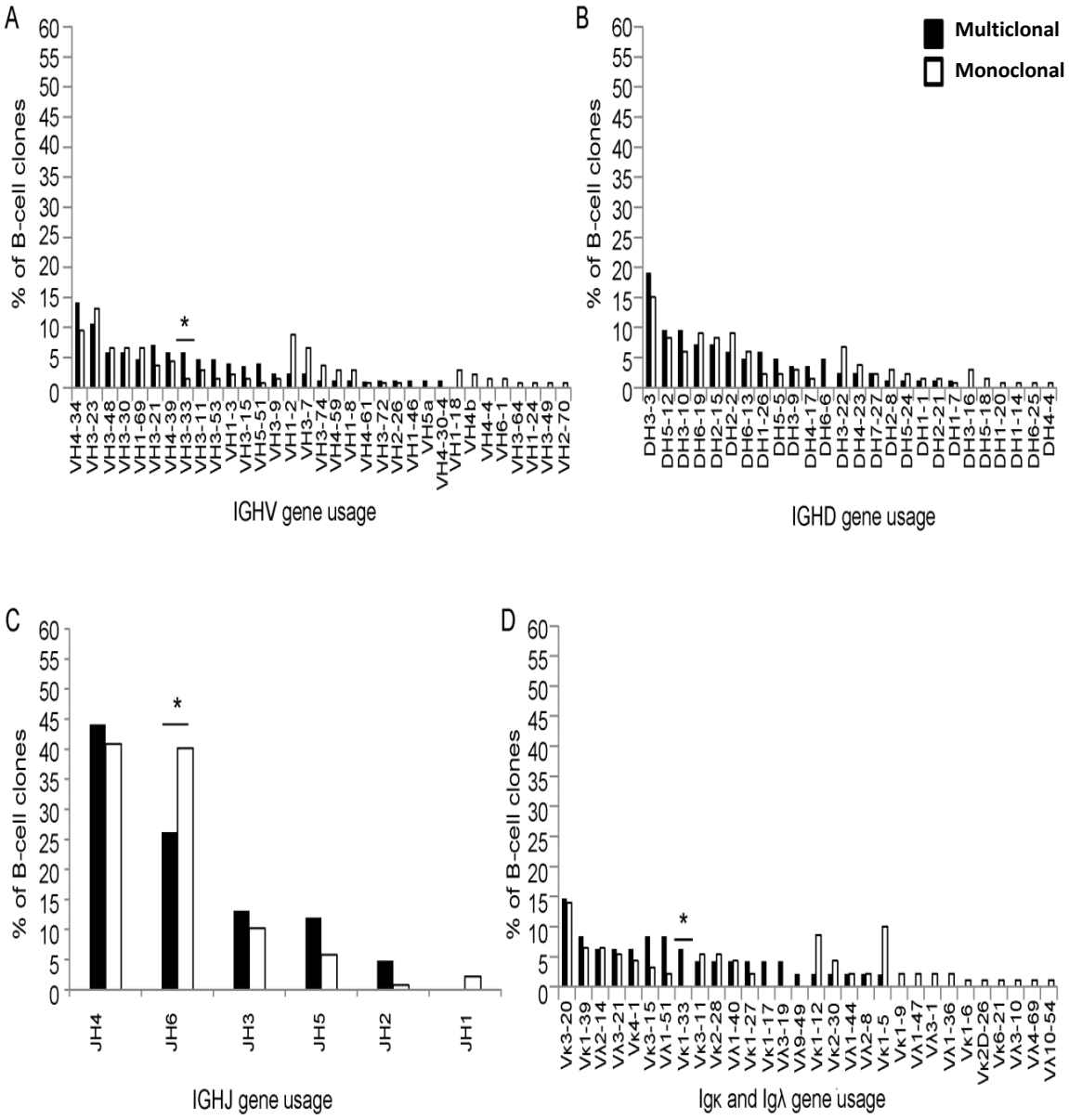


Figure 9. Frequency of *IGHV* (panel A), *IGHD* (panel B), *IGHJ* (panel C) and both *IGKV* and *IGLV* (panel D) genes in multiclonal and monoclonal CLL versus non-CLL like B cell clones. Diagrams show the relative frequency of each *IG* gene in multiclonal compared to monoclonal B-cell clones (black and white bars, respectively). *Statistically significant differences were found between the multiclonal vs. monoclonal subgroups (*P* <0.05).

3.3.5. Molecular features of phylogenetically related BCRs of B-cell clones from multiclonal cases

Thirty-two of the 85 B-cell clones from individual multiclonal cases were phylogenetically closely related and had exactly the same *IGHV* family (*IGHV3* in 28 B-cell clones and *IGHV4* in 4 B-cell clones) (Figure 10). Of note, this subgroup of B-cell clones frequently showed *IGHV3* gene usage (28/85, 33%) and they displayed shorter HCDR3 sequences than other (multiclonal and monoclonal) B-cell clones − 13 (6-25) vs. 17(9-26) and 16 (8-32) aa; *P* =0.001 and *P* =0.004, respectively −; in addition, they also showed a higher frequency of del(13q14.3) compared to B-cell clones from multiclonal cases expressing phylogenetically unrelated *IGHV* families (41% vs. 17%, respectively; *P* =0.05). Moreover, a slightly higher frequency of multiclonal cases whose coexisting clones were cytogenetically altered was found among phylogenetically closely related clones vs phylogenetically unrelated clones from multiclonal cases (53% vs. 34%, respectively; *P* =0.06). Interestingly, a trend towards an increased percentage of *IGHV* mutated B-cell clones among phylogenetically related B-cell clones from multiclonal cases compared to other B-cell clones from multiclonal cases, was also found (70% vs. 54%, respectively; *P* =0.1). Interestingly, most of the co-existing phylogenetically related clones had a CLL-like phenotype (10/16 cases, identified in Supplemental Table 3 with the ¶ symbol), while in 4/16 multiclonal cases, one CLL-like B-cell clone coexisted with one non-CLL B-cell clone, (2 MZL, 1 MALT lymphoma and 1 HCL clones from cases 29, 32, 37 and 38, also identified in Supplemental Table 3 with the ¶ symbol). In a minority of cases (2/16), the two co-existing phylogenetically related clones were both non-CLL like, their phenotype being consistent with FL (case 16¶, in Supplemental Table 3) and MALT lymphoma (case 34¶, in Supplemental Table 3), respectively.

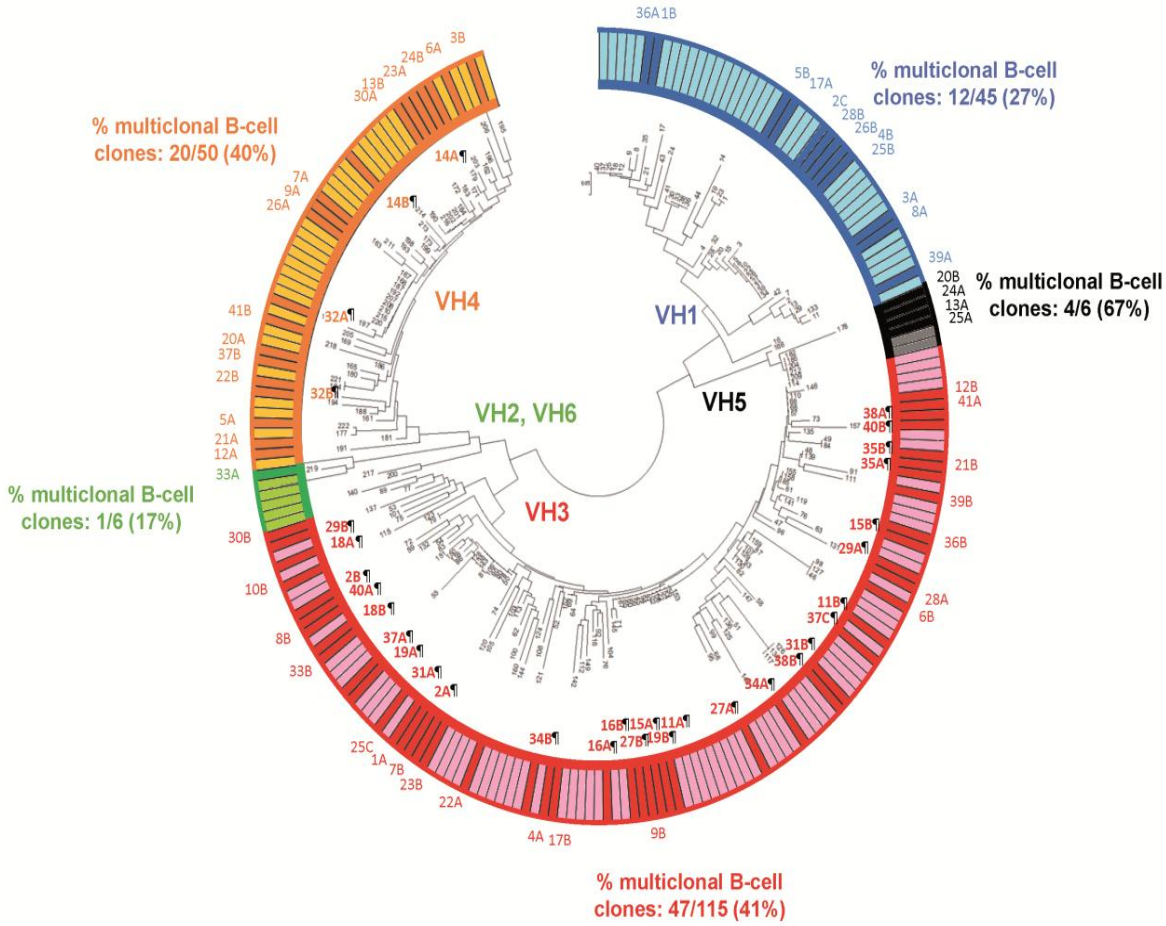


Figure 10. Sequence distance cladogram of *IGHV* gene usage in CLL-like and non-CLL like B-cell clones from multiclonal (dark colored bars in the outside circle) and monoclonal (light colored bars in the outside circle) cases. Five major branches were found in the sequence distance cladogram (i.e *VH1, VH5, VH3, VH2-VH6, VH4*). B-cell clones from individual multiclonal cases are represented by numbers; from them, those phylogenetically closely related B-cell clones, which share the same *IGHV* family, are specifically identified by bold numbers in the inner part of the circle and the symbol ¶. Of note, B-cell clones from multiclonal cases 14¶, 16¶ and 35¶ belong to closely located sub-branches of the cladogram, having their *IGHV* sequences an aa identity of 79%, 76% and 69%, respectively. In turn, B-cell clones from the multiclonal case 32¶ belong to the *VH4* major branch with *IGHV* sequences whose aa identity is of 69%. Finally, the other B-cell clones from multiclonal cases – cases 2¶, 11¶, 15¶, 18¶, 19¶, 27¶, 29¶, 31¶, 34¶, 37¶, 38¶ and 40¶– belong to the *VH3* major branch, having *IGHV* sequences with an aa identity which is > 60% (–68%, 73%, 73.4%, 61%, 79%, 70%, 63%, 77%, 69.9%, 70%, 72% and 68.4%–, respectively).

3.3.6. Homology of the HCDR3 region between B-cell clones coexisting in multiclonal cases versus non-coexisting (monoclonal) B-cell clones

The HCDR3 aa sequence from coexisting B-cell clones had the same length or it just differed in one aa in 8/41 multiclonal cases analyzed (19%) (Table 15A). The homology of all these case-paired HCDR3 regions was calculated as the number of identical aa or aa with an analogous side-chain polarity (excluding the anchor second-CYS104 (**C_**) and the J-TRP 118

(**_W**) aa positions that delineate the HCDR3 region) divided by the corresponding HCDR3 length (Table 15A). It is worth noting that the aa composition of HCDR3 sequences of the same length (±1 aa) that belonged to the same or evolutionary, highly-related, *VH* families (e.g. *VH3-48, VH3-21, VH3-11*)[239] (n=57) from monoclonal cases (Supplemental Table 6) showed a tendency towards a lower homology than that of multiclonal cases: median of 37% (range: 11% to 71%) vs. 50% (range: 26% to 64%), respectively; (P =0.1). Since stereotyped sequences are widely represented in CLL [487], we further analyzed the frequency of stereotyped HCDR3 sequences in multiclonal cases (Table 15B) vs. monoclonal cases (Table 15C). Interestingly, the number of multiclonal cases showing the same or highly similar stereotyped HCDR3 sequences was significantly higher than that of monoclonal cases: 8/41 (19%) vs. 11/143 (8%), respectively, (*P* =0.001). Furthermore, the aa composition of HCDR3 sequences from monoclonal cases with stereotyped HCDR3 sequences showed clearly less identical and/or conserved positions than those found among multiclonal cases (underlined aa in Table 15B and Table 15C).

Table 15. Multiclonal cases with coexisting B-cell clones sharing HCDR3 features (A). Multiclonal (B) and monoclonal (C) cases with B-cell clones showing stereotyped HCDR3 aa sequences.

A. Multiclonal cases with coexisting B-cell clones showing identical or one aa differing length of HCDR3 regions, and analogous composition of aa in some parallel positions.

Multiclonal Case ID	*VH* families	aa composition of HCDR3 (length)	% homology[#]
8A	*V1-3*	**C**_ARDRVVIIPDTTTINWFDP_**W** (19)	26
8B	*V3-53*	**C**_ATHPTNIYTRWPYVSDMDV_**W** (19)	
11A	*V3-23*	**C**_ANRGETRGMDV_**W** (11)	54
11B	*V3-48*	**C**_VRDGFHYYGFDI_**W** (11)	
14A	*V4-34*	**C**_ARGPDRLYSGSYTRFDY_**W** (17)	47
14B	*V4-34*	**C**_ARREDDNFWSGFYMDV_**W** (16)	
22A	*V3-74*	**C**_ARDLDGSGSGVFDW_**W** (14)	64
22B	*V4-59*	**C**_ARGWRSTDSYYGMDV_**W** (15)	
29A	*V3-48*	**C**_VRELWFGNGGDY_**W** (12)	42
29B	*V3-15*	**C**_ATAGQGSADFLY_**W** (12)	
31A	*V3-33*	**C**_ARGELLHNWFDP_**W** (12)	58
31B	*V3-23*	**C**_AKDGFPYYGFDI_**W** (12)	
32A	*V4-39*	**C**_ARQTGWLAPSDY_**W** (12)	54
32B	*V4-34*	**C**_ARRDSSGWYYFDY_**W** (13)	
33A	*V2-26*	**C**_AGTNIPRQFDFWSGSSPNWFDP_**W** (22)	32
33B	*V3-53*	**C**_ARAGGYCNSGSCRGAPRWYFDL_**W** (22)	

*Amino acids (aa) with analogous side-chain polarity (highlighted in gray): case 8 (I, V and F, M), case 11 (A, V and M, F), case 14 (L, F), case 22 (L, W; S, T and F, M), case 29 (A, V), case 31(L, F and H, Y), case 32 (T, S), case 33 (W, C) (EMBL-EBI Cambridge, UK). All cases had coexisting B-cell clones with CLL-like phenotype, except case 29 (one B-cell clone corresponded to a MALT lymphoma-like phenotype), cases 32 and 33 (both had one B-cell clone with a MZL-like phenotype). MALT, lymphoma of mucosa-associated lymphoid tissue; MZL, marginal zone lymphoma. [#]Number of aa with analogous side-chain polarity (excluding the delineating **C**_ and _**W** positions)/HCDR3 length*100.*

B. Multiclonal cases with B-cell clones showing stereotyped HCDR3 sequences.

Multiclonal Cases ID	*V(D)J* rearrangement	AA composition of HCDR3
1	*V3-30(D3-9)J6*	**C**_AKYGGVKLRYFDWLLYGDYYYGMDV_**W**
2	*V3-30(D3-9)J6*	**C**_AKYGGVKLRYFDWLLYGDYYYGMDV_**W**
9	*V3-23(D5-12)J6*	**C**_ANRGETRGMDV_**W**
15	*V3-23(D3-22)J6*	**C**_ANRGESWGMDV_**W**
21	*V3-21(D2-2)J6*	**C**_ARDANGMDV_**W**
35	*V3-21(D2-2)J6*	**C**_ARDANGMDV_**W**
22	*V3-74(D3-10)J4*	**C**_ARDLDGSGSGVFDW_**W**
40	*V3-21(D4-23)J4*	**C**_ARDLDGGNSVFDC_**W**

Cases 1 and 2; 9 and 15; 21 and 35; 22 and 40 showed a highly similar HCDR3 sequence; the underlined aa were different. All the listed B-cell clones had a CLL-like phenotype.

C. Monoclonal cases with B-cell clones showing stereotyped HCDR3 sequences.

Monoclonal Cases ID	*V(D)J* rearrangement	AA composition of HCDR3
117	*V3-7(D3-3)J4*	**C**_VRENELWSGGWGLDG_**W**
134	*V3-7(D3-3)J4*	**C**_VRENEFWSGGWGLDG_**W**
207	*V4-39(D2-2)J6*	**C**_ARHRLGYCSSTSCYYYYGMDV_**W**
208	*V4-39(D2-2)J6*	**C**_ARHRLGYCSSTSCYYYYGMDV_**W**
210	*V4-39(D2-2)J6*	**C**_ARDRLGYCSSTSCYYYYGMDV_**W**
187	*V4-b(-)J4*	**C**_ARSWIQLWSEFDY_**W**
215	*V4-b(D5-5)J4*	**C**_ARAWIQLWSDFDY_**W**
180	*V1-2/D6-19/J4*	**C**_ARLQWLGISHFDY_**W**
204	*V1-2/D6-19/J4*	**C**_ARAQWLVLENFDY_**W**
196	*V4-34/D3-16/J6*	**C**_VRGYPSDYTERRYYYYGLDV_**W**
198	*V4-34/D4-23/J6*	**C**_ARGYGSTGETRRYYYYGMDV_**W**

Cases 117 and 134; 207, 208 and 210; 187 and 215; 180 and 204; 196 and 198 showed a highly similar HCDR3 region; the underlined aa were different. All the listed B-cell clones had a CLL-like phenotype.

3.4. Discussion

Multiclonal expansions of phenotypically aberrant B-cell clones (MBL^{low}) have been reported as frequently present in the general population;[19] of note, multiclonal expansions of immunophenotypically normal B-cells can also be found in non-malignant diseases, such as autoimmune disorders and inflammatory responses against several infectious agents (e.g. Helicobacter pylori, HCV).[488,489] Whether clonal expansions of aberrant B-cells found in otherwise healthy individuals (MBL^{low}) reflect a prominent reactive process against potent antigenic stimuli with unknown clinical relevance, or they represent an early (multi)clonal manifestation of a BCR-dependent neoplastic event, still remains to be established. In this regard, it should be noted that between 30% and 40% of such cases show cytogenetic changes shared by MBL and CLL, e.g. del(13q). Of note, among other large structural chromosomal alterations, clonal mosaicism involving del(13q14) has also been recently found in peripheral blood cell populations from otherwise healthy individuals, particularly among subjects with

more advanced age (around 2-3% in the elderly), but its potential relationship with MBL and CLL remains unknown.[490,491] Compared to the typical (monoclonal) MBL and B-CLPD, coexisting B-cell clones from multiclonal MBL and B-CLPD may potentially have a greater probability of interacting with common immunological determinants. However, there is still little information about the potential existence of shared BCR features in cases showing ≥2 coexisting B-cell clones vs. monoclonal cases.

In the present study, we analyzed for the first time the molecular and cytogenetic features of a large group (n=85) of coexisting, but unrelated, B-cell clones from a series of 41 multiclonal MBL and B-CLPD cases, in comparison to 143 monoclonal cases. Overall, the former clones more frequently showed cytogenetic and hematological features which are typical of the earliest MBL stages and/or initial phases of CLL.[468,488,489] Accordingly, B-cell clones from multiclonal cases more frequently corresponded to MBL cases, whereas B-cell clones from monoclonal cases were more frequently found to correspond to overt CLL. Of note, these findings do not contradict the apparent discrepancy between such association and our previous observation among CLL patients of a worse clinical outcome for multiclonal cases carrying non-CLL clones,[21] as this latter study was restricted to overt CLL cases. In addition, multiclonal cases were also associated with lower clonal B-cell counts in peripheral blood, a lower number of cytogenetically altered clones, particularly of those carrying del(13q), and a decreased frequency of clones with ≥2 alterations. Of note, clonal expansions of non-CLL like B-cell clones were also more frequently observed in multiclonal than in monoclonal cases, such expansions corresponding mainly to indolent lymphomas (e.g. MZL) which have been associated with chronic immune responses.[492,493]

Altogether, these results support the notion that the presence of multiple B-cell clones in the same individual more closely reflects the earlier stages of the disease. If this holds true and chronic antigen stimulation is involved in the onset of MBL and B-CLPD – as it has been recently suggested for MBL, based on epidemiological studies[204] –, it could be hypothesized that B-cell clones coexisting in multiclonal cases would show more closely related BCR features than B-cell clones from monoclonal cases. In this regard, our results point out the existence of a slightly higher level of HCDR3 homology among B-cell clones from multiclonal vs monoclonal cases. In fact, in around one fifth of all multiclonal cases, the co-existing B-cell clones showed a high homology in their HCDR3 aa sequences; this also hold true when we compared the homology of the HCDR3 sequences of these multiclonal against those of monoclonal cases whose B-cell receptors were restricted to the same and/or ontogenetically related IGHV families. In addition, the frequency of stereotyped HCDR3 was also higher in multiclonal vs. monoclonal cases. Such more closely related BCR features would be found independently of

whether common antigens or superantigens are specifically involved, although the former would potentially lead to a higher HCDR3 homology, whereas superantigens could contribute to a greater frequency of usage of specific *IGHV*, *IGHD* and/or *IGHJ* genes.[22,244]

In the present study, we found a similar frequency of *IGHV* gene usage between coexisting multiclonal and non-coexisting monoclonal B-cell clones in association with a lower frequency of *DH1, DH4* and *DH7* as well as *JH6* families in multiclonal vs. monoclonal B-cell clones. Overall, these results suggest that no single Ag or superantigen is involved in common in MBL and B-CLPD. This is further supported by the relatively low percentage of alignment (≈50%) of the *IGHV* aa sequences observed among the different clonal B-cell populations analyzed, since such potential antigens – including superantigens – would require interaction with highly conserved sites at the IGHV/HCDR3 regions of the BCR.[494] Interestingly however, the higher representation of *DH1, DH4, DH7* and *JH6 IGH* gene segments in monoclonal vs multiclonal B-cell clones, together with the slightly higher levels of HCDR3 homology observed among coexisting (multiclonal) vs non-coexisting (monoclonal) B-cell clones from MBL, CLL and other B-CLPD cases, would indicate that still non-random selection of specific HCDR3*, DH* and *JH* segments could exist in the MBL and CLL repertoire of both multiclonal and monoclonal cases, which could reflect antigen-driven selection and expansion of specific B-cell clones, both at the MBL and/or CLL stages.[64]

In this regard, based on the phylogenetic proximity of their BCR, we could further identify within the B-cell clones from multiclonal cases, a considerably represented subgroup of B-cell clones showing preferential usage of *IGHV3* genes and shorter HCDR3 sequences carrying a significantly higher number of *IGHV* mutations vs the unrelated clones. These results further support the involvement of a common antigen, at least in this specific subset of cases.[495] Interestingly, these "phylogenetically-related" B-cell clones coexisting in multiclonal cases showed a significantly higher frequency of del(13q) than B-cell clones expressing other *IGHV* genes. These observations further suggest that the BCR features of this subset of coexisting multiclonal B-cell clones could also contribute to determine the probability and/or type of cytogenetic progression occurring at the earliest stages of the disease, as previously suggested by our group[468] and others.[479,496] Further long-term, longitudinal studies are required to confirm this hypothesis, since multiple productive *IGHV* gene rearrangements may also underline clonal drift leading to selection for more aggressive clones whose proportions would change over time.[497]

In summary, based on the molecular features of the BCR and the cytogenetic profile of B-cell clones from the multiclonal vs monoclonal MBL, CLL and other B-CLPD cases here analyzed, it may be concluded that multiclonality is typically associated with early stages of B-

CLPD, at the same time it appears to more closely reflect an antigen-driven nature of MBL and B-CLPD, with potential involvement of multiple and diverse antigenic determinants.

Chapter 4 | Subjects with chronic lymphocytic leukemia-like B-cell clones with stereotyped B-cell receptors frequently show MDS-associated phenotypes on myeloid cells

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4.1. Abstract

An increasing body of evidences suggests the potential occurrence of antigen encounter by the cell of origin in CLL and CLL-like MBL. However, the scenario in which this event might occur, remains unknown. In order to gain insight into this scenario we investigated the molecular, cytogenetic and hematological features of 223 CLL-like (n=84) and CLL (n=139) clones with stereotyped (n=32) vs. non-stereotyped (n=191) *IGHV* aa sequences. Overall, stereotyped CLL-like MBL and CLL clones showed a unique *IGHV* profile, associated with higher *VH1* and lower *VH3* gene usage (*P* =0.03), longer HCDR3 sequences (*P* =0.007) and unmutated *IGHV* (*P* <0.001) vs. non-stereotyped clones. Whilst the overall size of the stereotyped B-cell clones in PB did not appear to be associated with the CLL-related cytogenetic profile of B-cells (*P* >0.05) it did show a significant association with the presence of myelodysplastic syndrome (MDS)-associated immunophenotypes on PB neutrophils and/or monocytes (*P* =0.01). Altogether these results, point to the potential involvement of different selection forces in the expansion of stereotyped vs. non-stereotyped CLL and CLL-like MBL clones, the former being potentially favored by an underlying altered hematopoiesis.

4.2. Materials and methods

4.2.1. CLL patients and MBL subjects

A total of 200 subjects (119 males and 81 females; mean age of 69±12 years; range: 35-89 years) presenting one or more CLL-like and/or CLL clonal B-cell populations, were included in this study. Fifteen cases (7%) corresponded to healthy individuals with MBL^{low} (<200 CLL-like B-cells/μL in PB), 41 (20%) were MBL^{high} (≥200 and <5 000 CLL-like B-cells/μL in PB), 131 (66%) had newly-diagnosed untreated CLL, 9 (4%) were CLL patients who had previously received chemotherapy for CLL, and 4 (2%) had other B-CLPD in association with ≥1 CLL-like MBL B-cell population(s). PB samples were obtained from each subject after written informed consent was given, and the study was approved by the local ethics committees of the participating centres. Diagnosis of MBL and CLL was based on the WHO 2008 criteria[466] and clinical staging of CLL subjects was established according to the Binet classification.[498] Immunophenotypic evaluation of the B-cell clones in MBL cases and both CLL and other B-CLPD patients was performed as previously described.[192,468,499]

Overall, 37/200 subjects (18%) showed co-existence of two or three phenotypically different aberrant B-cell populations with a CLL-like (n=33) or other B-CLPD phenotype (n=4). For this study, analysis was focused only on those aberrant B-cell populations displaying a typical CLL-like MBL and CLL phenotype, for a total of 228 B-cell clones. The distribution of all clonal B-cell populations analyzed in the distinct diagnostic categories was as follows: 27 corresponded to CLL-like MBL^{low}, 59 to CLL-like MBL^{high} and 142 to CLL (see Table 16).

Table 16. Distribution according to diagnosis of subjects included in the study and the corresponding CLL and CLL-like MBL clones.

			Diagnostic subgroups			
		No. of cases	**MBL^{low}**	**MBL^{high}**	**CLL**	**Other B-CLPD**
Subjects	*Monoclonal*	163	13 (87%)	33 (80%)	117 (84%)$^{\#}$	-
	Multiclonal	37†	2 (13%)*	8 (19%)*	23 (16%)*,$^{\#}$	4 (100%)*,$^{\#}$
	Total	**200**	**15**	**41**	**140**$^{\#}$	**4**$^{\#}$
B-cell clones	*From monoclonal cases*	163	13 (48%)	33 (56%)	117 (82%)	-
	From multiclonal cases	65	14 (52%)	26 (44%)	25 (18%)	-
	Total	**228**	**27**	**59**	**142**	**-**

**For multiclonal CLL and CLL-like MBL cases as well as for B-CLPD cases other than CLL, only CLL-like clones were considered; the later B-CLPD cases included the following diagnoses: HCL, hairy cell leukemia; SMZL/MALT, splenic marginal zone B-cell lymphoma/ extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue lymphoma. CLL, chronic lymphocytic leukemia; MBL, monoclonal B-cell lymphocytosis; B-CLPD, B-cell chronic lymphoproliferative disorders.*

†The number of clones per multiclonal case was of two in all diagnostic subgroups, except in three tri-clonal subjects corresponding to one CLL patient, one MBL^{high} case and one patient with a B-CLPD other than CLL.

$^{\#}$Six monoclonal and four multiclonal subjects had received conventional chemotherapy (see Supplemental Table 7).

4.2.2. Cytogenetic and molecular studies

Cytogenetic analyses were performed by multicolor interphase fluorescence *in situ* hybridization on slides containing FACS-purified and fixed aberrant B-cells, as previously described in detail.[253,468,499] In parallel, analysis of the patterns of rearrangement of the *IGHV* was performed for each FACS-purified B-cell clone.[468,471,472,499] Forward and reverse sequences were aligned into a single resolved sequence and then aligned with germline sequences using the IMGT database and tools (http://imgt.org). Only B-cell clones showing in-frame rearrangements were finally evaluated (n=223). Sequences containing >2% deviation from the germline sequence were considered as being somatically mutated. Those *IGHV-IGHD-IGHJ* rearrangements with HCDR3 sequences that matched stereotyped CLL subsets, as previously defined by Agathangelidis[13] and Stamatopoulos *et al*.,[237] were classified as stereotyped *IGHV* sequences; all other *IGHV* sequences were identified as being non-stereotyped.

4.2.3. Immunophenotypic analyses of PB myeloid cells

Neutrophils were recognized as displaying MDS-associated phenotypes when they lacked or expressed low levels of CD10 and/or showed low SSC properties (e.g. granularity) vs. their normal counterpart;[500] in turn, presence of CD56 expression on PB monocytes was also defined as an MDS-associated phenotype.[500]

4.2.4. Statistical methods

Conventional descriptive and comparative statistics –nonparametric Kruskal-Wallis and Mann-Whitney U tests (for continuous variables), or the Pearson's $\chi 2$ and Fisher exact tests (for categorical variables) – were performed using the SPSS software program (SPSS, version 20.0; SPSS software, IBM, Armonk, NY, USA). *P* values <0.05 were considered to be associated with statistical significance.

4.3. Results

4.3.1. Molecular and cytogenetic features of CLL and MBL B-cell clones with stereotyped versus non-stereotyped IGHV amino acid sequences

Thirty-two of the 223 B-CLPD clones (14%) analyzed showed stereotyped HCDR3 aa sequences. These stereotyped HCDR3 sequences corresponded to 19 different stereotyped CLL subsets, as previously defined by Stamatopoulos and Agathangelidis *et al*..[13,237] From the 32 stereotyped clones, 26 (81%) corresponded to B-cell clones from monoclonal B-CLPD, whereas 6 (19%) derived from multiclonal B-CLPD cases (Table 17 and Table 18), the latter representing a slightly lower frequency than that found among non-stereotyped clones (19% vs. 30%, *P* =0.2)(Table 18). Further analysis of the specific *IGHV* gene sequences revealed a higher *VH1* gene usage in stereotyped vs. non-stereotyped B-cell clones; in contrast, the *VH3* gene was less frequently found among the stereotyped clones (*P* =0.03) (Table 19). Moreover, stereotyped B-cell clones displayed longer median HCDR3 sequences and lower percentages of *IGHV* mutated aa sequences than non-stereotyped B-cell clones (*P* =0.007 and *P* <0.001, respectively) (Table 19).

Table 17. Phenotypic, haematological, molecular and cytogenetic features of B-CLPD cases whose CLL-like B-cell clones had stereotyped HCDR3 sequences defined according to previously reported stereotypic CLL profiles.[13,237]

Clone No	Subset No.	Clone Phenotype	% Clone	Abs. No. Clone (x10^6/L)	MAP	Binet Stage*	IGHV MS	IGHV-D-J gene rearrangements	HCDR3 sequence (length)	iFISH (% of altered cells)
1	**1**	CLL	88	120 102	No	B	U	V1-2/D6-19/J4	CARAQWLVLENFDYW (13)	ND
2	**1**	CLL-like MBL^{low}	3.1	112	No	-	U	V1-2/D6-19/J4	CARLQWLGISHFDYW (13)	ND
3	**1**	CLL	87.6	79 839	**Yes**	B	U	V1-3/D6-19/J4	CARWQWLVPSRFDYW (13)	-13q14.3 (94%)
4	**1**	CLL	35	5 085	No	A	U	V1-18/D6-19/J4	CARKQWLGMYYFDYW (13)	-13q14.3 (13%); t(14q32) (13%)
5B†	**2**	CLL-like MBL^{high}	9.8	2 078	**Yes**	-	M	V3-21/D2-15/J6	CARDANGMDVW (9)	ND
6B	**2**	CLL-like MBL^{high}	2.3	250	**Yes**	-	M	V3-21/D2-15/J6	CARDANGMDVW (9)	ND
7	**2**	CLL-like MBL^{high}	33.2	4 424	No	-	M	V3-21/D1-26/J6	CARDANGMDVW (9)	ND
8	**3**	CLL	45.8	9 545	**Yes**	NA	U	V4-34/D2-2/J6	CARADLLVVPAAIYYYYYGMDVW (21)	-13q14 (90%);-13q14.3 (90%);-17p13.1 (83%)
9	**6**	CLL	87	20 880	No	A	U	V1-69/D3-16/J3	CARGGNYDYIWGSYRPNDAFDIW (21)	-11q23 (24%)
10	**7**	CLL	88	75 170	No	A	U	V1-69/D3-3/J6	CARADGGYDFWSGYSTVNYYGMDVW (23)	t(14q32) (91%)
11	**7**	CLL	72	71 516	No	B	U	V1-69/D3-3/J6	CARGPSSYDFWSGYYTGRDYYYYMDVW (25)	-13q14 (12%);-13q14.3 (12%); t(14q32) (60%)
12	**7**	CLL	43	8 544	**Yes**	A	U	V1-69/D3-3/J6	CAREGGADKDYDFWSGYYPNYYYYGMDVW (27)	ND
13	**7**	CLL	68.3	30 093	No	A	M	V1-69/D3-3/J6	CARAEQYYDFWSGHKGVDYYYYMDVW (24)	+12 (33%); -11q22.3 (24%)
14B	**8**	CLL-like MBL^{high}	3	700	**Yes**	-	U	V4-39/D6-13/J5	CASVQGYSSSWYGGDNWFDPW (19)	+12 (93%)
15	**8**	CLL	62.7	24 647	No	NA	U	V4-39/D6-13/J5	CATQTGYSSSWYAVNWFDPW (18)	t(14q32) (94%)
16	**10**	CLL-like MBL^{high}	44.6	4 594	**Yes**	-	U	V4-39/D2-2/J6	CARHRLGYCSSTSCYYYYYGMDVW (22)	ND
17	**10**	CLL	40.6	17 214	No	A	U	V4-39/D2-2/J6	CARHRLGYCSSTSCYYYYYGMDVW (22)	ND
18	**10**	CLL	86	22 704	No	B	U	V4-39/D2-2/J6	CARDRLGYCSSTSCYLYYYGMDVW (22)	ND
19A	**11**	CLL	33.7	6 807	No	NA	U	V4-34/D3-10/J4	CARGLYYYGSGVYFDYW (15)	t(14q32) (98%)
20	**11**	CLL	64.2	19 568	No	C	U	V4-34/D3-10/J4	CARGLIGAYGSGSYYPFPFDYW (20)	+12 (73%); t(14q32) (82%)
21	**12**	CLL-like MBL^{high}	15.3	1 454	NA	-	M	V1-2/D3-22/J4	CARDLARYDSGGSYKRKMFDYW (20)	ND
22	**16**	CLL	90	186 012	**Yes**	B	M	V4-34/D2-15/J6	CAGRFYCSGDTCHLPLYHYYYGLDVW (24)	ND
23	**21**	CLL	42	11 147	NA	A	U	V3-23/D3-3/J6	CAKHQLTYYDFWSGYYTEYYYYYGMDVW (26)	-13q14.3 (99%)
24	**22**	CLL-like MBL^{high}	18	2 531	No	-	U	V3-11/D3-3/J6	CARDRRDDFWSGYRIYYYYYGMDVW (23)	+12 (84%)
25	**22**	CLL	66.9	17 488	**Yes**	A	M	V3-23/D3-3/J6	CARDLTHHNFWSAYYETSYCGMDVW (23)	ND
26A†	**23**	CLL	62.9	49 600	**Yes**	B	U	V3-30/D3-9/J6	CAKYGGVKLRYFDWLLYGDYYYGMDVW (25)	ND
27	**25**	CLL	65.6	33 003	No	A	U	V1-8/D3-3/J6	CARGPSYYDFWSGPFDNYGMDVW (21)	-13q14.3 (97%)
28	**31**	CLL-like MBL^{high}	19.5	1 661	No	-	U	V3-48/D3-3/J6	CARSPGYDFWSGYPDYYGMDVW (20)	+12 (84%)
29	**34**	CLL	24.9	5 578	**Yes**	A	U	V1-18/D3-9/J6	CARGAYYDILTGYRYYYGMDVW (20)	+12 (75%)
30A	**38**	CLL-like MBL^{high}	23	3 174	**Yes**	-	U	V4-39/D3-3/J5	CARHTSLYDFWSGYYRGWFDPW (20)	-13q14 (15%);-13q14.3 (15%)
31	**77**	CLL	91	219 537	No	A	M	V4-59/D6-19/J4	CARGPDISGWNGLDYW (14)	-13q14.3 (82%)
32	**201**	CLL	65.4	11 523	No	A	M	V4-34/D5-12/J3	CARREEDWKRSGRDSFDIW (17)	Biallelic -13q14.3 (79%)

**Specified for CLL cases only. Gray shadowed lines correspond to stereotyped CLL-like B-cell clones derived from multiclonal B-CLPD cases. U, unmutated; M, mutated; CLL, chronic lymphocytic leukemia; MBL, monoclonal B-cell lymphocytosis; B-CLPD, B-cell chronic lymphoproliferative disorders; MAP, myelodysplasia-associated phenotype; MS, mutational status. ND, not detected. NA, not available. †Patients that received chemotherapy for CLL.*

Table 18. Peripheral blood B-cell counts, distribution of CLL-like MBL^{low}, MBL^{high} and CLL B-cell clones and of CLL clinical stages according to the expression of non-stereotyped *vs.* stereotyped *IGHV* amino acid sequences.

	Non-stereotyped B-cell clones n=191	**Stereotyped B-cell clones n=32**
*N. of PB clonal B cells(x10^6/L)**	7,408 (0.09-369 289)	11,336 (112-219 537)
*% of PB clonal B cells from WBC**	37% (0.001%-97%)	45% (2.3%-91%)
% of multiclonal B-cell clones	58/191 (30%)	6/32 (19%)
CLL-like B-cell clones and CLL clones:		
CLL-like MBL^{low} B-cell clones	25/191 (13%)	2/32 (6%)
CLL-like MBL^{high} B-cell clones	49/191 (26%)	8/32 (25%)
CLL B-cell clones	117/191 (61%)	22/32 (69%)
CLL-stage A clones	62/106 (59%)	12/19 (63%)
CLL-stages B clones	27/106 (25%)	6/19 (32%)
CLL-stages C clones	17/106 (16%)	1/19 (5%)
% of cases with MAP (MDS-associated phenotypes)	**27/154 (17%)**	**12/30 (40%)****

*Results expressed as number of B-cell clones and percentage between brackets or as *median value (range). PB, peripheral blood; WBC, white blood cells; IGHV, immunoglobulin heavy chain variable region genes; CLL, chronic lymphocytic leukemia; MBL, monoclonal B-cell lymphocytosis; MDS, myelodysplastic syndromes. **Statistically significant differences (P <0.05).*

The frequency of cytogenetically altered stereotyped B-cell clones (59%) was similar to that found among non-stereotyped B-cell clones (58%). Likewise, the proportion of B-cell clones showing coexistence of ≥2 cytogenetic alterations was also similar in both groups (22% and 26%, respectively). Regarding each specific cytogenetic alteration, similar frequencies and percentages of cytogenetically altered cells were observed in stereotyped vs. non-stereotyped B-cell clones (Table 19).

Table 19. Molecular and cytogenetic features of CLL-like MBL and CLL clones with non-stereotyped versus stereotyped *IGHV* amino acid sequences.

Molecular and cytogenetic features	Non-stereotyped clones n=191	Stereotyped clones n=32
VH subgroups		
VH1	**37/191 (19%)**	**12/32 (38%)****
VH3	**101/191 (53%)**	**8/32 (25%)****
VH4	43/191 (23%)	12/32 (38%)
VH2,VH5, VH6	10/191 (5%)	0/32 (0%)
HCDR3 length, aa*	**16 (6-32)**	**20 (9-27)****
IgH mutated clones	**122/191 (64%)**	**9/32 (28%)****
No. of genetically altered CLL-like clones	110/191 (58%)	19/32 (59%)
No. of CLL-like clones with ≥2 alterations	50/191 (26%)	7/32 (22%)
Type of cytogenetic alterations		
No. of del(13q)$^+$ clones (%)	74/191 (39%)	9/32 (28%)
*% del(13q)$^+$ cells **	82% (10%-99%)	79% (15%-90%)
No. of del(13q14.3)$^+$ clones (%)	71/191 (37%)	9/32 (28%)
*% del(13q14.3)$^+$ cells **	76% (5%-99%)	82% (12%-99%)
No. of del(13q14)$^+$ clones (%)	28/190 (15%)	3/32 (9%)
*% del(13q14)$^+$ cells **	80% (7%-99%)	15% (12%-90%)
No. of del(13q)$^+$ clones with another alteration (%)	40/191 (21%)	5/32 (16%)
No. of trisomy 12$^+$ clones (%)	30/191 (16%)	6/32 (19%)
*% trisomy 12$^+$ cells **	76% (8%-97%)	80% (33%-93%)
No. of trisomy 12$^+$ clones with another alteration (%)	14/191 (7%)	2/32 (6%)
No. of t(14q32)$^+$ clones (%)	20/180 (11%)	6/32 (19%)
*% t(14q32)$^+$ cells **	27% (6%-91%)	87% (13%-98%)
No. of del(11q)$^+$ clones (%)	8/186 (4%)	2/32 (6%)
*% del(11q)$^+$ cells **	68% (20%-98%)	24% (24%-24%)
No. of del(11q22.3)$^+$ clones (%)	8/186 (4%)	1/32 (3%)
*% del(11q22.3)$^+$ cells **	82% (20%-98%)	24% (-)
No. of del(11q23)$^+$ clones (%)	3/176 (2%)	1/32 (3%)
*% del(11q23)$^+$ cells **	64% (40%-93%)	24% (-)
No. of del(17p13.1)$^+$ clones (%)	6/188 (3%)	1/32 (3%)
*% del(17p13.1)$^+$ cells **	56% (33%-88%)	83% (-)

*Results expressed as number of CLL and CLL-like MBL clones with cytogenetic alterations from all CLL and CLL-like MBL clones in the corresponding group (percentage) or as *median values (range). In eight clones, biallelic del(13q14.3) was detected and polysomy was found in 1 (multiclonal) clone. **Statistically significant differences (P < 0.05).*

4.3.2. Haematological features of CLL and CLL-like MBL B-cell clones with stereotyped versus non-stereotyped IGHV amino acid sequences

Within the two groups of B-cell clones classified according to the presence of stereotyped vs non-stereotyped *IGHV* aa sequences, a similar distribution of CLL-like MBL^{low}, MBL^{high} and CLL B-cell clones was found, albeit the stereotyped group included a slightly higher, but statistically not significant, ratio of CLL/CLL-like MBL B-cell clones (CLL/ CLL-like MBL ratio of 2.2 vs. 1.6, respectively; *P* =0.3) (Table 18). Similarly, the overall distribution of CLL B-cell clones per clinical stage showed a predominance of Binet stage A (63% and 59% of the clones, respectively) vs. Binet stage B (32% and 25%, respectively) and Binet stage C (5% and 16%, respectively) among both the stereotyped and the non-stereotyped CLL clones. Of note, the median PB percentage and absolute count of stereotyped B-cell clones showed a tendency towards higher values than that of non-stereotyped B-cell clones: 45% vs. 37% of all white blood cells (*P* =0.1) and 11,336 clonal B-cells/µl vs. 7,408 clonal B-cells/µl (*P* =0.1), respectively (Table 18).

Despite all the above similarities, non-stereotyped B-cell clones which were *IGHV* unmutated displayed significantly higher PB (clonal) B-cell counts than the *IGHV* mutated ones (14,076 cells/µl vs. 5,354 cells/µl, *P* =0.006) (Figure 11A). Likewise, the non-stereotyped B-cell clones which carried ≥2 cytogenetic alterations showed higher PB (clonal) B-cell counts than the the cytogenetically unaltered or minimally altered (isolated cytogenetic alteration) non-stereotyped B-cell clones (17,310 cells/µl vs 4,841 cells/µl and 6,701 cells/µl, respectively; *P* ≤0.007) (Figure 11B). In contrast, *IGHV* stereotyped B-cell clones did not show significant differences in their overall size when grouped according to their *IGHV* mutational status or to their cytogenetic profile (*P* >0.05; Figure 11).

Similarly to what has been recently reported for multiple myeloma patients,[501] 29 of 140 (21%) CLL and 10 of 41 (24%) MBL^{high} cases, displayed MDS-associated immunophenotypic profiles on PB neutrophils (97%) and/or monocytes (46%). Most interestingly, 40% of all CLL and MBL cases carrying stereotyped B-cell clones showed MDS-associated phenotypic alterations on PB neutrophils and/or monocytes, a frequency which was significantly higher than that found among cases which had non-stereotyped B-cell clones (17%) (*P* =0.01) (Table 18).

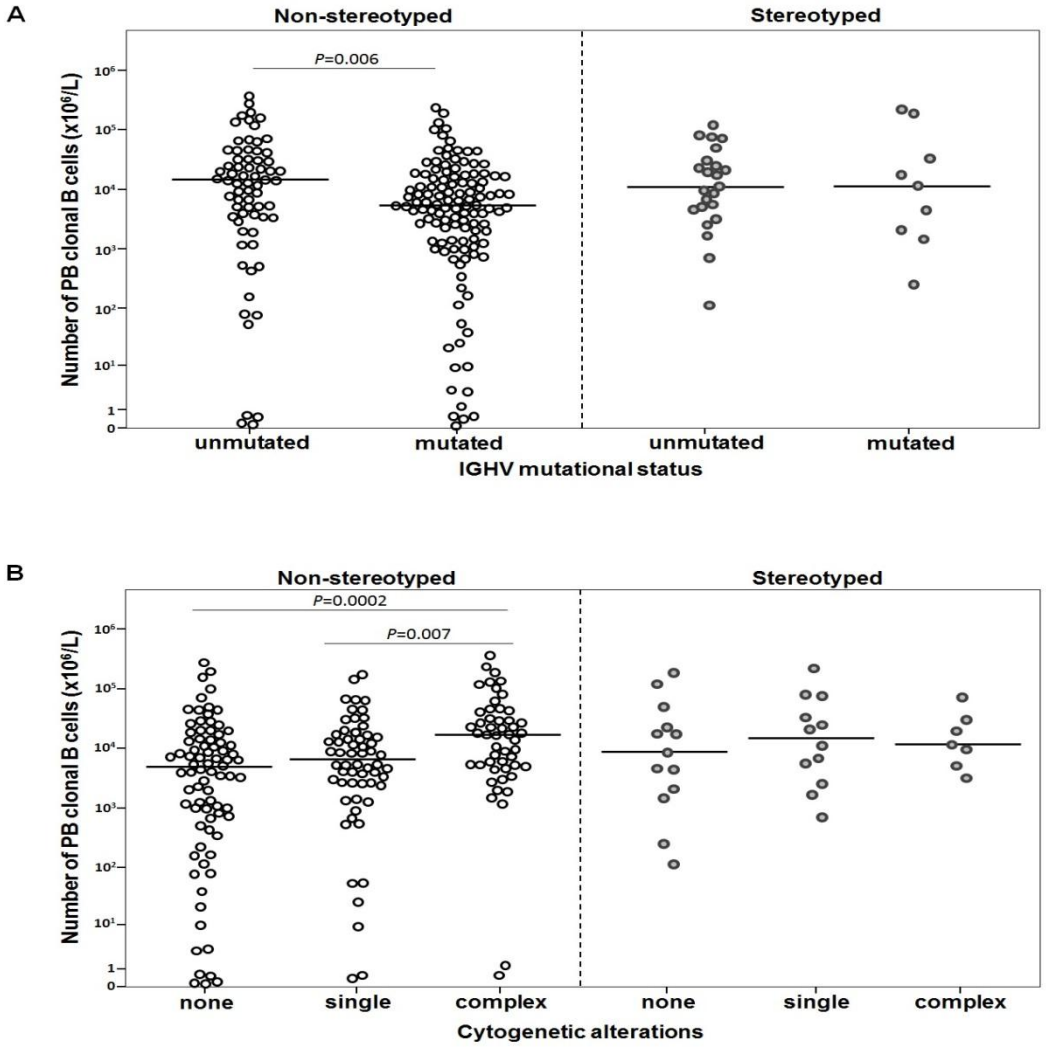

Figure 11. Number of clonal B-cells in the PB from IGHV stereotyped vs. non-stereotyped B-cell clones grouped according to their IGHV mutational status (panel A) and cytogenetic profile (panel B). Diagrams show the number of PB clonal B cells (x10^6/L) with both stereotyped and non-stereotyped IGHV sequences according to their mutational status: unmutated (≤2% deviation from the germline sequence) vs. mutated (>2% deviation from the germline sequence) and the absence versus the presence of one or more than one cytogenetic abnormalities. Circles represent individual cases, and horizontal lines indicate median values.

4.4. Discussion

Despite increasing evidences about the potential occurrence of Ag encounter by the cell of origin in CLL exist,[319] the precise B-cell scenario in which this event might occur, remains unknown. In order to gain insight into such potential scenario, here we investigated the molecular and biological features of stereotyped vs. non-stereotyped CLL and CLL-like MBL clones. Overall, stereotyped CLL and CLL-like MBL clones showed a unique *IGHV* profile, associated with higher *VH1* gene usage (an *IGHV* subgroup that is particularly related with early stages in the evolution of the *IGHV* repertoire),[40,502] longer HCDR3 sequences and

unmutated *IGHV* as compared to non-stereotyped clones. More interestingly, the overall size of the stereotyped B-cell clones in PB did not appear to be associated with the CLL-related cytogenetic profile of B-cells, whereas it did show a significant association with the presence of MDS-associated immunophenotypes on PB myeloid cells (neutrophils and monocytes).

In recent years, immunophenotyping has been progressively introduced in clinical research and diagnostic laboratories for the identification of altered phenotypes in bone marrow precursors and myeloid (e.g. neutrophil, monocytic and erythroid lineage) cells, being currently considered in the WHO 2008 classification of hematological malignancies as a co-criterion for the diagnosis of MDS.[466] More recently, we have shown that altered MDS-like immunophenotypes are frequently observed on residual maturing neutrophils and monocytes (and to a lesser extent also on erythroid cells) from "de novo" AML and both MGUS and MM patients, where such aberrant myeloid phenotypes can be detected in >80% [503] and around 15% [501] of de novo AML and MGUS/MM cases, respectively. Of note, in both groups of diseases ("de novo" AML and MGUS/MM), the MDS-associated phenotypes predicted for MDS-associated cytogenetic changes (but not e.g. MGUS/MM-related genetic alterations) and the presence of an underlying clonal hematopoiesis.[501] Based on these findings, we hypothesize that in those CLL and MBL cases in which we here found PB neutrophils and monocytes displaying the same MDS-associated phenotypes, an underlying clonal hematopoiesis, potentially associated with specific genetic changes that still remain to be identified, might also exist. Under these circumstances it could be speculated that, due to the close association between the presence of such MDS-associated phenotypes on myeloid cells and stereotyped B-cell clones, expansion of such stereotyped B-cell clones could more likely be favored by the altered local BM microenvironment than by chronic antigen stimulation outside the BM. This could also explain, at least in part, the greater rate of *IGHV* unmutated clones with longer HCDR3 sequences among stereotyped vs non-stereotyped cases. Similarly, it might also explain the lack of relationship observed between the presence and number of CLL-associated cytogenetic changes and the size of the stereotyped CLL and MBL PB clones, in contrast to what we observed among non-stereotyped cases. In line with these findings, it should be noted that within non-stereotyped cases, the presence and number of CLL-associated cytogenetic changes was significantly associated with the size of the B-cell clone in PB, only among those cases that did not show MDS-associated phenotypes on myeloid cells (data not shown).

Altogether, our results support the notion that selective genetic and/or BM microenvironment forces may favor expansion of specific *VDJ* clones. This might occur already at the HSC level, particularly among *IGHV* stereotyped cases; such forces could initially lead to

the expansion of B-cell clones in an Ag-independent way, as recently proposed by Kikushige *et al*. [329]. These early expansions would therefore, more likely translate into an oligoclonal MBL^{low} stable condition, in which clonality will not directly translate into malignancy;[468,479] further clonal B-cell expansion might depend on additional micro-environmental factors (e.g. infections)[204] and/or acquisition of specific (additional) genetic changes by the expanded B-cells.[468] Alternatively, chronic Ag-driven B-cell stimulation could act as the primary triggering factor in other cases (e.g. non-stereotyped B-cell clones from subjects who have a normal haematopoietic BM background/environment). However, due to the stable nature of most MBL^{low} clones, very long-term longitudinal studies are required to determine the exact pathways and factors involved in clonal CLL and CLL-like B-cell expansions potentially leading to neoplastic transformation.[468]

In line with all the above, our study also revealed a greater *VH1* and a lower *VH3* gene usage in stereotyped vs. non-stereotyped B-cell clones. These findings would further support the existence of different ontogenic pathways for both categories of CLL-like and CLL clones.[239] *VH3* genes belong to the ancestral phylogenetic clan III, which shows the greatest nucleotide conservation within the FR1 and FR3 intervals (versus *IGHV1/5/7* and *IGHV2/4/6* genes); because of this, usage of *VH3* genes has been more frequently associated with the production of autoantibodies (e.g. anti-DNA antibodies) and direct FR3-ligand interaction with superantigens or self-antigens recognized in a pattern-specific way;[40] this is further supported by the polyreactivity of some monoclonal antibodies produced by CLL cells[247,318] which react with molecular structures present on apoptotic cells and bacteria.[245,247] Fully in line with this hypothesis, we observed a lower incidence of multiclonality and a lower frequency of *IGH* mutated clones with longer HCDR3 sequences among stereotyped vs. non-stereotyped B-cell clones, pointing out the potential involvement of different types of BCR-triggers in stereotyped (e.g. survival promoting antigens like vimentin and calreticulin found on stromal cells[504] or apoptotic cells[245,246]) vs. non-stereotyped clones (e.g. superantigens and autoantigens). Such hypothesis would be supported by recent observations which show that coexistence of phylogenetically-related B-cell clones that frequently share *IGHV3* gene usage and that show both shorter HCDR3 sequences and a greater proportion of *IGHV* mutations and del(13q14.3), are more frequently seen in multiclonal MBL, CLL and other B-CLPD than in unrelated B-cell clones from monoclonal cases.[499] Altogether these findings suggest that non-stereotyped B-cell clones with mostly mutated *IGHV* sequences and shorter HCDR3 sequences, could be more closely associated with chronic expansions driven by antigens in the periphery, whereas stereotyped B-cell clones with mostly unmutated *IGHV* sequences and longer HCDR3

sequences could derive from B-cells which are specifically selected in the bone marrow microenvironment.

The association between stereotypy and MDS-associated immunophenotypic features of myeloid cells, further suggests the potential occurrence of increased cytokine-mediated intramedullary apoptosis reported to occur in specific subtypes of MDS.[505] The potential contribution of intramedullary apoptosis to the expansion of stereotyped CLL and CLL-like MBL clones carrying unmutated *IGHV* sequences reactive against molecular structures present on apoptotic cells,[245,247,296] would be in line with the highly-repetitive HCDR3 sequences of these stereotyped B-cell clones selected in such BM microenvironment; further investigations are required to confirm this hypothesis.

In summary, in this study we report for the first time a significant association between stereotyped *IGHV* HCDR3 B-cell clones from both MBL subjects and CLL patients and coexistence of phenotypically altered (MDS-like) myeloid cells, suggesting that the emergence and/or expansion of CLL-like MBL and CLL clones in these cases could be favored by an underlying altered hematopoiesis; the precise significance of the MDS-like altered myeloid phenotypes, the potential underlying genetic lesions and the specific antigens involved, remain to be defined.

Chapter 5 | CONCLUDING REMARKS

In general, detailed molecular analysis of the *IGHV* genes, has significantly contributed to unravel the pathogenesis of human B-CLPD. Thus, immunogenetic analysis of the clonogenic BCR offers valuable insight into both the ontogenesis of CLL and other B-CLPD and their precursor (e.g. MBL) states, and the potential involvement of Ag selection in the onset and development of the disease. In this regard, multiple studies have shown that CLL and other B-CLPD exhibit a biased repertoire together with SHM patterns in their *IGHV* genes, both findings being generally considered as evidence for the involvement of a limited set of Ags and/or superantigens in the development of the disease. For CLL, the involvement of Ags in leukemogenesis is further supported by the identification of closely homologous Ag binding sites among unrelated cases ("stereotyped" BCR), which are characterized by a non-random combination of specific *IGHV* genes and homologous HCDR3, also associated in some instances, with a restricted selection of *IGKV/IGLV* light chains; of utmost relevance, such IGHV profiles are strongly associated with the clinical course of the disease. Despite all the above findings, at present it still remains unclear whether a single or multiple normal precursors are stimulated in parallel to evolve into CLL and at what stage(s) this potentially occurs. The recognition of MBL and particularly, the high frequency at which MBL cases show expansions of multiple B-cell clones, highlight a potential scenario where some individuals develop oligoclonal expansions of B-cells (e.g. CLL-like) from which only some will be selected to progress to overt disease. The specific driving forces involved in the origin, expansion, selection and malignant transformation of these B-cell clones, still remain largely unknown.

In order to gain insight into the precise mechanisms leading to the development and progression of MBL into CLL and other B-CLPD, here we investigated the BCR features of a relatively large series of MBL and B-CLPD cases, particularly focusing on CLL-like MBL^{low}, CLL-like MBL^{high} and CLL subjects. For the purpose of the study we focused on three major goals: 1) the investigation of the potential associations between the specific IGHV repertoires and unique cytogenetic and mutational profiles of MBL vs. CLL cases; 2) the comparison of the immunogenetic, cytogenetic and hematological features of B-cell clones from monoclonal vs. multiclonal MBL, CLL and other B-CLPD, and; 3) the molecular and cytogenetic characteristics of MBL and CLL cases carrying stereotyped vs. non-stereotyped BCR. Based on the results of the work performed during this doctoral thesis, the following major conclusions can be drawn:

- Regarding the potential existence of unique cytogenetic and mutational profiles associated with specific IGHV repertoires in MBL versus CLL:

– MBL^{low}, MBL^{high} and CLL B-cell clones display three major distinct, but partially overlapping, patterns of *IGHV* gene usage, *IGHV* mutational status and cytogenetic

alterations, suggesting that the combination of specific *IGHV* genes and *IGHV* mutational status of CLL-like B-cell clones may modulate the type of cytogenetic alterations acquired, their rate of acquisition and/or potentially also the distinct clinical behavior of such clones.

- With respect to the phenotypic, cytogenetic and molecular characterization of expanded B-cell clones from multiclonal versus monoclonal B-CLPD:

– Multiclonal MBL, CLL and other B-CLPD display molecular, cytogenetic and hematological features which are typically associated with early MBL stages and/or initial phases of disease, at the same time they appear to more closely reflect an antigen-driven nature of MBL and B-CLPD with potential involvement of multiple and diverse antigenic determinants.

- Regarding the molecular and cytogenetic characteristics of stereotyped CLL and CLL-like MBL clones and the potential coexistence of myelodysplasia-associated phenotypes on myeloid cells:

– Stereotyped CLL and CLL-like MBL clones show unique *IGHV* profiles associated with unmutated *IGHV* sequences, longer HCDR3 and preferential usage of the *VH1* vs. *VH3* genes; these BCR features may reflect a distinct origin for MBL and CLL B-cells with stereotyped vs. non-stereotyped BCR. In line with this hypothesis, stereotyped CLL and CLL-like MBL B-cell clones more frequently showed myelodysplasia-associated immunophenotypes on PB myeloid cells. Altogether, these results point out the coexistence of an underlying altered hematopoiesis with potential involvement of HSC in the development and/or expansion of the CLL and CLL-like MBL B-cell clones from a significant fraction of cases carrying stereotyped BCR, the precise pathogenic role of such myelodysplasia-associated alterations of hematopoiesis deserving further investigations.

Chapter 6 | REFERENCES

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SUPPORTING INFORMATION

Supplemental Table 1. Informative parameters of the CLL-like/CLL B-cell clones included in the three major groups graphically visualized with APS view of the Infinicyt™ software.

		Continuous parameters included for multivariate analysis based on PCA											
Groups	**ID**	**Percentage of del(13q14.3)$^+$ cells**	**Percentage of biallelic del(13q14.3)$^+$ cells**	**Percentage of del(13q14)$^+$ cells**	**Percentage of trisomy 12$^+$ cells**	**Percentage of t(14q32)$^+$ cells**	**Percentage of del(11q22.3)$^+$ cells**	**Percentage of del(11q23.3)$^+$ cells**	**Percentage of del(17p13.1)$^+$ cells**	**Absolute number of clonal B cells/µL**	***IGHV* gene repertoire**	***IGHV* mutational status***	**Binet Stage****
1	127_MBL^{low}_V3-23_UM_del13q.xlsx	0	73	0	0	0	0	0	0	54	3-23	>98%	
1	134_ MBL^{low}_V3-23_UM_0	0	0	0	0	0	0	0	0	0.11	3-23	>98%	
1	135_ MBL^{low}_V3-23_UM_0	0	0	0	0	0	0	0	0	0.17	3-23	>98%	
1	136_ MBL^{low}_V3-23_UM_0	0	0	0	0	0	0	0	0	0.47	3-23	>98%	
1	137_ MBL^{low}_V3-23_UM_0	0	0	0	0	0	0	0	0	0.57	3-23	>98%	
1	41_ MBL^{low}_V3-21_UM_0.xlsx	0	0	0	0	0	0	0	0	80	3-21	>98%	
1	42_ MBL^{low}_V3-21_M_0.xlsx	0	0	0	0	0	0	0	0	250	3-21	<98%	
1	138_ MBL^{low}_V3-21_M_0	0	0	0	0	0	0	0	0	3.05	3-21	<98%	
1	77_ MBL^{low}_V4-34_M_0.xlsx	0	0	0	0	0	0	0	0	39	4-34	<98%	
1	140_ MBL^{low}_V4-34_M_bidel13q	0	91	0	0	0	0	0	0	9.31	4-34	<98%	
1	35_ MBL^{low}_V3-11_M_0.xlsx	0	0	0	0	0	0	0	0	10	3-11	<98%	
1	36_ MBL^{low}_V3-11_M_0.xlsx	0	0	0	0	0	0	0	0	200	3-11	<98%	
1	93_ MBL^{low}_V1-69_M_0.xlsx	0	0	0	0	0	0	0	0	115	1-69	<98%	
1	94_ MBL^{low}_V1-69_UM_0.xlsx	0	0	0	0	0	0	0	0	158	1-69	>98%	
1	48_ MBL^{low}_V1-2_UM_0.xlsx	0	0	0	0	0	0	0	0	112	1-2	>98%	
1	69_ MBL^{low}_V3-7_M_0.xlsx	0	0	0	0	0	0	0	0	0.09	3-7	<98%	
1	72_ MBL^{low}_V3-30_M_0.xlsx	0	0	0	0	0	0	0	0	3	3-30	<98%	
1	26_MBL^{high}_V3-23_M_0.xlsx	0	0	0	0	0	0	0	0	986	3-23	<98%	
1	27multi_ MBL^{high}_V3-23_UM_0.xlsx	0	0	0	0	0	0	0	0	2900	3-23	>98%	
1	28_ MBL^{high}_V3-23_M_0.xlsx	0	0	0	0	0	0	0	0	3281	3-23	<98%	
1	43multi_ MBL^{high}_V3_21_M_0.xlsx	0	0	0	0	0	0	0	0	2078	3-21	<98%	
1	44_ MBL^{high}_V3-21_M_0.xlsx	0	0	0	0	0	0	0	0	4424	3-21	<98%	
1	78multi_ MBL^{high}_V4-34_M_0.xlsx	0	0	0	0	0	0	0	0	346	4-34	<98%	
1	79_ MBL^{high}_V4-34_M_0.xlsx	0	0	0	0	0	0	0	0	1352	4-34	<98%	
1	80multi_ MBL^{high}_V4-34_M_0.xlsx	0	0	0	0	0	0	0	0	2047	4-34	<98%	
1	81_ MBL^{high}_V4-34_M_0.xlsx	0	0	0	0	0	0	0	0	2311	4-34	<98%	
1	95multi_ MBL^{high}_V1-69_UM_0.xlsx	0	0	0	0	0	0	0	0	430	1-69	>98%	
1	96multi_ MBL^{high}_V1-69_UM_0.xlsx	0	0	0	0	0	0	0	0	511	1-69	>98%	
1	49_ MBL^{high}_V1-2_M_0.xlsx	0	0	0	0	0	0	0	0	999	1-2	<98%	
1	50_ MBL^{high}_V1-2_M_0.xlsx	0	0	0	0	0	0	0	0	1454	1-2	<98%	
1	70_ MBL^{high}_V3-7_M_0.xlsx	0	0	0	0	0	0	0	0	1264	3-7	<98%	
1	73_ MBL^{high}_V3-30_M_0.xlsx	0	0	0	0	0	0	0	0	3986	3-30	<98%	
1	128multi_ MBL^{high}_V3-30_M_del13q.xlsx	0	95	0	0	0	0	0	0	914	3-30	<98%	
1	57multi_ MBL^{high}_V1-3_UM_0.xlsx	0	0	0	0	0	0	0	0	3458	1-3	>98%	
1	89_ MBL^{high}_V3-48_UM_0.xlsx	0	0	0	0	0	0	0	0	1170	3-48	>98%	
1	63_ MBL^{high}_V4-39_UM_0.xlsx	0	0	0	0	0	0	0	0	4594	4-39	>98%	
1	39_ MBL^{high}_V5-51_UM_0.xlsx	0	0	0	0	0	0	0	0	2000	5-51	>98%	
1	31_CLL_V3-23_UM_0_A.xlsx	0	0	0	0	0	0	0	0	5200	3-23	>98%	
1	32multi_CLL_V3-23_M_0_A.xlsx	0	0	0	0	0	0	0	0	5680	3-23	<98%	
1	33_CLL_V3-23_M_0_A.xlsx	0	0	0	0	0	0	0	0	17488	3-23	<98%	
1	34_CLL_V3-23_M_0_A.xlsx	0	0	0	0	0	0	0	0	28710	3-23	<98%	

(**Supplemental Table 1**, continued)

		Continuous parameters included for multivariate analysis based on PCA											
Groups	**ID**	**Percentage of del(13q14.3)$^+$ cells**	**Percentage of biallelic del(13q14.3)$^+$ cells**	**Percentage of del(13q14)$^+$ cells**	**Percentage of trisomy 12$^+$ cells**	**Percentage of t(14q32)$^+$ cells**	**Percentage of del(11q22.3)$^+$ cells**	**Percentage of del(11q23.3)$^+$ cells**	**Percentage of del(17p13.1)$^+$ cells**	**Absolute number of clonal B cells/µL**	***IGHV* gene repertoire**	***IGHV* mutational status***	**Binet Stage****
1	47_CLL_V3-21_M_0_A.xlsx	0	0	0	0	0	0	0	0	37985	3-21	<98%	A
1	83_CLL_V4-34_M_0_A.xlsx	0	0	0	0	0	0	0	0	7103	4-34	<98%	A
1	84multi_CLL_V4-34_M_0_A.xlsx	0	0	0	0	0	0	0	0	7597	4-34	<98%	A
1	85_CLL_V4-34_M_0_A.xlsx	0	0	0	0	0	0	0	0	8653	4-34	<98%	A
1	130multi_CLL_V4-34_M_del13q_A.xlsx	0	96	0	0	0	0	0	0	8382	4-34	<98%	A
1	131multi_CLL_V4-34_M_del13q_A.xlsx	0	91	0	0	0	0	0	0	8398	4-34	<98%	A
1	132_CLL_V4-34_M_2del13q_A.xlsx	14	79	0	0	0	0	0	0	11523	4-34	<98%	A
1	37_SLL_V3-11_UM_0_A.xlsx	0	0	0	0	0	0	0	0	2404	3-11	>98%	A
1	38_CLL_V3-11_UM_0_A.xlsx	0	0	0	0	0	0	0	0	14076	3-11	>98%	A
1	98_CLL_V1-69_UM_del11q_A.xlsx	0	0	0	0	0	0	24	0	20880	1-69	>98%	A
1	56_CLL_V1-2_M_0_A.xlsx	0	0	0	0	0	0	0	0	9524	1-2	<98%	A
1	71_CLL_V3-7_M_t14q_del17p_A.xlsx	0	0	0	0	18	0	0	33	3055	3-7	<98%	A
1	58_CLL_V1-3_M_0_A.xlsx	0	0	0	0	0	0	0	0	7220	1-3	<98%	A
1	59multi_CLL_V1-3_M_0_A.xlsx	0	0	0	0	0	0	0	0	7833	1-3	<98%	A
1	61_CLL_V1-3_UM_0_A.xlsx	0	0	0	0	0	0	0	0	20229	1-3	>98%	A
1	92_CLL_V3-48_M_t14q_A.xlsx	0	0	0	0	22	0	0	0	64726	3-48	<98%	A
1	64multi_CLL_V4-39_UM_0_A.xlsx	0	0	0	0	0	0	0	0	6800	4-39	>98%	A
1	65_CLL_V4-39_UM_0_A.xlsx	0	0	0	0	0	0	0	0	17214	4-39	>98%	A
1	68multi_CLL_V4-39_UM_0_A.xlsx	0	0	0	0	0	0	0	0	71485	4-39	>98%	A
1	87_CLL_V4-34_M_0_B.xlsx	0	0	0	0	0	0	0	0	186012	4-34	<98%	B
1	133multi_CLL_V4-34_UM_del13q_C.xlsx	0	85	0	0	0	0	0	0	66142	4-34	>98%	C
1	53_CLL_V1-2_UM_0_B.xlsx	0	0	0	0	0	0	0	0	45081	1-2	>98%	B
1	54_CLL_V1-2_UM_0_B.xlsx	0	0	0	0	0	0	0	0	120102	1-2	>98%	B
1	74_SLL_V3-30_UM_0_B.xlsx	0	0	0	0	0	0	0	0	3509	3-30	>98%	B
1	76multi_CLL_V3-30_UM_0_B.xlsx	0	0	0	0	0	0	0	0	49600	3-30	>98%	B
1	129_CLL_V3-30_M_2del13q_C.xlsx	5	95	0	0	0	0	0	0	17175	3-30	<98%	C
1	66_CLL_V4-39_UM_0_B.xlsx	0	0	0	0	0	0	0	0	22704	4-39	>98%	B
2	24_ MBL^{low}_V3-23_M_2del13q.xlsx	86	0	86	0	0	0	0	0	1	3-23	<98%	
2	115_ MBL^{low}_V4-34_M_del13q.xlsx	96	0	0	0	0	0	0	0	26	4-34	<98%	
2	139_ MBL^{low}_V3-7_M_del13q	22	0	0	0	0	0	0	0	0.4	3-7	<98%	
2	141_ MBL^{low}_V3-48_M_del13q_tris12	19	0	0	41	0	0	0	0	0.57	3-48	<98%	
2	142_ MBL^{low}_V3-48_M_del13q	70	0	0	0	0	0	0	0	0.57	3-48	<98%	
2	29_ MBL^{high}_V3-23_M_del13q.xlsx	21	0	0	0	0	0	0	0	4040	3-23	<98%	
2	101_ MBL^{high}_V3-23_M_del13q.xlsx	88	0	0	0	0	0	0	0	3044	3-23	<98%	
2	116multi_ MBL^{high}_V4-34_M_del13q.xlsx	83	0	0	0	0	0	0	0	551	4-34	<98%	
2	102multi_ MBL^{high}_V3-11_M_del13q.xlsx	87	0	0	0	0	0	0	0	2692	3-11	<98%	
2	51_ MBL^{high}_V1-2_M_del13q.xlsx	23	0	0	0	0	0	0	0	4623	1-2	<98%	
2	103_ MBL^{high}_V1-2_M_2del13q.xlsx	96	0	96	0	0	0	0	0	4371	1-2	<98%	
2	107multi_ MBL^{high}_V3-7_M_2del13q.xlsx	98	0	98	0	0	0	0	0	2750	3-7	<98%	
2	88multi_ MBL^{high}_V3-48_M_del13q.xlsx	18	0	0	0	0	0	0	0	680	3-48	<98%	
2	62multi_ MBL^{high}_V4-39_UM_2del13q.xlsx	15	0	15	0	0	0	0	0	3174	4-39	>98%	
2	118_CLL_V4-34_M_del13q_A.xlsx	99	0	0	0	0	0	0	0	13167	4-34	<98%	A
2	119_CLL_V4-34_M_2del13q_A.xlsx	97	0	95	0	0	0	0	0	105594	4-34	<98%	A
2	104_CLL_V1-2_UM_2del13q_A.xlsx	84	0	93	0	0	0	0	0	7683	1-2	>98%	A
2	111multi_CLL_V3-30_M_del13q_A.xlsx	80	0	0	0	0	0	0	0	7920	3-30	<98%	A

(**Supplemental Table 1**, continued)

		Continuous parameters included for multivariate analysis based on PCA											
Groups	**ID**	**Percentage of del(13q14.3)$^+$ cells**	**Percentage of biallelic del(13q14.3)$^+$ cells**	**Percentage of del(13q14)$^+$ cells**	**Percentage of trisomy 12$^+$ cells**	**Percentage of t(14q32)$^+$ cells**	**Percentage of del(11q22.3)$^+$ cells**	**Percentage of del(11q23.3)$^+$ cells**	**Percentage of del(17p13.1)$^+$ cells**	**Absolute number of clonal B cells/µL**	***IGHV* gene repertoire**	***IGHV* mutational status***	**Binet Stage****
2	112_CLL_V3-30_M_del13q_A.xlsx	53	0	0	0	0	0	0	0	13524	3-30	<98%	A
2	113_CLL_V3-30_UM_2del13q_del11q_A.xlsx	60	0	60	0	0	98	0	0	32063	3-30	>98%	A
2	114_CLL_V3-30_M_del13q_A.xlsx	88	0	0	0	0	0	0	0	33146	3-30	<98%	A
2	105_CLL_V1-3_UM_2del13q_t14q_del11q_A.xlsx	73	0	58	0	86	0	21	0	11419	1-3	>98%	A
2	123_CLL_V3-48_UM_2del13q_2del11q_A.xlsx	19	0	55	0	0	51	64	0	369289	3-48	>98%	A
2	45_CLL_V3-21_M_del13q_B.xlsx	0	0	61	0	0	0	0	0	5314	3-21	<98%	B
2	46multi_CLL_V3-21_M_del13q_C.xlsx	30	0	0	0	0	0	0	0	15309	3-21	<98%	C
2	97_CLL_V1-69_UM_del13q_del11q_B.xlsx	25	0	0	0	0	95	0	0	17445	1-69	>98%	B
2	125_CLL_V1-69_UM_2del13q_del17p_C.xlsx	91	0	93	0	0	0	0	44	23134	1-69	>98%	C
2	126_CLL_V1-69_M_del13q_C.xlsx	40	0	0	0	0	0	0	0	45737	1-69	<98%	C
2	110_CLL_V3-7_M_2del13q_B.xlsx	81	0	81	0	0	0	0	0	237817	3-7	<98%	B
2	120_CLL_V3-48_M_del13q_t14q_C.xlsx	64	0	0	0	19	0	0	0	16670	3-48	<98%	C
2	121_CLL_V3-48_M_2del13q_C.xlsx	77	0	66	0	0	0	0	0	26880	3-48	<98%	C
2	122multi_CLL_V3-48_M_2del13q_C.xlsx	87	0	95	0	0	0	0	0	27200	3-48	<98%	C
2	106_CLL_V4-39_M_2del13q_del11q_C.xlsx	51	0	47	0	0	68	0	0	190555	4-39	<98%	C
3	2_ MBL^{high}_V3-23_M_tris12.xlsx	0	0	0	91	0	0	0	0	1358	3-23	<98%	
3	3multi_ MBL^{high}_V3-23_UM_tris12.xlsx	0	0	0	87	0	0	0	0	3360	3-23	>98%	
3	4multi_ MBL^{high}_V3-23_M_tris12_del11q.xlsx	0	0	0	95	0	93	0	0	3443	3-23	<98%	
3	5_ MBL^{high}_V3-23_M_tris12_del11q.xlsx	0	0	0	93	0	20	0	0	4851	3-23	<98%	
3	19_ MBL^{high}_V3-48_UM_tris12.xlsx	0	0	0	84	0	0	0	0	1661	3-48	>98%	
3	11multi_ MBL^{high}_V4-39_UM_del13q.xlsx	0	0	0	93	0	0	0	0	700	4-39	>98%	
3	7multi_ MBL^{high}_V5-51_UM_tris12_t14q.xlsx	0	0	0	76	52	0	0	0	1900	5-51	>98%	
3	8multi_ MBL^{high}_V5-51_M_tris12.xlsx	0	0	0	93	0	0	0	0	2659	5-51	<98%	
3	10_CLL_V3-21_UM_tris12_A.xlsx	0	0	0	75	0	0	0	0	30739	3-21	>98%	A
3	17multi_CLL_V4-34_M_tris12_A.xlsx	0	0	0	75	0	0	0	0	5482	4-34	<98%	A
3	21_CLL_V1-69_UM_tris12_A.xlsx	0	0	0	87	0	0	0	0	14470	1-69	>98%	A
3	99_CLL_V1-69_UM_tris12_del11q_A.xlsx	0	0	0	33	0	24	0	0	30093	1-69	>98%	A
3	100_CLL_V1-69_UM_t14q_A.xlsx	0	0	0	0	91	0	0	0	75170	1-69	>98%	A
3	22_CLL_V1-69_UM_tris12_t14q_B.xlsx	0	0	0	73	82	0	0	0	46393	1-69	>98%	B
3	23_CLL_V1-69_UM_tris12_t14q_B.xlsx	0	0	0	97	30	0	0	0	119647	1-69	>98%	B
3	6_SLL_V3-11_UM_tris12_B.xlsx	0	0	0	84	0	0	0	0	2531	3-11	>98%	B
3	18_CLL_V4-34_UM_tris_t14q_C.xlsx	0	0	0	73	82	0	0	0	19568	4-34	>98%	C
3	13_CLL_V3-7_M_tris12_C.xlsx	0	0	0	78	0	0	0	0	9093	3-7	<98%	C
3	14_CLL_V3-7_M_tris12_C.xlsx	0	0	0	85	0	0	0	0	15257	3-7	<98%	C
3	15multi_CLL_V3-30_UM_tris12_del11q_B.xlsx	0	0	0	93	0	91	0	0	5412	3-30	>98%	B
3	20_CLL_V3-48_M_tris12_t14q_B.xlsx	0	0	0	82	91	0	0	0	10807	3-48	<98%	B
3	12_CLL_V4-39_UM_tris12_del17p_B.xlsx	0	0	0	80	0	0	0	35	22028	4-39	>98%	B
3	9_CLL_V5-51_UM_tris12_B.xlsx	0	0	0	76	0	0	0	0	175686	5-51	>98%	B

**The unmutated status of the IGHV genes (U) was defined as those with >98% identity with the most similar germline gene, while mutated one (M) was those in which CLL-like B cell clones displayed IGHV genes with <98% identity with the most similar germline gene. **only applicable to CLL cases.*

Supplemental Table 2. *IGHV* sequences of CLL-like MBL and CLL B-cell clones analyzed by the IMGT-V-QUEST tool.

B-cell clon from #case number	V-GENE and allele	Functionality	% Identity V-REGION	J-GENE and allele	D-GENE and allele	D-REGION reading frame	CDR3 length	AA JUNCTION
MBL^{low} from (monoclonal) #1	IGHV4-34*01	Productive	91.84%	IGHJ4*02	IGHD2-15*01	2	20	CARGPPYCSGDSCSWGGILDYW
MBL^{low} from (monoclonal) #2	IGHV3-23*01	Productive	100.00%	IGHJ6*02	IGHD3-10*01	1	11	CANRGETRGMDVW
MBL^{low} from (monoclonal) #3	IGHV3-7*01	Productive	96.39%	IGHJ4*02	IGHD6-19*01	1	12	CVRDKYDSGSMDYW
MBL^{low} from (monoclonal) #4	IGHV3-21*01	Productive	92.34%	IGHJ6*02	IGHD6-25*01	1	20	CARHHPVRESSATGHYYGMDVW
MBL^{low} from (monoclonal) #5	IGHV3-23*01	Productive	98.42%	IGHJ6*02	IGHD5-12*01	1	11	CANRGETRGMDVW
MBL^{low} from (monoclonal) #6	IGHV3-48*03	Productive	90.36%	IGHJ3*02	IGHD5-12*01	3	12	CVRDGFHYYGFDIW
MBL^{low} from (monoclonal) #7	IGHV3-23*01	Productive	95.98%	IGHJ4*02	IGHD2-15*01	2	15	CAKHGSYSPDPYYFDYW
MBL^{low} from (monoclonal) #8	IGHV1-8*01	Productive	97.86%	IGHJ4*02	IGHD2-21*02	2	13	CARGLGSASQSRDSW
MBL^{low} from (monoclonal) #9	IGHV4-34*01	Productive	96.57%	IGHJ4*02	IGHD4-11*01	2	13	CARLGGDDSDYGFYW
MBL^{low} from (monoclonal) #10	IGHV3-7*03	Productive	85.54%	IGHJ4*03	IGHD3-3*01	2	15	CVRENEFWSGGWGLDGW
MBL^{low} from (monoclonal) #11	IGHV3-23*01	Productive	99.6%	IGHJ6*02	IGHD5-12*01	1	11	CANRGETRGMDVW
MBL^{low} from (monoclonal) #12	IGHV3-30*02	Productive	88.54%	IGHJ3*02	IGHD1-26*01	3	20	CANLGESRGGGSYPAPDTFDIW
MBL^{low} from (monoclonal) #13	IGHV1-2*02	Productive	100.00%	IGHJ4*02	IGHD6-19*01	3	13	CARLQWLGISHFDYW
MBL^{low} from (multiclonal) #14A	IGHV3-23*01	Productive	99.6%	IGHJ6*02	IGHD5-12*01	1	11	CANRGETRGMDVW
MBL^{low} from (multiclonal) #14B	IGHV3-48*03	Productive	90.76%	IGHJ3*02	IGHD5-12*01	3	12	CVRDGFHYYGFDIW
MBL^{low} from (multiclonal) #15B	IGHV3-23*01	Productive	97.99%	IGHJ6*02	IGHD6-19*01	1	22	CANAPTPYSSGWNPWDYYYGMDVW
MBL^{low} from (multiclonal) #16B	IGHV3-11*03	Productive	96.79%	IGHJ4*02	IGHD2-15*01	2	17	CAREEYCDGGTCYRLFDYW
MBL^{low} from (multiclonal) #17A	IGHV3-74*01	Productive	90.32%	IGHJ4*02	IGHD3-10*01	2	14	CARDLDGSGSGVFDWW
MBL^{low} from (multiclonal) #17B	IGHV4-59*03	Productive	91.8%	IGHJ6*01	IGHD3-10*01	2	15	CARGWRSTDSYYGMDVW
MBL^{low} from (multiclonal) #18B	IGHV5-a*01	Productive	100.00%	IGHJ6*03	IGHD6-19*01	2	21	CARHVAVAGTTWGPYYYYYMDVW
MBL^{low} from (multiclonal) #19B	IGHV3-23*01	Productive	100.00%	IGHJ4*02	IGHD2-2*01	2	15	CAKDHGEQFIGGCFDYW
MBL^{low} from (multiclonal) #20B	IGHV1-69*01	Productive	100.00%	IGHJ3*02	IGHD3-3*01	2	22	CARDNPKYYDFWSGYYAPPAFDIW
MBL^{low} from (multiclonal) #21C	IGHV1-69*13	Productive	94.84%	IGHJ4*02	IGHD4-11*01	2	15	CAREGKSRDNSNPFDYW
MBL^{low} from (multiclonal) #22B	IGHV3-21*04	Productive	94.98%	IGHJ6*02	IGHD2-15*01	3	9	CARDANGMDVW
MBL^{low} from (multiclonal) #23B	IGHV4-34*01	Productive	96.68%	IGHJ4*02	IGHD7-27*01	1	6	CAHLSGYW
MBL^{low} from (multiclonal) #23C	IGHV3-11*04	Productive	97.57%	IGHJ6*03	IGHD2-21*01	2	18	CARKTCASITNYYYYYMDVW
MBL^{low} from (multiclonal) #24B	IGHV3-21*01	Productive	99.6%	IGHJ4*02	IGHD6-13*01	2	11	CARVGAATGMDYW
MBL^{high} from (monoclonal) #25	IGHV3-23*01	Productive	94.76%	IGHJ3*02	IGHD1-7*01	3	18	CAKDLPSTYNWNSGGAFDIW
MBL^{high} from (monoclonal) #26	IGHV3-23*01	Productive	90.36%	IGHJ4*02	IGHD5-12*01	3	16	CTKDPRDTGYGGDAFDYW
MBL^{high} from (monoclonal) #27	IGHV3-23*01	Productive	99.58%	IGHJ6*02	IGHD3-3*01	2	22	CAKDNKYYDFWSGYYPVGTGMDVW
MBL^{high} from (monoclonal) #28	IGHV3-53*01	Productive	92.31%	IGHJ3*02	IGHD3-10*01	3	16	CARGPPQSRPVGDTFEIW

(**Supplemental Table 2**, continued)

B-cell clon from #case number	V-GENE and allele	Functionality	% Identity V-REGION	J-GENE and allele	D-GENE and allele	D-REGION reading frame	CDR3 length	AA JUNCTION
MBL^{high} from (monoclonal) #29	IGHV2-26*01	Productive	91.64%	IGHJ4*03	IGHD5-18*01	3	15	CTRTRGYPYGDRYFDSW
MBL^{high} from (monoclonal) #30	IGHV1-2*02	Productive	94.44%	IGHJ3*02	IGHD4-17*01	2	13	CARGLNTDYGAFDIW
MBL^{high} from (monoclonal) #31	IGHV1-2*02	Productive	91.88%	IGHJ3*02	IGHD2-21*02	3	18	CARDRSVIVVTYILDAFDMW
MBL^{high} from (monoclonal) #32	IGHV3-23*01	Productive	91.97%	IGHJ5*01	IGHD6-13*01	1	8	CSKGGWGDSW
MBL^{high} from (monoclonal) #33	IGHV3-74*01	Productive	94.51%	IGHJ5*02	IGHD2-8*02	3	9	CARQLDMYSLW
MBL^{high} from (monoclonal) #34	IGHV4-34*01	Productive	92.68%	IGHJ6*02	IGHD3-16*01	2	20	CVRGYPSDYTERRYYYYGLDVW
MBL^{high} from (monoclonal) #35	IGHV3-30*04	Productive	90.76%	IGHJ3*02	IGHD2-2*01	2	18	CTRPHCSMSSCSWNDAFAIW
MBL^{high} from (monoclonal) #36	IGHV3-23*01	Productive	91.94%	IGHJ3*02	IGHD3-22*01	2	14	CAKFYDDIQPNAFDIW
MBL^{high} from (monoclonal) #37	IGHV1-2*02	Productive	91.7%	IGHJ4*02	IGHD5-18*01	3	15	CARDLEMRYSQGSFDSW
MBL^{high} from (monoclonal) #38	IGHV3-11*01	Productive	99.2%	IGHJ6*02	IGHD3-3*01	2	23	CARDRRDDFWSGYRIYYYYYGMDVW
MBL^{high} from (monoclonal) #39	IGHV3-21*01	Productive	97.88%	IGHJ6*02	IGHD1-26*01	3	9	CARDANGMDVW
MBL^{high} from (monoclonal) #40	IGHV3-7*03	Productive	97.19%	IGHJ4*02	IGHD6-19*01	1	9	CARGGWYGDYW
MBL^{high} from (monoclonal) #41	IGHV4-39*01	Productive	100.00%	IGHJ6*02	IGHD2-2*01	2	22	CARHRLGYCSSTSCYYYYYGMDVW
MBL^{high} from (monoclonal) #42	IGHV3-15*01	Productive	91.39%	IGHJ4*02	IGHD2-8*01	3	12	CTTDSMVYVDMDYW
MBL^{high} from (monoclonal) #43	IGHV3-48*02	Productive	100.00%	IGHJ6*02	IGHD2-2*01	3	23	CARDNTANDIVVVPADYYYYGMDVW
MBL^{high} from (monoclonal) #44	IGHV3-23*01	Productive	90.76%	IGHJ3*02	IGHD4-17*01	3	17	CAKDRTLATVIQKDTFDIW
MBL^{high} from (monoclonal) #45	IGHV5-51*01	Productive	100.00%	IGHJ6*02	IGHD3-3*01	2	19	CARRDFRGDFWSGYYYGMDVW
MBL^{high} from (monoclonal) #46	IGHV3-23*01	Productive	95.32%	IGHJ6*02	IGHD2-2*01	2	14	CAFHCCRISCYGVDFW
MBL^{high} from (monoclonal) #47	IGHV4-34*01	Productive	93.67%	IGHJ4*01	IGHD1-1*01	3	14	CARVIGDKGGYYLTYW
MBL^{high} from (monoclonal) #48	IGHV3-48*01	Productive	99.59%	IGHJ6*02	IGHD3-3*01	2	20	CARSPGYDFWSGYPDYYGMDVW
MBL^{high} from (monoclonal) #49	IGHV1-2*02	Productive	96.02%	IGHJ4*02	IGHD3-22*01	2	20	CARDLARYDSGGSYKRKMFDYW
MBL^{high} from (multiclonal) #50B	IGHV3-11*03	Productive	95.32%	IGHJ4*02	IGHD6-19*01	1	10	CAKVRSHYFDYW
MBL^{high} from (multiclonal) #51B	IGHV3-21*04	Productive	96.65%	IGHJ6*02	IGHD2-15*01	3	9	CARDANGMDVW
MBL^{high} from (multiclonal) #52B	IGHV3-33*01	Productive	99.6%	IGHJ4*02	IGHD3-9*01	1	21	CARDPRVLRYFDWLLSPPPFDYW
MBL^{high} from (multiclonal) #53A	IGHV3-15*01	Productive	89.03%	IGHJ4*02	IGHD2-21*01	2	12	CTTESGWYSASDHW
MBL^{high} from (multiclonal) #53B	IGHV3-30*03	Productive	94.27%	IGHJ4*02	IGHD2-15*01	2	18	CAKDTWGHCSGGFCSHFDSW
MBL^{high} from (multiclonal) #54B	IGHV3-48*03	Productive	90.95%	IGHJ3*02	IGHD3-3*01	2	20	CVRDDRSCSSNNCHALRSFDMW
MBL^{high} from (multiclonal) #55B	IGHV3-53*01	Productive	92.55%	IGHJ6*02	IGHD2-8*01	1	19	CATHPTNIYTRWPYVSDMDVW
MBL^{high} from (multiclonal) #56A	IGHV3-23*01	Productive	100.00%	IGHJ6*02	IGHD3-3*01	1	11	CAKDWESWGMDVW
MBL^{high} from (multiclonal) #57A	IGHV4-39*01	Productive	100.00%	IGHJ5*02	IGHD6-13*01	1	19	CASVQGYSSSWYGGDNWFDPW
MBL^{high} from (multiclonal) #57B	IGHV3-23*01	Productive	100.00%	IGHJ6*02	IGHD3-10*01	1	11	CANRGETRGMDVW

(**Supplemental Table 2**, continued)

B-cell clon from #case number	V-GENE and allele	Functionality	% Identity V-REGION	J-GENE and allele	D-GENE and allele	D-REGION reading frame	CDR3 length	AA JUNCTION
MBL^{high} from (multiclonal) #58A	IGHV4-39*01	Productive	100.00%	IGHJ5*02	IGHD3-3*01	2	20	CARHTSLYDFWSGYYRGWFDPW
MBL^{high} from (multiclonal) #58B	IGHV1-69*01	Productive	100.00%	IGHJ4*02	IGHD5-18*01	2	17	CAREAGSIQLWPPGFFDYW
MBL^{high} from (multiclonal) #59A	IGHV3-7*01	Productive	93.57%	IGHJ3*02	IGHD5-12*01	2	9	CARGRYVYDIW
MBL^{high} from (multiclonal) #60A	IGHV4-34*01	Productive	96.48%	IGHJ4*02	IGHD5-24*01	1	19	CARAEGQATLLSVWEYYFDSW
MBL^{high} from (multiclonal) #60B	IGHV3-33*01	Productive	96.67%	IGHJ2*01	IGHD6-19*01	1	17	CARDILITGGRGDWYFDLW
MBL^{high} from (multiclonal) #61B	IGHV3-72*01	Productive	95.92%	IGHJ5*02	IGHD2-2*01	2	13	CVRSSTGWTDWFDPW
MBL^{high} from (multiclonal) #62B	IGHV4-34*01	Productive	96.81%	IGHJ6*03	IGHD3-3*01	2	16	CARREDDNFWSGFYMDVW
MBL^{high} from (multiclonal) #63B	IGHV3-23*01	Productive	95.16%	IGHJ4*02	IGHD2-15*01	2	18	CAKLSTPCGGGSCYSSLDYW
MBL^{high} from (multiclonal) #64B	IGHV4-34*01	Productive	95.12%	IGHJ4*02	IGHD6-19*01	1	13	CARRDSSGWYYFDYW
MBL^{high} from (multiclonal) #65A	IGHV5-51*01	Productive	92.2%	IGHJ4*01	IGHD5-18*01	3	14	CGRRRTGYNDGEIDYW
MBL^{high} from (multiclonal) #65B	IGHV4-30-4*01	Productive	97.81%	IGHJ4*01	IGHD2-2*01	2	16	CARHPSCSRTSCYFFDYW
MBL^{high} from (multiclonal) #66A	IGHV5-51*01	Productive	100.00%	IGHJ4*02	IGHD3-3*01	2	21	CARHGTYYDFWSGYYLPGFFDYW
MBL^{high} from (multiclonal) #66B	IGHV1-69*01	Productive	100.00%	IGHJ6*02	IGHD6-13*01	1	26	CARQGAGSSWYGIVKGWFEYYYYGMDVW
MBL^{high} from (multiclonal) #66C	IGHV3-33*01	Productive	98.81%	IGHJ3*02	IGHD3-3*01	1	22	CARGNGGALRFLEWLLYHDAFDIW
MBL^{high} from (multiclonal) #67A	IGHV1-3*01	Productive	97.96%	IGHJ6*02	IGHD3-3*01	2	23	CARADGGYDFWSGYSTVNYYGMDVW
MBL^{high} from (multiclonal) #67B	IGHV3-9*01	Productive	93.57%	IGHJ4*02	IGHD1-26*01	3	14	CARVESGSYFWPSDYW
CLL from (monoclonal) #68	IGHV3-23*01	Productive	93.57%	IGHJ4*02	IGHD4-23*01	1	12	CAKGRQLWSYLDYW
CLL from (monoclonal) #69	IGHV1-2*02	Productive	99.59%	IGHJ3*02	IGHD5-12*01	3	12	CARDGDYFDAFDIW
CLL from (monoclonal) #70	IGHV3-11*01	Productive	98.8%	IGHJ4*02	IGHD3-3*01	2	22	CARDPRYYDFWSGYYLPDDKFDYW
CLL from (monoclonal) #71	IGHV3-7*01	Productive	93.98%	IGHJ4*02	-	-	8	CASGSHVDYY (TRP 118 not identified)
CLL from (monoclonal) #72	IGHV4-b*02	Productive	100.00%	IGHJ4*02	IGHD5-18*01	2	13	CARSWIQLWSEFDYW
CLL from (monoclonal) #73	IGHV4-4*02	Productive	91.57%	IGHJ6*02	IGHD5-12*01	1	25	CARGSRNVDIVATITFIGFYYYGMDVW
CLL from (monoclonal) #74	IGHV1-69*06	Productive	97.96%	IGHJ6*03	IGHD3-3*01	2	24	CARAEQYYDFWSGHKGVDYYYYMDVW
CLL from (monoclonal) #75	IGHV3-11*01	Productive	97.91%	IGHJ4*02	IGHD3-10*01	2	16	CARGPDPYYYGSGTPSYW
CLL from (monoclonal) #76	IGHV3-21*01	Productive	97.21%	IGHJ5*02	IGHD3-9*01	1	22	CARDRRNGNFDWLEDPLYNWFDPW
CLL from (monoclonal) #77	IGHV3-9*01	Productive	90.91%	IGHJ6*02	IGHD4-23*01	1	16	CAKDRSNTWPLWGGMDVW
CLL from (monoclonal) #78*	IGHV1-2*02	Productive	99.57%	IGHJ4*02	IGHD6-19*01	3	13	CARAQWLVLENFDYW
CLL from (monoclonal) #79	IGHV4-34*01	Productive	85.43%	IGHJ3*02	IGHD5-12*01	2	17	CARREEDWKRSGRDSFDIW
CLL from (monoclonal) #80	IGHV1-69*01	Productive	95.1%	IGHJ4*02	IGHD3-3*01	2	13	CAKGPYYDFWSGDYW
CLL from (monoclonal) #81	IGHV4-39*01	Productive	99.21%	IGHJ4*02	IGHD3-16*02	2	19	CARHTYYDYVWGSYRTPFDYW
CLL from (monoclonal) #82	IGHV3-21*01	Productive	100.00%	IGHJ4*02	IGHD2-2*02	2	21	CAREGGLGYCSSTSCYTTLFDYW

(**Supplemental Table 2**, continued)

B-cell clon from #case number	V-GENE and allele	Functionality	% Identity V-REGION	J-GENE and allele	D-GENE and allele	D-REGION reading frame	CDR3 length	AA JUNCTION
CLL from (monoclonal) #83	IGHV3-72*01	Productive	89.41%	IGHJ4*02	IGHD6-13*01	3	15	CVRSSMGAEQTIACDYW
CLL from (monoclonal) #84	IGHV3-30*01	Productive	95.2%	IGHJ4*02	IGHD6-19*01	1	15	CARDDYSSGVGTRLSYW
CLL from (monoclonal) #85	IGHV4-4*03	Productive	92.77%	IGHJ6*02	IGHD2-2*01	2	21	CARAPYCGSNTCYSYYYYGMDVW
CLL from (monoclonal) #86	IGHV4-34*01	Productive	89.72%	IGHJ6*02	IGHD2-15*01	2	24	CAGRFYCSGDTCHLPLYHYYYGLDVW
CLL from (monoclonal) #87	IGHV3-23*01	Productive	94.78%	IGHJ6*04	IGHD3-3*01	2	23	CARDLTHHNFWSAYYETSYCGMDVW
CLL from (monoclonal) #88	IGHV3-48*02	Productive	95.58%	IGHJ6*03	IGHD5-24*01	3	18	CARQGEDYNNRGYYCYMDVW
CLL from (monoclonal) #89	IGHV1-2*02	Productive	98.29%	IGHJ6*02	IGHD3-10*01	2	17	CARDPGGGDYYYYYGMDVW
CLL from (monoclonal) #90	IGHV4-39*01	Productive	92.97%	IGHJ4*02	IGHD6-13*01	3	10	CARHEQQLADYW
CLL from (monoclonal) #91	IGHV4-34*01	Productive	95.98%	IGHJ6*02	IGHD4-23*01	3	20	CARGYGSTGETRRYYYYGMDVW
CLL from (monoclonal) #92	IGHV4-59*01	Productive	100.00%	IGHJ4*02	IGHD3-3*01	2	19	CARVVHYLDFWSGYTYYFDYW
CLL from (monoclonal) #93	IGHV3-64*01	Productive	95.58%	IGHJ6*02	IGHD6-19*01	2	9	CAVDRTGMDVW
CLL from (monoclonal) #94	IGHV6-1*01	Productive	92.19%	IGHJ4*02	IGHD6-19*01	1	18	CARSPSRYSNGWYERDFDCW
CLL from (monoclonal) #95	IGHV1-69*01	Productive	100.00%	IGHJ6*02	IGHD6-19*01	2	21	CAREVVYGVAGTYYYYYYGMDVW
CLL from (monoclonal) #96	IGHV4-34*08	Productive	85.96%	IGHJ4*02	IGHD3-22*01	2	13	CARGFHWGGYYLDFW
CLL from (monoclonal) #97	IGHV3-23*01	Productive	94.4%	IGHJ4*02	IGHD2-15*01	2	18	CAKLSTPCGGGSCYSSLDYW
CLL from (monoclonal) #98	IGHV1-8*01	Productive	87.5%	IGHJ6*02	IGHD3-3*01	2	21	CARGPSYYDFWSGPFDNYGMDVW
CLL from (monoclonal) #99*	IGHV1-2*02	Productive	100.00%	IGHJ4*02	IGHD6-19*01	3	13	CARAQWLVLENFDYW
CLL from (monoclonal) #100	IGHV3-7*01	Productive	94.38%	IGHJ3*01	IGHD3-16*01	1	16	CASALRYLPYADTAFDLW
CLL from (monoclonal) #101	IGHV3-7*03	Productive	85.77%	IGHJ4*03	IGHD3-3*01	2	15	CVRENEFWSGGWGLDGW
CLL from (monoclonal) #102	IGHV1-69*13	Productive	99.59%	IGHJ6*02	IGHD3-3*01	3	22	CATTTITIFGVVTVYYYYYGMDVW
CLL from (monoclonal) #103	IGHV4-34*01	Productive	100.00%	IGHJ4*02	IGHD3-10*01	2	20	CARGLIGAYGSGSYYPFPFDYW
CLL from (monoclonal) #104	IGHV1-69*01	Productive	100.00%	IGHJ6*02	IGHD3-3*01	2	23	CARADGGYDFWSGYSTVNYYGMDVW
CLL from (monoclonal) #105	IGHV3-48*03	Productive	91.06%	IGHJ3*02	IGHD5-12*01	3	12	CVRDGFHYYGFDIW
CLL from (monoclonal) #106	IGHV3-30*03	Productive	97.93%	IGHJ6*03	IGHD3-3*01	2	30	CAKDQEQGPRPRYYDFWSAPPPWYYYYYMDVW
CLL from (monoclonal) #107	IGHV1-69*01	Productive	98.29%	IGHJ5*02	IGHD3-3*01	2	15	CATDKKYYDFWSGYYLW
CLL from (monoclonal) #108	IGHV4-59*02	Productive	91.5%	IGHJ4*02	IGHD1-14*01	3	13	CARHLRNDKYYLDFW
CLL from (monoclonal) #109	IGHV3-48*03	Productive	100.00%	IGHJ6*02	IGHD3-3*01	2	21	CARDYDFWSGYYSYYYYYGMDVW
CLL from (monoclonal) #110	IGHV5-51*01	Productive	99.6%	IGHJ6*03	IGHD2-2*01	2	23	CARYCSSTSCMTGTMGYYYYYMDVW
CLL from (monoclonal) #111	IGHV3-48*02	Productive	92.34%	IGHJ4*02	IGHD6-13*01	1	14	CARDLGGNSWPTFDFW
CLL from (monoclonal) #112	IGHV3-30*03	Productive	89.56%	IGHJ6*02	IGHD6-19*01	2	19	CAKIGMAGDFLEFRYYGMDVW
CLL from (monoclonal) #113	IGHV1-3*01	Productive	99.18%	IGHJ6*02	IGHD3-22*01	2	25	CARDLTYYYDSSGYYYFNYYYYGMDVW

(**Supplemental Table 2**, continued)

B-cell clon from #case number	V-GENE and allele	Functionality	% Identity V-REGION	J-GENE and allele	D-GENE and allele	D-REGION reading frame	CDR3 length	AA JUNCTION
CLL from (monoclonal) #114	IGHV3-7*03	Productive	85.54%	IGHJ4*03	IGHD3-3*01	2	15	CVRENEFWSGGWGLDGW
CLL from (monoclonal) #115	IGHV4-59*01	Productive	91.94%	IGHJ4*02	IGHD6-19*01	1	14	CARGPDISGWNGLDYW
CLL from (monoclonal) #116	IGHV4-34*01	Productive	91.46%	IGHJ5*02	IGHD6-13*01	3	13	CATNSRESQGWFDPW
CLL from (monoclonal) #117	IGHV3-15*01	Productive	91.32%	IGHJ6*02	IGHD2-15*01	2	23	CVTGPGYCSGGGCSSRGYYYGMDVW
CLL from (monoclonal) #118	IGHV3-30*04	Productive	100.00%	IGHJ6*02	IGHD3-3*01	2	22	CARDLKTAYYDFWSGYYGDGMDVW
CLL from (monoclonal) #119	IGHV3-9*01	Productive	98.8%	IGHJ6*02	IGHD3-3*01	2	26	CAKDKYYDFWSGYSHLGVLYYYYGMDVW
CLL from (monoclonal) #120	IGHV1-3*01	Productive	97.94%	IGHJ6*02	IGHD3-3*01	2	23	CARADGGYDFWSGYSTVNYYGMDVW
CLL from (monoclonal) #121	IGHV1-18*01	Productive	100.00%	IGHJ6*02	IGHD2-15*01	3	9	CARDANGMDVW
CLL from (monoclonal) #122	IGHV1-69*01	Productive	100.00%	IGHJ3*02	IGHD3-16*02	2	21	CARGGNYDYIWGSYRPNDAFDIW
CLL from (monoclonal) #123	IGHV1-69*02	Productive	100.00%	IGHJ6*03	IGHD2-15*01	3	22	CARSQAHIVVVVAATYYYYYMDVW
CLL from (monoclonal) #124	IGHV1-18*01	Productive	100.00%	IGHJ4*02	IGHD7-27*01	3	13	CARKNWGPDYYFDYW
CLL from (monoclonal) #125	IGHV4-39*01	Productive	100.00%	IGHJ6*01	IGHD2-2*01	2	22	CARHRLGYCSSTSCYYYYYGMDVW
CLL from (monoclonal) #126	IGHV1-69*01	Productive	98.76%	IGHJ1*01	IGHD3-22*01	2	22	CARGSSTYYYDSSVYGVAEYFQHW
CLL from (monoclonal) #127	IGHV3-30*03	Productive	98.39%	IGHJ4*02	IGHD3-22*01	2	21	CARGPNVSHTYYYDNSGSHFDYW
CLL from (monoclonal) #128	IGHV3-74*01	Productive	92.98%	IGHJ4*02	IGHD2-2*01	3	15	CARVDIEVDGGGHFDNW
CLL from (monoclonal) #129	IGHV1-2*02	Productive	96.97%	IGHJ4*02	IGHD2-8*01	1	15	CGRDVELRYWQGYFDLW
CLL from (monoclonal) #130	IGHV1-18*01	Productive	100.00%	IGHJ6*02	IGHD6-13*01	1	19	CARDLSLSSNWFTPPYGMDVW
CLL from (monoclonal) #131	IGHV1-2*02	Productive	100.00%	IGHJ5*01	IGHD3-3*01	2	32	CARAPRGDYDTEAGGAYSYGLEVWRLRRNRFDSW
CLL from (monoclonal) #132	IGHV4-39*07	Productive	100.00%	IGHJ6*02	IGHD2-2*01	2	22	CARDRLGYCSSTSCYLYYYGMDVW
CLL from (monoclonal) #133	IGHV4-b*01	Productive	94.14%	IGHJ1*01	IGHD2-15*01	2	20	CARLPHCTASRCYGGGRYVDQW
CLL from (monoclonal) #134	IGHV1-18*01	Productive	99.57%	IGHJ6*02	IGHD3-9*01	2	20	CARGAYYDILTGYRYYYGMDVW
CLL from (monoclonal) #135	IGHV4-34*01	Productive	95.49%	IGHJ4*02	IGHD2-15*01	2	11	CARGSAGSRLDYW
CLL from (monoclonal) #136	IGHV3-21*01	Productive	94.63%	IGHJ6*02	-	-	9	RTKDANGMDVW (2nd-CYS 104 not identified)
CLL from (monoclonal) #137	IGHV3-7*01	Productive	95.98%	IGHJ4*02	IGHD2-2*01	2	16	CGSQCSTTSCPSSISEYW
CLL from (monoclonal) #138	IGHV1-69*01	Productive	100.00%	IGHJ6*03	IGHD7-27*01	3	21	CARDTGLMTNWGYYYYYYYMDVW
CLL from (monoclonal) #139	IGHV3-7*03	Productive	92.59%	IGHJ4*02	IGHD3-22*01	2	15	CARVSDETTGYGNFDYW
CLL from (monoclonal) #140	IGHV4-b*01	Productive	99.59%	IGHJ4*02	IGHD5-18*01	2	13	CARAWIQLWSDFDYW
CLL from (monoclonal) #141	IGHV4-34*01	Productive	99.19%	IGHJ6*02	IGHD2-2*01	3	21	CARADLLVVPAAIYYYYYGMDVW
CLL from (monoclonal) #142	IGHV3-48*03	Productive	91.13%	IGHJ6*02	IGHD6-19*01	1	8	CSRRGRLDIW
CLL from (monoclonal) #143	IGHV3-30*03	Productive	96.37%	IGHJ4*02	IGHD3-10*01	2	19	CANRGDTSGLGTCCQGIGDSW
CLL from (monoclonal) #144	IGHV4-61*02	Productive	91.63%	IGHJ5*02	IGHD5-12*01	3	14	CAKRYGDHGEGWFDPW

(**Supplemental Table 2**, continued)

B-cell clon from #case number	V-GENE and allele	Functionality	% Identity V-REGION	J-GENE and allele	D-GENE and allele	D-REGION reading frame	CDR3 length	AA JUNCTION
CLL from (monoclonal) #145	IGHV3-30*01	Productive	93.15%	IGHJ4*02	IGHD3-10*01	3	17	CASGSMIGGVILPPGFDYW
CLL from (monoclonal) #146	IGHV3-53*01	Productive	100.00%	IGHJ6*02	IGHD3-22*01	2	25	CAREGYYDSSGYSEAPHYYYYYGMDVW
CLL from (monoclonal) #147	IGHV2-70*11	Productive	96.21%	IGHJ4*02	IGHD1-20*01	3	14	CARMQHRYHWNDSDSW
CLL from (monoclonal) #148	IGHV1-3*01	Productive	90.2%	IGHJ5*01	IGHD3-9*01	1	19	CARGIRYSGWLLYGSDWYDSW
CLL from (monoclonal) #149	IGHV3-23*01	Productive	99.58%	IGHJ6*02	IGHD5-12*01	1	11	CANRGETRGMDVW
CLL from (monoclonal) #150	IGHV4-39*01	Productive	100.00%	IGHJ5*02	IGHD6-13*01	1	18	CATQTGYSSSWYAVNWFDPW
CLL from (monoclonal) #151	IGHV1-24*01	Productive	100.00%	IGHJ6*02	IGHD3-9*01	2	24	CATDGYDILTGYYKGPGAYYYGMDVW
CLL from (monoclonal) #152	IGHV3-48*03	Productive	99.16%	IGHJ3*02	IGHD5-12*01	3	12	CARDGFHYYGFDIW
CLL from (monoclonal) #153	IGHV4-61*02	Productive	92.65%	IGHJ4*02	IGHD7-27*01	3	12	CARDNWGFEGFDSW
CLL from (monoclonal) #154	IGHV3-74*01	Productive	93.15%	IGHJ3*01	IGHD4-23*01	3	14	CARGHKVVNPGSFDLW
CLL from (multiclonal) #155A	IGHV4-34*01	Productive	93.06%	IGHJ4*02	IGHD3-3*01	3	11	CARPNVGAVFVFW
CLL from (multiclonal) #156A	IGHV4-34*01	Productive	96.69%	IGHJ2*01	IGHD4-23*01	2	13	CARAGGYSDWYFDLW
CLL from (multiclonal) #157A	IGHV3-9*01	Productive	100.00%	IGHJ3*02	IGHD3-3*01	2	19	CAKDRYYDFWSGYYTAAFDIW
CLL from (multiclonal) #158A	IGHV4-39*01	Productive	98.47%	IGHJ6*02	IGHD3-3*01	1	18	CGILGEWLSFYFFFYGMDVW
CLL from (multiclonal) #159A	IGHV3-30-3*01	Productive	91.13%	IGHJ4*02	IGHD5-12*01	3	16	CARGKGRNSGYDYLLHYW
CLL from (multiclonal) #160A	IGHV1-3*01	Productive	94.03%	IGHJ5*02	IGHD5-18*01	1	19	CARDRVVIIPDTTTINWFDPW
CLL from (multiclonal) #161A	IGHV3-48*02	Productive	96.34%	IGHJ4*02	IGHD4-17*01	2	12	CARSSGDDSLIDYW
CLL from (multiclonal) #162A	IGHV4-34*01	Productive	97.56%	IGHJ6*02	IGHD3-10*01	2	17	CARGFDYYGSGSANGLDVW
CLL from (multiclonal) #163A	IGHV1-46*01	Productive	100.00%	IGHJ4*01	IGHD3-3*01	2	21	CARAHYYDFWSGYVYPRLAFDYW
CLL from (multiclonal) #164A	IGHV3-53*01	Productive	100.00%	IGHJ6*02	IGHD3-22*01	2	25	CAREGYYDSSGYSEAPHYYYYYGMDVW
CLL from (multiclonal) #165A	IGHV1-3*01	Productive	96.2%	IGHJ3*01	IGHD1-26*01	3	17	CARGLRSGTFYGADAFDFW
CLL from (multiclonal) #165B	IGHV4-34*01	Productive	92.93%	IGHJ3*02	IGHD2-15*01	3	26	TARGGLFVETEIAGVGYRSGTGTLFDSW (2nd-CYS 104 not identified)
CLL from (multiclonal) #166A	IGHV3-33*01	Productive	91.5%	IGHJ6*02	IGHD3-10*01	2	18	CARDDNRDGSGNYKGGMDFW
CLL from (multiclonal) #166B	IGHV3-21*01	Productive	94.8%	IGHJ4*02	IGHD4-23*01	2	13	CARDLDGGNSVFDYW
CLL from (multiclonal) #167A	IGHV3-52*01(P)	Productive**	96.06%	IGHJ3*02	IGHD2-21*02	2	19	CMTVLWANRGGDCPGDAFDIW
CLL from (multiclonal) #168A	IGHV4-34*01	Productive	98.78%	IGHJ4*02	IGHD1-26*01	3	17	CARGPDRLYSGSYTRFDYW
CLL from (multiclonal) #169A	IGHV3-48*02	Productive	93.6%	IGHJ4*02	IGHD3-10*01	1	12	CVRELWFGNGGDYW
CLL from (multiclonal) #170A	IGHV3-30*03	Productive	99.22%	IGHJ6*02	IGHD3-9*01	1	25	CAKYGGVKLRYFDWLLYGDYYYGMDVW
CLL from (multiclonal) #171A	IGHV3-33*01	Productive	99.61%	IGHJ5*02	IGHD1-26*01	2	12	CARGELLHNWFDPW
CLL from (multiclonal) #171B	IGHV3-23*01	Productive	99.18%	IGHJ3*02	IGHD5-12*01	3	12	CAKDGFPYYGFDIW
CLL from (multiclonal) #172A	IGHV2-26*01	Productive	91.91%	IGHJ5*02	IGHD3-3*01	2	22	CAGTNIPRQFDFWSGSSPNWFDPW

(**Supplemental Table 2**, continued)

B-cell clon from #case number	V-GENE and allele	Functionality	% Identity V-REGION	J-GENE and allele	D-GENE and allele	D-REGION reading frame	CDR3 length	AA JUNCTION
CLL from (multiclonal) #173A	IGHV3-30*03	Productive	99.6%	IGHJ6*02	IGHD3-9*01	1	25	CAKYGGVKLRYFDWLLYGDYYYGMDVW
CLL from (multiclonal) #174A	IGHV4-39*01	Productive	98.76%	IGHJ3*01	IGHD1-14*01	3	17	CASHRNTQTYNNRAAFDVW
CLL from (multiclonal) #175A	IGHV5-51*01	Productive	100.00%	IGHJ4*02	IGHD2-15*01	2	19	CARIPVAGYCRGGSCYPFDYW
CLL from (multiclonal) #175B	IGHV4-34*01	Productive	99.16%	IGHJ4*02	IGHD3-10*01	2	14	CARTKTYGSGPPGKYW
CLL from (multiclonal) #176A	IGHV4-34*01	Productive	99.59%	IGHJ4*02	IGHD3-10*01	2	15	CARGLYYYGSGVYFDYW

**B-cell clones from #78 and #99 corresponded to the same untreated CLL patient at recruitment and after 1-year evaluation, respectively; **however the closest V is a pseudogene. Grey shadowed cells highlight those CDR3 of the IGHV genes identical or highly homologous. Scripts in cells indicate IMGT/JunctionAnalysis giving no results for that JUNCTION.*

Supplemental Table 3. Diagnosis, differential immunophenotypic/*IGHV* features and cytogenetic alterations of the coexisting aberrant B-cell populations from multiclonal MBL, CLL and other B-CLPD cases (n=41).

Case ID	Phenotype of population 1 (% from WBC; compatible diagnosis) *V(D)J* rearrangement-MS[†]	iFISH	Phenotype of population 2 (% from WBC; compatible diagnosis) *V(D)J* rearrangement-MS[†]	iFISH*
1	FSC/SSC^{lo} CD19+κ+ CD5+ CD20^{lo} (62.9%; **CLL**) V3-30(D3-9)J6-UM	**ND**	FSC/SSC^{int} CD19+κ^{hi} CD5- CD20+ (4.1%; **Non-CLL-like MBL^{high} MZL**) V1-2(D5-5)J4-UM	**ND**
2¶	FSC/SSC^{lo}κ^{lo} FMC7^{lo} CD23+ CD5+ CD43^{lo} CD11c^{lo} (33%; **CLL**) V3-30(D3-9)J6-UM	**+12** (93%) **del(11q22.3)** (91%)	FSC/SSC^{lo}κ^{lo} FMC7^{lo} CD23+ CD5+ CD43+ CD11c+ (21%; **CLL-like MBL^{high}**) V3-23(D2-15)J4-M	**+12** (95%) **del(11q22.3)** (93%)
3	FSC/SSC^{lo} CD19+ κ^{lo} CD5+ CD79b^{lo} FMC7- (26.5%; **CLL**) V1-3(D1-26)J3-M	**ND**	FSC/SSC^{int} CD19^{lo}λ+ CD5^{het} CD79b- FMC7+ (25.7%; **CLL**) V4-34(D2-15)J3-M	**ND**
4	FSC/SSC^{int}κ^{lo} CD20^{lo} CD79b^{lo}CD5+ (20.6%; **CLL**) V3-53(D3-22)J6-UM	**+12** (49%) **polysomy**	FSC/SSC^{lo}λ^{lo} CD20^{lo} CD79b^{lo}CD5+ (0.6%; **CLL-like MBL^{low}**) V1-69(D3-3)J3-UM	**ND[a]**
5	FSC/SSC^{lo}λ^{lo}CD20^{lo} CD5+ CD43+ CD23+ (44.2%; **CLL**) V4-34(D3-3)J4-M	**biallelic del(13q14.3)** (99%)	FSC/SSC^{lo}κ^{hi}CD20+ CD5- CD43- CD23- (1.4%; **Non-CLL-like MBL^{low} MZL**) V1-8(D3-3)J5-UM	**ND**
6	FSC/SSC^{lo}κ^{lo} CD20^{lo} FMC7- CD5+ CD23+ (33%; **CLL**) V4-34(D4-23)J2-M	**del(13q14.3)** (96%)	FSC/SSC^{lo}λ^{lo} CD20^{lo} FMC7- CD5+ CD23+ (10.6%; **CLL-likeMBL^{high}**) V3-11(D6-19)J4-M	**del(13q14.3)** (96%)
7	FSC/SSC^{lo}CD19+ κ^{lo} CD20^{lo} CD5+ (34.6%; **CLL**) V4-39(D3-3)J6-UM	**ND**	FSC/SSC^{lo}CD19+ λ^{lo} CD20^{lo} CD5+ (9.4%; **CLL-likeMBL^{high}**) V3-33(D3-9)J4-M	**ND**
8	FSC/SSC^{lo} CD19^{lo} CD43- CD5+ CD25+ IgM+ CD27+ CD11c+/het (50.2% **CLL**) V1-3(D5-5)J5-M	**ND**	FSC/SSC^{lo} CD19+ CD43+ CD5^{hi} CD25^{hi} IgM^{hi} CD27^{hi} CD11c+ (5% **CLL-like MBL^{high}**) V3-53(D2-8)J6-M	**ND**
9	FSC/SSC^{lo}κ+ CD5+ CD20^{lo} CD43- (12.6%; **CLL-like MBL^{high}**) V3-23(D5-12)J6-UM	**+12** (87%)	FSC/SSC^{lo}κ+ CD5+ CD20^{lo} CD43+ (3%; **CLL-like MBL^{high}**) V4-39(D6-13)J5-UM	**+12** (93%)
10	FSC/SSC^{lo}λ^{lo} CD22^{lo} CD23+ CD5+ (54.6%; **CLL**) Not found	**del(13q14.3)** (96%)	FSC/SSC^{lo}κ^{lo} CD22^{lo} CD23+ CD5+ (4.1%; **CLL-like MBL^{high}**) V3-72(D2-2)J5-M	**ND**
11¶	FSC/SSC^{lo} CD19+ κ^{lo} CD5+ CD79b^{lo} FMC7- (0.6%; **CLL-like MBL^{low}**) V3-23(D5-12)J6-UM	**ND**	FSC/SSC^{lo} CD19+ λ+ CD5+ CD79b^{lo} FMC7- (1.6%; **CLL-like MBL^{low}**) V3-48(D5-12)J6-M	**del(13q14.3)**(19%) **+12** (41%)
12	FSC/SSC^{lo}CD19+λ^{lo} CD5+ CD20^{lo} (89%; **CLL**) V4-39(D1-7)J3-UM	**ND**	FSC/SSC^{lo}CD19+ κ^{lo} CD5+ CD20^{lo} (0.1%; **CLL-like MBL^{low}**) V3-21(D6-13)J4-UM	**ND**
13	FSC/SSC^{lo}CD19+ κ^{lo} CD20^{lo} FMC7- CD5+ (49.9%; **CLL**) V5-51(D2-15)J4-UM	**ND**	FSC/SSC^{lo}CD19+ λ^{lo} CD20^{lo} FMC7- CD5+ (40.8%; **CLL**) V4-34(D3-10)J4-UM	**ND**
14¶	FSC/SSC^{lo}λ+d CD20^{lo} CD5+ CD22^{lo} CD23+ FMC7- (84%; **CLL**) V4-34(D1-26)J4-UM	**biallelic del(13q14.3)** (85%);	FSC/SSC^{lo}κ^{lo}CD20$^{lo/het}$ CD5+ CD22^{lo} CD23+ FMC7- (0.7%; **CLL-like MBL^{high}**) V4-34(D3-3)J6-M	**del(13q14.3)** (83%)
15¶	FSC/SSC^{lo}CD19^{lo}κ+ CD5- (11%; **CLL-like MBL^{hi}**) V3-23(D3-22)J6-UM	**ND**	FSC/SSC^{lo}CD19+ λ^{lo}CD5+ (1.1%; **CLL-like MBL^{low}**) V3-11(D2-15)J4-M	**ND[a]**
16¶	FSC/SSC^{hi} CD19^{lo}CD38++CD10+ cBcl2+ sIg- cλ+ (20.6%; **Non-CLL FL**) V3-11(D1-1)J3-M	**t(14q32)**(95%); **t(14;18)** (96%); **+18q21** (95%) **+8q24** (92%)	FSC/SSC^{lo} CD19+CD38-CD10-λ+ (2.5%; **Non-CLL FL**) V3-23(D5-12)J6-UM	**t(14;18)** (90%); **+8q21** (90%) **+18q21** (87%)
17	FSC/SSC^{lo}CD19+ λ+ CD5+ CD20^{lo} CD11c- (59.3%; **CLL**) V1-46(D3-3)J4-UM	**ND**	FSC/SSC^{lo} CD19+ Ig- CD5- CD20^{hi} CD11c^{hi} (0.5%; **Non-CLL-like MBL^{low} MZL**) V3-53(D1-26)J4-M	**ND**

(**Supplemental Table 3**, continued)

Case ID	Phenotype of population 1 (% from WBC; compatible diagnosis)	iFISH	Phenotype of population 2 (% from WBC; compatible diagnosis)	iFISH*
	V(D)J rearrangement-MS†		V(D)J rearrangement-MS†	
18¶	FSC/SSC^{lo}CD19+ CD5+ λ^{lo} (15.6%; **CLL-like MBL^{high}**)	**ND**	FSC/SSC^{lo}CD19+ CD5+ κ^{lo} (10.1%; **CLL-like MBL^{high}**)	**+12** (19%); **del(13q14.3)** (32%)
	V3-15(D3-3)J4-M		V3-30(D5-12)J4-M	
19¶	FSC/SSC^{lo}CD19+λ^{lo} CD20^{lo} CD5+ (44%; **CLL**)	**del(13q14.3)** (80%)	FSC/SSC^{lo}CD19+ κ^{lo}CD20^{lo} CD5+ (0.3%; **CLL-like MBL^{low}**)	**biallelic del(13q14.3)** (73%)
	V3-30(D5-12)J4-M		V3-23(D6-19)J6-UM	
20	FSC/SSC^{lo}CD19+ κ^{lo}CD20^{lo} CD5+ (35.6%; **CLL**)	**+12** (75%);	FSC/SSC^{lo}CD19+ λ^{lo} CD20^{lo} CD5+ (0.5%; **CLL-like MBL^{low}**)	**ND**[a]
	V4-34(D3-10)J6-M		V5-a(D6-19)J6-UM	
21	FSC/SSC$^{lo}\kappa^{hi}$ FMC7+ CD5- CD23- CD43- (5.2%; **Non-CLLMZL**)	**ND**	FSC/SSC$^{lo}\kappa^{lo}$ FMC7- CD5+ CD23+ CD43+ (2.3%; **CLL-like MBL^{low}**)	**ND**
	V4-61(D7-27)J4-M		V3-21(D2-2)J6-M	
22	FSC/SSC$^{lo}\kappa$+ CD5+ CD20^{lo} CD79b^{lo} (6.4%; **CLL-like MBL^{low}**)	**ND**[a]	FSC/SSC$^{lo}\kappa^{lo}$CD5+ CD20^{lo} CD79b- (0.6%; **CLL-like MBL^{low}**)	**ND**
	V3-74(D3-10)J4-M		V4-59(D3-10)J6-M	
23	FSC/SSC^{lo}CD19+ κ^{lo} CD22^{lo} CD23+ CD5+ (11.5%; **CLL-like MBL^{high}**)	**ND**	FSC/SSC^{lo}CD19+ λ^{lo} CD22^{lo} CD23+ CD5+ (3.8%; **CLL-like MBL^{high}**)	**ND**
	V4-34(D5-24)J4-M		V3-33(D6-19)J2-M	
24	FSC/SSC$^{lo}\kappa^{lo}$ CD20^{lo} FMC7- CD5+ CD23+ (21.1%; **CLL-like MBL^{high}**)	**+12** (93%)	FSC/SSC$^{lo}\lambda^{lo}$ CD20^{lo} FMC7- CD5+ CD23+ (8.7%; **CLL-like MBL^{high}**)	**ND**
	V5-51(D5-5)J4-M		V4-30-4(D2-2)J4-M	
25	FSC/SSC^{lo}CD19+ κ^{lo} CD5^{het} CD20^{lo} (17.8%; **CLL-like MBL^{high}**)	**+12**(76%); **t(14q32)** (52%)	FSC/SSC^{lo}CD19+ λ^{lo} CD5^{lo} CD20^{lo} (2.6%; **CLL-like MBL^{high}**)	**ND**
	V5-51(D3-3)J4-UM		V1-69(D6-13)J6-UM	
26	FSC/SSC$^{lo}\kappa$+ CD5+ CD20^{lo} CD43+ (23%; **CLL-like MBL^{high}**)	**del(13q14)** (15%); **del(13q14.3)** (15%)	FSC/SSC$^{lo}\kappa^{lo}$ CD5+ CD20^{lo} CD43- (3.7%; **CLL-like MBL^{high}**)	**ND**
	V4-39(D3-3)J5-UM		V1-69(D5-5)J4-UM	
27¶	FSC/SSC$^{lo}\kappa^{lo}$CD20^{lo} CD79b^{lo} CD5+ (10%; **CLL-like MBL^{high}**)	**del(13q14)** (98%); **del(13q14.3)** (98%)	FSC/SSC$^{lo}\lambda^{lo}$CD20^{lo} CD79b^{lo} CD5+ (0.6%; **CLL-like MBL^{low}**)	**ND**
	V3-7(D5-12)J3-M		V3-23(D6-6)J4-M	
28	FSC/SSC^{lo}CD19+ λ+ CD5+ CD20^{lo} CD22- (68%; **CLL**)	**del(13q14)** (95%); **del(13q14.3)** (87%)	FSC/SSC^{lo}CD19+ κ+ CD11c+ CD5^{het} CD20^{hi} CD22+ (1.1%; **Non-CLL-like MBL^{high} MZL**)	**ND**
	V3-48(D4-17)J4-M		V1-69(D6-6)J4-M	
29¶	FSC/SSC$^{lo}\kappa^{lo}$ CD19^{lo}, CD20^{lo} CD79b- CD43+ (36.4%; **CLL**)	**ND**	FSC/SSC$^{lo}\kappa^{hi}$ CD19+, CD20^{hi} CD79b+ CD43- (16.7%; **Non-CLL MALT**)	**del(17p13)** (91%)
	V3-48(D3-10)J4-M		V3-15(D4-17)J4-M	
30	FSC/SSC$^{lo}\lambda^{lo}$FMC7^{lo} CD5+ CD79b- CD23+ CD43+ (33.7%; **CLL**)	**t(14q32)** (98%)	FSC/SSC$^{lo}\lambda$+ CD5- CD11c- FMC7^{hi}CD79b^{het} CD23- CD43- (8.4%; **Non-CLL MALT**)	**ND**
	V4-34(D3-10)J4-UM		V3-15(D6-6)J6-M	
31¶	FSC/SSC$^{int}\kappa^{lo}$ CD23+ CD5+ CD11c+ (24%; **CLL**)	**ND**	FSC/SSC$^{lo}\kappa^{lo}$ CD5+ CD23^{het} CD11c^{lo} (20%; **CLL**)	**ND**
	V3-33(D2-15)J5-UM		V3-23(D5-12)J3-M	
32¶	FSC/SSC^{int}CD19+ κ+ CD20+ CD5- (17.7% **Non-CLL MZL**)	**ND**	FSC/SSC^{lo}CD19+ κ^{lo}CD5+ CD20^{lo} (3.2%; **CLL-like MBL^{high}**)	**ND**
	V4-39(D6-19)J4-M		V4-34(D6-19)J4-M	
33	FSC/SSC^{lo}CD19+dλ^{lo} CD5+ CD20^{lo} (65.3%; **CLL**)	**del(13q14.3)** (81%)	FSC/SSC^{lo}CD19$^{hi}\lambda$+ CD5+ CD20^{hi} (12.5%; **Non-CLL MZL**)	**t(14q32)** (28%)
	V2-26(D3-3)J5-M		V3-53(D2-15)J2-M	
34¶	FSC/SSC$^{lo}\lambda$+ CD19+ CD20^{hi} CD22+ CD38- CD11c- CD25+ (65.8%; **Non-CLL MALT**)	**ND**	FSC/SSC$^{lo}\kappa$+ CD19+ CD20^{hi} CD22+ CD38- CD11c- CD25+ (13.2%; **Non-CLL MALT**)	**ND**
	V3-7(D2-21)J4-M		V3-23(D2-2)J2-M	
35¶	FSC/SSC$^{lo}\kappa^{lo}$ CD20^{lo} FMC7- CD5+ CD23+ (55.3%; **CLL**)	**ND**	FSC/SSC$^{lo}\lambda^{lo}$ CD20^{lo} FMC7- CD5+ CD23+ (9.8%; **CLL-likeMBL^{high}**)	**ND**
	V3-9(D3-3)J3-UM		V3-21(D2-2)J6-M	

(**Supplemental Table 3**, continued)

Case ID	Phenotype of population 1 (% from WBC; compatible diagnosis)	iFISH	Phenotype of population 2 (% from WBC; compatible diagnosis)	iFISH*
	V(D)J rearrangement-MS†		**V(D)J rearrangement-MS†**	
36	FSC/SSC^{int}κ^{hi}CD5+ CD11c+ FMC7^{lo} (46%; **Non-CLL MZL**)	**ND**	FSC/SSC^{lo}κ^{hi} CD5- CD11c- FMC7^{hi} (40.9%; **Non-CLL MALT**)	**ND**
	V1-2(D6-6)J5-UM		V3-48(D1-26)J4-UM	
37¶	FSC/SSC^{hi} CD103+ CD25+ CD11c+ (21%; **Non-CLL HCL**)	**NA**	FSC/SSC^{lo}λ^{lo}CD5+ (0.8%; **CLL-like MBL^{low}**)	**ND[a]**
	V3-30(D3-3)J5-UM		V3-11(D4-17)J6-M	
38¶	FSC/SSC^{lo}CD19^{lo}λ+ CD5+ CD20+ CD23- (38.6%; **Non-CLL MZL**)	**+3q27** (89%)	FSC/SSC^{lo}CD19^{hi}κ^{lo} CD5+ CD20^{lo} CD23+ (6.3%; **CLL-likeMBL^{high}**)	**del(13q14.3)** (18%)
	V3-21(D6-13)J6-UM		V3-48(D3-3)J3-M	
39	FSC/SSC^{lo}κ^{lo} CD19+, CD20^{lo} CD79b- CD43+ (24%; **CLL-like MBL^{high}**)	**ND**	FSC/SSC^{lo}λ^{lo} CD19+ CD20^{lo} FMC7- CD5+ CD23+ (14%; **CLL-like MBL^{high}**)	**del(13q14.3)** (65%); **t(14q32)** (31%)
	V1-3(D3-3)J6-UM		V3-9(D1-26)J4-M	
40¶	FSC/SSC^{lo}κ^{lo}CD20^{lo} CD5+ CD22^{lo} CD23+ FMC7- (46.7%; **CLL**)	**biallelic del(13q14.3)** (95%)	FSC/SSC^{lo}λ^{lo}CD20$^{lo/het}$ CD5+ CD22^{lo} CD23+ FMC7- (43.1%; **CLL**)	**del(13q14.3)** (30%)
	V3-33(D3-10)J6-M		V3-21(D4-23)J4-M	
41	FSC/SSC^{int}λ^{lo} CD5+ CD19+ CD11c- (87.1%; **Non-CLL MCL**)	**t(11;14)** (97%)	FSC/SSC^{lo}λ+ CD11c^{hi} CD19^{hi} (1.3%; **Non-CLL-like MBL^{low} MZL**)	**ND**
	V3-21(D3-3)J6-UM		V4-34/D3-10/J5-M	

*CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; MZL, marginal zone lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MALT, B-cell lymphoma of mucosa-associated lymphoid tissue. + indicates antigen expression at normal levels; -, absence of expression; het, heterogeneous antigen expression; hi, high antigen expression; lo, low antigen expression; int, intermediate scatter; c, cytoplasmatic antigen expression. †MS, mutational status (UM, unmutated; M, mutated), *FISH was performed on interphase nuclei from FACS-purified cells; ND: no chromosomal alterations detected by iFISH for the probes studied; NA: not analyzed. An additional B-cell clone (**population 3)** was detected by interphase FISH in **case 2** (FSC/SSC^{lo}λ^{lo} CD23^{lo} CD5^{lo} CD43^{lo} CD11c^{lo}; 0.7%, **CLL-like MBL^{low}; ND[a]**), **case 25** (FSC/SSC^{lo}CD19+ κ+ CD5^{hi} CD20^{lo}; 2.4%, **CLL-like MBL^{high}; +12**(66%)) and **case 37** (FSC/SSC^{lo}κ^{lo} CD5+; 0.2%, **CLL-like MBL^{low}; ND[a]**)[a]The percentage of B-cells from this subpopulation was very low and only allowed analysis for some iFISH probes (13q14, 13q14.3, 17p13.1 and 11q22.3). Genetic abnormalities present in one population were always evaluated also in the other coexisting population.*

¶Cases whose clones had IGHV aa sequences phylogenetically closer than those found in the rest of multiclonal cases.

Supplemental Table 4. Peripheral blood B-cell counts and BCR features of multiclonal versus monoclonal CLL-like and non-CLL-like B-cell clones.

	CLL-like B-cells		**Non-CLL-like B-cells**	
	Multiclonal B-cells n=66 clones	**Monoclonal B-cells n=128 clones**	**Multiclonal B-cells n=19 clones**	**Monoclonal B-cells n=15 clones**
*N. of PB clonal B cells (x10^6/L)**	**2,675 (0.6-71,485)[a]**	**10,956 (0.1-369,288)**	4,375 (85-156,168)	4,771 (54-41,221)
*% of PB clonal B cells from WBC**	**13% (0.1%-89%)[a]**	**45% (0.001%-97%)**	13% (0.5%-87%)	41% (1%-73%)
MBL^{low} B-cell clones	**14/66 (21%)[a]**	**13/128 (10%)**	3/19 (16%)	2/15 (13%)
MBL^{high} B-cell clones	**26/66 (39%)[a]**	**26/128 (20%)**	2/19 (10%)	2/15 (13%)
CLL B-cell clones	**26/66 (39%)[a]**	**89/128 (69%)**	NA	NA
CLL-stage A clones	**12/66 (18%)[a]**	**53/128 (41%)**	NA	NA
CLL-stage B/C clones	**8/66 (12%)[a]**	**36/128 (28%)**	NA	NA
Non-CLL B-cell clones	NA	NA	14/19 (74%)	11/15 (73%)
IGHV mutated B-cell clones	40/66 (61%)	76/124 (61%)	11/19 (58%)	8/15 (53%)
% alignment of IGHV aa sequences between coexisting B-cell clones	51% (38%-79%)	NA	62% (46%-76%)[#]	NA
% alignment of IGHV aa sequences between each B-cell clone and the other clones	**52% (31%-100%)[a]**	**50% (29%-100%)**	**51% (32%-89%)[a]**	**49% (33%-86%)**

*Results expressed as number of B-cell clones and percentage between brackets or as *median value (range). PB, peripheral blood; WBC, white blood cells; CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL^{low}, low count monoclonal B-cell lymphocytosis; IGHV, immunoglobulin heavy chain variable region genes; CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL^{high}, clinical monoclonal B-cell lymphocytosis; aa, amino acids. NA, not appropriate.[#]Both coexisting B-cell clones showed Non-CLL-like phenotype (n=4 cases) or the majority B-cell clone showed Non-CLL-like phenotype (n=4 cases). [a]Statistically significant differences (P <0.05) found between clones from multiclonal vs. monoclonal cases.*

Supplemental Table 5. Cytogenetic features of non-CLL like B-cell clones from monoclonal cases.

Clone type	**Type of cytogenetic changes / % aberrant B-cells analyzed by iFISH**
Non-CLL-like MBL^{high} (MZL)	trisomy 12^{+} (80%); and t(14q32)$^{+}$ (35%)
Non-CLL (FL)	del(13q14.3)$^{+}$ (18%); 3 copies of *IGH* gene (85%)*
Non-CLL (FL)	t(14;18)$^{+}$ (89%); and t(8;14)$^{+}$ (78%); polysomy
Non-CLL (MCL)	t(11;14)$^{+}$ (93%)
Non-CLL (MALT)	t(14q32)$^{+}$ (94%)
Non-CLL (MZL)	t(14q32)$^{+}$ (94%); 3 and 4 copies of *IGH* gene (53% and 37%, respectively)

*Only cytogenetically altered clones are shown; cytogenetically non-altered clones from monoclonal cases included non-CLL MZL (n=5; 2 MBL^{low}, 1 MBL^{high} and 2 MZL), MALT-lymphoma (n=1), MCL (n=1), DLBCL (n=1) and LPL (n=1). CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL, monoclonal B-cell lymphocytosis; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone B-cell lymphoma; MALT, B-cell lymphoma of mucosa-associated lymphoid tissue; * t(14;18)$^{+}$ by molecular studies; ND, not detected ; NA, not analyzed.*

Supplemental Table 6. Monoclonal cases with B-cell clones sharing HCDR3 sequences of the same length (± 1 amino acid) and belonging to identical or evolutionary highly-related *VH* families.

Monoclonal Case ID	*VH* families	3HCDR length	aa composition of HCDR3	(% homology)[#]
149	*V3-48*	8	**C**_SRRGRLDI_**W**	(25)
158	*V3-11*	8	**C**_ARGSYFDY_**W**	
66	*V3-7*	9	**C**_ARGRYVYDI_**W**	(44)
136	*V3-7*	9	**C**_ARGGWYGDY_**W**	
113	*V3-74*	9	**C**_ARQLDMYSL_**W**	(11)
121	*V3-64*	9	**C**_AVDRTGMDV_**W**	
92	*V3-23*	12	**C**_AKGRQLWSYLDY_**W**	(33)
153	*V3-23*	12	**C**_AKDGFTKDVFDI_**W**	
16	*V1-2*	13	**C**_ARGLNTDYGAFDI_**W**	(31)
18	*V1-2*	13	**C**_ARAQWLVLENFDY_**W**	
201	*V4-34*	13	**C**_ARGFHWGGYYLDF_**W**	(23)
206	*V4-34*	13	**C**_ATNSRESQGWFDP_**W**	
190	*V4-34*	14	**C**_APARYYDFSAPIDY_**W**	(29)
214	*V4-34*	14	**C**_ARVIGDKGGYYLTY_**W**	
205	*V4-59*	14	**C**_ARGPDISGWNGLDY_**W**	(50)
218	*V4-61*	14	**C**_AKRYGDHGEGWFDP_**W**	
124	*V3-23*	14	**C**_AKFYDDIQPNAFDI_**W**	(29)
145	*V3-23*	14	**C**_AFHCCRISCYGVDF_**W**	
24	*V1-2*	15	**C**_ARDLEMRYSQGSFDS_**W**	(60)
35	*V1-2*	15	**C**_GRDVELRYWQGYFDL_**W**	
125	*V3-7*	16	**C**_ASALRYLPYADTAFDL_**W**	(31)
147	*V3-7*	16	**C**_GSQCSTTSCPSSISEY_**W**	
105	*V3-23*	16	**C**_TKDPRDTGYGGDAFDY_**W**	(35)
142	*V3-23*	17	**C**_AKDRTLATVIQKDTFDI_**W**	
142	*V3-23*	17	**C**_AKDRTLATVIQKDTFDI_**W**	(35)
104	*V3-23*	18	**C**_AKDLPSTYNWNSGGAFDI_**W**	
151	*V3-30*	17	**C**_ASGSMIGGVILPPGFDY_**W**	(18)
120	*V3-30*	18	**C**_TRPHCSMSSCSWNDAFAI_**W**	
132	*V3-30*	19	**C**_AKIGMAGDFLEFRYYGMDV_**W**	(37)
150	*V3-30*	19	**C**_ANRGDTSGLGTCCQGIGDS_**W**	
96	*V3-21*	20	**C**_ARHHPVRESSATGHYYGMDV_**W**	(45)
155	*V3-48*	20	**C**_ARSPGYDFWSGYPDYYGMDV_**W**	
196	*V4-34*	20	**C**_VRGYPSDYTERRYYYYGLDV_**W**	(40)
202	*V4-34*	20	**C**_ARGLIGAYGSGSYYPFPFDY_**W**	
198	*V4-34*	20	**C**_ARGYGSTGETRRYYYYGMDV_**W**	(50)
202	*V4-34*	20	**C**_ARGLIGAYGSGSYYPFPFDY_**W**	
155	*V3-48*	20	**C**_ARSPGYDFWSGYPDYYGMDV_**W**	(71)
130	*V3-48*	21	**C**_ARDYDFWSGYYSYYYYGMDV_**W**	
96	*V3-21*	20	**C**_ARHHPVRESSATGHYYGMDV_**W**	(35)
114	*V3-21*	21	**C**_AREGGLGYCSSTSCYTTLFDY_**W**	

(**Supplemental Table 6**, continued)

Monoclonal Case ID	*VH* families	3HCDR length	aa composition of HCDR3	(% homology)[#]
130	*V3-48*	21	**C**_ARDYDFWSGYYSYYYYYGMDV_**W**	(48)
114	*V3-21*	21	**C**_AREGGLGYCSSTSCYTTLFDY_**W**	
22	*V1-69*	21	**C**_AREVVYGVAGTYYYYYYGMDV_**W**	(67)
39	*V1-69*	21	**C**_ARDTGLMTNWGYYYYYYYMDV_**W**	
22	*V1-69*	21	**C**_AREVVYGVAGTYYYYYYGMDV_**W**	(48)
31	*V1-69*	21	**C**_ARGGNYDYIWGSYRPNDAFDI_**W**	
31	*V1-69*	21	**C**_ARGGNYDYIWGSYRPNDAFDI_**W**	(43)
39	*V1-69*	21	**C**_ARDTGLMTNWGYYYYYYYMDV_**W**	
143	*V3-30*	21	**C**_ARGPNVSHTYYYDNSGSHFDY_**W**	(28)
138	*V3-30*	22	**C**_ARDLKTAYYDFWSGYYGDGMDV_**W**	
114	*V3-21*	21	**C**_AREGGLGYCSSTSCYTTLFDY_**W**	(42)
110	*V3-21*	22	**C**_ARDRRNGNFDWLEDPLYNWFDP_**W**	
114	*V3-21*	21	**C**_AREGGLGYCSSTSCYTTLFDY_**W**	(42)
157	*V3-21*	22	**C**_ARGRLSAWLLMEGIYYYYGMDV_**W**	
110	*V3-21*	22	**C**_ARDRRNGNFDWLEDPLYNWFDP_**W**	(36)
157	*V3-21*	22	**C**_ARGRLSAWLLMEGIYYYYGMDV_**W**	
110	*V3-21*	22	**C**_ARDRRNGNFDWLEDPLYNWFDP_**W**	(41)
93	*V3-11*	22	**C**_ARDPRYYDFWSGYYLPDDKFDY_**W**	
157	*V3-21*	22	**C**_ARGRLSAWLLMEGIYYYYGMDV_**W**	(32)
93	*V3-11*	22	**C**_ARDPRYYDFWSGYYLPDDKFDY_**W**	
26	*V1-69*	22	**C**_ATTTITIFGVVTVYYYYYGMDV_**W**	(32)
34	*V1-69*	22	**C**_ARGSSTYYYDSSVYGVAEYFQH_**W**	
26	*V1-69*	22	**C**_ATTTITIFGVVTVYYYYYGMDV_**W**	(50)
27	*V1-69*	23	**C**_ARADGGYDFWSGYSTVNYYGMDV_**W**	
34	*V1-69*	22	**C**_ARGSSTYYYDSSVYGVAEYFQH_**W**	(23)
27	*V1-69*	23	**C**_ARADGGYDFWSGYSTVNYYGMDV_**W**	
129	*V3-11*	23	**C**_ARDRRDDFWSGYRIYYYYGMDV_**W**	(48)
141	*V3-48*	23	**C**_ARDNTANDIVVVPADYYYYGMDV_**W**	
27	*V1-69*	23	**C**_ARADGGYDFWSGYSTVNYYGMDV_**W**	(69)
15	*V1-69*	24	**C**_ARAEQYYDFWSGHKGVDYYYYMDV_**W**	

Amino acids with an analogous side-chain polarity (highlighted in gray): L,F; A,V; I,L; K,R; L,V; L,M; L,C; T,S; A,I; D,E; I,M; V,C; L,F; M,F; I,V; H,Y; I,C; F,W. [#]*Number of aa with an analogous side-chain polarity (excluding the delineating **C**_ and _**W** positions)/HCDR3 length*100.*

Supplemental Table 7. Haematological features of B-CLPD cases who received chemotherapy.

	No. of case	Abs. No. Clonal B-cells (x10^6/L)	Binet Stage	MAP
Monoclonal Subjects	33	12 640	A	**Yes**
	34	23 134	C	No
	35	5 314	B	No
	36	44 428	B	**Yes**
	37	18 600	B	**Yes**
	38	29 376	B	**Yes**
Multiclonal Subjects	5	Clone A: 11 724 Clone B: 2 078	B	**Yes**
	26	Clone A: 49 600 Clone B: 350	B	**Yes**
	39	Clone A: 5 438 Clone B: 158	C	No
	40*	Clone A, HCL: 1 029 Clone B, CLL-like MBL^{low}: 39 Clone C, CLL-like MBL^{low}: 10	-	No

**This case corresponded to a hairy cell leukemia patient. Gray shadowed clonal B-cells represent multiclonal B-CLPD cases with stereotyped CLL-like B-cell clones. CLL, chronic lymphocytic leukemia; MBL, monoclonal B-cell lymphocytosis; HCL, hairy cell leukemia; B-CLPD, B-cell chronic lymphoproliferative disorders; MAP, myelodysplasia-associated phenotype; -, not applicable.*