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Determinants of progression and screening of Monoclonal B-cell lymphocytosis

Dissertação no âmbito do Mestrado em Biotecnologia Farmacêutica, orientada pelo Professor Doutor Hugo Prazeres e pelo Professor Doutor Luís Almeida e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro de 2022

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Abstract

Monoclonal B-cell lymphocytosis (MBL) is an asymptomatic condition in which individuals have increased blood levels of particular subtypes of monoclonal B lymphocytes. These cells share some characteristics with clones circulating in chronic lymphocytic leukemia (CLL). In turn, CLL is a type of heterogeneous leukemia characterized by the accumulation of clonal B cells such as CD5, CD19 and CD23 in peripheral blood, bone marrow and other lymphoid tissues. Much of the heterogeneity is associated with the characteristics of the tumor B-cell receiver (BCR). It is currently established that the CLL is preceded by the MBL but the progression from low MBL to high MBL and CLL is reduced. The prevalence of low MBL is significantly higher than the others, with about 3 to 14% in the population. The key question is how to know which MBL cases may or may not have the risk of progressing to CLL.

In order to develop methods to screen the progression of MBL to CLL, we combined two different molecular markers with a relevant weight in the patient's outcome: a) the mutational status of the IGHV genes, determined using Sanger Sequencing; and b) the presence of del(17p), analyzed by Copy Number Variation at the TP53 *locus* . Through these methods it was possible to determine the IGHV rearrangements of about 91% of the samples. The most frequent HV gene families were V1 and V3, with 50% and 40%, respectively. IGHV1-69 and IGHV1-2 were the most prevalent genes, mostly associated with unmutated status and, consequently, worse prognosis and reduced OS. Almost 50% of the patients had del(17p) and about 75% of cases correspond to patients with unmutated CLL, presenting unfavorable prognosis with the aggravating factor that chemoimmunotherapy may not be recommended because they do not have a good chance of responding effectively to treatment. The complementarity of these two methods allows the knowledge of a more complete prognosis, involving TTFT, OS, and advice about treatment. Overall, these methods could be used for a population screening pilot and for ascertaining the genetic factors contributing to the identification of patients at risk of progression from MBL to CLL, allowing early diagnostics/surveillance and better treatment outcomes.

Resumo

A linfocitose monoclonal de células B (MBL) é uma condição assintomática em que os indivíduos têm níveis sanguíneos elevados de subtipos particulares de linfócitos B monoclonais. Estas células partilham algumas características com clones que circulam na leucemia linfocítica crónica (CLL). Por sua vez, a CLL é um tipo de leucemia heterogénea caracterizada pela acumulação de células B clonais como CD5, CD19 e CD23 no sangue periférico, medula óssea e outros tecidos linfoides. A maior parte da heterogeneidade está associada às características do recetor de células B do tumor (BCR). Está atualmente estabelecido que a CLL é precedida pela MBL, mas a progressão de MBL baixo para MBL alto e CLL é reduzida. A prevalência de baixo MBL é significativamente maior do que as outras, com cerca de 3 a 14% da população. A questão-chave é como saber quais os casos de MBL que podem ou não ter o risco de progredir para o CLL.

Com o objetivo de desenvolver métodos de rastreio da progressão de MBL para CLL, combinamos dois marcadores moleculares diferentes com grande relevância no prognóstico do doente: a) o estado mutacional dos genes IGHV, determinado através da Sequenciação de Sanger; b) a presença da del(17p), analisado pelo método das Variações do Número de Cópia no *locus* TP53. Através destes métodos foi possível determinar os rearranjos IGHV de cerca de 91% das amostras. As famílias de genes VH mais frequentes foram V1 e V3, com 50% e 40%, respetivamente. Os genes IGHV1-69 e IGHV1-2 foram os mais prevalentes, associados ao estado não-mutado e, consequentemente, pior prognóstico e menor tempo médio de vida. Cerca de 50% dos doentes apresentaram del(17p) e cerca de 75% dos casos correspondiam a doentes com CLL não-mutado, tendo um prognóstico desfavorável com o fator agravante de que a quimioimunoterapia não poder ser recomendada devido à baixa taxa de resposta ao tratamento.

A complementaridade destes dois métodos permite o conhecimento de um prognóstico mais completo, com informação acerca do início e tipo de tratamento e a taxa de sobrevivência. Como tal, estes resultados poderiam ser usados para um piloto de rastreio populacional e para determinar os fatores genéticos que contribuem para a identificação de doentes em risco de progressão de MBL para CL, permitindo um diagnóstico/vigilância precoce e melhores resultados de tratamento.

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List of Acronyms

A – Adenine

BCL6 - B-cell lymphoma 6

BCR - B cell receptor

C – Cytosine

CDR - Complementarity Determining Region

CLL - Chronic lymphocytic Leukemia

CNV – Copy Number Variations

DNA – Deoxyribonucleic Acid

dNTP - Deoxyribonucleotide triphosphate

ERIC - European Research Initiative in CLL

ESCCA - The European Society for Clinical Cell Analysis

FDC - Follicular Dendritic Cells

FR – Framework region

G – Guanine

GC – Germinal Centre

H_2O – Purified Water

ICU - Intensive Care Unit

IG – Immunoglobulin

IGH - Immunoglobulin Heavy-chain

IGHC - Immunoglobulin Heavy-chain Constant region

IGHD - Immunoglobulin Heavy-chain Diversity region

IGHJ - Immunoglobulin Heavy-chain Joining region

IGHV - Immunoglobulin Heavy-chain Variable region

IL – interleukin

LF – Follicular lymphoma

M-CLL – Mutated CLL

MBL - Monoclonal B-cell lymphocytosis

MBLhi – High Count Monoclonal B-cell lymphocytosis

$MgCl_2$ – Magnesium chloride

MM - Multiple Myeloma

MZB – Marginal Zone B cells

OS – Overall Survival

PB – Peripherical blood

PCN – Predicted Copy Number

PCR – Polymerase Chain Reaction

qPCR – Quantitative Polymerase Chain Reaction

RCV – Raw Copy Value

RNA - Ribonucleic Acid

SARS-Cov2 - Severe Acute Respiratory Syndrome Coronavirus 2

SHM - Somatic Hypermutation status

SMZL - Splenic marginal-zone lymphoma

T – Thymine

TAE - Tris-acetate-EDTA

TD - T-cell dependent

TH cell – T Helper cell

TI - T-cell independent

TNF-α - Tumor Necrosis Factor Alpha

TTFT – Time to first treatment

U-CLL – Unmutated CLL

WBC - White blood cell

WHO - World Health Organization

ZAP70 - Zeta-chain-associated protein kinase 70

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CHAPTER 1 – INTRODUCTION

1.1 Ageing of the immune system & monoclonal B-cell lymphocytosis

The survival of the human being is strongly related to a well-functioning immune system, which protects the body from pathogens that may cause infections and diseases (Weyand and Goronzy, 2016). At birth, the immune system is equipped with a very diverse repertoire of T and B cells (Dorshkind et al., 2009). Naïve T cells have a half-life of 6 to 12 months and memory T cells 15 to 48 days (Czesnikiewicz-Guzik et al., 2008). Memory T cells, for example, are formed during the initial antigen encounter, have the ability to persist, and can provide a source of effectors that respond quickly when exposed to the same antigen (Salam et al., 2013). Multiple pathogen exposure over time results in a complex immune repertoire that includes a large number of memory cells (Nikolich-Žugich et al., 2012). Although the innate immune system is fast and efficient, it lacks long-term memory and discriminatory power (Warrington et al., 2001). The adaptive immune system works through specific antigen recognition, memory formation and adaptive proliferation of cells that offer antigen-specific immunity (Weyand and Goronzy, 2016). An ageing immune system shows a reduction in adaptive immunity and an increase in non-specific innate immunity, leaving older individuals vulnerable (Salam et al., 2013, Weyand and Goronzy, 2016).

In fact, ageing is linked with various morbidities that ultimately lead to the progressive deterioration of protective immunity, making older individuals more susceptible to cancers and infections, eventually leading to organ failure and death (Wertheimer et al., 2014).

Older people do not respond effectively to new or even to previously identified antigens (Montecino-Rodriguez et al., 2013). The first signs of decreased immune function occur after the age of 50 years and accelerate after 65 to 75 years (Goodwin et al., 2006). One of the first signs of immune ageing is reduced antibody production in response to vaccination (Whiting et al., 2015).

Another effect of ageing on the immune system is the rate of production of naïve B and T cells (Whiting et al., 2015). $CD4^+$and $CD8^+$ T cells behave differently in ageing (Longo et al., 2014); age-related loss of naïve T cells is much more pronounced in the $CD8^+$ compartment compared to $CD4^+$. Interestingly, $CD8^+$ T cells appear to age more rapidly (Longo et al., 2014). Although the number of naïve B and T cells migrating from primary to secondary lymphoid organs is reduced by ageing, B and T cell development does not cease entirely (Shao et al., 2021)

The production of lymphocytes continues, although at a reduced rate. The key to the elderly's loss of functional immunity is not the number of lymphocytes, but the composition and quality of their production, which tends to change dramatically as they age (Weyand and Goronzy, 2016).

$CD8^+$ T cells undergo oligoclonal expansions that can be easily detected with age and tend to be induced by chronic persistent viruses, but also from irregular homeostatic proliferation, showing impaired function (Akbar and Fletcher, 2005, Khan et al., 2002). In contrast, $CD4^+$cells do not show relevant percentages of oligoclonal expansions and, when present, are found in patients with autoimmune diseases (Czesnikiewicz-Guzik et al., 2008). These $CD4^+$ T cells show activation defects and increased differentiation into Th17 cells, which are T helper cells defined by the production of IL-17 (Montecino-Rodriguez et al., 2013).

1.1.1 The B Cell life's cycle

B cells are distinguished by their capacity to produce antibodies, act as antigen-presenting cells, and produce immunoregulatory cytokines (Wang et al., 2020). They are also in charge of developing an immunological memory capable of preventing infection recurrence (Sosa-Hernández et al., 2020). These cells also mediate/regulate humoral immune responses, both positively and negatively, through their cellular receptors (LeBien and Tedder, 2008).

Naïve B cells develop in the bone marrow, recirculate throughout the spleen and lymph nodes, and eventually return to the bone marrow until they die or have an encounter with a specific antigen (Franchina et al., 2018). There are two populations of B cells: B-1 and B-2 (Ghosn et al., 2011). B-1 cells are the primary B cells during fetal and neonatal development (Tangye, 2013), can renew themselves and are mostly located in the peritoneal and pleural cavities. B-1 cells produce most of the 'natural' antibodies and immunoglobulins M and A (Tarlinton et al., 1995). B-2 cells are, in turn, constantly produced from bone marrow precursors, circulating in the blood, and are found in secondary lymphoid tissues (Ghosn et al., 2011). They can be classified into follicular B cells, which respond immediately to pathogens, and as marginal zone B cells that are mediators of the immune response (Dorshkind and Montecino-Rodriguez, 2007). They respond to various types of antigens, producing antibodies with higher affinity during infections (Quách et al., 2016). B-2 cells initiate immunoglobulin heavy chain gene rearrangements in the bone marrow. Pro-B cells that successfully make these rearrangements are selected at the pre-B cell checkpoint receptor, proliferate and then rearrange the light chain as they differentiate into immature B cells (Pillai and Cariappa, 2009).

B cell activation is a dynamic process that leads to antibody class switching, somatic hypermutations and differentiation of plasma cells (Franchina et al., 2018). It is triggered by the binding of an antigen to the B cell receptor (BCR), thus initiating an intracellular signaling cascade leading to antigen internalization, and consequent processing and presentation to the cells (Lanzavecchia, 1985). BCR is a transmembrane protein on the B cell surface that is composed of a immunoglobulin membrane (Treanor, 2012).

The human IGH locus is located on the chromosome 14, at band 14q32.33 and it spans 1250 kilobases (kb) figure 1.1 (Croce et al., 1979). On chromosome 14q, the majority of the 5' IGHV genes are found close to the telomere, whereas the IGHC genes appear to be more centromeric in location (Lefranc, 2001).

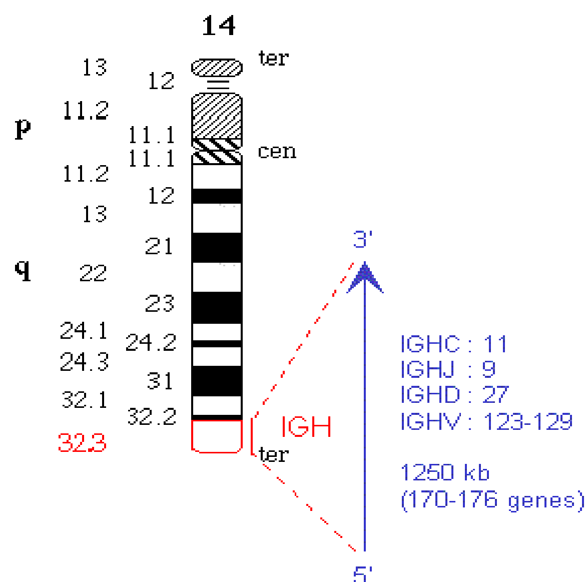

Figure 1.1 - Chromosomal localization of the human IGH locus at 14q32.33
The vertical red line indicates the localization of the IGH locus at 14q32.33. The arrow indicates the orientation 5) → 3) of the locus. The total number of genes in the locus is shown between parentheses. Adapted from (Lefranc, 2001).

Proteins encoded by the IGH locus are the immunoglobulin heavy chains. They are formed as a result of the recombination (or rearrangement) of three genes: IGHV, IGHD, and IGHJ, as well as the deletion of the intermediary DNA, to generate the IGHV-D-J gene (Chiu et al., 2019). When B cells differentiate in the lymph nodes, the rearranged variable genes will acquire somatic mutations, greatly increasing their diversity in comparison to germline genes (Wang et al., 2020). Inside the IGH group there are a total of 76-84 functional genes, where the variable region contains between 38 and 46 functional genes belonging to 6 or 7 subgroups. These subgroups are: IGHV1 with 9 genes, IGHV2 with 3 genes, IGHV3 with 18-20 genes, IGHV4 with 6-9 genes, IGHV5 with 1 gene, IGHV 6 with 1 gene and IGHV7 with 0 or 1 gene depending on allelic polymorphism by insertion/deletion (Pallarès et al., 1999).

The terminal region of these chains is characterized by long and variable sequences that have been acquired through gene recombination, nucleotide addition and somatic hypermutations. This region, the complementarity determining region (CDR), is responsible for the clonal specificity of antigen recognition by the receptor and the affinity of the ligand (Brezski and Monroe, 2008).

Rearrangement of antigen receptor genes, a characteristic of the adaptive immune system, is an essential mechanism for generating diversity in B cells from variable gene segments (V) through genetic recombination (LeBien and Tedder, 2008). Through this mechanism, B cells can create about 1011 B-cell receptors or antibodies (Wang et al., 2020). IGHV genes undergo somatic hypermutation during germinal center (GC) reaction, where diversity increases, and may change the affinity and sometimes the specificity of the antibody (Nussenzweig and Alt, 2004). GCs are transient microstructures that develop in secondary lymphoid tissues' follicles in response to specific pathogens and immunizations (Huang, 2020). The main function of GCs is to produce plasma cells that secrete antibodies with high affinity and memory cells (Camacho et al., 1998). Briefly, naïve B cells migrate to a T-cell-rich area called the T-cell zone. In these zones, B cells can then differentiate into antibody-secreting cells or GC precursor cells that then migrate to the primary follicle, which is a structure rich in circulating $IgM^{+}IgD^{+}$ B cells, where they interact with follicular dendritic cells (FDCs) (Klein and Dalla-Favera, 2008). After proliferation, two zones are denoted: a dark zone, consisting of centroblasts, which are compacted B cells that proliferate, and a light zone, with centrocytes (Liu et al., 1991). Somatic hypermutations occur in centroblasts, which is a process that modifies the immunoglobulin variable region, causing a great diversity of IGHV genes (Berek et al., 1991). In centrocytes, cells undergo class-switching recombination which is a mechanism whereby the heavy chain produced by an activated B cell changes from IgM or IgD to IgG, IgA or IgE (Klein and Dalla-Favera, 2008).

During the clonal expansion phase, Ig BCR expression is downregulated and only becomes upregulated again when the centroblasts become centrocytes and move into the clear zone, where they interact with FDCs and T helper cells (Camacho et al., 1998).

Studies support that the interaction that takes place in the clear zone is critical for affinity selection (Liu and Banchereau, 1996). During this process, most GC B cells undergo apoptosis and only those expressing a receptor with sufficient affinity for the antigen are selected to differentiate into memory cells or plasma cells (Smith et al., 1997).

Figure 1.2 illustrates a schematic of the B-cell reactions that occur in germinal centers.

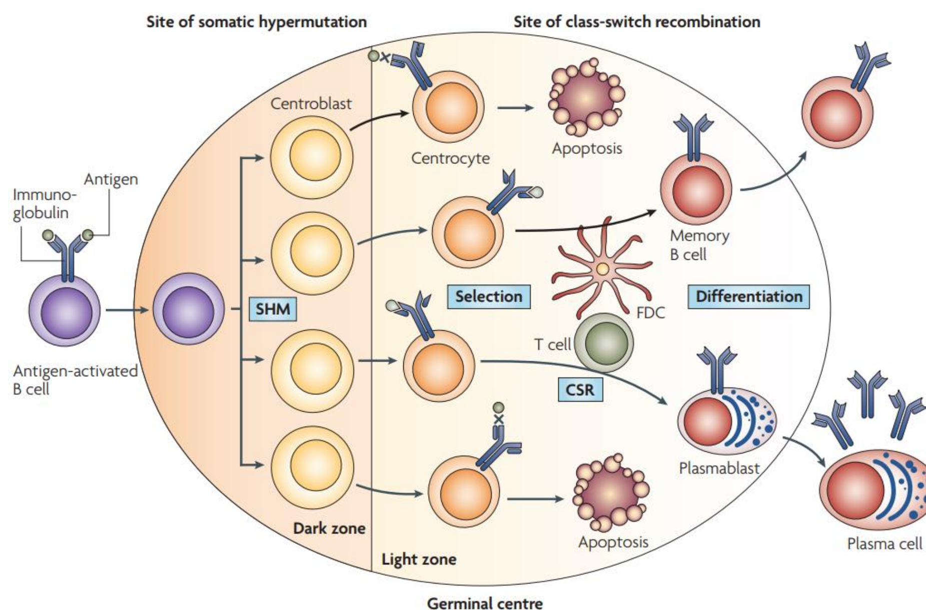

Figure 1.2 – The germinal center microenvironment

Germinal centers are structures formed within follicles of the secondary lymphoid tissues by the entry of antigen-activated B cells in which these cells undergo clonal expansion. These expansions occur in the GC dark zone where somatic hypermutation in the IGHV gene also takes place when antigen-activated B cells differentiate into centroblasts. Then, the mutated cells differentiate into centrocyte and migrate to the GC light zone and interact with $CD4^{+}$ TH cells and FDCs, gaining BCR affinity mutations. Cells with damaged mutations undergo apoptosis. Some B cells also go through class switch recombination of their IGHC. The cells that have acquired good affinity, undergo multiple rounds of proliferation, mutation, and selection until they differentiate in memory B cells or plasma cells and then leave the GC. Adapted from (Klein and Dalla-Favera, 2008).

Despite their benefits in maturing the immune response, somatic mutations in variable regions can increase the risk of oncogenic mutations, resulting in autoreactive B-cell clones and lymphomas (Gatto and Brink, 2010).

B cells may also be involved in pathogenic roles, namely in autoimmune diseases, and may exert antibody-independent responses that are normally based on the secretion of cytokines with either pro- or anti-inflammatory properties (Sosa-Hernández et al., 2020).

B-cell development and activation involves genetic alterations such as rearrangements of the VDJ region, somatic hypermutations of the immunoglobulin gene and recombinations (Meng et al., 2020). Abnormalities and errors in these genetic arrangements can cause chromosomal translocations and genomic mutations, leading to altered B-cell gene function and expression, leading to proliferation and consequently lymphomas (Meng et al., 2020).

B-cell lymphomas account for about 95% of lymphomas, which represent a diverse group of neoplastic disorders of lymphocytes (Teras et al., 2016). Monoclonal B-cell lymphocytosis is one type of lymphoma characterized by low B-cell counts (Fisher and Fisher, 2004).

1.1.2 MBL in the general population, an occult epidemic?

Monoclonal B-cell lymphocytosis is an asymptomatic condition where individuals have increased blood levels of specific subtypes of monoclonal B cell clones (Galigalidou et al., 2021). It is characterized by the presence of monoclonal B-cell populations (<5,000 clonal cells/mL) in the absence of clinical symptoms (Mowery and Lanasa, 2012).

MBL consists of two groups depending on the number of clonal B cells detected in the peripheral blood: low count MBL (<0.5x109 cells/L) and high count MBL (>0.5x109 cells/L) (Dagklis et al., 2008, Strati and Shanafelt, 2015). The rate of progression from low to high MBL is reduced as well as progression to malignant disease, however high MBL progresses at a risk rate of 1-2% per year (Criado et al., 2018b). Low MBL count is the most common diagnosis (Slager et al., 2013).

B cells have a particular immunophenotypic profile: clonal CD19 positive B cells, co expression of CD5 and CD23 and low density of CD20 (Scarfò et al., 2016). Monoclonal B cells are predominantly identified in the CD5+ and CD5- subsets of CD19+ B-lymphocytes. In addition, MBL have mature lymphocytes with a narrower border and a denser nucleus with no discernible nucleolus and partially aggregated chromatin (Maitre and Troussard, 2019).

The incidence of MBL is most common in Western countries (Criado et al., 2018a) with similar percentages to the United States, with less incidence in certain countries in Asia, namely China and Japan (Mukkamalla et al., 2022).

The disease mostly affects older people, particularly men, with an average age at diagnosis of 70 years (Slager et al., 2014, Smith et al., 2011). According to de Tute et al., the male gender also had a higher risk of contracting MBL disease than the female gender (de Tute et al., 2006). However, some studies show that women develop a more aggressive form of the disease compared to men (Slager et al., 2014, Smith et al., 2011). The disease is extremely uncommon in children, and its prevalence tends to rise with age (Hernández et al., 1995). Over the age of 40 years, the incidence is about 10% in all individuals, however, people over 90 years old the incidence can be as high as 75% (Criado et al., 2018b).

What makes it more difficult to detect this disease is the fact that it is asymptomatic, with no constitutional symptoms, no anemia, thrombocytopenia (low platelet count), no organomegaly (enlargement of organs) and the lymph nodes are smaller than 1.5cm in size (Mukkamalla et al., 2022). Recognition of MBL disease in apparently healthy individuals is derived from observations inspired by genetics, environmental exposure and ageing (Marti et al., 2007).

Very low levels of circulating monoclonal B-cell subpopulations have already been identified in individuals who appear to have a healthy life. According to Marti et al. MBL can be detected

in the absence of lymphocytosis and in individuals with a normal CD5+ cell ratio. Several laboratories have used various approaches to identify monoclonal B-cell lymphocytosis with minimal values, making comparisons geographically, ethnically and in different risk groups (Marti et al., 2005).

1.1.3 Prevalence and prognosis of MBL comparative to CLL

B cells share certain characteristics with the abnormal lymphocyte clones circulating in chronic lymphocytic leukemia thus individuals with these cells have the risk of developing CLL (Landgren et al., 2009). It is now well established that basically all cases of CLL are preceded by MBL and cases of high count MBL may progress from a pre-malignant phase to CLL disease (Maitre and Troussard, 2019).

According to the World Health Organization, chronic lymphocytic leukemia is defined as a lymphoproliferative disorder characterized by the accumulation of clonal B lymphocytes, such as CD5, CD20 and CD23 in the peripheral blood, bone marrow and lymphoid organs (Campo et al., 2011, Criado et al., 2018a). However, not all clonal cells necessarily express all the markers (Faguet et al., 1992). The fact that some patients require treatment soon after diagnosis, presenting a more progressive disease, while others have a more favorable prognosis, not requiring treatment for several years, shows how heterogeneous this disease can be (Zhang and Kipps, 2014). About 70% of patients undergo treatment with responses varying from complete and prolonged survival to worsened disease and consequent death (Criado et al., 2018b).

About 191000 cases and 61000 deaths are attributed to CLL around the world (Siegel et al., 2020). According to Cancer Research UK, lately CLL has been more frequently diagnosed in younger individuals, with around 15% of patients aged 55 and under. The frequency of MBL is estimated to be about 100 times more common than CLL (Maitre and Troussard, 2019). However, even as a precursor to CLL, a significant proportion of MBL cases may regress or remain stable (Marti et al., 2005).

As CD5+ and CD23+ cells may be a precursor of CLL, the possibility has been raised that MBL may be overrepresented in apparently healthy relatives of familial CLL cases (Brown, 2008). Epidemiological studies have already shown that the risk of MBL in relatives of patients with CLL is higher and it is likely that part of the disease is caused by genetic traits (Kleinstern et al., 2022). According to Rawstron et al., about 14% of apparently healthy family members of a CLL case tested positive for MBL compared to about 3% of control patients, representing a significant increase in risk (Rawstron et al., 2002). Marti et al., also reported an increased

risk of MBL in people with a family history of CLL (18% compared to 0.7% of controls), and also reported a family history of MBL in first-degree relatives of someone with CLL (Casabonne et al., 2012, Marti et al., 2003). The probability of a family member developing the disease also increases with age and male gender (Goldin et al., 2010).

In first-degree relatives with a family history of CLL, the relative risk of developing MBL is about 4 times higher than in the general population (de Tute et al., 2006).

Criado et al., observed that more than two-thirds of the low-count CLL-like MBL clones showed a significantly increased size in the blood after 7 years of follow-up while for the non-CLL-like clones there were more variable kinetics (Criado et al., 2018b). Additionally, they noticed a significant rise in the frequency of cytogenetic modifications over time, proving that B-cell clones are dynamic both in terms of clone size and in terms of their capacity to gain new cytogenetic variations. (Criado et al., 2018b). Regarding the prognosis, this study indicates that progression from MBL to CLL, although more delayed, can most definitely occur.

1.1.4 Subclasses and molecular traits

According to the American Cancer Society, the Binet staging system classifies CLL by the number of affected lymph nodes (neck, groin, underarm, spleen, and liver) and by whether the patient has an insufficient amount of red blood cells or blood platelets (Hallek, 2019). Stage A is characterized by increased lymph glands in less than 3 zones and a high-level white blood cell count. As for the B state, it is characterized by an enlargement of 3 or more areas and a high white blood cell count. Finally, the final and most critical state, state C, is characterized by a high white blood cell count, a low red blood cell or platelet count and enlarged lymph glands or spleen. Normally, stage B and C are treated straight away whereas stage A only needs to be treated if the patient's condition quickly decays or the symptoms worsen (Hallek, 2019, Zengin et al., 1997).

Based on the abnormal immunophenotypic profiles of clonal B cells, MBL is classified into three subclasses: CLL-type, which is the most common, atypical CLL-type and non-CLL-type (Maitre and Troussard, 2019).

Regarding the immunophenotypic analysis of these three subclasses, the CD19 marker showed no differences between the groups. The B-cell memory marker CD27 showed higher expression levels in the MBL CLL-type subclass compared to atypical CLL-type and non-CLL-type. The marker CD23, a cell surface receptor that is widely used as a marker of B cell maturation, was expressed at higher levels in CLL-type MBL than in the other two subclasses

(Lanasa et al., 2011). However, CD79b, which is a cofactor associated with the B-cell receptor (BCR) that is required for signaling, was very poorly expressed in MBL CLL-type (Lanasa et al., 2011). IgM and IgD expression was very similar in the three groups, although slightly lower in MBL CLL-type (Lanasa et al., 2011).

Many of the established risk factors for CLL are also differentially expressed between the different subclasses of MBL. Markers associated with more aggressive CLL, such as CD38, ZAP70 and CD49 are more expressed in the atypical CLL-type subclass and very poorly expressed in CLL-type MBL (Rawstron, 2013). The markers CD22 and CD79 are more expressed in non-CLL-type (Galigalidou et al., 2021). Table 1.1 summarizes the expression of the main immunophenotypic markers for each MBL subclass.

Table 1.1 - Expression of the main immunophenotypic markers for each MBL subclass, namely CLL-type, atypical CLL-type and non-CLL-type. (+) represents higher expression; (-) represents equal expression.

	CLL-type	**Atypical CLL-type**	**Non-CLL-type**
CD19	+	+	+
CD27	+	-	-
CD23	+	-	-
CD79b	-	+	+
CD38	-	+	-
ZAP70	-	+	-
CD49	-	+	-
CD22	-	-	+

Atypical CLL-type MBL and non-CLL-type MBL account for less than 25% of all cases diagnosed with MBL (Maitre and Troussard, 2019). Non-CLL-type MBL tend to have a better prognosis compared to atypical CLL-type MBL, with a median progression time of about 84 months for the first and 45 months for the second (Maitre and Troussard, 2019).

The relationship between MBL CLL-type and Rai 0 CLL (early CLL state) is still being studied. A study carried out by Rossi et al. (2009) compared the clinical and biological profile of 123 patients with MBL with 154 patients with Rai 0 CLL. MBL patients had better humoral immunity scores and lower risk of infection, along with longer survival time if treatment needed. However, all MBL were projected to progress to Rai 0 CLL (Rossi et al., 2009). A

cohort study conducted by Landgren et al., determined that about 98% had pre-diagnosed B-cell clones in peripheral blood obtained 77 months prior to CLL diagnosis (Landgren et al., 2009). This means that MBL is most likely an earlier step in the progress towards CLL development. Another study performed by Kostopoulos et al. (2017) in 227 patients with MBL, showed that more than 1/3 progressed after a 76-month follow-up, regardless of MBL subclass (Kostopoulos et al., 2017).

Recently, several CLL stereotyped subsets with distinct IGHV rearrangements were characterized based on their B-cell receptors contained remarkably stereotyped CDR3 sequences (Murray et al., 2008). These subsets show significant differences in terms of age, sex, CD38 expression, cytogenetic aberrations and mutations and prognostic significance. Patients in the same subset generally follow the same clinical course, whereas patients in different subsets, despite of having the same IGHV and exhibiting similar immunoglobulin mutational status, show different TTFT, OS and clinical course. There is a wide variety of subsets, ranging from very indolent to aggressive disease (Baliakas et al., 2014a).

1.1.5 BCR response and IGHV mutation

As mentioned earlier, each B cell has a BCR that is formed by variable combinations of the V, D and J segments of the immunoglobulin heavy chain and the V and J segments of the light chain (Küppers, 2005). The expression of this B-cell receptor is poorer on MBL and CLL B-cell clones than on normal B-cells (Klein and Dalla-Favera, 2008).

Two distinct groups of IGHV genes can be identified: mutated (M-CLL) and unmutated (U-CLL) which have different clinical pathways (Damle et al., 1999). Patients with non-mutated IGHV have a worse prognosis, a higher need for treatment and a greater probability of progression to more advanced stages (Kröber et al., 2002, Thunberg et al., 2001). The presence or absence of clonal mutations in B cells is the most significant biological difference between these two groups (Damle et al., 1999). Both groups show different levels of ZAP70 protein and CD38 expression. ZAP70 is a protein coding gene that is involved in T cell signaling and is preferentially expressed on normal T lymphocytes (Rosenwald et al., 2001). With regard to CD38 expression, this is much higher in non-mutated cases, and mutated cases respond better to chemotherapy, with better survival rates (Damle et al., 1999). According to Hamblin et al., the CD38 marker presents an even worse prognosis than non-mutated genes (Hamblin et al., 1999). The main differences are represented in figure 1.3.

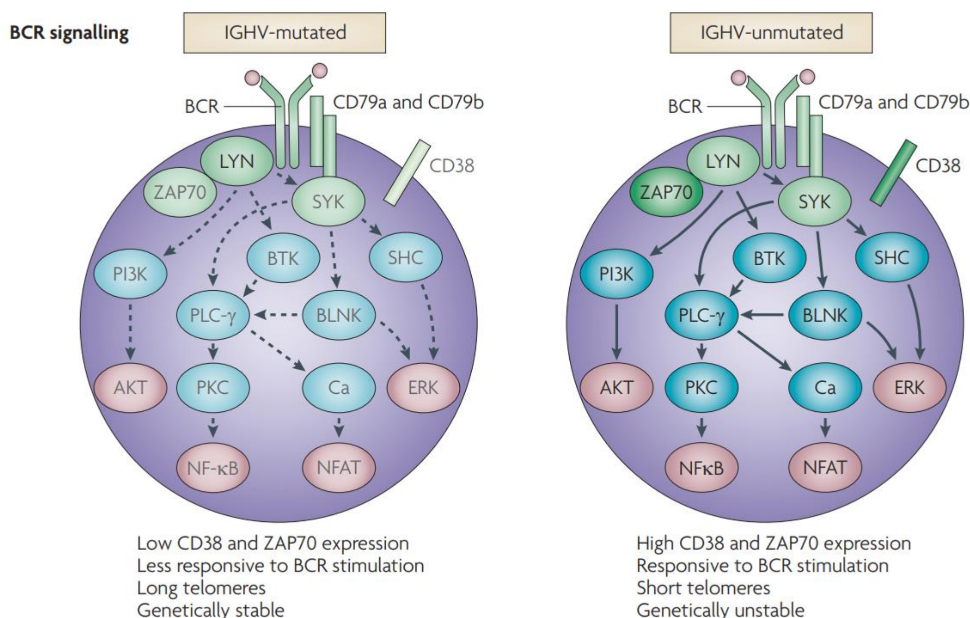

Figure 1.3 - CLLs with unmutated or mutated IGHV genes show markedly different biological and clinical behaviors.
The CLL cells can be differentiated into mutated and unmutated IGHV genes. These two groups present biological differences. IGHV-unmutated cells have higher levels of ZAP70 and CD38 expression, marked as dark green and they can have a better response to BCR activation through signal transduction pathways, which is represented by strong line arrows. Authors support that IGHV-unmutated have greater proliferative capacity with different telomere lengths than the mutated ones. Adapted from (Zenz et al., 2010).

It is not entirely certain where the IGHV-non-mutated and IGHV-mutated genes come from. Several studies point out that IGHV-mutated genes are derived from post-GC B cells (Klein et al., 2001, Zenz et al., 2010). A study from Klein et at. compared the gene expression profile of both mutated and unmutated CLL cells with naïve B cells, GC cells, post-GC memory cells and CD5 blood cells to understand where they were derived from. The results showed similarities between IGHV-mutated genes and post-GC memory cells (Klein et al., 2001). According to Capello et al., approximately one-third of IGHV-mutated cases have mutations in the BCL6 protein, whose expression is restricted to B-post GC cells. These results suggest that CLL cases presenting BCL6 mutations must derive from B cells that have already undergone mutations in GCs (Capello et al., 2000). A study conducted by Pasqualucci et al. also confirms this theory by the fact that analyses of the expression of the BCL6 protein showed that at least part of the CLL cells derive from a cell that was previously exposed to SHM (Pasqualucci et al., 2000). As for IGHV-non-mutated cells, these can also derive from antigen-activating B cells that have acquired memory B-cell characteristics. However, the origin of these B cells is not yet certain, and they may come from naïve B cells, CD5 B cells or marginal zone B cells. (Hoogendoorn et al., 2004, Zenz et al., 2010). On the other hand, it is not yet known whether the activation of these cells is part of the immune response

dependent or T-cell independent pathway (Pede et al., 2013). Some studies already pointed to the T-cell independent pathway as the pathway for IGHV-non-mutated genes (Zenz et al., 2010). This explanation is represented schematically in figure 1.4.

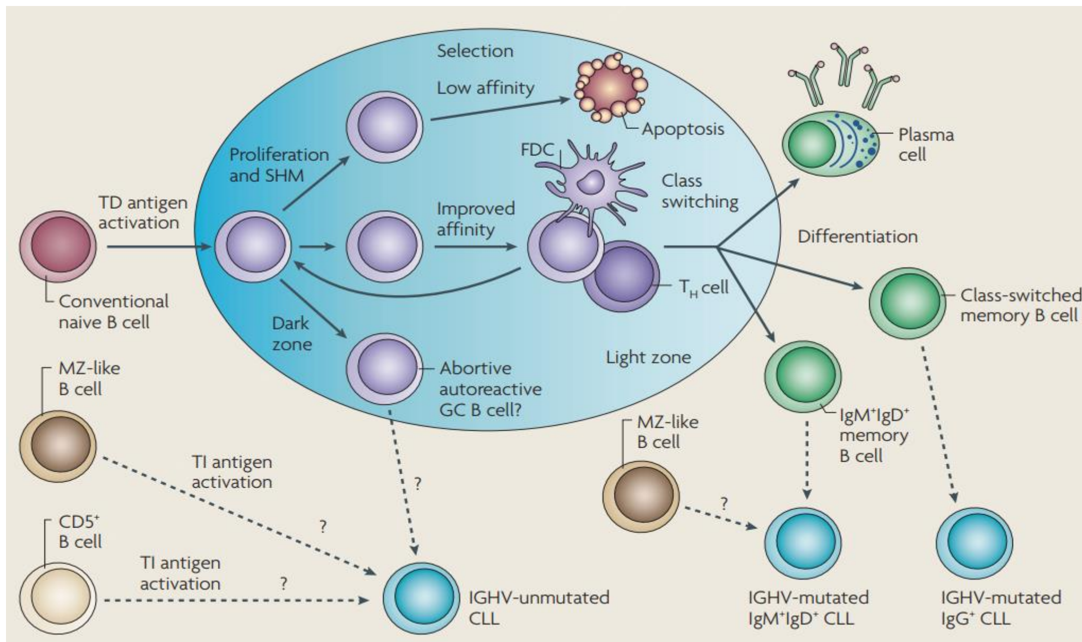

Figure 1.4 – Germinal center reaction and normal B cell differentiation
Several studies support that IGHV-mutated CLL cells could be derived from memory B cells and IGHV-unmutated CLL cells most likely derive from antigen-activated B cells. Adapted from (Zenz et al., 2010).

1.1.6 Oncogenic risk

The possibility of the existence of risks associated with the possible development of the disease has been studied and observed and is currently supported by several lines of evidence (Scarfò et al., 2016).

A large epidemiological study of Slager et al., with approximately 2500 cases of CLL, studied the patients' lifestyle habits, medical and family history and possible occupational risk factors, compared to a control group. The study concluded that the most pronounced and consistent risk factor is a family history of hematological diseases (Slager et al., 2014). The risk of family members developing MBL and CLL with a family history of lymphoproliferative tumors increases by about 1.8 and with leukemia by about 2.2 (Slager et al., 2013). In a population study comparing to controls, first-degree relatives of CLL patients showed a relative risk of 8.5 of developing CLL (Lynn et al., 2009).

In addition to the family background, there are also several infectious agents and environmental factors that may be involved in triggering the disease, through interaction with a compromised

immune system (Morton et al., 2008). Within these factors, contact with farm animals may play a role in controlling the risk of lymphoma, possibly through the transmission of biological agents from bovine, bird and sheep viruses (Cocco et al., 2013). Studies suggest that exposure to endotoxins, a component of the outer membrane of Gram-negative bacteria may play an important role (Morrison and Ryan, 1987). Endotoxins activate B cells through several mechanisms, including the TNF-α released from macrophages (Morrison and Ryan, 1987).

Some well-known risks include exposure to certain chemicals and radiation, as well as work involving contact with heavy solvents and benzene (Mukkamalla et al., 2022). Tobacco users had also a higher risk of developing MBL and CLL compared to non-smokers (Brown et al., 1992).

1.2 Risk of infection

Infections are a major cause of morbidity and mortality when acquired in an individual with a weakened immune system (Langerbeins and Eichhorst, 2021). As both MBL CLL-type disease and chronic lymphocytic leukemia itself are diseases that gradually weaken the immune system, the risk of getting infections is much higher and represents a major concern for patients.

According to Moreira et al., up to 50% of patients with CLL will develop complications due to infections during the course of the disease and between 30-50% of patient deaths with CLL are attributed to infections or their aggravation (Moreira et al., 2013). A risk factor for the development of MBL is exposure to infections and immunodeficiency, with a high mortality rate when admitted to hospital (Fazi et al., 2010).

However, the pattern of humoral and cellular response observed among CLL-type MBL patients does not show significant differences compared to the non-CLL population. Even though the magnitude is slightly lower than in the healthy population. Despite infection, people with MBL can develop durable B-cell or T-cell immunity responses (Blixt et al., 2022).

Dysregulation of this system is one of the key features of MBL CLL-type disease, with a tendency to worsen as it progresses. As a result, a set of deficiencies in the adaptive and innate immune systems can be seen in CLL-type MBL patients (Langerbeins and Eichhorst, 2021).

In this disease, alterations in the adaptive immune response encompass T-cell and B-cell deficiencies (Langerbeins and Eichhorst, 2021). T cells play a central role in the acquisition of immunity by activating or suppressing immune responses through the secretion of cytokines or the destruction of antigen-carrying cells. T cell activation requires the recognition of an antigen presented on the cell or targeting of cells through cytoskeleton-dependent complex processes (Billadeau et al., 2007).

However, T cells show changes in the early stages of the disease and are shown to have greater accumulation throughout disease progression (Forconi and Moss, 2015).

As for the quantitative levels of $CD8^+$ and $CD4^+$ T cells, a significant increase was noted at the beginning of the disease, increasing on average until stage 3 of the disease and a decreasing in the most advanced stage. However, the ratio between CD8 and CD4 T cells decreased (Forconi and Moss, 2015). The mechanism behind this increase in peripheral T-cell count is still partially unknown, though it might involve an increased mobilization of secondary lymphoid tissue or even an absolute increase in T cells that can be interpreted as a specific immune response to MBL CLL-type disease (Forconi and Moss, 2015). Despite these changes in the T-cell count, T-cells also show functional deficiency to modify the immune response (Görgün et al., 2005).

Differential expression of genes is also observed when compared to healthy T-cell donors and in the same age group. These analyses showed differentially expressed genes that are mostly involved in cell differentiation and cytoskeletal pathway formation in TCD4+ cells and in cytoskeletal formation, vesicle transport and cytotoxic pathways in TCD8+ cells (Ramsay et al., 2008). In other words, MBL CLL-type B cells induce changes in cytoskeleton formation and vesicle transport pathways in T cells through cell-cell contact (Görgün et al., 2005).

Hypogammaglobulinemia affects about 80% of patients with CLL in the first 7 years of diagnosis (Dearden, 2008, Langerbeins and Eichhorst, 2021). Immunoglobulin levels are lower than normal (according to age-related) in this condition, which can affect IgG, IgA, and IgM subclasses (Freeman et al., 2013). Patients with low immunoglobulin levels are at increased risk of severe and possibly recurrent infection, and the severity of infection tends to increase with disease progression (Gale et al., 1988), with IgG3 and IgG4 subclasses presenting a higher risk (Forconi and Moss, 2015). IgA and IgG4 deficiencies are most related to respiratory tract infections (Dearden, 2008).

Low immunoglobulin level may be related to cell-to-cell contact with malignant B cells (Dearden, 2008). There is a direct correlation between low IgG levels and the frequency and severity of bacterial infections, most frequently *Streptococcus pneumoniae* and *Haemophilus influenzae* (Dearden, 2008), both bacteria responsible for attacking the lungs and respiratory system.

A study conducted by Freeman et al. in CLL patients showed that of those with recurrent infections, about half had hypogammaglobulinemia, but all had at least one IgG subclass deficiency. In patients without hypogammaglobulinemia and with normal IgG levels, no infections were expressed (Freeman et al., 2013).

Although the levels of hypogammaglobulinemia do not differ much in U-CLL and M-CLL, the infection tends to be more severe in U-CLL cases (Sinisalo et al., 2004).
In a study carried out by Francis et al. with 231 patients, those with U-CLL had a higher morbidity and mortality rate for infections as well as a shorter first infection time when compared to M-CLL cases (Francis et al., 2006).
According to Rossi et al., a study with untreated patients showed that U-CLL tumors were related to a shorter time to initiation of recurrent infections. Overall, infections are functionally rather than quantitatively associated with U-CLL (Rossi et al., 2008).
Although the risk of progression from MBL to CLL has been well characterized, it is not known whether other more serious complications associated with CLL also occur in individuals with MBL (Moreira et al., 2013).

1.2.1 Risk of SARS-CoV-2 severe disease

Studies have shown that MBL was significantly less common among individuals vaccinated against influenza or pneumonia (Casabonne et al., 2012). This raised the possibility of linking monoclonal B-cell lymphocytosis to the SARS-Cov2 pandemic.

A study by Sosa-Hernández indicates that the frequency of CD19 B cells is increased in the most severe cases of COVID-19 relative to less severe cases. Transitional B cells increase in moderate cases, but also decrease in severe cases of the disease. The memory B-cell compartment decreases, and the antibody-secreting cells increase with aggressiveness of the disease. The worsening of COVID-19 is accompanied by changes in both immature and differentiated B-cell sub-populations. Some researchers point out that the existing relationship between B cell subsets and their expression levels could suggest that these lymphocytes could be used in the antiviral adaptive response against SARS-COV-2 (Sosa-Hernández et al., 2020).

Furthermore, several hospitalized patients with severe COVID-19 exhibit high levels of cytokines such as IL-2, IL-6, IL-7, IL-10 and TNFα which are associated with lymphopenia and cytokine storm (Sosa-Hernández et al., 2020).
Due to the induction of lymphocyte apoptosis, lymphopenia is one of the most commonly observed "symptoms" in COVID-19 patients, and it is associated with more severe disease and mortality. Patients with more severe disease show a lower total level of lymphocytes and B cells than patients with moderate disease. This was also observed in some cases with pneumonia caused by the same virus (He et al., 2005).

Hence, COVID-19 may accelerate the course of B-cell derived malignancies. Although the lung is the most affected organ, lesions have been reported in other organs, notably the spleen and bone marrow. The bone marrow microenvironment in patients with multiple myeloma may be affected by SARS-Cov2 due to the high production of IL-6, which promotes T-cell expansion and activation, B-cell differentiation and regulates the phase-acute response, thus creating a favorable microenvironment for oncogenesis (Blixt et al., 2022).
It is therefore possible to presume that, in MM patients who have developed COVID-19 and have not been correctly treated, IL-6 production may play a crucial role in activating a disease in partial remission and initiating uncontrolled production of cells (Blixt et al., 2022).
Patients with CLL may not respond effectively to SARS-Cov2 vaccination. People with hematological disorders and CLL may be more vulnerable, and their condition may worsen if infected with SARS-Cov2 (Blixt et al., 2022).
A study by the European Research Initiative in CLL (ERIC) identified 190 patients with CLL who were positive for SARS-Cov2. Of this set, almost 80% were diagnosed with severe Covid-19, that is, requiring oxygen and intensive care. Most of those with severe disease were of advanced age. Of the patients hospitalized, about 33% did not resist and eventually died (Scarfò et al., 2020).

1.2.2 Risk of hospital acquired infection

Hospital-acquired infections are nosocomial infections (i.e. only acquired at the hospital) (Tikhomirov, 1987). The most frequent are catheter-associated urinary tract infections, bloodstream infections, surgical site infections, ventilator-associated pneumonia or even air-acquired pneumonia, and infections caused by *Clostridium difficile* bacteria, causing diarrhea and inflammation of the colon (Monegro et al., 2022).

Most infections are manifested 48 hours admission of patient at the hospital (Majumdar and Padiglione, 2012). Risk factors for nosocomial infection include advanced age, the duration of hospitalization, associated comorbidities, the use of ventilator supports, invasive procedures, staying in the intensive care unit, and chemotherapy and a weakened immune system (Sydnor and Perl, 2011). These hospital-acquired infections contribute to functional disability and emotional destabilization of the patient, and in some cases can lead to conditions that reduce quality of life (Ponce-de-Leon, 1991).
According to World Health Organization 2002, these infections are as common in developing as in developed countries and occur all over the world. The incidence is higher in developing countries, particularly in the eastern Mediterranean and south-east Asia, at more than 12%,

than in developed countries, where the rate is 5-10%. It is also stated that approximately 1.4 million people worldwide suffer from complications related to hospital infections.

Moreover, nosocomial infections affects a massive number of patients globally, raising mortality rating and increasing morbidity in hospitalized patients (Khan et al., 2017).

According to recent prevalence studies carried out in Europe, 45% of patients in ICUs have a nosocomial infection and 21% of the remaining infections are from ICU-acquired infections (Vincent, 2000).

In 2012, a study on the prevalence of infection and antimicrobial consumption in hospitals was carried out in Europe, revealing that 10.5% of hospitalized patients in Portugal had acquired an infection during hospitalization, while the percentage in all European countries was 6.1% (DGS, 2017, Figure 1.5).

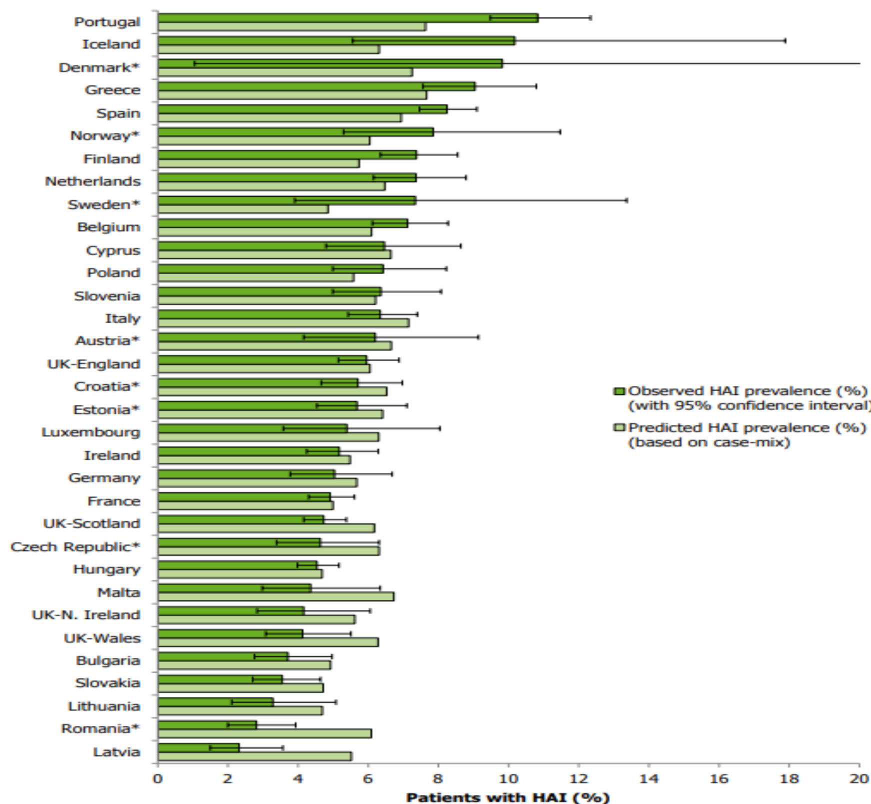

Figure. 1.5 - Prevalence of Healthcare-Associated Infections in ICU in the years 2011-2012 and corresponding predicted prevalence for each European country with 95% confidence interval. Dark green represents the observed prevalence of infections in ICUs and light green represents the predicted prevalence of infections based on hospital characteristics (Adapted from ECDC, 2013)

In 2017 new studies of infection prevalence were carried out in Hospitals and ICUs, with lower values of about 7.8% (DGS, 2017).

Based on what has been previously explained about MBL disease, most patients who carry the disease are asymptomatic and therefore appear to be healthy individuals. It is in this hospital

setting that many patients end up developing nosocomial infections, worsening their conditions and may even die from the infection rather than the disease itself. Early diagnosis could help combat or at least reduce the risk of contracting these infections in hospital, in particular, if health professionals are informed of the patient's condition, particularly on their weak immune system.

1.3 Early detection & biomarkers in CLL/ MBL

The possibility of determining the rate of progression from low count MBL to high count MBL and from high count MBL to CLL through genetic alterations is being increasingly investigated. Genetic alterations are considered diagnostic markers of cancer (Zenz et al., 2011). It is possible to state that certain genetic alterations together with BCR mutational status can increase the rate of disease progression, allowing the creation of an accurate disease prognosis. The use of specific IGVH subsets has been reported: for high-count MBL and for M-CLL cells, namely IGHV3-23 and IGHV4-34; and for U-CLL cells, with IGHV1-69, one of the first genes discovered to be more expressed in CLL patients (Dagklis et al., 2008). MBL CLL-type cells have a higher incidence of IGHV3 genes followed by IGHV4 genes, where the IGHV3-21 gene alone is associated with a more aggressive disease (Dagklis et al., 2008) The most frequent IGHV genes in CLL were IGVH4-34, VH3-30, VH1-69, VH3-48, VH4-39, VH1-2 and VH3-7 (Henriques et al., 2013).

Only a small percentage of cytogenetic alterations occurs at relatively high frequencies (Fabbri et al., 2011). These alterations encompass del(13q14) which is reported in almost half of CLL cases, trisomy 12 which is present in about one third, del(11q) and del(17p) which occur in 5-15% of patients (Döhner et al., 2000, Zenz et al., 2011). According to Henriques et al. (2013), high count MBL clones have shown the type of chromosomal alterations seen in CLL (Henriques et al., 2013).

The most common cytogenetic alteration in CLL is deletion of the long arm of chromosome 13 (del13q14), which is found in 50% of all CLL cases and has previously been associated with a good prognosis (Shanafelt et al., 2006, Stilgenbauer et al., 2007). This genetic alteration appears at earlier stages of the disease (Zenz et al., 2011).

Trisomy is found in 10-20% of patients with CLL and usually appears as a single alteration (40-60%). It is most commonly associated with the atypical CLL-type MBL subclass (Matutes et al., 1996). In terms of prognosis, it is more aggressive than (del13q14) but more favorable than the others (Del Giudice et al., 2012).

Deletion of the long arm of chromosome 11 (del11q22) occurs in about 10-25% of CLL patients and is typically associated with poor prognosis, rapid progression, and reduced survival rates (Döhner et al., 1999, Döhner et al., 1997, Grever et al., 2007). About one fifth of patients who require treatment carried this deletion (Zenz et al., 2011). Moreover, there is a strong association between this deletion and unmutated IGVH status (Henriques et al., 2013).

Patients with CLL and deletion of the short arm of chromosome 17 (17p13) are always included in the unfavorable risk group, presenting rapid disease progression, poor response to treatment and consequently poor survival rates (Stilgenbauer et al., 2009). This deletion is present in about 4-9% of CLL cases at diagnosis (Zenz et al., 2010). The 17p deletion is often associated with mutation of the TP53 gene, which encodes the p53 protein (Zenz et al., 2011). This tumor-suppressor protein is responsible for the regulation of the cell cycle and apoptosis, being involved in the DNA repair process, also triggering apoptosis (Campo et al., 2018). According to Zenz et al., relatively few cases with the 17p deletion have a functional p53 pathway (Zenz et al., 2011).

TP53 aberrations can result from gene mutations such as missense mutations, insertions or deletions, nonsense mutations, or splice-site mutations as well as deletion of the TP53 locus on chromosome 17 (Campo et al., 2018). Figure 1.6 represents the possible combinations for TP53 and del(17p) mutations in CLL compared to the normal karyotype of a person without the disease.

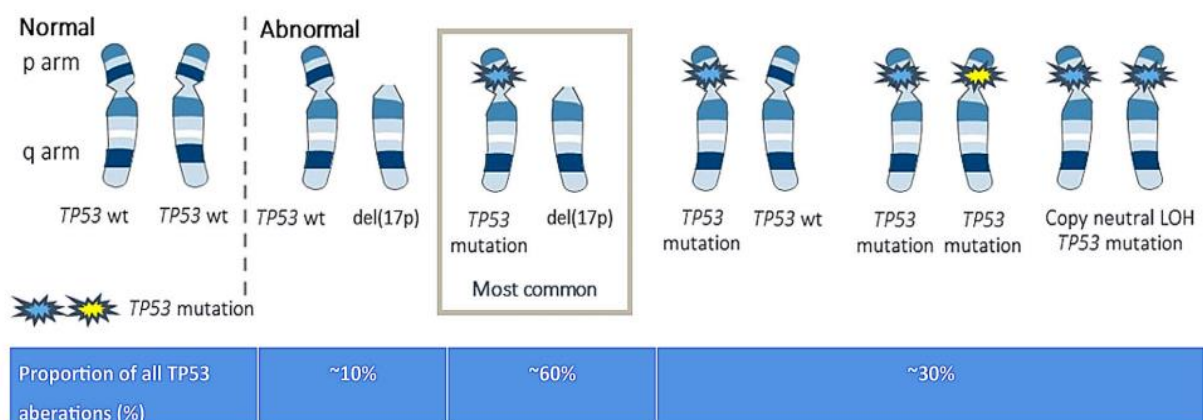

Figure 1.6- Representation of del(17p) and/or TP53 mutations in various combinations, being the most common cause of TP53 aberration in CLL (about 60%) the result of TP53 mutation and del(17p). Adapted from (Campo et al., 2018).

All the alterations previously described can function as biomarkers of disease progression and death, however only del 17p/ TP53 mutation has predictive prognostic value related to the effectiveness of a particular therapy. (Yun et al., 2020). According to Campo et al., clinical trials have already demonstrated the importance of diagnosis based on TP53 mutations as well as deletions in 17p, to select the appropriate treatment regimen for a better patient outcome (Campo et al., 2018).

As such, low count MBL clones have shown lower levels of CLL-associated genetic alterations than high count MBL (Henriques et al., 2013). The t(14q32) and del(11q22.3) were exclusively found in high count MBL and CLL whereas del(17p), del(11q23) and the NOTCH1 gene mutation were only present in CLL clones. The NOTCH1 gene is preferentially detected in the non-mutated clones (Del Giudice et al., 2012).

High-count MBL cases have molecular features associated with a good prognosis of CLL both in terms of chromosomal alterations and IGHV gene repertoire, particularly cases with mutations (Lanasa et al., 2011). According to Vardi et al., low count MBL exhibit a status of somatic hypermutations with some similarities to high count MBL, but with a different repertoire of IGVH genes (Vardi et al., 2013). As progression between the two classes of MBL and subsequently to CLL occurs, the number of cytogenetic alterations also tends to increase and the profile of clonal cells becomes increasingly heterogeneous (Henriques et al., 2013).

One of the most expressed markers is CD38, whose expression is dynamic and high values are associated with IGHV-non-mutated and poor prognosis (Patten et al., 2008). The ERIC and The European Society for Clinical Cell Analysis (ESCCA) have rated some of the 35 potential diagnostic markers for CLL as 'required' and 'recommended'. These 35 markers were selected based on the literature and the WHO classification panels. The "required" markers are: CD19, CD5, CD20, CD23, Kappa and Lambda while the "recommended" markers for more differential diagnosis are: CD43, CD79b, CD81, CD10 and CD200 (Rawstron et al., 2018). Figure 1.7 shows the graph with the ratings attributed to each of the 35 selected markers.

Most of these markers of CLL discussed in this chapter are also seen in MBL and can be used to identify and diagnose patients who are more likely to develop CLL, thus worsening the disease.

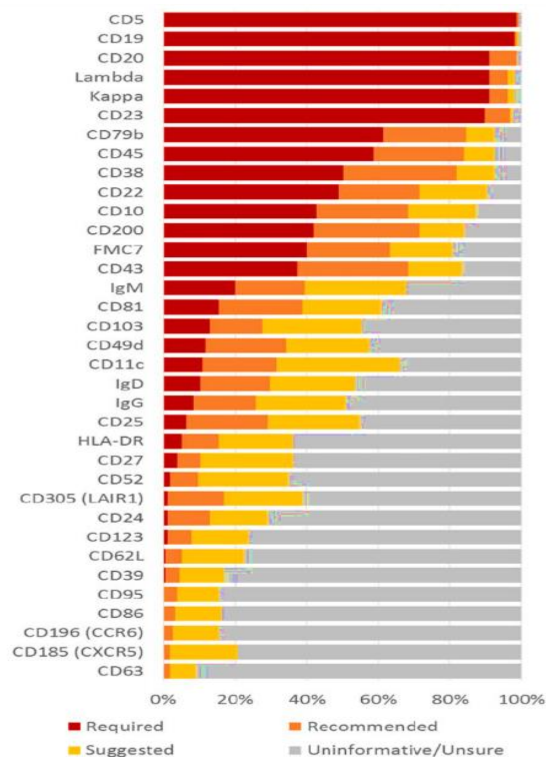

Figure 1.7 – Percentages of participants who assigned the classification according to the legend (necessary, recommended, suggested, not sure) to each of the 35 CLL markers. Adapted from (Rawstron et al., 2018)

1.4 Emerging prognostic methods

As already known, both CLL and MBL diseases are highly heterogeneous, leading to diverse prognoses and, consequently, decisions regarding the need or not for treatment. Thus, to guide and assist in decisions involving the patient's entire clinical process, biomarkers and classification systems are used (Yun et al., 2020).

In recent years an extraordinary amount of progress has been made in identifying diagnostic markers that can accurately predict patient survival time and disease progression, even response to therapy (Yun et al., 2020).

Immunophenotypic markers (CD38, ZAP70...), IGHV mutational status, chromosomal aberrations (del13q, del17p...), and genetic mutations are the most well-known prognostic biomarkers (TP53, ATM, SFB1) (Yun et al., 2020). Regarding immunophenotypic markers, these can assess TTFT in the Binet Stage classification and provide information about OS and disease progression according to the expression levels of a particular marker. The most frequent method of analyzing these markers is by flow cytometry or immunofluorescence microscopy (Yun et al., 2020). With regard to the mutational status of IGHV, knowledge is more extensive, allowing for more complete prognostics regarding OS, progression, and even

classifying some of the mutations into subsets with specific characteristics useful for patient follow-up. The standard method recommended by the ERIC guidelines is sequencing (Yun et al., 2020).

Chromosomal alterations and gene mutations are very much interconnected, since most mutated genes are located on the same chromosomes. They provide valuable information in prognosis, OS, evolution and mainly with concerns to making treatment decisions and designing clinical trials. The most prominent methods for detecting these biomarkers have been fluorescence in situ hybridization (FISH), karyotype analysis, and next-generation sequencing (Yun et al., 2020).

To avoid repetition of information regarding the prognosis and to facilitate analysis, some parameters defined as the most essential and relevant have been selected, involving genetic, clinical, and biological information (Wierda et al., 2007). The currently most used scoring prognostic system is the CLL International Prognostic Index (CLL-IPI), which combines both clinical parameters and cytogenetic factors into a prognostic model (Hallek, 2019). Currently the most important prognostic markers are age, clinical stage, del17p/TP53 status, IGHV mutational status and serum β2-microglobulin (Lancet Oncol., 2016).

CHAPTER 2 – STUDY OBJECTIVES

The main objective of this study is to develop methods to screen the progression of monoclonal B-cell lymphocytosis to CLL, based on the complementarity of two different molecular markers with a lot of weight in the patient's outcome:

- The mutational status of the IGHV gene
- Deletion of 17p, at the TP53 *locus*, by a Copy Number analysis method.

The combined analysis of both methods is expected to provide a broader and adequate prognosis information. The developed methods will be used for a population screening pilot study and for ascertaining the genetic factors contributing to the identification of patients at risk of progression from MBL to CLL, thus allowing early diagnostics/surveillance and better treatment outcomes.

CHAPTER 3 – STUDY DESIGN, MATERIAL AND METHODOLOGIES

3.1 Study population

The baseline study was performed using 18 peripherical blood (PB) samples containing DNA from purified cells corresponding to 11 adult patients with CLL, MBL, LF or SMZL from the Banco Nacional de ADN Carlos III from the University of Salamanca. Cell sorting was performed on each sample to separate the normal B cells from the pathologic clones. All samples initially went through an immunophenotypic flow cytometry, performed to determine the percentage of the clone size of each sample, comparing the cells with aberrant phenotype to the total cellularity of the sample.

The following 2 tables, namely table 3.1 and table 3.2 contain information about the diagnosis of patients and the information about follow up outcomes.

Table 3.1 – Information about the diagnosis of the samples used in the study and their corresponding characteristics, namely if the DNA are from pathological or normal clones as well as the diagnosis of the disease, the Binet class, clone size. *Clone size – percentage of cells with aberrant phenotype compared to the total cellularity of the sample.

Patient	Diagnosis	Binet at diagnosis	Clone size*	Cells
A	CLL	B	79,9	Pathological and normal DNA
B	CLL	A	72,69	Pathological and normal DNA
C	CLL	A	90,25	Pathological and normal DNA
D	Atypical CLL	-	88,4	Pathological and normal DNA
E	MBLhi	-	31,2	Pathological and normal DNA
F	MBLhi	-	43,27	Pathological and normal DNA
G	SMZL	-	86,33	Pathological and normal DNA
H	LF	-	88,63	Pathological DNA
I	CLL	B	72,4	Pathological DNA
J	CLL	C	95,94	Pathological DNA
K	CLL	A	97	Pathological DNA

Table 3.2 – Basic information about each of the patients and about the follow-up after diagnosis, namely sex, age at diagnosis, duration of study post diagnosis in years, first relapse and time of first relapse in less than 11 years post diagnosis, last follow up in years and the vital status (<11 years from diagnosis).

Patient	Sex	Date of birth	Age at diagnosis	Age at study	Study post diagnosis (years)	First relapse (*)	Time of first relapse (*)	Last Follow up (years)	Vital Status
A	M	01/1953	59	59	>5 years	Yes	0,4	5,3	Alive
B	M	09/1950	55	61	5-10	No	-	7,2	Deceased
C	F	04/1941	63	68	5-10	Yes	4,9	8,9	Alive
D	F	08/1958	-	-	-	-	-	-	-
E	M	08/1940	-	-	-	-	-	-	-
F	M	12/1944	-	-	-	-	-	-	-
G	M	08/1978	-	-	-	-	-	-	-
H	M	01/1946	-	-	-	-	-	-	-
I	M	05/1931	80	80	<5	Yes	0,5	6,9	Alive
J	M	03/1970	33	41	5-10	Yes	6,7	11	Alive
K	M	04/1961	45	49	<5	Yes	3,4	11	Alive

3.2 DNA quantification

A quantitative analytical method was used to determine the DNA concentration of the patient samples. The NanoDrop™ One with Acclaro Sample Intelligence software was used. The concentration is given in ng/µL and the purity indicator is measured using the wavelength ratios of 260/280 and 260/230.

3.3 Analysis of clonality

As the purpose of this study is to evaluate a method for detecting the progression from MBL to CLL, all of the patients analyzed had already been diagnosed. Therefore, the following results are intended to support and confirm the previously established diagnosis.

Assessment of clonality is an important factor to detect neoplastic lesions (Perren and Komminoth, 2001). Neoplasm cells are known to be clusters of cells that share the same characteristics and possibly originate from the proliferation of a single common precursor, which makes it expected that most cells will contain similar DNA sequences. This designation is called monoclonality (Gazzola et al., 2014). The distinction between monoclonal and

polyclonal is based on their origin, that is, polyclonal cells are secreted by different B cell lineages while monoclonal cells come from a single B cell lineage (Singh et al., 2014).

During the development of B cells, the fragments V, D and J of the Ig genes become rearranged, originating specific and unique sequences. The lymphoid cells that then result from mutations of these modified cells have clonal IG regions and can later be used as specific markers of cancer cells (Tonegawa, 1983).

It is common to identify clonal rearrangements of IGHV genes in B-cell pathologies and can thus be characterized by the expansion of these cells that have undergone changes during differentiation. According to our current knowledge, there are a leader region, four generally conserved framework regions (FR1, FR2, FR3, and FR4) and three hypervariable regions known as complementary determining regions (CDR1, CDR2, and CDR3) (Nikiforova et al., 2007). For the identification of most V/D/J rearrangements, amplification is performed using a leader primer or FR1, FR2, or FR3 primer (forward) and a JH primer (reverse) placed in the FR4 region. Leader primers originate the biggest PCR products (up to 500bp), giving the most reliable amplification. The FR1-JH combination of primers produces the second biggest PCR products (up to 400 bp), the FR2-JH combination generates up to 280 bp, and the FR3-JH combination generates the shortest PCR products (70–170 bp) (Nikiforova et al., 2007).

Figure 3.1 shows a global view of the IGH gene with the constituent regions V, D, and J. The arrows illustrate what each primer mix amplifies. Leader primers allow the amplification of the entire rearranged IGHV-IGHD-IGHJ gene sequence.

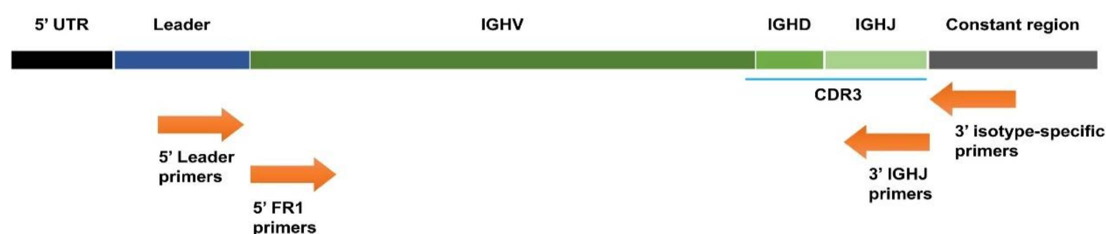

Figure 3.1 – Schematic representation of an IGHV-IGHD-IGHJ gene rearrangement with primer annealing sites indicated. IGHV 5' leader primers anneal to the leader sequence found upstream of the IGHV coding region. 5' IGHV framework (FR) primers anneal to the beginning of the FR1 region within the rearranged IGHV gene, whereas 3' IGHJ primers anneal to the ending of the rearranged IGHJ gene. UTR: Untranslated region. Adapted from (Abayomi et al., 2021, Agathangelidis et al., 2018).

According to Gazzola et al., the distance between primers must not pass 500bp with the aim of being able to distinguish clearly between monoclonal and polyclonal Ig gene rearrangements (Gazzola et al., 2014). On agarose gel electrophoresis, a well-defined band will appear if a significant population of cells contains the same unique IGH rearrangement. Unlike monoclonal B cells, polyclonal B cells produce a ladder or smear of bands or peaks (Nikiforova et al., 2007).

The clonality analysis was performed using two different methods that will be discussed and compared later. Therefore, the tests are divided into Part I and Part II.

3.3.1 Part I

3.3.1.1 PCR amplification

To analyze the clonality of the DNA samples, it was performed a PCR using the commercial kit assay from Master Diagnostica named "IgH REARRANGEMENTS MOLECULAR ANALYSIS KIT" following the procedure proposed by the manufacturer. Using multiple primers that hybridize with conserved gene regions, this kit amplifies rearranged VDJ segments in the hypervariable region of the immunoglobulin heavy chain (IGH), allowing the detection of clonality in B cell lymphoproliferative processes. The DNA was amplified using four mixtures of primers: one for each of the fragments FR1, FR2 and FR3 and an internal control mix.

In the electrophoresis analysis, clonal rearrangement of the IGH gene is indicated by the presence of a single, strong band of the expected size. A sample is considered positive (monoclonal) when either or both of the two test amplification mixes (FR1-JH, FR2-JH or FR3-JH) detect a band with these characteristics. When there is no clonal rearrangement and the test sample consists of a heterogeneous polyclonal population of B cells, it will form a heteroduplex and electrophoresis reveals multiple bands. The expected size ranges of amplified fragments for each amplification mix are shown in Table 3.3.

Table 3.3 - Expected size range of amplified fragments for each amplification mix

Primer Mix	Size Range	Clonal Positive Control
FR1-JH	310-360 bp	338 bp
FR2-JH	250-295 bp	274 bp
FR3-JH	100-170 bp	133 bp

To each mixture was added 0,4µL of DNA polymerase (Phire® Hot Start II) and 1,5µL of the same DNA sample was added to the four different mixtures. A polyclonal and monoclonal DNA were used as positive controls.
The PCR conditions are shown in table 3.4.

Table 3.4 – Thermocycle conditions for the PCR amplification of FR1, FR2 and FR3 fragments and internal control.

Cycle step	Temperature	Duration	Cycle number
Initial denaturation	98°C	2 min	1
Denaturation	98°C	10 s	35
Annealing	60°C	10 s	
Extension	72°C	15 s	
Final extension	72°C	1 min	1

3.3.1.2 Electrophoresis

PCR products obtained in the previous steps were then revealed by electrophoresis gel with 4% agarose covered with TAE 1X buffer. The electrophoresis gel was stained by 3% GreenSafe Preminum (Nzytech, Portugal) containing TAE 1X buffer. The gel ran at 140 mV for 50 min, with visualizations at each 25 min interval.

3.3.2 Part II

3.3.2.1 PCR amplification

Clonality was tested using a protocol described by Agathangelidis et al. published on jove. Instead of using a pre-made kit, all primers were prepared individually (Agathangelidis et al., 2018). Two PCRs were performed with 2 different specific forward primer sets, one for the FR1 region and another for the leader region, and a set of common to both containing JH primers (reverse). The primers used in the amplification reactions are shown in Table 3.5.

Table 3.5– Primer sequences used for PCR amplification of the clonotypic IGHV-IGHD-IGHJ gene rearrangement

5'IGHV FR1 primers	Primer sequence
IGHV1	CAGGTGCAGCTGGTGCAGTCTGG
IGHV2	CAGGTCAACTTAAGGGAGTCTGG
IGHV3	GAGGTGCAGCTGGTGGAGTCTGG
IGHV4	CAGGTGCAGCTGCAGGAGTCGGG
IGHV5	GAGGTGCAGCTGTTGCAGTCTGC
IGHV6	CAGGTACAGCTGCAGCAGTCAGG
5'IGHV leader primers	
IGHV1aL	AAATCGATACCACCATGGACTGGACCTGGAGG
IGHV1bL1	AAATCGATACCACCATGGACTGGACCTGGAGA
IGHV1bL2	AAATCGATACCACCATGGACTGGACCTGGAGC
IGHV2aL	AAATCGATACCACCATGGACACACTTTGCTMAC
IGHV2bL	AAATCGATACCACCATGGACATACTTTGTTCCAC
IGHV3aL	AAATCGATACCACCACCATGGAGTTTGGGCTGAGC
IGHV3bL	AAATCGATACCACCACCATGGARYTKKGRCTBHGC
IGHV4L	AAATCGATACCACCATGAAACACCTGTGGTTCTT
IGHV5L	AAATCGATACCACCATGGGGTCAACCGCCATC
IGHV6L	AAATCGATACCACCATGTCTGTCTCCTTCCTC
3'IGHJ primers	
IGHJ1-2	TGAGGAGACGGTGACCAGGGTGCC
IGHJ3	TGAAGAGACGGTGACCATTGTCCC
IGHJ4-5	TGAGGAGACGGTGACCAGGGTTCC
IGHV6	TGAGGAGACGGTGACCGTGGTCCC

It was performed a mixture with reagents for the amplification reactions in a sterile 1,5mL microtube. The reaction conditions were determined for a final volume of 50µL, with the addition of 2µL of DNA for each sample. Table 3.6 shows all the reagents used in the mixture as well as their corresponding concentrations.

Table 3.6 – Reagents concentrations used for the PCR amplification of the clonotypic IGHV-IGHD-IGHJ gene rearrangement to a final volume of 50µL

Reagents	Final concentration
Reaction buffer (10x)	1x
$MgCl_2$ (50mM)	1,5mM
dNTPs (10mM)	200µM
Primer IGHV leader/FR1 mix (100µM)	10µM
Primer IGHJ mix (100µM)	10µM
Taq DNA polymerase (5U/µL)	2,5 U
H_2O	--

The PCR run on BIO-RAD T100 Thermal Cycler and the conditions are shown in table 3.7.

Table 3.7 - Thermocycle conditions for the PCR amplification of the clonotypic IGHV-IGHD-IGHJ gene rearrangement

Cycle step	Temperature	Duration	Cycle number
Initial denaturation	94°C	5 min	1
Denaturation	94°C	1 min	39
Annealing	59°C	1 min	
Extension	72°C	1,5 min	
Final extension	72°C	10 min	1
Preservation	18 °C	∞	1

3.3.2.2 Electrophoresis

PCR products obtained in earlier steps were then visualized on an agarose gel containing 2% agarose and 1X TAE electrophoresis buffer. The electrophoresis gel was stained with 3% GreenSafe Preminum (Nzytech, Portugal) and ran at 140 mV for 60 minutes, with visualizations occurring every 20 minutes, so that the separation of the monoclonal samples from the polyclonal background could be observed. A DNA size marker was added to identify the size of the different amplicons.

The results obtained led us to modify the initial protocol such as: the concentration of DNA added, the percentage of the agarose gel and the volume of $MgCl_2$. Regarding DNA concentration, a 1:4 dilution was made, keeping the same volume and the volume of $MgCl_2$ was reduced by half.

3.4 Somatic hypermutation

3.4.1 PCR product purification

According to the most recent guidelines published by ERIC, it recommends the use of IGHV leader primers to analyze the IG gene sequence (Rosenquist et al., 2017). Leader primers allow the amplification of the entire rearranged IGHV-IGHD-IGHJ gene sequence allowing more accurate detection of clonality and, consequently, of somatic hypermutation. Therefore, only PCR products containing the leader primers that showed clonality were selected to be sequenced.

The samples were then purified to eliminate polyclonal background from normal B cells within the CLL sample as well as primer dimers that could lead to suboptimal sequencing.

The EXOSAP (Exonuclease I and Shrimp Alkaline Phosphatase, Thermo Scientific, USA) method was used. It was added to each sample: 0.5uL of Exo and 1uL of Sap and placed on the thermocycle for 30 minutes at 37°C to incubate the reactions followed by 15 minutes at 80°C to inactivate the enzyme. Then, it was added 2,5µL of the sequencing primer, 2,5µL of sterile water and 5µL of DNA sample. For regions with more than 1 primer, a combination of the two was made, replacing the volume of sterile water with the other primer.

3.4.2 Sanger Sequencing

The samples were sequenced at STABVIDA using the Sanger Sequencing method.

The sequencing primer was designed specifically to bind to the IGHV leader primers, with the following sequence: 5'-AAATCGATACCACCATGGACTGGACCTGGAG-3'. However, this primer only identified mutations in the IGHV1 region so the samples that failed to sequence with the previously used primer were sequenced again with different primer sets. There were used leader primers from the amplification step, as shown in table 3.5, encompassing the other 5 missing variable regions under study.

3.5 Copy Number Variations

Del(17p) is one of the most common abnormalities affecting the TP53 gene, about two-thirds of the cases, whereby approximately 80% of patients harboring del(17p) also carry TP53 mutations in the second allele (Campo et al., 2018).

The analysis of the p53 gene copy number was performed, to find out whether the gene contains deletions or gains compared to the reference copy number. As so, it was used a method of relative quantitation named TaqMan™ Copy Number Assays from Applied Biosystems™ (USA). The predesigned TaqMan Copy Number Assay collection consists of human and mouse assay libraries for CNV analysis.

TaqMan Copy Number Assays are run together with a TaqMan Copy Number Reference Assay in a duplex qPCR reaction. The reference assay identifies a sequence that is known to be present in two copies in the diploid genome, while the copy number assay detects the target sequence.

3.5.1 PCR amplification

It was followed the protocol TaqMan™ Copy Number Assays from Applied Biosystems™, according to the indications of the manufacture. Each sample was diluted to 5 ng/μL with nuclease–free water. The PCR used was Step One by Applied Biosystems™ and the conditions are shown in table 3.8.

Table 3.8- Thermocycle conditions for the PCR amplification

<table>
<tr><th>Cycle step</th><th>Temperature</th><th>Duration</th><th>Cycle number</th></tr>
<tr><td>Initial denaturation</td><td>95°C</td><td>10 min</td><td>1</td></tr>
<tr><td>Denaturation</td><td>95°C</td><td>15 sec</td><td rowspan="2">40</td></tr>
<tr><td>Annealing/ Extension</td><td>60°C</td><td>1 min</td></tr>
</table>

The results were analyzed by the relative quantitation method using Applied Biosystems™ CopyCaller Software. The number of copies of the target sequence in each test sample is determined by the measurement of the Ct difference (ΔCt) between the target and reference sequences. Then, the software compares the ΔCt values of test samples to a calibrator sample known to have two copies of the target sequence.

CHAPTER 4 – RESULTS

4.1 DNA quantification

In measurements of absorbance, every molecule in the sample that absorbs at the targeted wavelength is considered. The absorbance ratio 260/280 is considered a useful indicator of protein contamination and it is generally used to evaluate the purity of DNA and RNA. As we are using DNA, when the ratio is ≥ 1.8, it indicates a pure DNA sample. A secondary metric for determining the purity of nucleic acids is the ratio 260/230. The values for "pure" nucleic acid are frequently greater than the respective 260/280 values. Expected 260/230 values are generally represented between 2.0-2.2.

Table 4.1 show the concentrations and absorbance ratios corresponding to the set of purified DNA samples.

Table 4.1 – Representation of concentrations (ng/µL), wavelength ratios of 260/280 and 260/230 from each sample (1-18).

Patient	Sample	Type of Sample	Concentration (ng/µL)	A260/A280	A260/A230
A	1	Pathological	39.62	1.93	1.13
	17	Normal	60.99	1.95	1.23
B	2	Pathological	50.36	1.91	1.15
	14	Normal	75.36	1.91	1.17
C	10	Pathological	42.43	1.93	0.90
	3	Normal	44.68	1.90	0.96
D	4	Pathological	25.56	1.83	0.75
	8	Normal	55.26	1.86	1.13
E	5	Pathological	38.72	1.87	0.88
	12	Normal	67.48	1.90	1.43
F	6	Pathological	89.14	1.86	1.34
	7	Normal	81.46	1.86	1.30
G	15	Pathological	39.48	1.90	0.97
	13	Normal	36.93	1.89	0.90
H	9	Pathological	26.12	1.89	0.72
I	11	Pathological	54.79	1.90	1.18
J	16	Pathological	32.70	1.95	0.98
K	18	Pathological	42.77	1.89	1.05

Sample 6 had the highest concentration (89.14 ng/µL) and samples 4 and 9 had the lowest concentration (25.56 ng/µL and 26.12 ng/µL, respectively). The average concentration was 50.21 ng/µL ± 17,89.

As observed in table 1, ratio 260/280 is in line with what is expected, indicating that the sample has no contaminating proteins and can be considered a pure sample. However, the ratio 260/230 is lower than expected, particularly smaller than 1.8. These could be a strong indicator of any contaminants that absorb at 230 nm. The contamination could be cause by organic compounds or chaotropic agents, namely EDTA, carbohydrates, phenol, TRIzol reagent and guanidine HCL which all have absorbance near 230 nm.

4.2 Analysis of clonality

4.2.1 Part I

The aim of this test was to detect the clonality of the B cells in peripherical blood and was performed with 18 samples from 11 patients aged between 44 and 91, as described in table 3.1 and 3.2.

The results obtained by PCR amplification were then visualized by electrophoresis on a 4% agarose gel, as represented in figure 4.1.

The negative control didn't show any amplification, which means it appeared as a blank space for all the mixed tested, including internal control. As can be observed in figure 4.1, the positive polyclonal control showed multiple bands within the expected size range for each amplification mix. The clonal positive control gave a single band for each mix, meaning both positive controls were correctly operating. The internal control was valid in all the samples, showing an intense band of 274 base pairs, indicating that the sample handling and DNA quality were adequate.

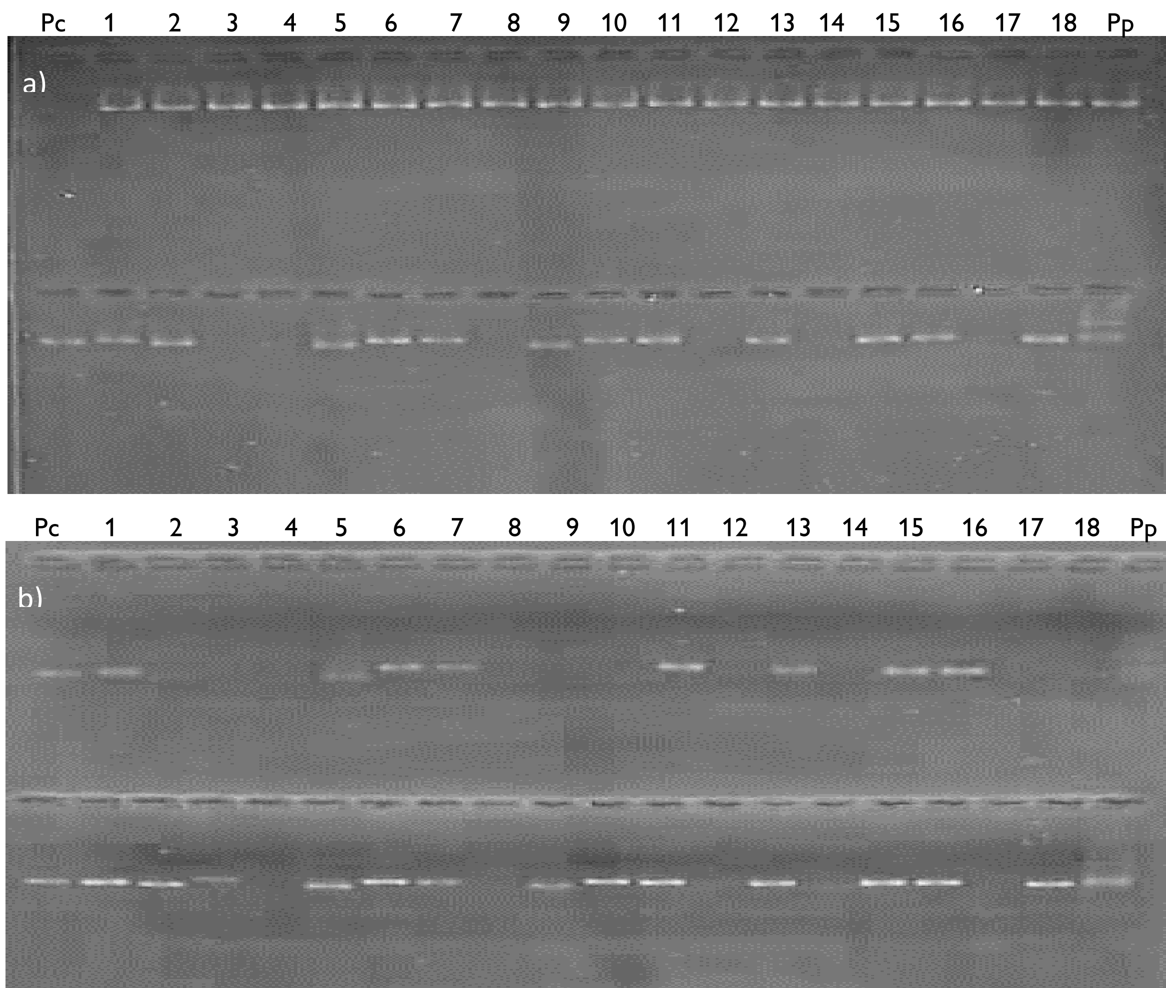

Figure 4.1- Illustration of the obtained electrophoresis gels corresponding to the IG gene amplification by PCR in 18 samples. In image **a)**, the first line represents the internal control and the second one represents the amplification of the FR2-JH region. In image **b)**, the first line represents the amplification of the FR3-JH and the second line represents the amplification of FR1-JH. **(1)** Patient A-Pathological DNA; **(2)** Patient B-Pathological DNA; **(3)** Patient C-Normal DNA; **(4)** Patient D-Pathological DNA; **(5)** Patient E-Pathological DNA; **(6)** Patient F-Pathological DNA; **(7)** Patient F-Normal DNA; **(8)** Patient D-Normal DNA; **(9)** Patient H-Pathological DNA; **(10)** Patient C-Pathological DNA; **(11)** Patient I-Pathological DNA; **(12)** Patient E-Normal DNA; **(13)** Patient G-Normal DNA; **(14)** Patient B-Normal DNA; **(15)** Patient G-Pathological DNA; **(16)** Patient J-Pathological DNA; **(17)** Patient A-Normal DNA; **(18)** Patient K-Pathological DNA; **(Pc)** Clonal B positive control DNA; **(Pp)** Polyclonal positive control DNA

As we can see in figure 4.1, samples 1, 2, 5, 6, 7, 11, 13, 15 and 16 presented clonality in the three amplified regions, with all bands appearing at the size corresponding to the clonal positive. Samples 4, 8, 12, 14 and 17 exhibited no amplification for any of the regions under study. Sample 3 appears positive only on amplification with FR1-JH and the clonality of samples 9, 10 and 18 cannot be detected on amplification of the FR3-JH region. The only sample showing polyclonality is sample 5, being detected in FR3-JH but with FR1 and FR2 shows monoclonality. The summary of these results is represented and organized in table 4.2.

Table 4.2 – Representation of the PCR amplification results obtained in the agarose gel of the 18 samples tested for each mix: FR1-JH, FR2-JH and FR3-JH.

Patient	Sample	Type of Sample	FR1-JH	FR2-JH	FR3-JH
A	1	Pathological	Monoclonal	Monoclonal	Monoclonal
	17	Normal	-	-	-
B	2	Pathological	Monoclonal	Monoclonal	Monoclonal
	14	Normal	-	-	-
C	10	Pathological	Monoclonal	Monoclonal	-
	3	Normal	Monoclonal	-	-
D	4	Pathological	-	-	-
	8	Normal	-	-	-
E	5	Pathological	Monoclonal	Monoclonal	Polyclonal
	12	Normal	-	-	-
F	6	Pathological	Monoclonal	Monoclonal	Monoclonal
	7	Normal	Monoclonal	Monoclonal	Monoclonal
G	15	Pathological	Monoclonal	Monoclonal	Monoclonal
	13	Normal	Monoclonal	Monoclonal	Monoclonal
H	9	Pathological	Monoclonal	Monoclonal	-
I	11	Pathological	Monoclonal	Monoclonal	Monoclonal
J	16	Pathological	Monoclonal	Monoclonal	Monoclonal
K	18	Pathological	Monoclonal	Monoclonal	-

4.2.2 Part II

In this test the same samples were used as in the previous test, namely 18 samples from 11 patients, as described in table 3.1 and 3.2, to detect clonality.

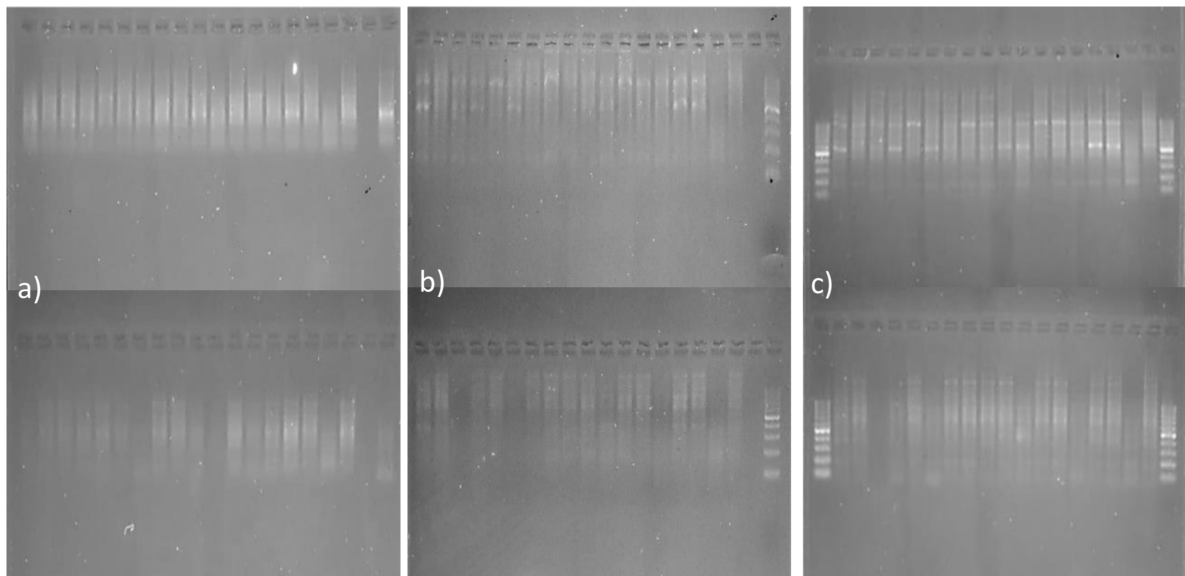

Figure 4.2- Electrophoresis gel representation of the optimization process of PCR amplification of the IGHV-IGHD-IGHJ gene. Image a) illustrates the first attempt with 2% agarose gel following the methodology. Image b) illustrates the second attempt with 4% agarose gel and with DNA dilution of 1:4. Image c) illustrates de third attempt with 2% agarose gel, and with DNA dilution of 1:4.

As we can see in figure 4.2 a), the first electrophoresis gel obtained did not show clear results. So, to improve the quality of the results, an optimization process was conducted by changing the percentage of agarose and the concentration of the DNA. There was an improvement in the results between the three electrophoresis gels, presenting thinner and sharper bands, being able to better differentiate the bands of different sizes. As in figure 4.2 a) the gel showed a smear in all bands, the sample could have excess DNA, so this was diluted 1:4. The 2% agarose gel showed more intense fluorescence compared to the 4% agarose gel (figure 4.2 a) and 4.2 b)). However, in figure 4.2 c) it was still possible to see a smear that made it difficult to visualize the bands, so a new PCR was performed, changing the Mg2+ concentration.

The final results obtained by new PCR amplification were visualized by electrophoresis on a 2% agarose gel, as represented in figure 4.3.

The existence of a single, strong band of the expected size of each primer mix on electrophoresis indicates a clonal rearrangement of the IGH gene. A sample is considered positive (monoclonal) when a 500bp band is detected for the leader primers and a 350bp band for the FR1 primers. When there is no clonal rearrangement and the test sample consists of a heterogeneous polyclonal population of B cells, electrophoresis reveals multiple bands, and the result is polyclonal.

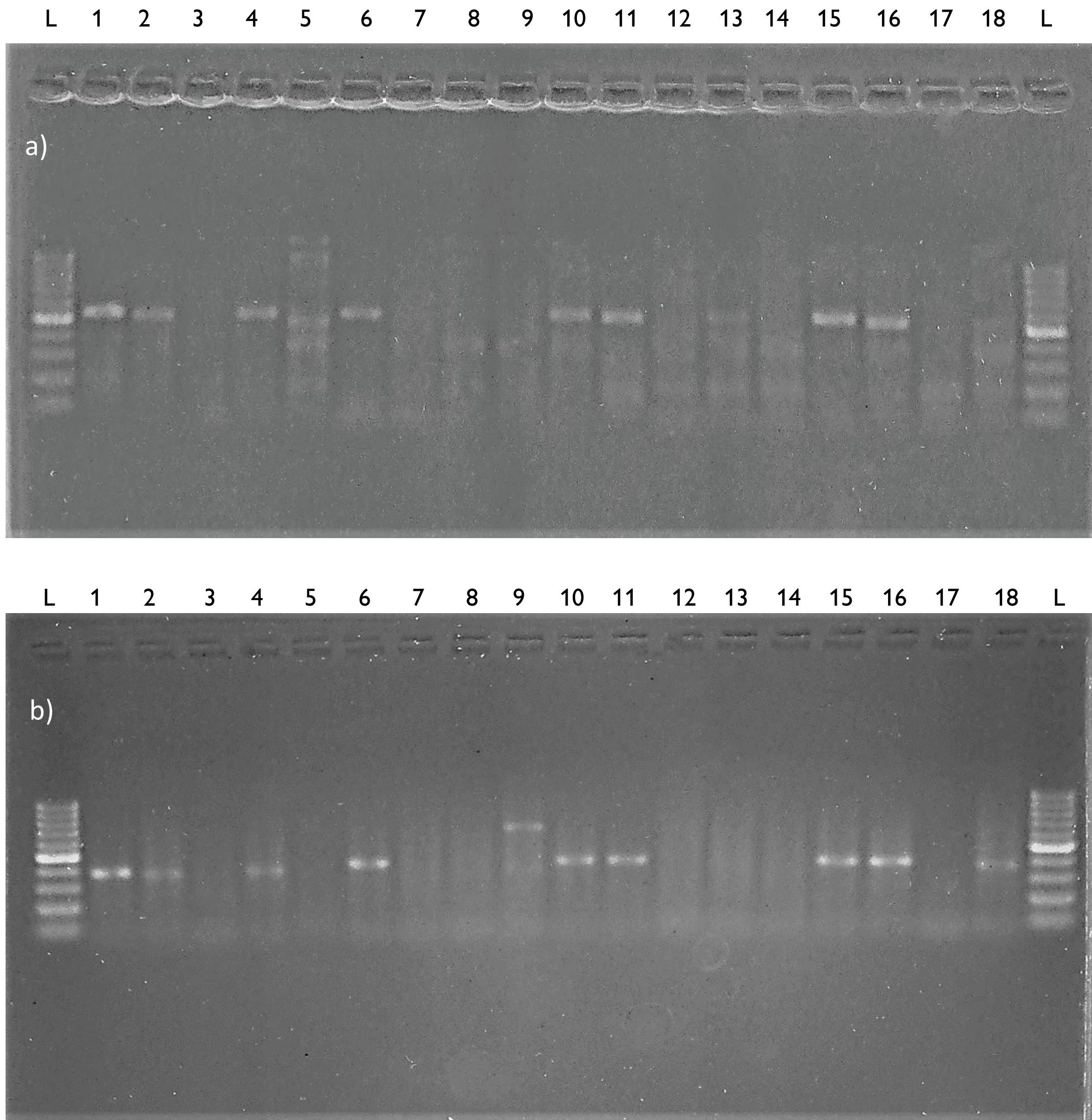

Figure 4.3- Illustration of the obtained electrophoresis gels corresponding to the IGHV-IGHD-IGHJ gene PCR amplification in 18 samples. Image **a)** represents the amplification of the IGHV-IGHD-IGHJ gene rearrangement using 5' IGHV leader primers. Image **b)** represents the amplification IGHV-IGHD-IGHJ gene rearrangement using IGHV FR1 primers. **(1)** Patient A-Pathological DNA; **(2)** Patient B-Pathological DNA; **(3)** Patient C-Normal DNA; **(4)** Patient D-Pathological DNA; **(5)** Patient E-Pathological DNA; **(6)** Patient F-Pathological DNA; **(7)** Patient F-Normal DNA; **(8)** Patient D-Normal DNA; **(9)** Patient H-Pathological DNA; **(10)** Patient C-Pathological DNA; **(11)** Patient I-Pathological DNA; **(12)** Patient E-Normal DNA; **(13)** Patient G-Normal DNA; **(14)** Patient B-Normal DNA; **(15)** Patient G-Pathological DNA; **(16)** Patient J-Pathological DNA; **(17)** Patient A-Normal DNA; **(18)** Patient K-Pathological DNA; **(L)** 100bp DNA ladder;

The negative control showed no amplification, validating the test. The samples amplified by the leader primers are displayed in figure 4.3 a). In samples 1,2,4,6,10,11,15 and 16, were observed a clear band with strong fluorescence with a size of 500bp, thus confirming monoclonality. If observed closely, samples 13 and 18 appear to exhibit a very weak band at

size 500bp. Sample 5 showed no amplification with FR1 primers but two bands ranging in size between 500 and 400bp appeared using leader primers, and thus can be classified as polyclonal. However, as the two bands are repeated in sizes between 900 and 1000, meaning that they have duplicated bands, it can be classified as oligoclonal.

Samples amplified with the FR1 primers are depicted in figure 4.3 b). Samples 1,2,4,6,10,11, 15,16 e 18 were amplified at a size of 350bp, being classified as monoclonal. Sample 13 showed no amplification with primers FR1 and sample 9 showed a band in the range of approximately 700-800bp. By comparing with amplification with leader primers, sample 5 showed neither monoclonality nor polyclonality with the FR1 primers.

The summary of the results illustrated in figure 4.3 regarding the monoclonality of the samples is shown in table 4.3 for each primer mix.

Table 4.3 - Representation of the PCR amplification results obtained in the agarose gel of the 18 samples tested for Leader primers and FR1 primers.

Patient	Sample	Type of Sample	Leader	FR1-JH
A	1	Pathological	Monoclonal	Monoclonal
	17	Normal	-	-
B	2	Pathological	Monoclonal	Monoclonal
	14	Normal	-	-
C	10	Pathological	Monoclonal	Monoclonal
	3	Normal	-	-
D	4	Pathological	Monoclonal	Monoclonal
	8	Normal	-	-
E	5	Pathological	Oligoclonal	-
	12	Normal	-	-
F	6	Pathological	Monoclonal	Monoclonal
	7	Normal	-	-
G	15	Pathological	Monoclonal	Monoclonal
	13	Normal	Monoclonal	-
H	9	Pathological	-	-
I	11	Pathological	Monoclonal	Monoclonal
J	16	Pathological	Monoclonal	Monoclonal
K	18	Pathological	Monoclonal	Monoclonal

4.3 Analysis of somatic hypermutations

For sequence evaluation, it was used IgBLAST and IMGT/V-QUEST. IMGT/V-QUEST is a highly customized and integrated system for the standard analysis of immunoglobulin (IG) nucleotide

sequence rearrangements. IMGT/V-QUEST identifies the variable (V), diversity (D), and joining (J) genes and alleles through sequence alignment with the germline IG and TR gene and allele sequences of the IMGT reference directory (Agathangelidis et al., 2018). The percentage of identity between the submitted sequence and the closest IGHV gene and allele is crucial, as it determines the status of the SHM. This value can be obtained from the first nucleotide of the V-region to its 3' end, excluding the CDR3. Less than 2% divergence from the closest germline sequence indicates the absence of mutation (Abayomi et al., 2021).

Both IMGT and IgBLAST can determine whether a sequence is productive or unproductive, however only IMGT warns when the sample is not fit enough to determine the IGHV rearrangement. A rearranged sequence is productive if the V-D-J region of VH contains no stop codons or out-of-frame regions (Lasabova et al., 2011).

Table 4.4 shows the sequencing results and mutation status determined by IMGT/V-QUEST for the 10 samples with positive results for clonality, as well as the percentage of sequence identity using IgBlast and IMGT. Number of base pair indicates the primer used for sequencing, being where the size between 450-550 refers to the sequencing performed with the leader primer while the one between 300-350 refers to the use of the FR1 primer. Sample 9, which showed a weak band in test 1, and sample 13, which was positive in all regions of test 1 and in the leaders of test 2, are not shown due to the lack of sequencing results.

Table 4.4- Sequencing results and mutation status identified by PCR from peripheral blood in 10 samples for V regions, with the respective sequence identity (%) by IgBLAST and by IMGT, number of base pair as well as the consequent result of mutation or unmutational status. Note that all the samples were sequenced by a leader primer except from sample 18 which was sequenced by FR1 primer. (M) – mutated; (U) – unmutated:

Patient	Sample	Somatic hypermutation	Sequence identity (%) IgBLAST	Sequence identity (%) IMGT	Mutational Status
A	1	IGHV1-69*01	100	100	U
B	2	IGHV3-33*01 IGHV3-33*06	92,6	92,36	M
C	10	IGHV3-9*01	100	100	U
D	4	IGHV3-53*01	91,8	91,58	M
E	5	IGHV4-34*01	89,4	89,12	M
F	6	IGHV1-69*01	100	100	U
G	15	IGHV1-2*04	96,9	96,88	M
I	11	IGHV1-2*02	100	100	U
J	16	IGHV1-8*01	100	100	U
K	18	IGHV3-7*01	94,4	94,32	M

We observed that the sequence identity (%) is different in the mutated cases using different tools, namely IgBLAST and IMGT. The comparison by IMGT is performed from the position 1 to the position 104 of the second conserved cysteine. The comparison by IgBLAST was performed to the closest germline V sequence what included up to 6 nucleotides following the anchor second cysteine. This can explain the different number of mutations reported as the mutation could be placed behind the second cysteine, in those 6 extra nucleotides.

All analyzed PCR products were productive rearrangements.

The most preferred subfamilies found were VH1 (50%) and VH3 (40%) and only one sample showed mutations in IGHV4, which is represented in the graphic of the figure 4.4. Sample 2 showed the same identity for the IGHV3-33 mutation for both allele 01 and allele 06. Furthermore, sequencing analyses of patient's IGHV genes showed a tendency of certain IGHV subfamilies to have either a mutational or unmutational status. Two samples, sample 1 and sample 6, had IGHV1-69 mutations, and both were non-mutated with 100% sequence identity percentages. However, this is not the case for IGHV1-2 mutations, which are non-mutated in sample 11 and mutated in sample 15, with a sequence identity percentage of around 97%. Of the 10 samples with productive rearrangements, 5 are mutated sequences and 5 are unmutated. The VH1 family showed a tendency to have non-mutated genes (80%), whereas of the 5 cases only 1 had mutated genes. On the other hand, the VH3 family showed a higher frequency of mutated genes (75%), of which only 1 exhibited non-mutated genes.
Approximately 50% of the IGHV genes analyzed had less than 2% difference from the most similar germline gene (unmutated), 10% had between 2% and 5% mutations and 40% had more than 5% mutations. Sample 5 was the one with higher percentage difference between the sequence analyzed of the patient and the comparative germline sequence.

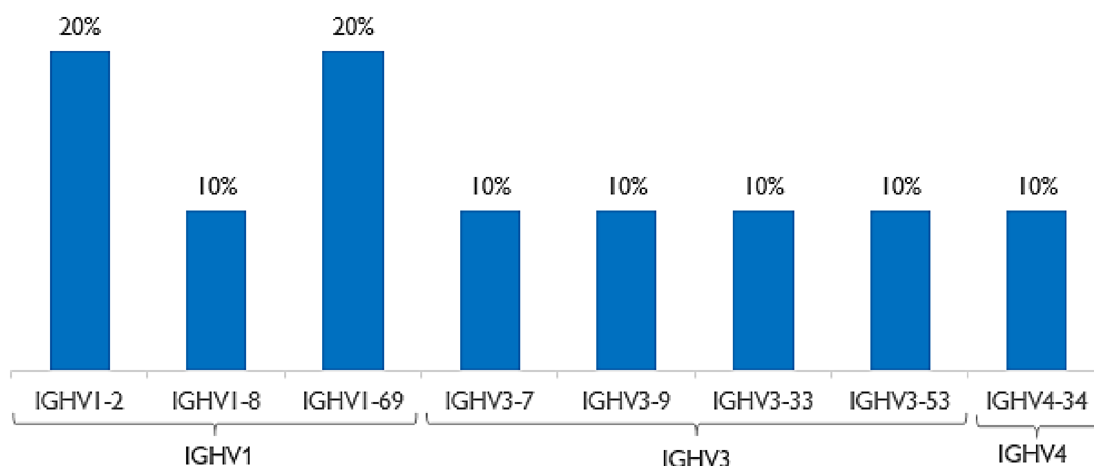

Figure 4.4- Graphical representation of the frequency of IGHV specific genes, grouped by V family.

Table 4.5 represents all the mutations of the 10 samples in the 3 IGH regions, namely V, D and J region, according to IMGT software analysis.

Table 4.5 – IG rearrangement in 10 samples from peripheral blood for V region, J region and D region.

Patient	Sample	V region	J region	D region
A	1	IGHV1-69*01	IGHJ5*02	IGHD3-16*02
B	2	IGHV3-33*01 IGHV3-33*06	IGHJ4*02	IGHD3-16*01
C	10	IGHV3-9*01	IGHJ6*02	IGHD3-16*02
D	4	IGHV3-53*01	IGHJ6*02	IGHD3-22*01
E	5	IGHV4-34*01	IGHJ4*02	IGHD3-10*01
F	6	IGHV1-69*01	IGHJ6*02	IGHD3-3*01
G	15	IGHV1-2*04	IGHJ1*01	IGHD3-3*01
I	11	IGHV1-2*02	IGHJ6*02	IGHD3-3*01
J	16	IGHV1-8*01	IGHJ6*02	IGHD2-2*01
K	18	IGHV3-7*01	IGHJ3*01	IGHD3-16*01

We identified DH genes in all of the B-CLL cases examined. D3 family genes were found most frequently in B-CLL cells (90%), followed by D2 (10%). The most frequently used D3 segment

was D3-16, which was found in 44,4% of the identifiable D genes (4/9 cases) and D3-3 which was found in 33,3% (3/9cases). There was an overall predominance of the JH6 gene (50%). Interestingly, the two V1-69 gene cases did not use the same pattern of JH and DH, though the two V1-2 gene cases only differed in the use of JH family. These results are graphically represented in figure 4.5.

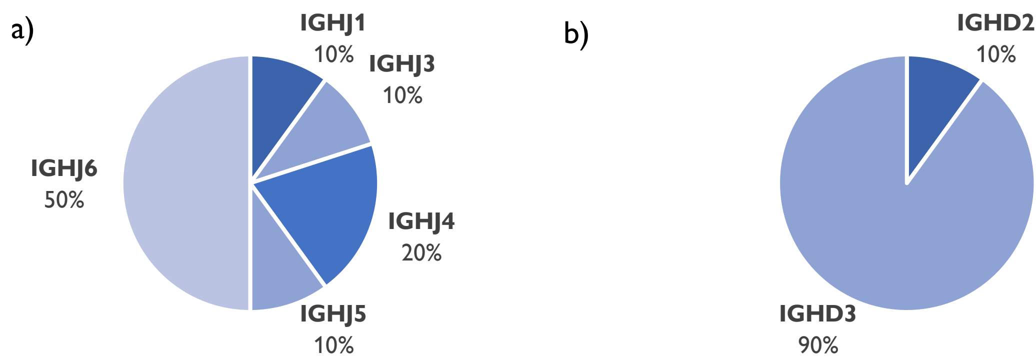

Figure 4.5 – a) Graphic representing the percentages of JH family genes found on the 10 samples. b) Graphic representing the percentages of DH family genes found on the 10 samples

4.4 Analysis of CNVs

Copy number variation is a type of structural variation larger than 1 kilobase pair and involves unbalanced rearrangements resulting in gains or losses in the DNA content, being a potential valuable prognostic marker (Niu et al., 2020).

Applied Biosystems CopyCaller™ Software v2.1 was used to determine the copy number status of each target region, namely p53 gene. Calculation of the CNV was made according to an algorithm belonging to the software (Park et al., 2012). Raw copy value (RCV) represents a non-integer number of copy calculated, although predicted copy number (CNP) is defined as an integer number of copy determined by the algorithm (0, 1, 2, or 3+) (Park et al., 2012). Copy number gain is defined as PCN higher than 2, and PCN lower than 2 is interpreted as loss (Park et al., 2012).

Table 4.6 represents the RCV and CNP obtained by CoppyCaller™ Software v2.1 for the 18 samples analyzed regarding the gene p53.

Table 4.6 - Analysis of CNV by Applied Biosystems CopyCaller™ Software v2.1of 18 samples for the gene p53. RCV-Raw copy value; CNP-Predicted copy number.

Patient	Sample	Type of Sample	RCV	CNP
A	1	Pathological	1.88	2
	17	Normal	2.06	2
B	2	Pathological	2.06	2
	14	Normal	2.00	2
C	10	Pathological	2.01	2
	3	Normal	2.31	2
D	4	Pathological	1.98	2
	8	Normal	1.92	2
E	5	Pathological	1.97	2
	12	Normal	2.39	2
F	6	Pathological	1.84	2
	7	Normal	1.96	2
G	15	Pathological	2.04	2
	13	Normal	1.85	2
H	9	Pathological	1.86	2
I	11	Pathological	2.08	2
J	16	Pathological	1.99	2
K	18	Pathological	1.87	2

It was possible to determine the copy number value for all the 18 samples. The negative showed no amplification in qPCR, and therefore no contamination, indicating that the results are valid. All samples are compared with the chosen reference gene, which in this case is RNase P, since all humans have 2 copies of this gene.

The predictive values for copy number were approximately 2 for all samples. However, the analysis of the raw RCV, demonstrated some oscillations. The highest value corresponds to sample 12, with 2.39 in the RCV. To consider gain/insertion on chromosome 17, the rounded value would have to be 3. However, as the RCV is close to 2.5, it should be considered, possibly indicating a slight modification in the p-arm of chromosome 17. The lowest value of

1.84 belongs to sample 6. The pathological samples that showed RCV below 2 were sample 1, 4, 5, 6, 9, 16, and 18. Only 4 pathological samples did not show values below 2, namely sample 2, 10, 15 and 11.

Regarding the variations in the RCV of normal clones and pathological clones from the same patient, some differences were observed. Figure 4.6 represents the RCV of the 7 patients who had the two sample types under study, namely normal clones, and pathological clones.

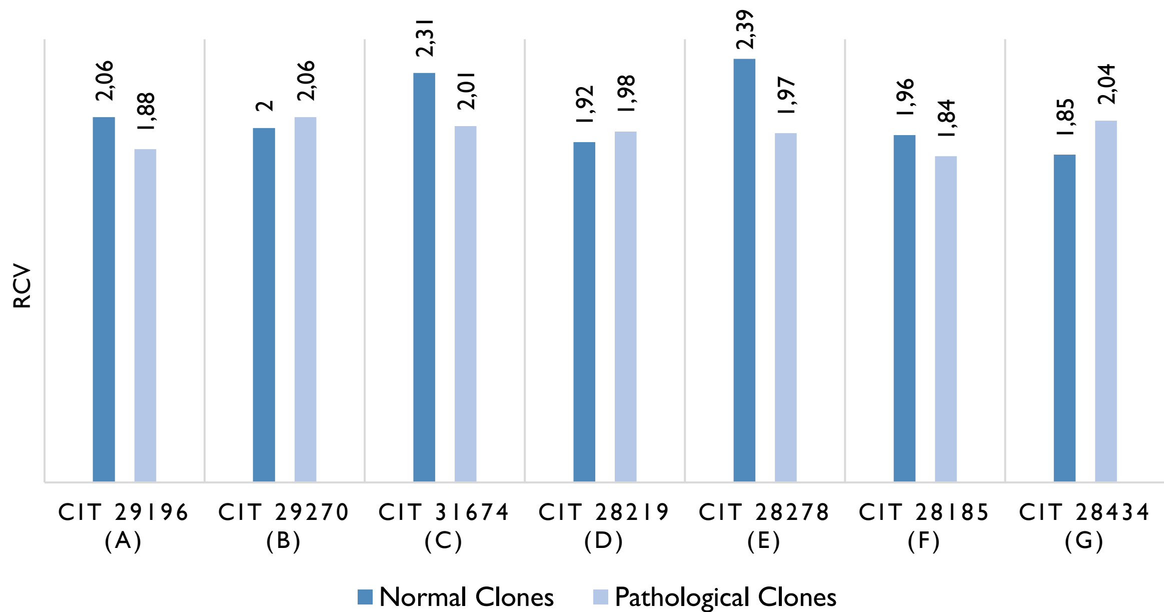

Figure 4.6 - Graphical representation of RCV from patients with both normal clones and pathological clones in analysis, as described in table 4.6**.** The dark columns represent the samples with normal clones and the lighter columns represents the samples with pathological clones. Sample 9, 11, 16 and 18 are not represented because they don't have a sample with normal clones from the same patient to compare with.

Patient (B) and (D) were the ones with the closest values between the normal clones and the pathological clones, having only a slight and insignificant difference of 0.6. The highest difference observed between normal and pathological was on patient (E), which presented a value of 2.39 in the normal clones (sample 12) against 1.97 in the pathological ones (sample 5).

CHAPTER 5 – DISCUSSION AND MAIN CONCLUSIONS

One of the most important requirements in clinical oncology is early and accurate cancer diagnosis, as well as monitoring disease progression/response to therapy.

In the present study, we dealt with the technical aspects concerning detecting IGVH gene mutational status in patients with CLL at diagnosis, aiming at predicting a more accurate prognosis.

The study considered the recommendations of ERIC guidelines on how to conduct and interpret IGHV gene mutational analysis in CLL due to the significance of determining IGHV gene mutational status in clinical practice (Rosenquist et al., 2017).

Clonality and subsequently mutational status were analyzed using DNA samples (Table 3.1) from a total of 18 normal and pathological samples of 11 diseased individuals. The initial phase of the study started at the University of Salamanca where they performed flow cytometry of the subjects' B cells to assess the clone size.

In 11 patients, approximately 82% are male, which is in line with a study by de Tute et al. that correlates a higher incidence of both MBL and CLL disease with male gender and according to the Cancer Research UK, 37% of chronic lymphocytic leukemia cases in the UK are in females and 63% are in males (de Tute et al., 2006).

In the first phase of work, the clonality of the 18 samples was tested in two ways. First, it was used a prefabricated kit in order to have a first line of analysis of the clonality of the samples. As the kit is an already approved and commercialized test on the market, the results obtained served as a possible means of comparison for the manually designed experimental protocol carried out later.

Therefore, for test 1 the samples were amplified using 3 different regions of the gene, namely FR1, FR2 and FR3, referred to as BIOMED-2 primers. In test 2 the samples were amplified using a specific mix of leader primers and another one with the FR1 primers. Comparing the two tests, it is possible to note some differences, particularly in the clonality of the samples of normal clones, being that about 43% presented monoclonality in the first test. This can be explained by the presence of pathological clones mixed with the normal cells in the sample since they were separated by cell sorting. Furthermore, test 1 possibly has a higher sensitivity than test 2 and the appearance of band on the agarose gel means that the primer in question detected these clones. This statement could also be support by sample 9 which showed amplification in test 1 and not in any primers of test 2. This sample is characterized as having pathological clones, however, when the sequencing analysis was performed there was no valid results. The percentage of cells with aberrant phenotype compared to the total cellularity of

the sample was 88.63%, however the sample concentration was relatively low, around 26.12 ng/µL. A plausible justification is that the low cell concentration does not allow for efficient detection or, for some reason external to our work, sample 9 did not come in the appropriate condition.

Sample 13 on the other hand, was the only one from the category of samples classified as normal cells that showed positive results for monoclonality in the second test with the leader primers having also been sequenced. However, the results showed no clonal rearrangements or mutations. This may suggest that the separation between pathological clones and healthy clones may not have worked properly since the percentage of cells with an aberrant phenotype compared to the sample cellularity was about 86.33%, which is a high value. Therefore, amplification detected a light band with the leader primers, but not strong enough to identify a mutation since the sample concentration was 36.93 ng/µL.

As described in table 4.3, only one pathological DNA sample (sample 4) did not show any result indicating clonality with any of the 3 primers used in the first test, which does not correspond to the previously diagnosis of the patient that confirms Atypical CLL. The percentage of aberrant clones compared to normal was a high value, about 88% but the concentration was too low, around 25,56 ng/µL. However, with the second test, sample 4 showed a monoclonal result with both leader and FR1. An experimental error may have occurred with this sample, either during the preparation of the PCR or even when pipetting the sample onto the agarose gel since test 1 seems to have a higher sensitivity.

Sample 5 showed polyclonal results with the FR3 primer on the first test. In the second one, with the design primers, it showed no amplification with the FR1-JH, however with the leader primers the outcome was different, exhibiting more than one band, which may indicate an oligoclonal result. These results are validated by the literature, with sample 5 corresponding to a patient previously diagnosed with MBLhigh with low clone size (31.2%). According to Lanasa et al., many MBL cases are frequently oligoclonal rather than monoclonal, in particular, MBL with a small absolute clone size. This article also defends that MBL's status is not entirely clear because the majority of cases do not progress to CLL being unsure whether MBL is a monoclonal, biologically silent form of CLL or a stage between healthy B cells and CLL (Lanasa et al., 2010). A study performed by Dagklis et al., found 6 polyclonal in 86 low-count MBL cases from Italy (Dagklis et al., 2008).

Although the sample size is small, it is possible to see that patients diagnosed with MBLhi have a lower percentage of clones than those diagnosed with CLL, with percentages below 45% and above 70%, respectively.

Next, it was studied the hypermutational pattern of IGHV genes in 10 patients with pathological clones, which encompasses one of the most reliable prognostic biomarkers and do not change over time (Giudice and Foà, 2019). Mutations were mostly obtained in IGHV1 and IGHV3 families and one case in IGHV4, which correspond to previous reported studies. Several studies had the higher representation of family IGHV3, followed by IGHV1 and IGHV4 (Donisi et al., 2006, González-Gascón et al., 2014). The lack of presence of mutations in other families, namely, V2, V5, V6 and V7, might be explained in part by the small number of samples analyzed in this study. As the sample size is small, it is reasonable that only the families with the highest incidence of mutation are detected.

In this study was found 50% cases of mutated CLL and 50% of unmutated CLL. The mean age was 55 years in the mutated group and 65 years in the unmutated group. Of the 5 cases of U-CLL, 4 of them had first relapse shortly after diagnosis. For the patient with sample 1 and the patient with sample 11 the relapse period was less than one year after diagnosis, both diagnosed with CLL type B on the Binet scale. Of all the patients in study, patient A was the one who presented the earliest first relapse with 4 months after diagnosis. Stage B in the Binet scale is the intermediate group, and associated with the non-mutated gene, in terms of prognosis it may eventually evolve to stage C. For samples 10 and 16 the period varied between 5 and 7 years after diagnosis, being diagnosed with CLL type A and CLL type C, respectively. There is no additional information about possible relapses for the patient corresponding to sample 6. These results are in agreement with previous studies that have already demonstrated a link between unmutated IGHV status and poor survival in CLL patients and more advanced stages (Emelie Curovic et al., 2020, González-Gascón et al., 2014, Hamblin et al., 1999). Studies also confirms that patients with unmutated IGHV genes have a remarkably more malignant disease and shorter OS and TTFT than those with somatic mutations (González-Gascón et al., 2014).

Compared to the patients with the M-CLL, the patient carrying sample 2 had no relapse after diagnosis during the follow-up time of about 7 years, being diagnosed with CLL type A on the Binet scale. However, according to recent knowledge, the patient is already deceased. This does not fully correspond with the literature previously described which states that M-CLL has a more favorable prognosis with better survival rates (Chiorazzi and Efremov, 2013). By analyzing the hypermutation of the IGHV region of this patient, V3-33 was identified. This mutation has not yet been extensively investigated, knowing only that it does not belong to any of the risk groups identified so far and there is no information about the detailed prognosis.

Of the other 4 samples with mutated genes, there is only information on sample 18 whose patient had a first relapse 3.4 years after diagnosis, being diagnosed with MBLhi type B on the Binet scale. There is not enough information about these study patients with a mutation in the gene, and it is not possible to confirm whether those with a mutation actually exhibited a better prognosis and consequently less need for treatment.

Most of the hypermutations in the V1 family are U-CLL, except for sample 15 in which the IGHV1-2 gene is mutated. Both IGHV1-69 genes detected in two samples showed U-CLL which agrees with previous studies stating that the vast majority of V1-69 cases are associated with U-CLL (González-Gascón et al., 2014, Thunberg et al., 2001). Sample 6 had V1-69 combined with segment J6, which corresponds with previously published studies (Potter et al., 2003) and was joined to D3-3 segment as in the study of Irina et al. (Irina et al., 2007). Regarding the prognosis of patients with V1-69, it is interesting to analyze potential differences between these two. Despite having the same V1-69 gene, both differ in the D and J segment. As said earlier, patient of sample 1 had been previously diagnosed with CLL type B and together with unmutated IGHV1-69, the prognosis is not favorable, being characterized by a more aggressive state of the disease, with the possibility of developing secondary neoplasias and consequently less OS, with a higher probability of TTFT. The same can be stated for sample 6, with the particularity of having been previously diagnosed with MBLhi raising the possibility of moving closer to CLL due to the severeness and rapid development with which the IGHV1-69 gene is associated.

As for the V1-2 gene present in samples 11 and 15, it should be noted that the former is unmutated and the second is mutated, respectively. According to the literature, the V1-2 gene is mostly non-mutated (Hojjat-Farsangi et al., 2009, Murray et al., 2008)

Patient G is diagnosed with SMZL and according to several studies, this disease has an IG gene repertoire of only 3 Ig genes, one of which is IGHV1-2 (Algara et al., 2002, Bahler et al., 2002). A more extensive study of Baliakas et al. shows that approximately 90% of patients that carried the IGHV1-2 cases had the usage of a certain polymorphic variant of this gene, namely allele *04. These data are in line with the results of our study in which the patient with sample 15 also has allele *04. Baliakas et al. also notes that the *04 allele is used at very low frequency in other B-cell lymphomas which also corresponds with our findings, since no other sample presents this allele (Baliakas et al., 2014b). Comparing the two samples with the same V1-2 gene, as sample 11 is mutated the prognosis is better and consequently so is OS.

Sample 16 was the last one belonging to the V1 family with the V1-8 gene. As said above, this patient was diagnosed with CLL type C and may be one of the least promising diagnoses of all

the patients who took part in this study. Having an unmutated gene with the presence of anemia and/or thrombocytopenia automatically directs the course of the disease towards a more deteriorated clinical state. However, this patient has age on his side, being diagnosed early, at the age of 33, which means that the immune system is not as aged and can help fight the disease more effectively. There's not much specific information about the V1-8 gene however this case had an interesting characteristic. All the Ig sequences from the 11 patients were submitted to the ARResT/AssignSubsets Tool and sample 16 was the only one assigned to CLL stereotyped, namely subset #3, with average confidence. According to a study from Maura et al., this subset is characterized by unmutated IGHV and is associated with an aggressive clinical course with TTFT being around 2,7. Patients with subset #3 have an increased risk of developing autoimmune anemia compared with patients with other subsets (Maura et al., 2013). In proportion to the whole CLL community, the size of this subset is about 0.6 (Sutton et al., 2016). Sample 16 had the V1-8 gene but the most common gene present in this subset is V1-69. Nevertheless, the J segment linked to our result is J6 which corresponds to that described by the literature. Sutton et al. also points the SF3B1 mutation in about 46% of cases in subset #3 (Sutton et al., 2016).

Several studies report that the V3 family is the most frequently used subgroup in CLL and is known to mark disease subsets characterized by completely different clinical courses (Dal-Bo et al., 2011, Fais et al., 1998) Sample 18 had the IGHV3-7 gene which is one of the most frequently used genes in leukemia (Dal-Bo et al., 2011, González-Gascón et al., 2014, Shi et al., 2020), also being among genes with the highest mutational load in CLL. In our results it presented a percentage of difference of about 94% with respect to the original germline. González-Gascón et al. identified in their study that patients with IGHV3-07 were associated with a lower WBC count and a nonprogressive disease (González-Gascón et al., 2014).

The two genes remaining to be discussed in the V3 family are V3-53 mutated and V3-9 unmutated in sample 4 and 10, respectively. There is no specific information in the literature about these two genes, therefore the prognosis is based on the mutational status of the gene and the class in which the diagnosis is made. Based on our results, the patient in sample 4 diagnosed with Atypical CLL with mutation in the gene will have a more favorable prognosis, with a higher probability of OS than the patient in sample 10 who, although having CLL type A on the Binet scale, the gene is not mutated, and may have a faster disease progression and need for TTFT.

The V4 family was the subgroup from which only one gene was found in a sample, namely V4-34 in sample 5. However, IGHV4-34 has been previously reported to be one of the most

frequently used genes in CLL rearrangements (Dal-Bo et al., 2011, González-Gascón et al., 2014, Shi et al., 2020, Sutton et al., 2016). Our results show a mutated gene which is in line with previous studies that found this gene predominantly in the M-CLL cases (González-Gascón et al., 2014, Stanganelli et al., 2022, Sutton et al., 2016). This gene is characterized by association to a longer TTFT and better outcome (González-Gascón et al., 2014). According to literature, part of the prognosis related to V4-34 gene relies on the subset assign, as so, a significant proportion of cases with this mutation are assigned to four majors stereotyped BCR subsets, namely #4, #16, #29 and #201. While subsets #29 and #201 have a more aggressive clinical course, the first two are linked to an indolent disease and a high SHM load (Stanganelli et al., 2022, Xochelli et al., 2015). Millaray et al. also says that patients without stereotyped BCRs have worse prognosis (Millaray et al., 2010). Sample 5 did not belong to none of these subsets, so the prognosis of this patient remains based on the mutational status, which provides good prognosis since the percentage of comparison with the original germline is 89% having a large SMH load.
According to Zhang et al. the presence of various clones in early stages is indicative of a potentially more aggressive disease in the future (Zhang et al., 2011). However, it is not possible to establish a relationship between the percentage of clones and disease severity in this study, since the sample with the highest percentage, 97% has the mutated IGHV3-7 gene, which is associated with good prognosis. On the other hand, the second sample with the highest percentage of clones, 95.94%, has the non-mutated V1-8 gene in class C on the Binet scale, presenting the worst prognosis.

As explained before, CLL disease is quite heterogeneous regarding prognosis, clinical outcome, and response to treatment. This heterogeneity is partially caused by genetic aberrations found in these cells such as mutations of *TP53* and/or deletions in chromosome 17p, resulting in loss of one *TP53* allele, which encodes the tumor-suppressor protein p53 (Campo et al., 2018). Regarding the analysis of TP53 aberrations, ERIC has created a certification program known as the TP53 Network, which recommends the use of Sanger Sequencing or NGS. Therefore, the status of chromosome 17p was studied using the copy number assay test, which is a faster and simpler test to perform. To differentiate between acquired CNVs in malignant cells and polymorphic CNVs in the human genome, CNV was conducted on DNA from pathological clone cells and from normal cells for 7 patients. On the other 4 patients, it was only performed the analysis on pathological cells.

All the CNP values were 2, but by analyzing the RCV values it is possible to draw some conclusions about the status of chromosome 17 and consequently to evaluate the disease and estimate the patient's prognosis.

Although the estimated normal value is 2, each individual has DNA segments ranging in size from one kilobase to several megabases that differ between individuals due to chromosomal alterations, being reported to be associated with aging (Iakoubov et al., 2013). Analyzing all the RCV values of the pathological clones from which we have information about the age of the patients, those who are older also have higher values. Patient I, diagnosed at age 80, has 2.08 RCV but there is no information about the RCV of the normal clones. Based on the literature, the probability of this value being lower than that of normal cells is very high, and consequently, of having del(17p) (Iakoubov et al., 2013). To support this theory, patient J and K are the younger cases of diagnosis and, despite the lack of information about the normal clones, the pathological ones had values above 2, confirming that older ages have a higher risk of CNV polymorphism.

All patients who had RCV values of pathological cells lower than normal cells may have del(17p), namely patient A, C, E and F. These alterations are associated with an aggressive disease with a poorer prognosis, lower OS, and a weaker response to chemotherapy, being considered one of the strongest markers regarding course of treatment in CLL.

Patient B shows very similar values from both normal and pathological clones, which may mean that he has no alterations on chromosome 17. Patient D, on the other hand, presents RCV values of the pathological clones slightly higher than those of the normal clones, both close to 2. In this case it may mean that either the patient has no alterations or that the patient may be in the beginning of the disease and the prevalence of these deletions is still very low. As stated by Chiorazzi, alterations as del(17p) might occur both as early or late events, but appear mostly in later stages of disease (Chiorazzi, 2007). According to studies, the prevalence of deletions in 17p region are around 3-8% but may rise up to 40% in patients undergoing chemotherapy, suggesting that these deletions may be triggered after treatment (Shahjahani et al., 2015). Patient G also showed normal clone values lower than pathological clones, but in this case the difference in values was higher, possibly signifying an insertion or gain on chromosome 17p.

The biggest differences concern patient C and E, both with normal clone values around 2.35, indicating that possibly the patients in question are already in a more advanced stage of the disease. The frequency of TP53 deletions is much higher in relapse than at the beginning of

treatment, increasing to about 20% (Edelmann et al., 2017), suggesting that clonal evolution may be accelerated by treatments, particularly chemotherapy.
Regarding the 4 samples from patients with no normal cells results to compare, patient H, J and K had values lower than 2, which could mean deletions in chromosome 17. However, it is not possible to draw a firm conclusion since there is no sample of normal clones to compare. It is possible to relate IGHV status to del(17p), providing a more accurate and complete prognosis. According to Guièze et al., Although they can occur in both mutated and unmutated CLL, TP53 mutations are commonly related to unmutated CLL (Guièze et al., 2015). Our results are in agreement, since of the 4 confirmed cases with p53 mutations, 3 of them correspond to non-mutated IGHV. All samples with del(17p) have a poor prognosis due to mutational status. The one with mutated IGHV is patient E whose already had a bad prognosis, so adding the del(17p) confirms and aggravates the condition. Advising treatment for these 4 patients is a risky challenge since, according to the prognosis, they do not have a good chance of responding effectively to treatment due to the presence of del(17p). According to Hallek, a patient with del(17p) is enough to not be indicated for chemoimmunotherapy, regardless of the mutational status (Hallek, 2019). However, without treatment, the mutational status already points to reduced OS. A study of Tam et al. points that if detected early, the average OS is about 4 to 5 years for diagnosis and 1 to 1.5 years for changes acquired during clonal evolution (Tam et al., 2009).

Overall, the objectives of the present study were achieved. The key question relied in the progressing of MBL to CLL and how to predict that. As such, this study aimed to create a methodology to understand this progression through specific molecular markers. With the combination of the mutational status of the IGHV genes and the presence of del(17p), at the TP53 locus, it was possible to construct a more solid and complete assessment of risk of disease progression and prognosis (OS, TTFT and response to treatment). Our results demonstrated that with this novel technique, it's possible to evaluate whether or not there is a deletion on chromosome 17 without major hurdles compared to the current approach regarding the *TP53* gene. Further work is needed to ascertain the effectiveness of both methods on a larger sample group. Since MBL disease is initially asymptomatic, the use of this methodology for pilot screening of the population above a certain age would be a great advance in preventing a more serious stage of the disease.

CHAPTER 6 – REFERENCES

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