Università degli Studi di Ferrara

DOCTORAL COURSE IN

"MOLECULAR MEDICINE AND PHARMACOLOGY"

CYCLE *XXXIII*

COORDINATOR Prof. Di Virgilio Francesco

CLINICAL AND BIOLOGICAL MEANING OF GENOMIC COMPLEXITY IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) AND COMPLEX KARYOTYPE (CK).

Scientific Disciplinary Sector (SDS) MED/15

Candidate
Dott.ssa Melandri Aurora

Supervisor
Prof. Cuneo Antonio

Years 2017/2020

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PhD Student: Aurora Melandri

Tutor: Prof. Antonio Cuneo

Chronic Lymphocytic Leukemia (CLL) is the most common form of leukemia in western countries. CLL's clinical course can range from an indolent condition, with a slow progression, to an aggressive form, which can lead to an early death. The discovery of new and more accurate prognostic markers is crucial. Complex Karyotype (CK) ($\geq$ 3 chromosome aberrations in the same clone) has emerged as an independent negative prognostic marker. CK includes a variety of cytogenetic aberrations, from numerical to structural abnormalities. In this study, it was performed a cytogenetic analysis on 90 treatment-naïve CLL patients with CK. It was evaluated the prognostic impact of each cytogenetic abnormality for the Overall Survival (OS) and Time To First Treatment (TTFT). Among all the abnormalities, unbalanced translocations had displayed an independent prognostic impact, with a worse OS and TTFT. It was performed a gene expression profile analysis on 23 patients, representative of the entire cohort, 11 with unbalanced translocation and 12 without. It emerged that the presence of unbalanced translocation identified a subset of patients with distinct molecular features.

Among the differentially expressed genes, the attention was focused on SLAMF1, a gene involved in lymphocyte activation, apoptosis, and cell cycle control. It was decided to analyze the expression of SLAMF1 in all CLL at onset (349) stored in the Ferrara laboratory from 2009 to 2018. Through a droplet digital PCR, it was studied the expression of SLAMF1. The housekeeping gene, β-actin, was used as a standard control. The patients were divided into three different groups based on SLAMF1 expression. Patients with an expression above 6.24 belonged in the high-expression group. A value below 2.8 was considered low, while the rest of the patients were classified as an intermediate group. This partition inversely overlaps with the division of patients based on their prognostic risks. Patients with lower expression of SLAMF1 had a shorter TTFT. and OS. In CLL miRNAs play an important role in the pathogenesis of the disease. The deletion of the miR-15a and miR-16-1, involved in the Bcl2 pathway, causes the development of CLL. It could be possible that miRNAs are responsible for the downregulation of SLAMF1 in CLL patients.

To clarify the role played by these miRNAs in the regulation of SLAMF1 expression it was performed a luciferase assay. The miRNAs selected as possibly responsible for SLAMF1 regulation were the ones up-regulated in CLL. The results of the luciferase assay show no difference in SLAMF1 expression based on the actions of miRNAs. This indicates that miRNAs are not responsible for the dysregulation of SLAMF1. The mechanisms remain unknown. Due to the clinical and molecular heterogeneity of CLL, a better understanding of the molecular pathway that regulates the development of CLL is important for highlighting new possible treatment targets. Although CK is a negative prognostic marker, in this study, it was proven that specific cytogenetic anomalies could worsen the CK prognostic significance. Also, the expression of SLAMF1, easy to measure in B lymphocytes, could represent a new prognostic marker. These findings will be helpful to separate the patients into risk categories and to select the best treatment.

To my family

For their sacrifices

For their support

For their love

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ACKNOWLEDGMENT

There are many people I have to thank for their contribution to this project and their support during this time.
First, I would like to express my thanks and gratitude to my tutor, Professor Antonio Cuneo for this excellent opportunity and the support and belief that he had showed in me during these years.
I would like to offer my special thanks to Prof. Gian Matteo Rigolin for helping me increase my knowledge and my skills.
I am extremely thankful to my supervisor Dr. Antonella Bardi for the knowledge and insightful. I am also deeply grateful for her support all these years.
I am also thankful to Prof. Carlo Croce for the opportunity to work in his laboratory and I want to extend my thanks to my tutor Veronica Balatti for her guidance.
I will forever be indebted to Dr. Luisa Ferrari for everything she had taught me, for her patience and her friendship. She has been an example in so many ways and I hope one day to be at least half as good as her.
I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement throughout all these years. This accomplishment would not have been possible without them. Thanks to my mother for always listening to me and consoling me and simply loving me. Thanks to my father who always pushes me and demand the best from me. Thanks to my brother, for his complicity, for the laughs, for always be there. Thanks to my uncles, who would do everything to make me happy.
A big thank you also goes to my colleagues: Eleonora, who never let anything put her down, for the constant banter and for having taught me something. Sometime. Nothing important though. Elisa, for listening to my (constant) complains, for the talks and for the passion she put in everything.
I want to thank my friends Valentina, Giulia e Samantha for stepping up for me and making sure that I did not feels alone when I was by myself on the other side of the world. I also want to thank Lara, who listened to my ramblings and provided advice and support when I was completely lost in the bureaucracy. Thank you to all the others American friends: Luisa, Rosario, Francesca, Emanuele, Gianni who made me feel at home away from home. Overall, for her friendship and support, I want to thank Marina, aka Marizon, who share the same values in life as mine: food, shopping and skin care. Last, but not least, a big thank you goes to Felice, for his gigantic patience and his enormous help with this thesis.

This accomplishment would not be possible without all of you.

Thank you.

1 INTRODUCTION

Cancer is a disease that affects human being and animals throughout recorded history[1]. The first description of cancer could be dated to Ancient Egypt, but it is the Greek physician Hippocrates who gave the name ‘cancer’ after he noticed that the blood vessels around a malignant tumor looked like the claws of a crab[2]. In 1827, the French physician Velpeau reported the first case of leukemia[3]. It is not until 20 years later that Bennett[4] first, and Virchow[5] later, describe leukemia as a clinical entity and a blood-related disease.

It is still Virchow that come up with the term leukemia[6] and the conclusion that it was not the result of an infectious process, but rather a separate disease derived from the tissue itself[7]. The combined work of several physicians brought, in 1913, to the classification of leukemia into four types: chronic lymphocytic leukemia, chronic myelogenous leukemia, acute lymphocytic leukemia, and acute myelogenous leukemia[8].

Leukemia is the product of a multistep process. An initial modification that confers a survival advantage to cells' sub-clones could lead to their uncontrolled growth and invasion of the local tissue. The advantage could be an increased proliferation or the ability to escape the immune system. In other cases, the alteration could involve genes with regulatory functions, such as tumor suppressor genes, causing tumor expansion. Alterations could manifest as mutations, insertions, deletions, amplification, translocations, and epigenetic changes[9].

Chronic leukemia is characterized by the accumulation of abnormal mature cells that slowly replace the healthy cells in the bone marrow until they spill over into the blood stream.

In this thesis, we focused our attention on Chronic Lymphocytic Leukemia (CLL) and the role played by chromosomal aberrations in the prognosis of the disease. CLL possess typical chromosome abnormalities that have been deeply investigated. Each of those related to a specific prognostic value. In recent years, it has risen the importance of the complex karyotype (CK), a cytogenetic category defined by the presence of 3 or more anomalies in the same clone. CK has always been associated with a negative prognostic value, without considering what kind of chromosomal abnormalities the CK harbors. It is important tough to better define the negative prognostic value of CK based on the types of abnormalities that it harbors.

Investigating different gene expression profile of CLL according to karyotype features has led us to the identification of a specific gene: SLAMF1. This gene has proven to be a very promising prognostic marker, easy to measure, and able to quickly separate the patients into risk categories. There are numerous prognostic markers for CLL, but not all laboratories are equipped to perform an analysis that requires expertise, money, and several days before obtaining the results. The identification of an efficient and reliable prognostic marker will help design a better therapeutic strategy for the patients.

Before deepen into the CLL topic, a brief introduction about the pathways that regulate normal B-cells is necessary.

1.1 PHYSIOLOGICAL DEVELOPMENT OF B-CELL

1.1.1 B-CELL MATURATION

B-cell development and committment begin in the fetal liver and continues in the bone marrow throughout life. The majority of B lymphocyte development occur in the bone marrow, deriving from hematopoietic stem cells (HSCs)[10].

Multipotent progenitors' cells (MPCs) originate from pluripotent HSC under the stromal cell stimulation. Stromal cells provide a hematopoietic-induced microenvironment (HIM). Growth and differentiation factors influence the development of HSC in common lymphoid progenitors (CLPs)[11].

It is at this stage that happens the formation of antigen receptors from gene segments termed: variable (V), diversity (D), and joining (J) segments[10] (fig1). These segments are located in the heavy (H) and light (L) chains that form the immunoglobulin (IG) loci. The variable region of the heavy chain (VH), together with the variable region of the light chain (VL) determines the specificity of the immunoglobulin.[12]

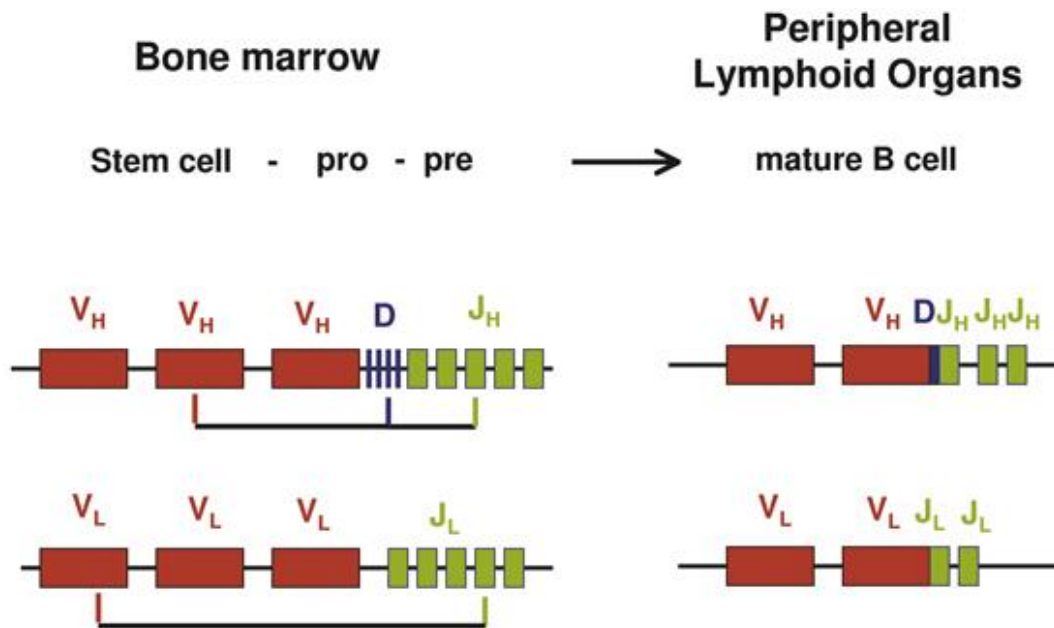

Figure 1 Rearrangement of the IGVH region[12]. (Berek)

A random rearrangement of the Ig variable region genes culminates in the development of a diverse repertoire of functional VDJ_H and VJ_L rearrangements encoding the B-cell receptor (BCR)[12, 13].

BCR is composed of one isotype of membrane-bound immunoglobulins (mIgA, mIgD, mIgE, mIgG, and mIgM) and a signal transduction moiety: a heterodimer called Ig-α/Ig-β (CD79), bound together by disulfide bridges[14].

Rearrangement of the IgH chain occurs first in pre-progenitors B-cells followed by V-DJ recombination in large pre-B-cells. The association of these two chains forms the pre-B-cell receptor (pre-BCR), expressed on the cell surface[15]. Therefore these pre-B-cells (arisen from pro-B cells) either express the pre-BCR or surface Ig[13,16].

Pro-B cells mature into pre-B cells through the stimuli provided by the bone marrow stromal cells. Pro-B cells require direct contact with the stromal cells, mediated by several adhesion molecules[17]. Once the contact is made, a receptor on the surface of the pro-B-cell, c-Kit, interacts with the Stem Cell factor (STF) on the surface of the stromal cell. This interaction causes pro-B-cells to differentiate into pre-B-cells, which no longer require direct contact with the stromal cells. The first functional BCR produced by pre-B-cells has a unique specificity that is expressed as IgM on the surface of immature B-cells.

Immature B-cells migrate from the bone marrow to the secondary lymphoid organs (SLOs), such as the spleen and lymph nodes[14]. Here they differentiate into long-lived mature follicular (FO) or marginal zone (MZ) B cells[15]. (fig. 2)

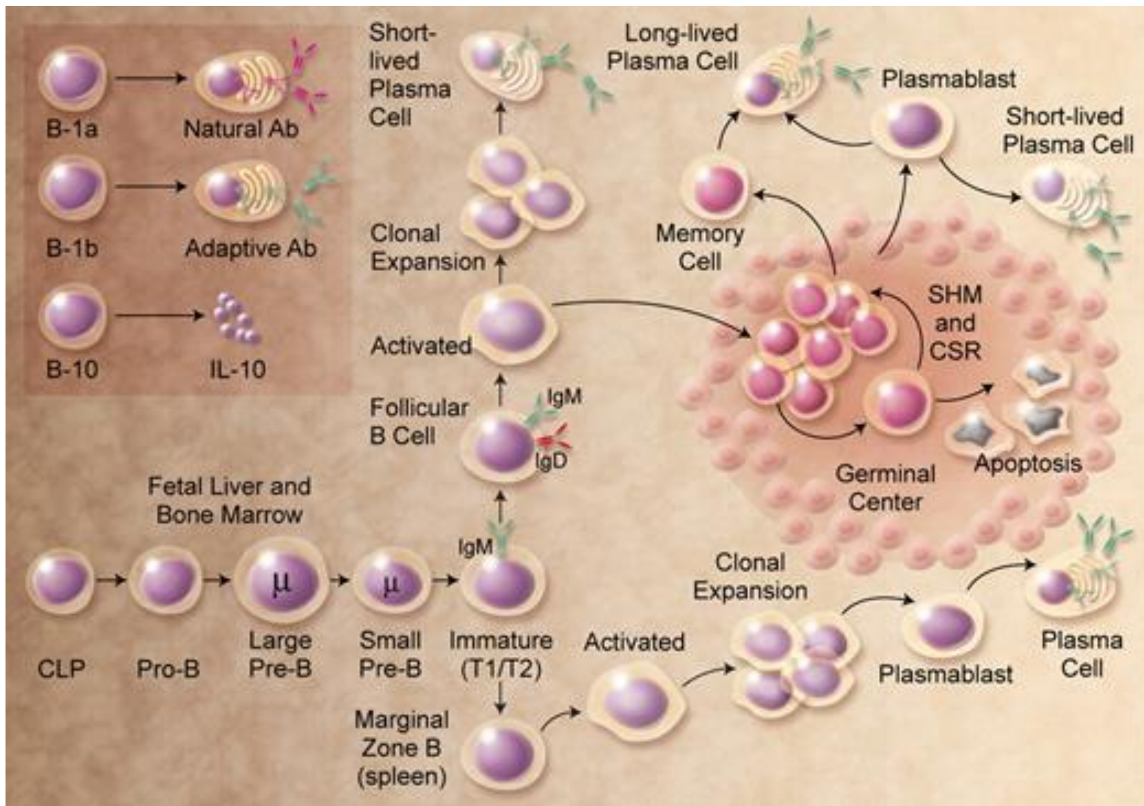

Figure 2.. A broad outline of B cell developmental stages. (Tucker, W. LeBien et al., 2008)

In the SLOs, mature B-cells encounter antigens in a T cell-dependent reaction that bound to BCR, activates the B-cells[18,19]. Activated B cells proliferate and either differentiate into plasma cells or enter germinal center (GC) reactions[20].

1.1.2 GERMINAL CENTER

GC is a specialized microstructure located in the SLOs. When a B-cell leaves the bone marrow after its maturation is called a naïve cell because it has yet to encounter the antigen. Mature B-cells with a functional BCR, migrate to the peripheral lymphoid tissue where they are exposed to an antigen[21]. It occurs about a week to form a GC, the expanding clonal B-cells push the naïve B-cell towards the outside creating a mantle zone around the forming GC. In the second week, GC mature in the definitive form divided into a light zone (LZ) and a dark zone (DZ), where the proliferation occurs[19] (fig. 3). When B-cells enter into the DZ of the GC they undergo the process of somatic hypermutation (SHM) of their immunoglobulin genes.

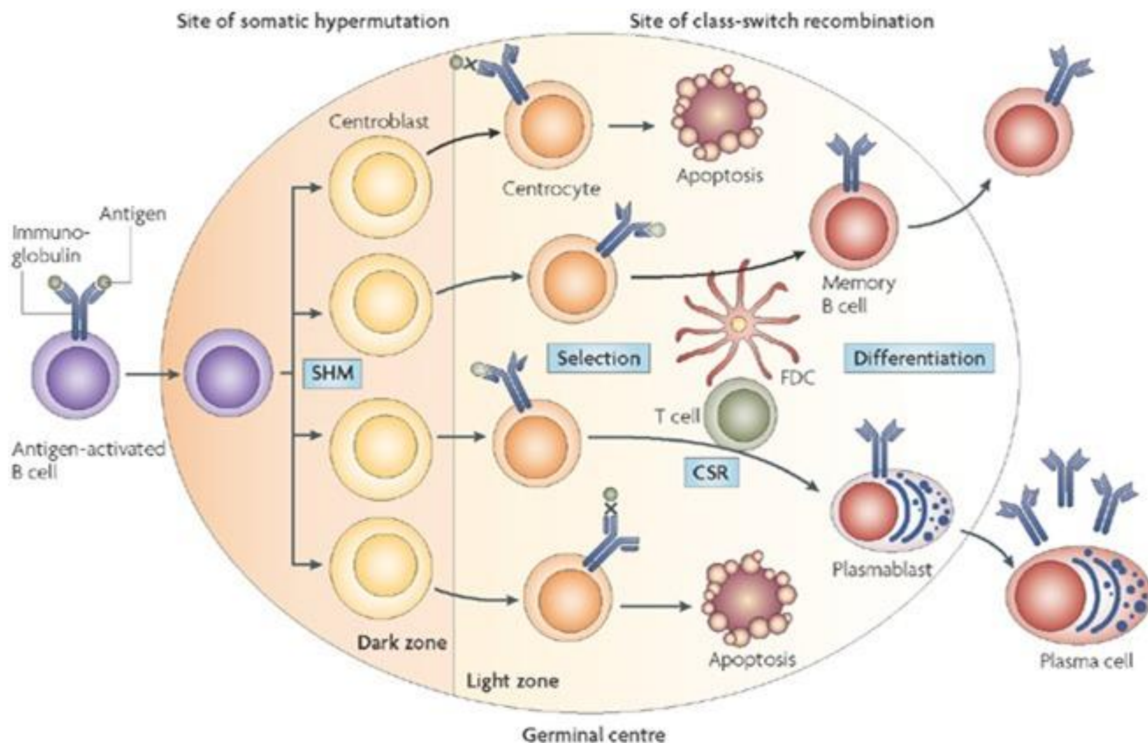

Figure 3. Antigen-activated B cells differentiate into centroblasts undergoing clonal expansion in the dark zone of the germinal center. (Klein, U. et al, 2008)

SHM happens when the B-cell encounters an antigen. A process mediated by T-cell CD4+ and follicular dendritic cells, called antigens presenting cells. DZ B-cells transit to the GC light zone where they are activated and selected through class-switch recombination (CSR) events, mediated by AID. After many rounds of CSR in the LZ, B-cells express high-affinity antibodies. Upon re-entering the DZ, they generate and differentiate into either antibody-secreting plasma cell or memory B-cells. The formation of GC is regulated by the transcriptional factor B-cell lymphoma 6 (BCL6). BCL6 is a potent transcriptional repressor that silences the anti-apoptotic BCL2 gene during the SHM process[21].

1.1.3 IGVH

The Ig binds antigens in the V region while the C domain has the function of activation of complement or binding to Fc receptors. Each V domain is divided into seven regions. Three of the sequence variability (complementarity determining regions or CDRs), and four regions of relatively constant sequence framework regions, or FRs (fig.4). The three CDRs of the H chain pair with the three CDRs of the L chain forming the antigen-binding site. Heavy chain C domains determines BCR isotypes (IgM, IgG, IgA, IgD, and IgE),[22,23] while the light region may be either a κ or a λ chain. The heavy chain and the two light chains are located in three different chromosomes and inherited in three unlinked gene

complexes. The immunoglobulin variable region heavy-chain gene (IGVH) is located on chromosome 14q32, k light chains gene on chromosome 2p11, and λ light chains genes on chromosome 22q11[23]. The heavy chains are composed of C-region genes which encode for the constant region of each immunoglobulin and variable-region genes (V_H genes), functional diversity (D_H) segments and functional joining region (J_H) genes. Together, they encode for the variable region of the immunoglobulin molecule[24].

The V regions of the antibody confer antigenic specificity while the C domain of the H chain defines effector function. They are generally defined as CH1-CH2-CH3 (IgG, IgA, IgD) with an additional domain (CH4) for IgM and IgE. To increase the affinity for the antigens, the variable domain undergoes an SHM[22].

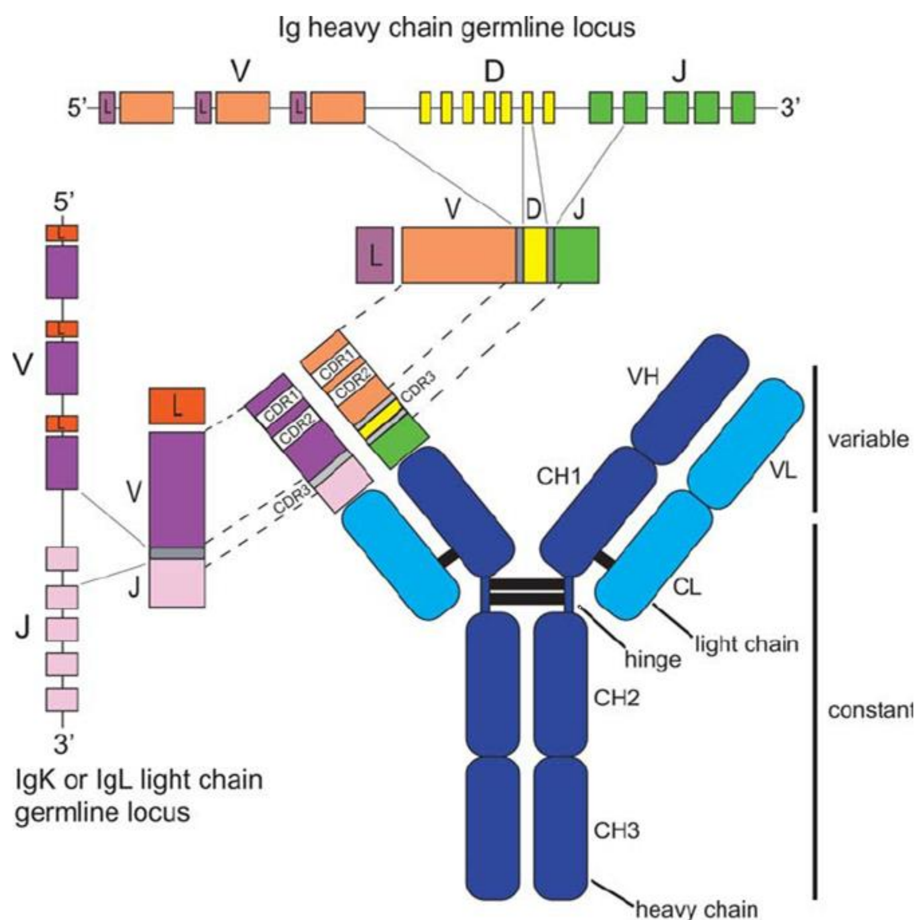

Figure 4. Antibody structure. The heavy chain locus is at the top of the picture showing V, D, and J gene segments, A germ line kappa or lambda light chain locus is depicted on the left-hand side, with unrearranged V and J segments. (Boyd et al.2014)

SHM frequency is about 10^{-3} per base pair per generation of the IgV, around one mutation per cell division. Upon encountering an antigen B-cell increase the affinity for that specific antigen by modifying the Ig genes encoding for the antibody. SHM causes a single point mutation selected to increase the affinity of the antigen in the variable regions of the Ig loci (IgV). AID will then initiate DNA double-strand breaks (DSBs) in the regions of the Ig

heavy chain gene (IgH). It will activate CSR and start the deamination of DNA cytosine bases to convert them to uracil. The mispairing is than processed by uracil DNA glycosylase (UNG) and targeted by repair pathways. This may lead to the replacement of the uracil with other bases or prompt further mutation in its vicinity that increase the spectrum of SHM, including mutations at A:T pairs, which AID cannot directly modify. The majority of mutations are clustered in complementary determining regions (CDRs) which are in direct contact with the antigens[25]. In CLL cases, there is a tendency of using certain IGHV genes (IGHV1-69, IGHV3-7, IGHV4-34) suggesting the selection of CLL progenitor cells by a restricted set of antigens[26].

The creation of immunoglobulin diversity is hierarchical. In pro-B-cells, $D_H \rightarrow J_H$ joining precedes $V_H \rightarrow DJ_H$ rearrangement and $V_L \rightarrow J_L$ joining occurs at the late pre-B-cell stage. In-frame the functional VDJ_H rearrangement allows the pro-B-cell to produce μ H chains, which defines the state of pre-B-cell[22].

1.2 CHRONIC LYMPHOCYTIC LEUKEMIA

CLL is the most common form of leukemia in western countries and affects mostly the elderly. CLL is a monoclonal B-cell malignancy, where a slow, unregulated clonal expansion of B-lymphocytes accumulates in the blood, bone marrow, and lymphoid tissues. At the time of the diagnosis of CLL, it is impossible to determine when the disease first started and for how long it has been developing. CLL could have been growing and evolved for months, if not years, before being discovered, accumulating genomic aberrations. This genetic heterogeneity reflects the different clinical aspects of the disease. CLL patients showed very different symptoms and outcomes. In some cases, CLL may present a rapid progression, while in others could be indolent and symptom-free for years. The main road of the study has been the discovery of prognostic markers that could help predict the development of the disease. In particular, we explored the role of chromosomal abnormalities, and the CK, as prognostic markers in CLL. Consequentially, we studied the role of a dysregulated gene found in patients with specific chromosomal abnormalities.

1.2.1 DIAGNOSIS

CLL symptoms are very heterogeneous such as involuntary weight loss, fatigue, night sweats, and an increase of infections. Moreover, CLL can show no symptoms at all. The

diagnosis of CLL in many cases is the result of a casual finding of lymphocytosis during a routine blood test.

A lymphocytosis could turn into CLL when clonal lymphocytes equal or surpass 5×10^9/L B-lymphocytes (5,000/μL) in the peripheral blood for at least 3 months. Most patients present an absolute count of over 10,000/μL[27]. With the increased number of lymphocytes in the blood, patients may present cytopenia, enlarged lymph nodes, hepatomegaly, and splenomegaly, which are palpable on physical examination[28,29]. The microscopic analysis of the peripheral blood cells is a fundamental step for the diagnosis of CLL. Leukemia cells found in the blood smear are characteristically small. Mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin. Mature lymphocytes may be found admixed with larger o atypical cells or prolymphocytes[28]. A typical characteristic of CLL is the presence of destroyed lymphocytes, called Gumprecht shadows or smudge cells: cells ruptured during peripheral blood smear preparation[30,31]. (Fig.5)

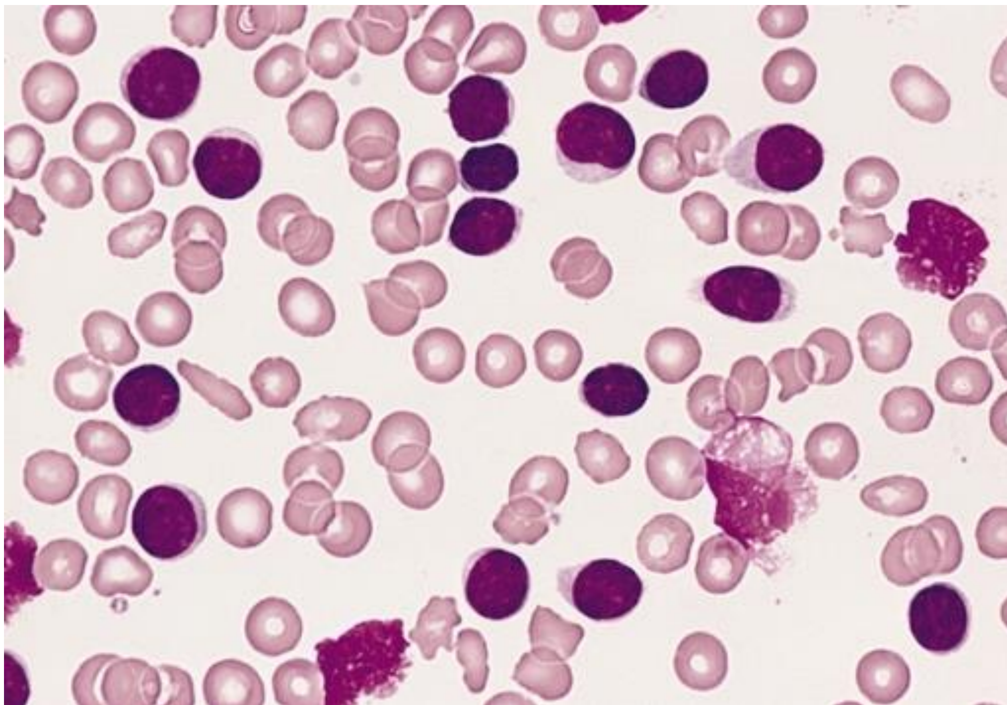

Figure 5. Image of smudge cells and pathological lymphocytes in CLL samples.

Although an altered blood count and the presence of Gumprecht shadows are not enough for the diagnosis of CLL, they arise a suspicion that leads to the request of other more sophisticated analysis, such as flow cytometry[31].

1.2.2 SCORE MATUTES

Immunophenotyping has a major role in the diagnosis of CLL. CLL's B-lymphocytes have a specific immunophenotype which distinguishes CLL from other B-cell malignancies[32].

In 1994 Matutes et al. studied the circulating cells of over 650 B-cell lymphoproliferative disorders using flow cytometry[33]. The result was a scoring system used to differentiate among the many B-cell lymphoproliferative disorders and effectively diagnose CLL. In the Score are not included the antigens used to identify B-lymphocytes, like the CD19. The scoring system consists of the evaluation of five membrane markers, namely: CD5, CD22, CD23, FMC7, and surface membrane immunoglobulin (SmIg). Each marker is worth one point, for a total score of 5, based on their characteristic expression on CLL[34]. In 1997, Moreau et al. replaced the CD22 with the CD79b in the scoring system[35,36].

Typical CLL cases are usually diagnosed with 4 or 5 of the total Score Matutes (Fig. 6), named after Matutes herself, while other mature B - cell lymphoid neoplasms score 3 or less[37].

	Score Point	
Marker	1	0
SmIg	Weak	Strong
CD5	Positive	Negative
CD23	Positive	Negative
FMC7	Negative	Positive
CD22 or CD79b	Weak	Strong

Figure 6. Illustration of the Score Matutes.

When B-lymphocytes activate, they express the antigen CD5 on their surface. Normally, the percentage of activate cells do not exceed 10% of the total of B-cells. The principal hallmark of CLL cell is the constant expression of CD5 on all its cells. The CD5 molecule is a 67 Kd glycoprotein member of the SRCR family (scavenger receptor cysteine-rich domains)[38]. It is a surface marker characteristic of T-cells. However, it can be found in other subtypes of lymphocytes at different stages of development and activation[39,40]. CD5+ B-cells are mostly expressed in the human fetal spleen and umbilical cord blood. Up to 80% of all B-cells in newborns are CD5+. The expression decreases progressively with age, until reaching 10-15% of B-cells (1% of the total number of lymphocytes) in a healthy adult peripheral blood. The majority of these cells are naïve. CD5 is considered an activation marker because about 25% of these CD19+CD5+ cells co-express the CD27, a

memory-associated molecule (Fig.7)[40]. B cells upregulate CD5 after interacting with an autoantigen or a T-independent antigen[38]. Due to the physical association between CD5 and BCR, when BCR is engaged in combination with other surface molecules and cytokines, it induces the expression of CD5 on B cells[40]. CD5 downregulates BCR, preventing further B-cell activation. At the same time enhances IL-10 production in human B cells, increasing B-cell survival[40]. Mantle cell lymphoma (MCL) can also express CD5 on the surface of the cells. Sometimes it is confused with atypical CLLs that have a Score Matutes of 3. To avoid this problem has been proposed a new implemented score. The addition of markers like CD43, and, in particular, CD200 help better differentiate CLL from other B-cell malignancies[37].

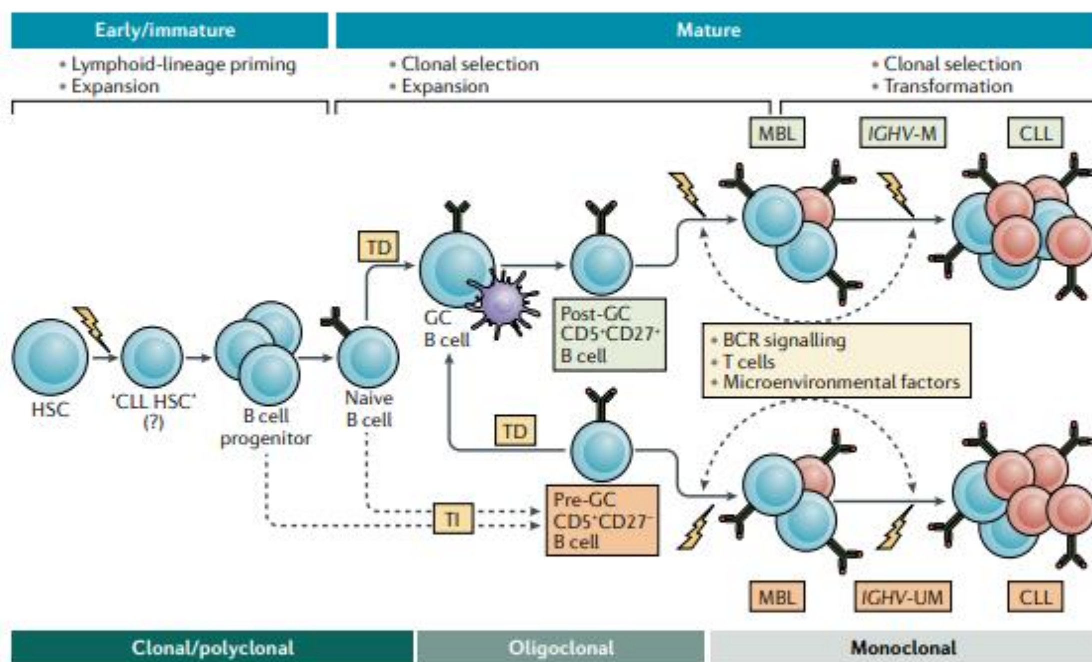

Figure 7. This image illustrates the current hypothesis of CLL development. Hematopoietic stem cells (HSCs) acquire genetic and epigenetic lesions, symbolized by lighting that may lead to a polyclonal expansion of B-cell progenitors. M-CLL seems to originate from cells, CD5+ CD27+, that went through immunoglobulin rearrangement in the germinal center (GC); while UM-CLL seems to derive from pre-GC cells, CD5+CD27- B-cells, it is not clear although whether from naive B-cells or another lineage (Bosch et al., 2019).

CD200 is a transmembrane type I glycoprotein belonging to the immunoglobulin superfamily and expressed by various cell types (B-cells, a subset of T cells including activated T cells, thymocytes, endothelial cells, and neurons). CD200 has differential expression in B‐cell neoplasms[42,43]. It is useful in distinguishing CLL from MCL and in discriminating hairy cell leukemia (HCL) from its variant form (v‐HCL)[37].

1.2.3 STAGING

There are two different staging systems for the evaluation of CLL: Rai and Binet. Named after the first authors of the original publications. The Rai staging system is more commonly used in the United States, while the Binet classification is more common in Europe. Both staging systems are simple, inexpensive, and rely on physical evidence and laboratory tests[44,45]. The Rai system does not take into consideration whether the lymph nodes are generalized or localized[42,43,46]. It dives the CLL in 5 stages:

Stage 0: Lymphocytosis (high blood count of lymphocytes) and no enlargement of the lymph nodes, spleen, or liver, and with near-normal red blood cell and platelet counts.

Rai stage I: Lymphocytosis plus enlarged lymph nodes. The spleen and liver are not enlarged, and the red blood cell and platelet counts are normal or only slightly low.

Rai stage II: Lymphocytosis plus an enlarged spleen (and possibly an enlarged liver), with or without enlarged lymph nodes. The red blood cell and platelet counts are normal or only slightly low.

Rai stage III: Lymphocytosis plus anemia, with or without enlarged lymph nodes, spleen, or liver.

Rai stage IV: Lymphocytosis plus thrombocytopenia, with or without anemia, enlarged lymph nodes, spleen, or liver.

This classification was then simplified into 3 stages:

- Stage 0 is low-risk.
- Stages I and II are intermediate risk.
- Stages III and IV are high risk.

The Binet system considered the number of areas affected by CLL based on the presence of enlarged lymph nodes greater than 1 cm in diameter or by the presence of anemia or thrombocytopenia. The areas of the body investigated are the head and neck, axillae, groin, palpable spleen, and palpable liver[47,48]. The Binet system is divided into three stages:

- Stage A: includes up to two of the areas described above being affected and hemoglobin (Hb) levels ≥ 10 g/dL and platelets ≥ 100 x 109/L.
- Stage B: is defined by at least three or more areas being affected and Hb ≥ 10 g/dL and platelets ≥ 100 x 109/L.
- Stage C: is defined as patients with Hb levels less than 10 g/dL and/or platelet count less than 100 x 109/L.

The Rai and Binet system has represented the basis for prognostication in CLL for more than 40 years. Due to the numerous discoveries of CLL biology, these staging systems have become insufficient, indicating the need for a new staging system. The result of an international project involving both the USA and Europe lead to the development of a new prognostic index: the Chronic lymphocytic Leukemia international prognostic index (CLL-IPI)[49]. It combines genetic, biochemical, and clinical parameters in a prognostic model. It has been published based on the results of a meta-analysis and subsequently validated in other publications[50,51,52]. The CLL-IPI includes five variables and assigns a score based on their prognostic impact: TP53 deletion and/or mutation (collectively called P53 dysfunction), IGHV mutational status, serum β2-microglobulin, clinical stage, and age[45,53]. One of the advantages of this staging system is the more precise identification of the patients who don't need therapy. Several studies have shown that treating patients with early-stage disease does not result in a survival benefit[48,54]. Patients should receive treatment only when they start to present symptoms.

1.2.4 EPIDEMIOLOGY

The average incidence of CLL varies in different geographical areas and races. It is the most common form of leukemia in western countries with an annual incidence of 5.82/100000 inhabitants in the USA[55], but less common in Asia and relatively rare in Japan and Korea. Interestingly, it is rare even among Japanese people who immigrate to western countries[28]. The median age at diagnosis is 72 years. With a higher incidence in Caucasian males (1.7:1), who are also more likely to relapse and to need therapy than females (Fig. 8)[55,56]. CLL is a disease of the elderly. The incidences increase with the advanced of age, and with the increase of the population's age, the rate of CLL is expected to increase[57].

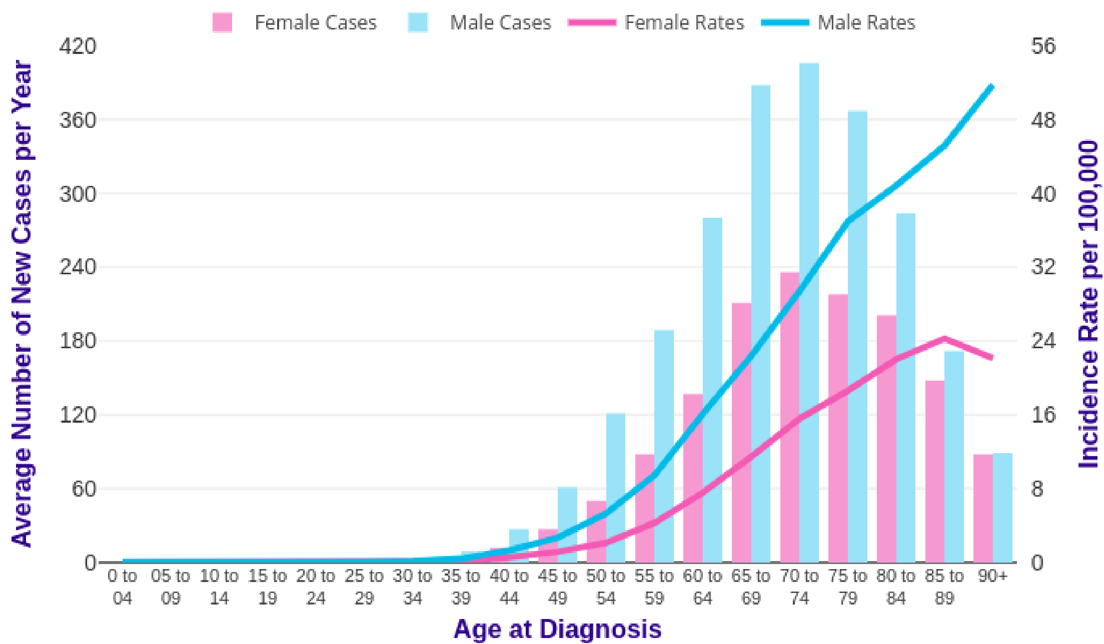

Figure 8.Representation of CLL incidence by age. (Cancer Research UK)

1.2.5 ETIOLOGY

The exact causes of CLL are still unknown, many studies have suggested a correlation with immune dysfunction, smoking, and infectious, but genetic factors due to family history are most likely the cause of CLL[58]. An increased risk of CLL has been seen in professional hairdressers and farm-related work. This indicates a correlation with exposition to heavy solvents and benzene, but these associations have not been firmly proven[57]. Several studies suggest a link between CLL and chemicals. There are evidence of an increase of CLL cases in people exposed to ionizing and non-ionizing radiation[57]. Atomic bomb survivors have shown an increased risk to develop different types of leukemia, however, no increase in the incidence of CLL/SLL has been noted[57].

1.2.6 EPIGENETICS OF CLL

CLL is reported to have a genetic basis and is known to run in families (familial CLL). Approximately 5% of patients with CLL reported a family history of leukemia[59]. Familiar cases generally have an earlier age of onset than sporadic cases. The age at diagnosis of the second-generation offspring is nearly 20 years younger as compared to the parent[57,60]. First-degree relatives (siblings, children, or parents) of CLL patients have a double risk for CLL. It seems that genetics play a part in CLL pathogenesis. It was investigated the role of hereditable mutations on creating a predisposition for the disease. Epigenetics describes heritable changes in a chromosome. Modifications of the DNA molecule that do not involve the nucleic acid sequence, but only the phenotype of the cells[61,62]. Epigenetic alterations in cell genomes could bear a predisposition to a malignant phenotype. These

changes can be transmitted from parental to daughter cells, "somatic transmission." Interestingly, these alterations can also occur in germline cells and inherited from one generation to another, augmenting the cancer risk in the next generation[63]. Several studies have found the gene promoters to be hypermethylated in CLL. DNA methylation consists in the addition of a methyl-group to the fifth position of a cytosine ring, commonly in the context of CpG dinucleotides. It is mediated by DNA methyltransferases (DNMTs)[64]. Physiologically, a copy of DNA is either fully methylated or demethylated. CLL cells show partially methylated copies. Methylation disorders are directly correlated with prognosis. The higher the instability, the more abnormalities are going to accumulate, the poorer the outcome will be. Methylation disorders have been associated with many types of cancers. Usually, the oncogenes are hypomethylated while the tumor suppressor genes are hypermethylated[62,65,66]. Aberrant promoter methylation, as well as CLL-associated microRNAs and long intervening noncoding RNAs, have been implicated in CLL pathogenesis. CLL genome results to be generally hypomethylated, with few exceptions of localized regions of hypermethylation. In CLL it has been observed abnormalities in methylation patterns. Most of them lying outside CpG islands, correlated to IGHV mutational status[67,68]. Methylation analysis of CLL correlated with normal B cell subpopulations has identified new clinical biological subtypes of CLL. The first are naive B-cells- like CLL (n-CLL), largely overlapping with U-CLL. The second are memory B-cell-like CLL (m-CLL), which carried mainly mutated IGHV genes. Suggesting that U-CLL and M-CLL most likely derive from different B-cell subpopulations. The third group has intermediate DNA methylation (i-CLL)[62].

Results of multivariate analyses have shown that the methylation imprint also influences TTFT. Methylation disorders preferentially compromise genes associated with stem cell biology. Potentially cause sub clonal diversification of leukemic cells[68]. U-CLL has distinctive methylation patterns than M.-CLL. This diversity, likely situated in the cells of origin of CLL cells, highlights the biological and phenotypic heterogeneity of this disease[29].

1.3 THE ORIGINS OF CLL

1.3.1 CELL OF ORIGIN

The identification of the cell of origins of any malignancy could be very useful. It could provide a better understanding of the alterations that caused the disease. The abnormal, but constant presence of CD5 on the surface of CLL B-lymphocytes led to the hypothesis that

CLL cells of origin derive from the B1 cells. The B1 lineage is involved in the innate immune system and can produce antibodies subsequently to a dependent T cell reaction[69,70]. After many studies, it has been concluded that CLL derives from mature B lymphocytes CD5 positive. In 50-80% of the cases, CLL cells present more than 2% of somatic mutations on IGHV genes which encode part of the BCR. Early gene expression profiling (GEP) analysis has revealed a relatively homogeneous gene expression profile in CLL. This expression profile is similar to human post germinal center (GC) CD5+ CD27+ memory B-cells. This implied that the IGHV- mutated (IGHV-M) CLLs are derived from antigen-experienced B-cells that have transited through the GC of secondary lymphoid organs, the site of immunoglobulin somatic hypermutation, mediated by the activation-induced cytidine deaminase (AID)[70,71,72].

It is supposed that if the IGHV-M CLL meets the antigen in a T-dependent reaction inside the germination center, then the unmutated IGHV (U-IGVH) derives from a subset of CD5+ CD27- stimulated outside the germination center by a stimulus unable to activate the process of somatic hypermutation of the Ig gene[21,70,71] It has been suggested that the earliest genetic and epigenetic events that lead to the development of the disease occur in HSCs[71]. Specific genetic lesions present in many lymphoid tumors have been found in HSCs in patients with CLL. The matter of the cell origins of CLL remains an open debate[69].

1.3.2 THE ROLE OF BCR IN CLL

CLL is a BCR-dependent malignancy. Normal B lymphocytes depend on the presence of an intact BCR signal transduction pathway for survival. This is likely due to a combination of "tonic" (antigen-independent) and antigen-induced BCR signaling. Recombinational and mutational patterns of the IGHV gene show a significant variability among patients with CLL, that correlates with the clinical outcome. Specifically, two major clinical subsets have been identified. One aroused from a B-cell which did not perform the somatic hypermutation in Ig variable (V) region genes. Defined unmutated CLL (U-CLL) because the CLL-BCRs have 98% or more identity with the germline IGHV sequence[73,74]. The other subset is defined mutated CLL (M-CLL), where the somatic mutations in the Ig genes occur during the maturation of the B-cell[75]. Around 30% of CLL patients express a particular skewed IGVH repertoire and BCRs with a virtually identical Ag-binding pockets, the so-called "stereotyped" receptors[76]. BCR signaling plays a central role in the proliferation of CLL cells. In the lymph node microenvironment of CLL patients it is the main activated pathway[73].

Antigen binding to the sIg induces the activation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmatic tails of CD79a and CD79b by Src family kinases Lyn (LYN), and spleen tyrosine kinase (SYK)[77].

The signal is further propagated by B-cell linker protein (BLNK) and it is downstream signaling components Bruton's tyrosine kinase (BTK) and phospholipase C gamma 2 (PLCγ2), components of the signalosome[78]. Lyn provides continued amplification of the BCR through the recruitment of protein tyrosine kinases as well as the formation of a complex with CD19 and other costimulatory molecules that reduce the threshold of B-cell activation[79]. LYN-dependent phosphorylation of the cytoplasmic domain of CD19 also recruits the p85 subunit of phosphoinositide 3-kinase (PI3K)[80,81,82].

In addition to its role in BCR activation, LYN also has a negative regulatory function. LYN can phosphorylate the immunoreceptor tyrosine-based inhibition motifs on CD22 and CD5. It also recruits the SH2domain-containing protein tyrosine phosphatase 1 (SHP1), thus contributing to attenuated BCR signaling. There are multiple layers of regulation to guarantee a tight modulation of LYN activity[74, 85]. (fig.9)

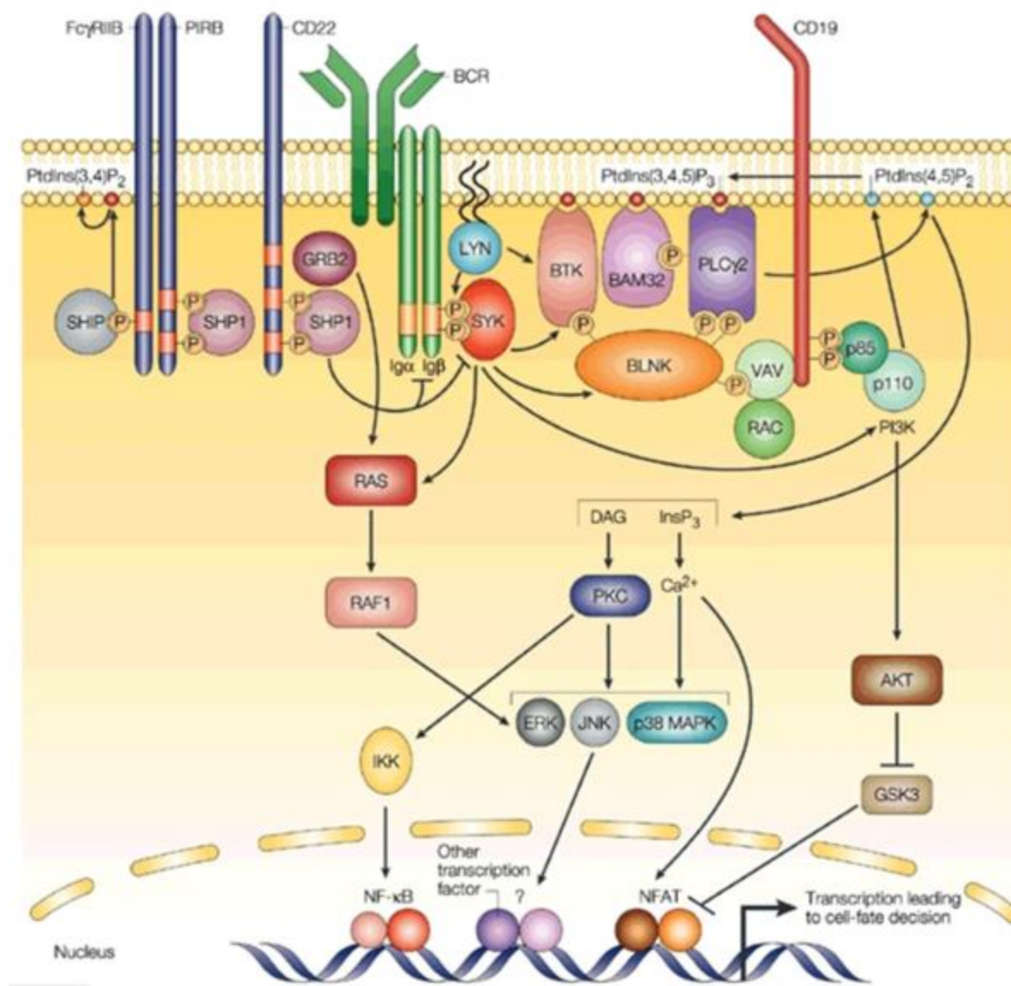

Figure 9. BCR-induced signal-transduction pathways. (Niiro, H. et al. 2002)

SYK kinase belongs to the SYK/ζ chain-associated protein kinase 70 (ZAP70) family of nonreceptor kinases. It is activated through the phosphorylation of ITAMs on CD79a and CD79b. Once activated, Syk propagates the BCR signal by phosphorylating the adaptor proteins BLNK, PI3K, and SHC. SHC serve as a scaffold for the recruitment of signaling molecules, chemokines, and integrin receptor, propagating the BCR signal[80,81].

A primary node in the BCR pathway is the PI3K/AKT cassette, involved in the recruitment of BTK[86]. The PI3Ks are a family of lipid kinases. They are divided into three different classes based on their sequence homology, structure, lipid substrate, and regulation. PI3Ks have been deeply studied because involved in AKT signaling. PI3Ks are further divided into two subfamilies, IA (α, β, and δ) and IB (γ)[80,81]. PI3Kα and PI3Kβ are ubiquitously expressed in several different tissues. PI3Kδ and PI3Kγ are predominantly found in immune and hematopoietic cells where the primary effectors of the BCR network are localized[82,84]. PI3Kδ is recruited to the cell membrane by SYK and CD19 where it is activated through a conformational change, producing the second messenger phosphatidylinositol-3, 4, 5-trisphosphate (PIP3). PIP3 promote cell survival by facilitating Akt phosphorylation and the recruitment of BTK[82,83]. Further downstream responses include calcium (Ca^{2+}) mobilization and activation of protein kinase C (PKC), the RAS–MAPK pathway, and nuclear factor-κB (NF-κB)[83]. BTK is a member of the nonreceptor tyrosine kinase Tec family. It is predominantly cytosolic and has a critical role in the amplification of BCR signaling. Upon BCR engagement, BTK translocate to the plasma membrane where it is firstly phosphorylated by both LYN and SYK kinases. This leads to autophosphorylation, resulting in the activation of PLCγ2, AKT, ERK kinases, and NF-κB signaling[85]. BTK is also involved in the regulation of migration and adhesion via CXCR4/CXCR5 and integrin signaling[81]. With the activation of phospholipase C gamma 2 (PLCγ2), PIP2 is hydrolyzed into secondary messenger inositol triphosphate (IP3). The result is the release of intracellular calcium stores, the propagation of the BCR signal, and upregulation of transcription factors such as nuclear factor κB (NF-κB)[86,87]. Nuclear transcription results in numerous outcomes, including the production and secretion of CCL3 and CCL4 chemokines, two important chemoattractants for lymphocytes and monocytes/macrophages[73].

BTK plays an important role in the interactions with the tissue microenvironment, as a receptor signaling for B-cell migration including cytokines, chemokine receptors, and adhesion molecules essential for the B-cell trafficking and tissue homing. The survival of CLL relies heavily on interaction with the microenvironment for homing and survival, both strongly affected by BTK signaling, which is in a protein expressed at higher levels in CLL

than in normal B-cells[88]. BTK and other Tec kinase family members are essential regulators of immune functions in both innate and adaptive immune cells and mediate signaling via G-protein coupled integrin, cytokine/chemokine, Toll-like, T-cell, and Fcγ receptors[89]. In summary, the combined BTK and PI3K pathways regulate DNA repair, cytoskeleton remodeling, B-cell metabolism, proliferation, survival, and migration[83]. (fig.10)

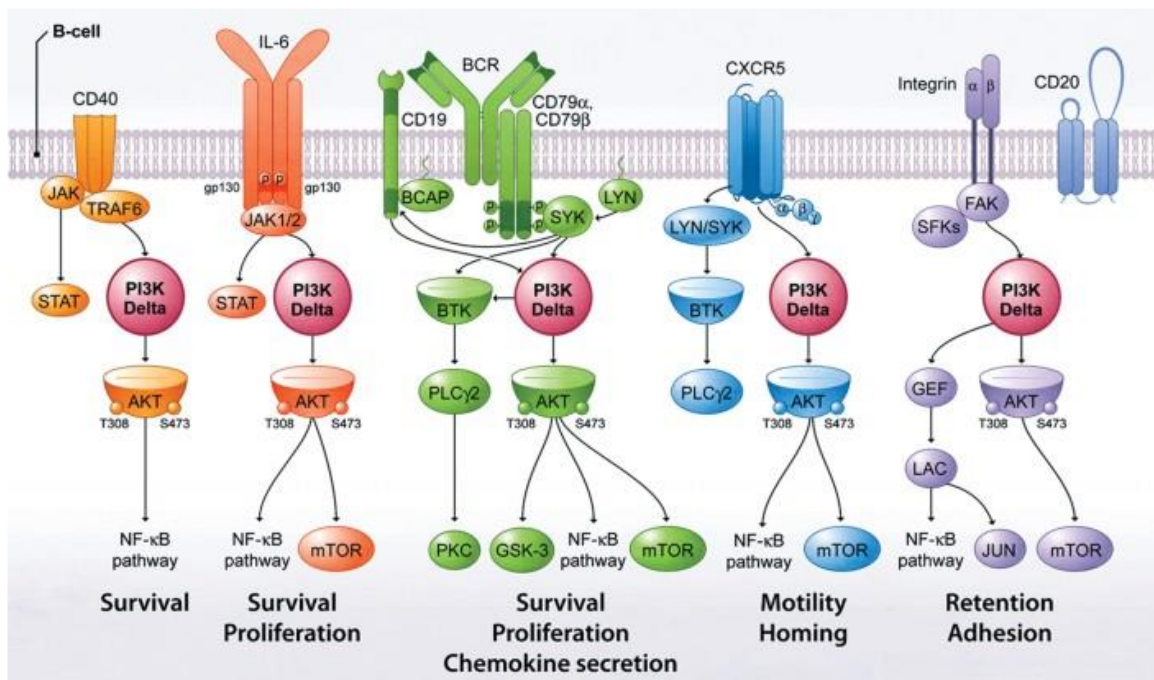

Figure 10. PI3k delta inhibitors tackle B-cell malignancies by interrupting B-cell receptor-mediated signaling and other key pathways needed for survival and homing. (Morrison, C.2014)

BCR increases the production of the T-cell chemokines CCL3 and CCL4, inducible in hematopoietic cells involved in adaptive immune responses. These chemokines have been seen among the strongest upregulated genes in CLL after BCR stimulation[90]. CCL3 and CCL4 secretion by CLL cells recruit accessory cells (T cells and monocytes/macrophages) for interaction with activated and proliferating CLL cells[91]. Increased CCL3 secretion by CLL cells causes attraction and homing of accessory cells to the malignant B-cells in the tissue microenvironments. This creates a favorable microenvironment that allows CLL cells to interact with T cells and NLC to receive survival- and proliferation-signals[92].

1.3.3 MICROENVIRONMENT ROLE IN CLL

CLL cells rapidly undergo apoptosis in vitro, indicating the crucial role of the microenvironment in the survival of CLL cells[93]. Homing to tissue is a tightly regulated process. It depends on the interaction between interleukins, for the survival and proliferation of the cell, and chemokines, secreted by stromal cells within the tissues.

Chemokines play a key role in attracting and retain CLL cells to tissue sites via corresponding chemokine receptors[94]. The proliferation of CLL cells occurs in tissue areas termed "pseudo follicles". The non-transformed elements of the microenvironment that promote the homing, retention, and proliferation of the CLL cells have been increasingly characterized. It includes stromal cells, monocyte lineage nurse-like cells (NLC), and T-cells[95]. These microenvironment components create niches that foster CLL proliferation. They protect CLL cells from spontaneous and drug-induced apoptosis through direct contact and paracrine signals[96]. NLCs are monocyte-derived cells similar to tumor-associated macrophages. They can be found in situ in lymphoid organs from CLL patients[95]. NLCs prevent apoptosis through the secretion of the chemokines CXCL12 and CXCL13, which attract CLL cells into the tissue microenvironment[81,97]. NLCs promote cell survival through the release of the cytokines BAFF (B-cell activating factor) and APRIL (proliferation-inducing ligand). They activate B-cell maturation. The results is a higher expression of important anti-apoptotic genes such as-as as BCL2, SURVIVIN, BCL2A1, and XIAP[95,98]. Chemokines and chemokines receptors on the surface of lymphocytes are fundamental for the mobilization of lymphocytes through the body, the secretion of chemokines in the lymphoid microenvironment is a tightly regulated mechanism[99]. CXCL12 (also known as SDF1 and PBSF) is crucial for the earliest stage of B-cell development. Chemokines are a large family of structurally related chemo attractive cytokines. Abnormal high levels of chemokine receptors in CLL patients have been reported to be implicated in CLL pathogenesis[100]. CXC-chemokine receptor 4 (CXCR4) and 5 (CXCR5) guide the lymphocyte and promote the homing into the bone marrow and lymph node by following CXCL12 and CXCL13 chemokine gradients, established by tissue stromal cells[95,101]. The interaction between ligand and receptor leads to chemotaxis of CLL cells into the tissue microenvironment and down-regulation of surface CXCR4[95]. Besides promoting cell migration, CXCR4 and CXCR5 activation also induce signals related to cell survival, such as extracellular signal-regulated kinase 1/2 (ERK1/2) and signal transducer and activator of transcription 3 (STAT3) signaling[80]. A higher level of chemokines is a marker for increased risk of lymphoid organ infiltration and inferior outcome. Indeed, CLL cells with high CXCR4 expression are more responsive to BCR stimulation. CLL cells expressing high levels of CD38 and CD49d display higher migratory potential towards CXCL12 and show a higher degree of extravasation in an in vitro model of CLL migration[81]. (fig.11)

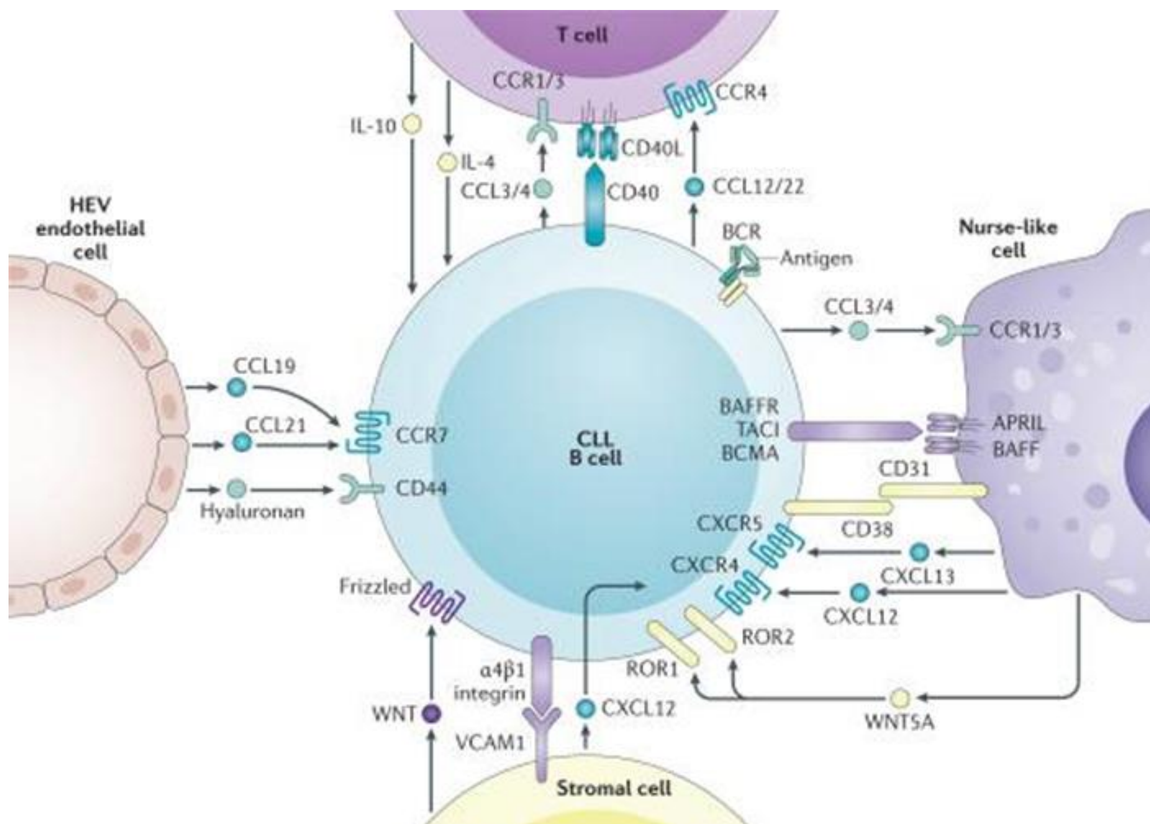

Figure 11. Role of chemokine in the migration of chronic lymphocytic leukemia (CLL) cells into the lymphoid tissue. (Kipps TJ, et al. 2017).

Additional cellular elements in the CLL microenvironment include endothelial cells and follicular dendritic cells (FDCs). They are essential for tissue homing and CLL attachment to tissues. FDCs protect CLL cells from spontaneous apoptosis by direct cell contact. They bond to CD44, upregulated on CLL cells, that cause the up-regulation of myeloid cell leukemia 1 (MCL1), a member of the BCL2 family of anti-apoptotic proteins[102]. The ligation of CLL cells to the stroma is mediated by several surface proteins, including CD38, CD49d, MMP-9, CD27, and ZAP-70. The binding of CD27 to ZAP-70 is crucial with the functional capacity of CLL cells to adhere to stromal cells. Inhibition of CD27 blocks CLL cells from binding to the stroma[103].

The ligation of BAFF and APRIL with their receptors on CLL cells activates survival and differentiation pathways. That includes upregulation of CD40 ligand (CD40L)[104]. The interaction of the CD40 molecule on the CLL cell surface with its ligand CD154 (CD40L), predominantly expressed on T-cells, plays an important role in CLL-cell biology. CD40 is a transmembrane receptor and member of the TNF superfamily that is expressed in activated CD4+ T cells[105]. Activation of CD40 results in downstream signaling, both dependent and independent of TNF-receptor associated factor adaptor protein (TRAF). (fig.12)

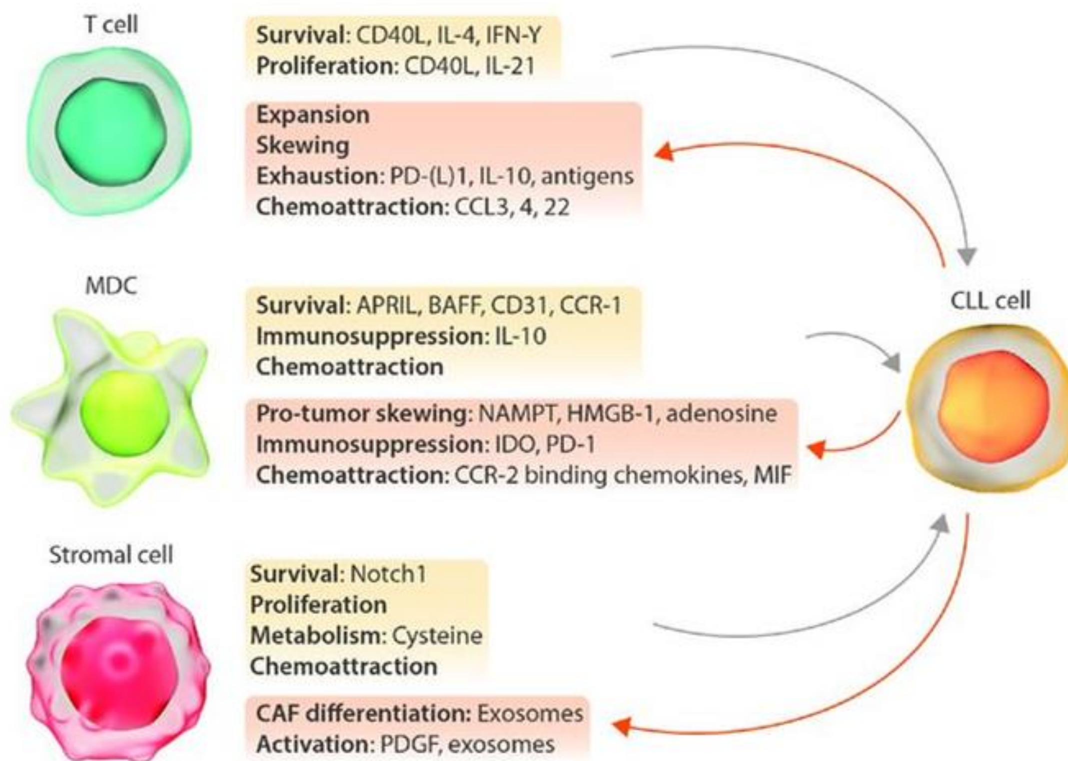

Figure 12. Interactions between chronic lymphocytic leukemia cells and other cells that contribute to the formation of a tumor-supportive microenvironment. (Van Attekum et al., 2016).

CD40-mediated signaling regulates apoptosis through modulation of the expression of pro-survival proteins, including BCL-xl, Mcl1, and surviving. CD40/IL-4 stimulation has also been shown to stimulate the PI3K/ AKT pathway. It has therefore been implicated in CLL-cell proliferation[105,106]. In CLL, the overall number of circulating T cells is increased, although their functionality seems to be compromised. T cells in CLL cases show an inability to form functional immune synapses. They display impaired motility and a higher expression of exhaustion markers including programmed cell death protein 1 (PD-1)[85]. The stimulus provided by CD40L gives CLL cells the capacity to chemoattract activated CD4+ T cells. This leads to the hypothesis that T cells may influence the proliferation of CLL cell populations. The stimulation of CD40 rescues blood CLL cells from apoptosis and induces their proliferation. The interaction between CLL and T-cells brought to the production, by both cell types, of several cytokines, such as IL-4, interferon (IFNα), and IFNγ, that may be involved in negative autocrine circuits able to inhibit CLL cell apoptosis[107,108].

1.3.4 NF-kB

Nuclear factor κ-light-chain (NF-κB) signaling plays essential roles in inflammation, immune responses, proliferation, and cell survival. In cancer cells, NF-κB promotes tumor growth by contributing to the maintenance/expansion of tumor-initiating cells and by

shaping the tumor microenvironment (fig.13). Deregulated NF-κB signaling is a common finding in most, if not all, B-lymphoid malignancies[109]. NF-κB signaling is a key factor in the development and evolution of CLL. There are two NF-κB pathways, the canonical and noncanonical pathways. The former start with BCR signaling through BTK. The latter is activated by members of the tumor necrosis factor (TNF) cytokine family[110,111]. In CLL, NF-κB is constitutively activated. This causes the up-regulation of antiapoptotic genes, which bring to an increased apoptosis resistance, a major feature of CLL cells. NF-κB signaling plays also a crucial role in the crosstalk between CLL cells and their protective microenvironments, such as the BCR pathway, the BAFF/BAFF-receptor axis, or the CD40L/CD40 axis[111].

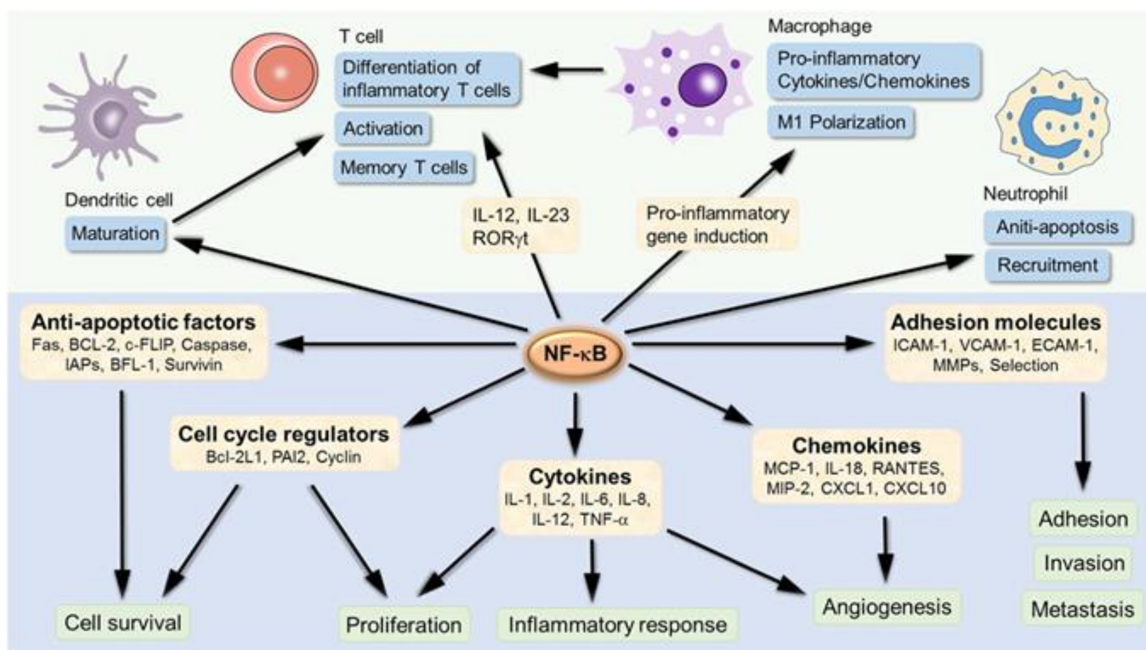

Figure 13. NF-κB target genes involved in inflammation development and progression. (Liu, T et al. 2017)

Besides being activated by BCR signaling, NF-κB receives additional activator signals from the micro-environment which bring to an activation of the Bcl-2 family, among the most important transcriptional targets and enhance CLL cell survival[109,110].

1.4 GENES INVOLVED IN CLL

1.4.1 BCL2

The physiological balance between cell survival and cell death is a tightly regulated mechanism. In cancer, it is often found altered in favor of cell proliferation. One way for cancer cells to shift this balance in their favor is to disrupt the pathways that regulate cell death. The first proto-oncogene to be described with anti-apoptotic function was B-cell

lymphoma 2 (Bcl-2). The key role in apoptosis played by BCL-2 made this gene the target of most compounds for CLL therapy (fig.14).

BCL-2 gene is located on chromosome 18q21.33, a region involved in t(14:18), a chromosomal abnormality very common in follicular lymphoma[112]. BCL-2 was the first found gene that does not stimulate cell proliferation, but rather increases the survival of the cells. High expression of BCL2 and impaired apoptosis are a hallmark of CLL[113]. In normal cells, BCL2 is regulated by miR-15a/16 that inhibits its function as an antiapoptotic protein. Deletion at locus 13q14 is the most frequent genetic abnormality in CLL[114]. Bcl-2 is a family of proteins localized in the mitochondria that regulate the intrinsic pathway of apoptosis[112], either inhibiting or activating the apoptosis pathway. They inhibit their respective functions by binding to each other in various combinations[115].

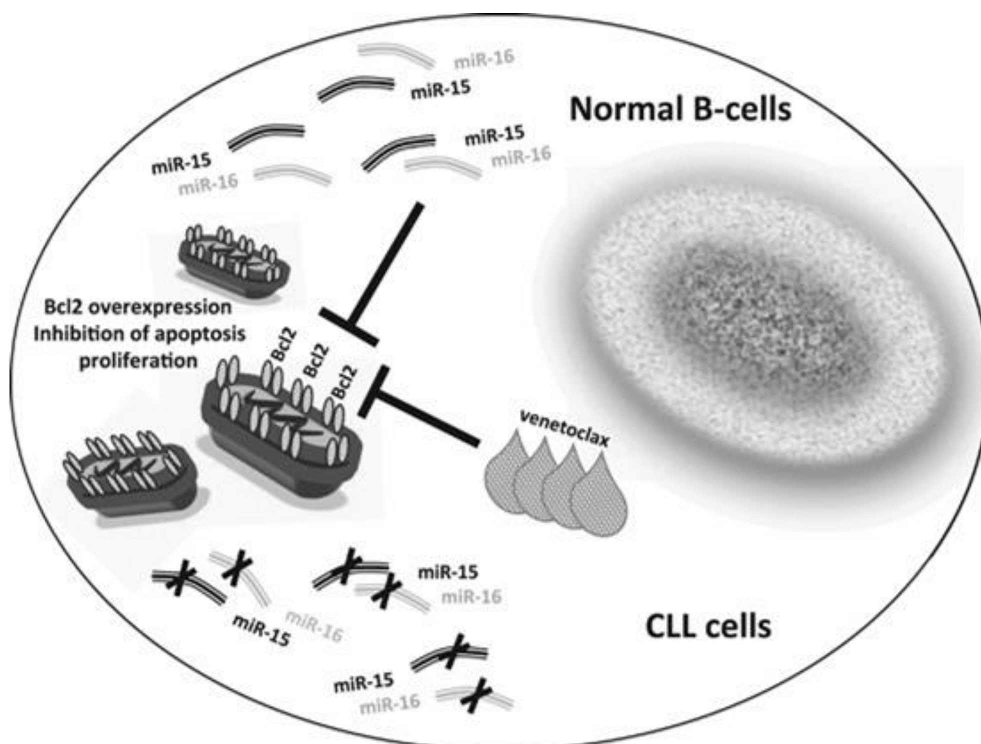

Figure 14. Therapeutic implications of miR-15/16 targeting BCL2. (Pekarsky et al, 2018)

Bcl2 shares four homology domains (BH 1-4) with the anti-apoptotic members of the BCL-2 family[115]. There are two clusters of pro-apoptotic members of the BCL-2 family. The first comprises multidomain effector proteins, such as BAX and BAK proteins. The second includes BH3-only proteins. Pro-apoptotic proteins (BAK and BAX) are inhibited by anti-apoptotic proteins (e.g., BCLXL and BCL2), whose function, in turn, is inhibited by BH3-only pro-apoptotic proteins[116]. When the intrinsic pathway of apoptosis is activated by an apoptotic stimulus, the pro-apoptotic multi-domain effector proteins BAK

and BAX change. They turn from their active forms to oligomers that permeabilize the outer mitochondrial membrane, causing the release of cytochrome c in the cytoplasm[117]. Cytochrome c then recruits caspase 9. Together they initiate the intrinsic pathway of apoptosis by binding to the protein Apoptotic Protease Activating Factor-1 (APAF-1)[118]. (fig.15.) The miR-15a/16 are not the only ones responsible for the regulation of Bcl2 expression, several factors contribute to it, including genetic, epigenetic, and micro environmental signaling[93]. In some CLL cases, the increased expression of BCL2 in CLL appears to be due to the hypomethylation of the BCL2 gene promoter. Several pro-apoptotic Bcl-2 genes are direct transcriptional targets for p53[119]. In CLL, as the disease progresses, the frequency of TP53 deletions and mutations increases, suggesting an augmentation of Bcl-2 family deregulation with advancing disease[120]. Besides, p53 is also capable of regulating the activity of Bax and Bcl-2 family proteins at both transcriptional and post-translational levels, such as through a protein-protein interaction in the cytosol[121].

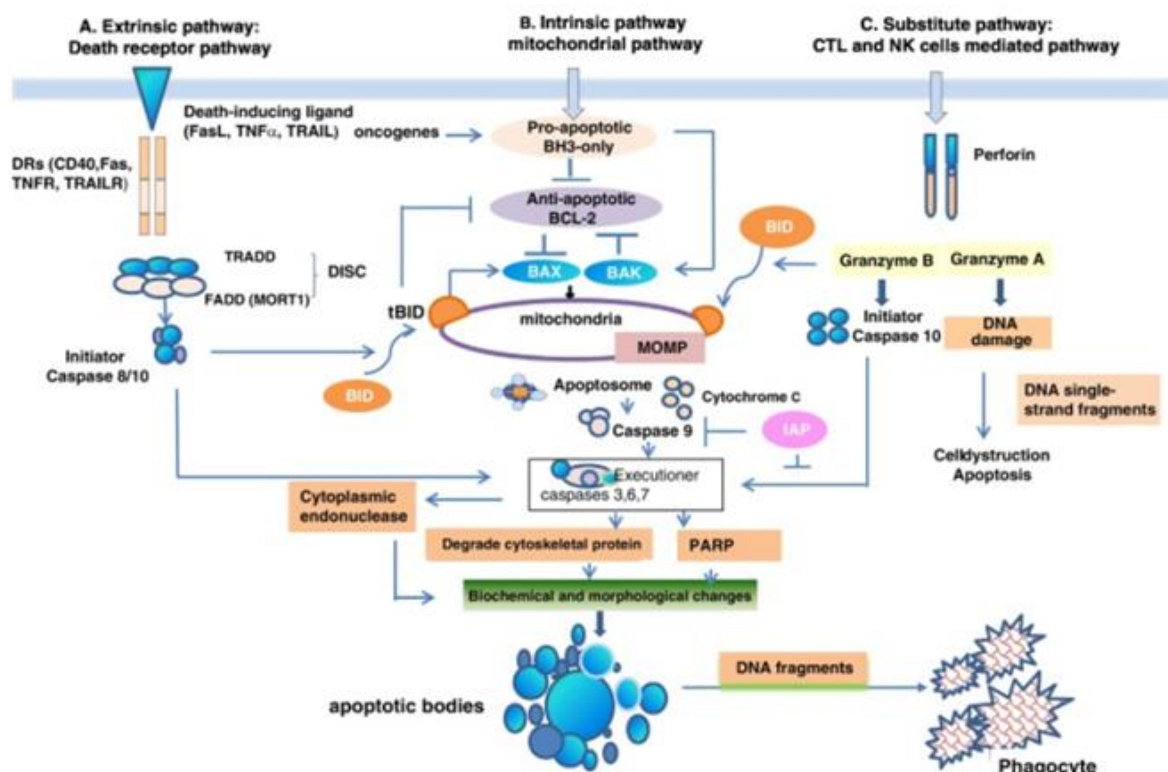

Figure 15. Illustration of major apoptotic signaling pathways. (A) The extrinsic pathway, (B) Intrinsic apoptotic pathway, (C) substitute pathway via activating interactions between BCL-2 members. (Wu et al., 2018)

The bcl-2 expression is an independent prognostic factor in CLL. The expression of prosurvival Bcl-2 family proteins could be induced by the contact of CLL cells with bone marrow stromal cells, nurse-like cells, and follicular dendritic cells[93]. CLL cells found in the peripheral blood have shown to have a lower expression of antiapoptotic Bcl-2 protein

compared with those located in lymph nodes. This finding suggests a correlation between Bcl2 and vascularity, leading to the hypothesis that endothelia cells play a role in the survival of CLL cells[122]. BCL-2 expression induced hematopoietic cell survival and cooperates with MYC, an oncogene commonly overexpressed in lymphomas, to induce proliferation of B-cell precursors, leading to tumor progression. High levels of Bcl-2 have been associated with shorter overall survival and increased chemo resistance to treatment in CLL patients[123].

1.4.2 NOTCH1

The most commonly mutated gene detected at diagnosis in patients with CLL is NOTCH1. Its frequency increase with the disease progression[124]. The NOTCH receptor genes encode a family of heterodimeric transmembrane proteins (NOTCH1 to NOTCH4) that function as ligand-activated transcription factors. When the NOTCH receptors interact with their ligands (JAGGED1 and JAGGED2) through the extracellular subunit, two consecutive proteolytic cleavages of the NOTCH proteins are initiated and lead to pathway activation[124,125]. Upon activation, the cleaved intracellular portion of the NOTCH receptors (ICN) translocates into the nucleus where it recruits a transcriptional complex that modifies the expression of many target genes, including MYC and NF-kB signaling components[125]. The majority of NOTCH1 mutations usually occur within the PEST domain, generating a truncated protein[126]. The PEST contains the amino acidic sequences recognized by the ubiquitin ligase FBXW7. FBXW7 induces NOTCH1 protein degradation through the proteasomal pathway[127]. Disruption of the PEST domain of the protein results in NOTCH1 impaired degradation, stabilization of the active ICN, and deregulated NOTCH signaling[128]. NOTCH1 mutations are significantly more common in U-CLL. They are enriched in CLL harboring +12 and are associated with short time to first treatment (TTFT) and poor overall survival[125,129]. A small percentage (3%) of CLLs that are devoid of NOTCH1 lesions harbor FBXW7-inactivating mutations that impair the ubiquitination of the NOTCH1 protein. This causes an analogous functional outcome via an abnormally stabilized NOTCH1 signaling[69,127]. Mutations of the NOTCH1 gene are present in approximately 10% of CLL patients at diagnosis. The frequency of NOTCH1 mutations varies according to the clinical stage in which it was analyzed[69,127]. ICN-positive CLL cells in NOTCH1 wild-type cases occur at similar frequencies in both IGHV CLL subtypes. NOTCH1 signaling in these tumors may be activated by additional extrinsic factors[130]. NOTCH1 has been seen abnormally activated even in the HSCs of patients with CLL,

regardless of its mutational status. The activation of NOTCH1 may contribute to the pathogenesis of CLL with the development of aberrant HSCs[71] (fig.16).

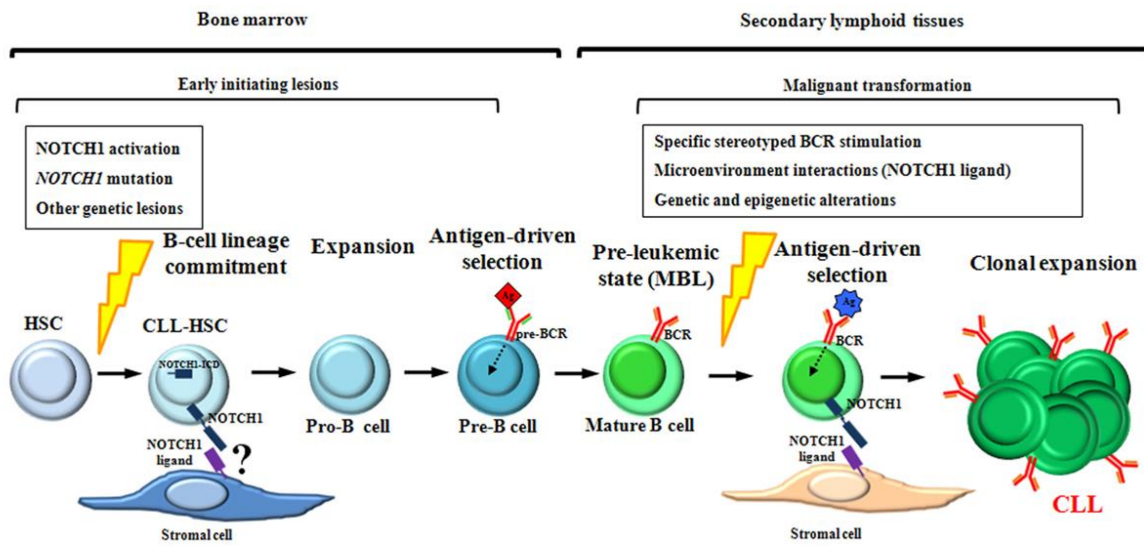

Figure 16. Schematic representation of NOTCH1 involvement during chronic lymphocytic leukemia (CLL) development. (Rosati et al., 2018)

CLL cells from NOTCH1-mutated cases are characterized by lower CD20 expression which makes them less sensitive to rituximab. CD20 expression is upregulated by the blockade of NOTCH1 signaling, exerted by specific small interfering RNAs[131]. These biological findings possibly reflect a deregulated epigenetic loop associated with the impaired function of histone deacetylase (HDAC) that is induced by NOTCH1 mutations and is partially restored by treatment with HDAC inhibitors[127,131].

1.4.3 BIRC3

BIRC3 is an E3 ubiquitin ligase member of the inhibitor of apoptosis (IAP) protein family. It acts as an inhibitor of the non-canonical NF-κB pathway. BIRC3 induces proteasomal degradation of MAP3K14, which is the major driver of non-canonical NFκB activation[132]. Therefore, disrupted BIRC3 could alter the ligand-independent activation of the constitutive NFκB pathway, inducing cell proliferation, and survival[133]. At the time of diagnosis, the BIRC3 gene is found mutated in less than 1% of CLLs. The frequency rises with disease progression and reaches more than 25% of cases that are refractory to fludarabine[134]. BIRC3 mutations are usually truncated and frequently associated with deletions of the 11q region, mapped close to ATM[125].

1.4.4 TOLL-LIKE RECEPTORS /MYD88

Toll-like receptors (TLR) are prototypic receptors of the innate immune system. They act as costimulatory molecules in the context of BCR stimulation[135]. In B-cells, Toll-like receptors are central in shaping the composition of the B-cell repertoire. They have also a crucial role in the BCR independent response to antigens by sensing a variety of pathogen-associated molecular patterns[125] (fig. 17). The TLR signaling require adaptor proteins for initiating, including the myeloid differentiation factor 88 (MYD88)[136].

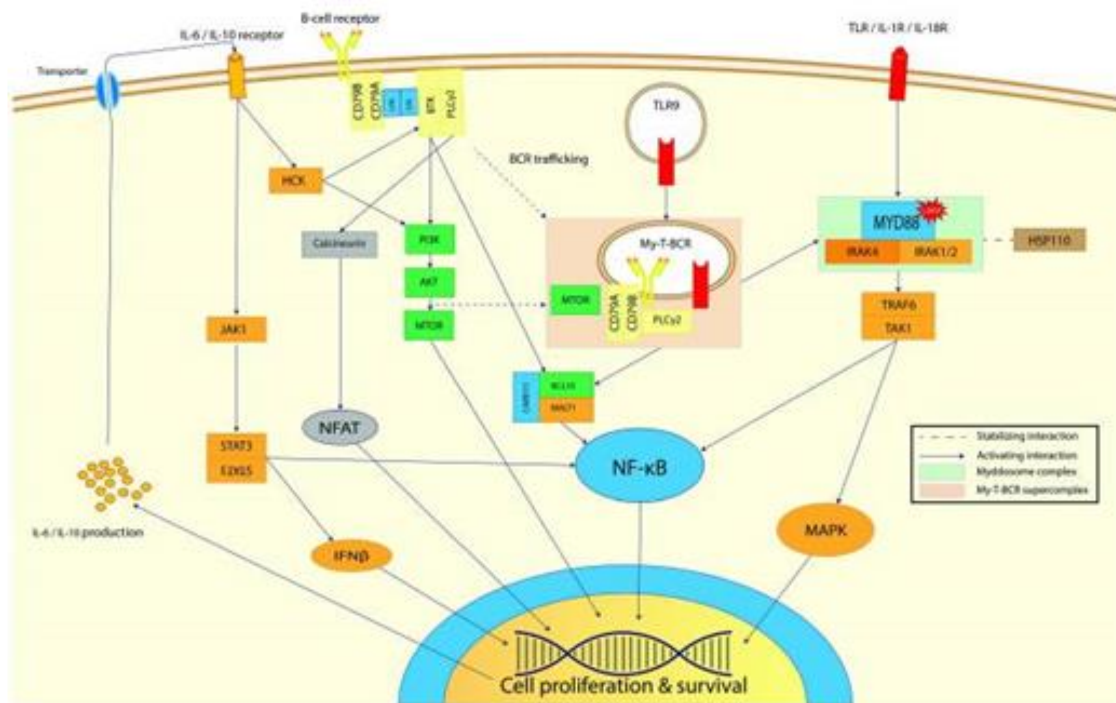

Figure 17. The role of MYD88 signaling in normal physiology and lymphomagenesis. (De Groen et al., 2019)

MYD88 is the canonical adaptor in the TLR signaling pathway. MYD88 has a death domain (DD) at the N terminus and a Toll-IL-1 receptor (TIR) domain at the C terminus. The DD domain recruits and activates interleukin-1 receptor-associated kinase 4 (IRAK4). It leads to spontaneous activation of a series of cascades and transcription factors, such as NF-kB, AP-1, and STAT3. In B-cell tumors, mutant MYD88 results in the uncontrolled formation of the MYD88/IRAK complex, which causes a rise in the NF-kB activity and cytokine secretion[125,137].

MYD88 undergoes an activating mutation in approximately 3% of CLL cases. The patients in these cases are usually young and harbor a M-CLL. MYD88 mutations are predominantly clonal. They are considered as drivers of CLL, highlighting the relevance of TLR signaling in CLL development and evolution[138]. The impact on the outcome is controversial, different results have come out from different studies[130].

1.4.5 SF3B1

Spliceosome Factor 3B (SF3B) is a heptameric protein complex. It is a critical component of the splicing machinery essential for creating a mature mRNA[139,140]. SF3B1 mutations in B-cells induce senescence through decreasing BCR signaling (fig.18). SF3B1 to generate a neoplastic transformation of the cells requires the concomitant inactivation of ATM[130].

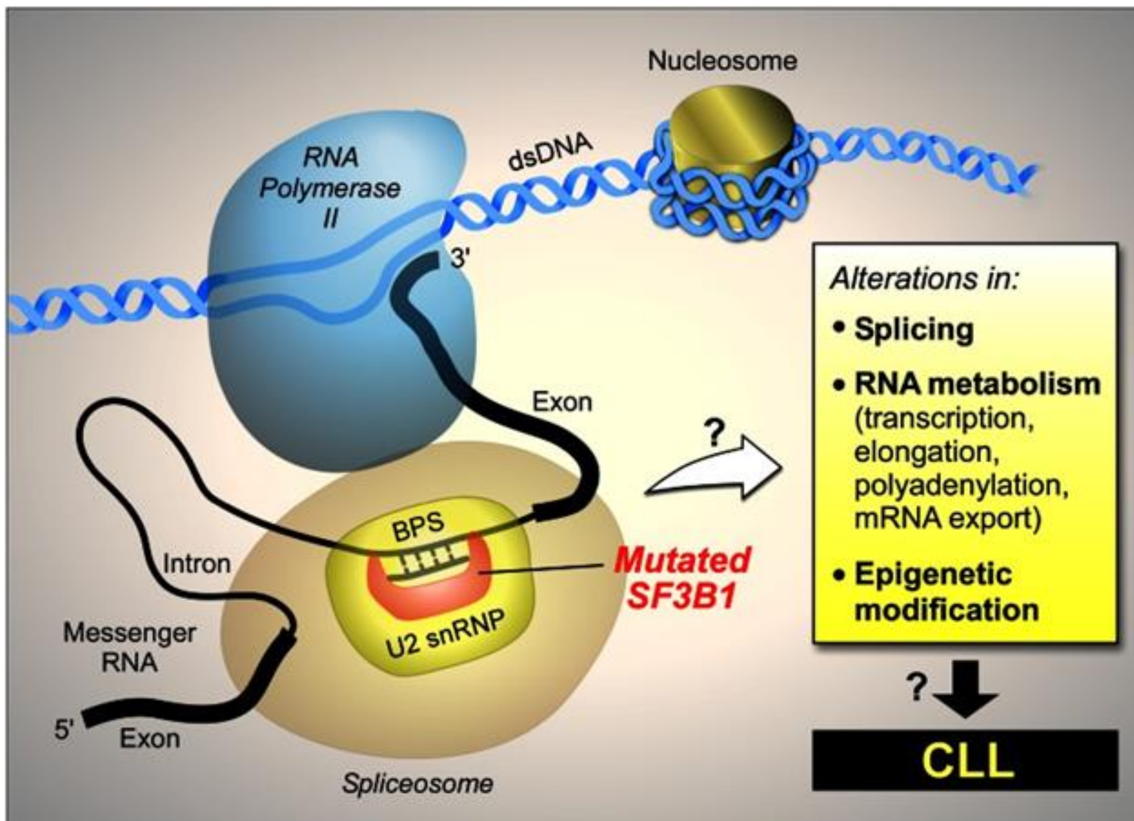

Figure 18. The potential impact of mutated SF3B1 on the pathobiology of CLL. (Wan et al., 2013)

SF3B1 mutations occur from 7 to 10% of unselected CLL. They are higher in cases harboring U-IGHV genes and tend to co-occur with ATM deletion or mutations. In CLL, the most frequent SF3B1 mutations are generally missense nucleotide changes that recurrently target hotspots[125,141].

1.5 OTHER MOLECULAR ALTERATION

1.5.1 COPY NUMBER VARIATION (CNV) AND LOSS OF HETEROZYGOSITY (LOH)

The copy number variation (CNV) represents a copy number change involving a DNA fragment. It could range from several kilobases (kb) to several megabases (Mb) in size[142]. CNV provides the raw material for gene family expansion and diversification, which is an

important evolutionary force. CNV is an important pathogenic factor in a range of common diseases, including infectious, autoimmune, and neuropsychiatric diseases and cancer[143]. In CLL, copy number aberrations (CNAs) tend to appear at an early stage in the disease. They usually remain stable, whereas the mutational heterogeneity may increase[144]. CNAs and copy number neutral loss of heterozygosity (CNLOH) act as oncogenic mechanisms that alter coding sequences and cause structural rearrangements. Oncogenes are frequently affected by copy number gains, while tumor suppressor genes tend to be deleted[145]. CNV is not necessarily pathologic, in every individual are present several benign CNVs[146]. CNVs often occur in regions containing, or be nearby, large homologous repeats or segmental duplications. Segmental duplications occur when a tandem repetition of a DNA segment undergoes a rearrangement that places the duplicated copies at different chromosomal loci[147]. Not all CNVs, however, are associated with segmental duplications. Subsets of CNVs may be formed or maintained by non-homology-based mutational mechanisms[142]. Approximately 80% of CLL harbors somatically acquired genomic copy number aberrations (aCNAs)[148]. A typical scenario for tumor suppressor genes is when an aCNA removes one allele of a critical gene or genes and the retained allele of the gene(s) is mutated. These are the cases of 17p for TP53 and at 11q for ATM in CLL. Observed LOH is usually associated with copy loss in CLL[149]. The number of aCNA is clinically important, two or more aCNA are detected in about 35 % of all cases, while three or more aCNA in about 20 % of CLL[150]. An elevated aCNA count is an indicator of an elevated genomic complexity. It highlights a CLL subset with progressive and aggressive disease and short survival. There is a strong association between an inability to maintain genomic stability and an aggressive CLL phenotype[150].

1.5.2 miRNA ROLE IN CLL

Almost 98% of human DNA is composed of what was called ‘junk DNA’, non-coding regions that until recently were considered useless[150]. A consistent part of this ‘junk DNA’ it was late discovered to be transcribed in non-coding RNA, which function is to silence functional RNA molecules[151]. Only 1% of the total RNA content in mammalian cells is composed of coding RNA, the rest is transcribed in several types of RNA: ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), as well as other small but fundamental RNA types, microRNAs (miRNAs)[152]. The miRNAs are 22nt-long non-coding RNA molecules present in all eukaryotic that regulate a wide array of biological processes, including carcinogenesis[151]. In cancer cells, miRNAs are heavily dysregulated[153]. miRNAs are usually clustered together into families sharing similar seed regions, a 2-7 nucleotides

region at the 5'end, and they are thought to act redundantly[154]. The miRNAs are the results of the transcription of longer RNA by the sequential action of RNA polymerase II, the nuclear nuclease Drosha, and the cytosolic nuclease Dicer.

The function of the miRNA could vary. They can either act as oncogenes or as tumor suppressor genes based on their level of expression. They often targets genes involved in cell cycle regulation, apoptosis, or angiogenesis[63]. Studies show that miRNA profiling may discriminate between different subgroups of tumors and predict outcome or response to therapy. miRNAs expression in chemo-resistant cancer cells is different from their parental chemosensitive cells, suggesting that they could be used as indicators for drug resistance[155].

If there is perfect complementarity between miRNA and the target it will cause the degradation of the mRNA target, in the case of imperfect complementarity, miRNAs cause translational repression of the mRNA target[156] (fig.20).

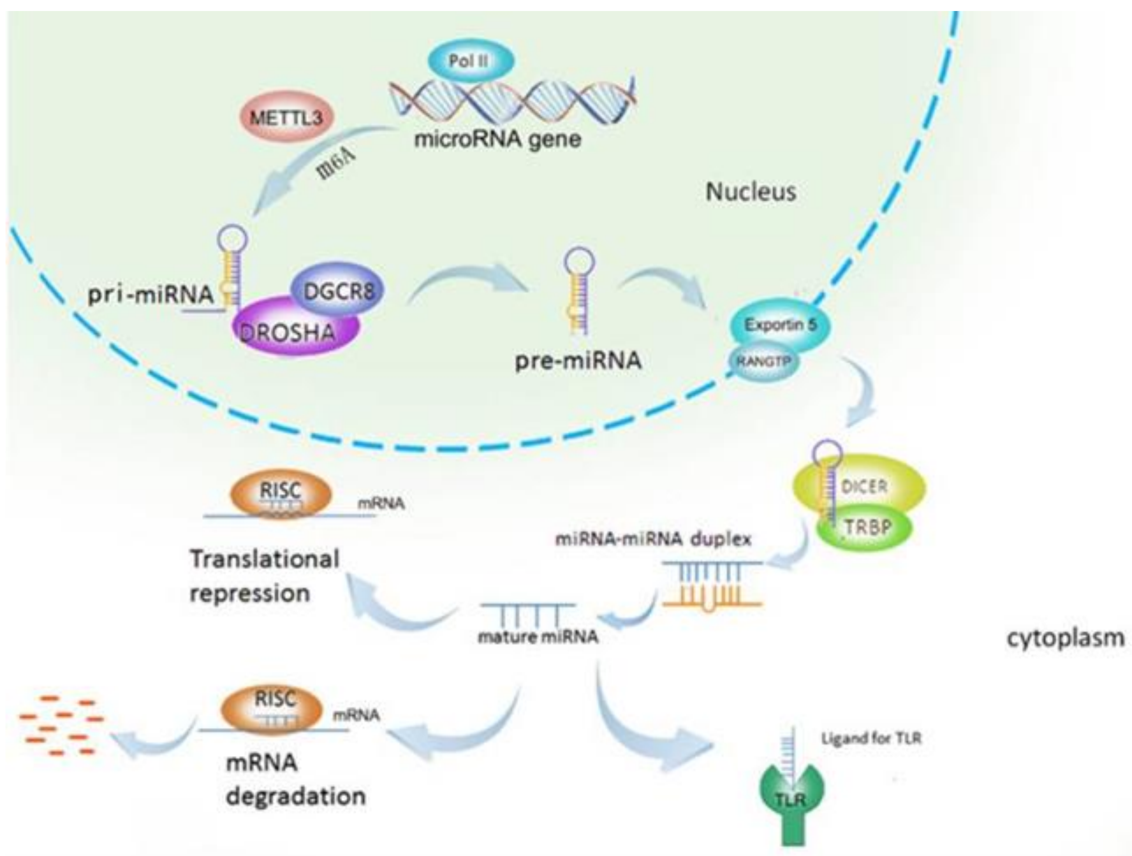

Figure 19. microRNA biogenesis. (Peng et al, 2016)

MicroRNAs can also act as protagonists in the control of the cellular methylation status by modifying the expression of the enzymes responsible for epigenetic control[157]. DNA demethylation and histone deacetylase inhibition can activate the expression of miRNAs that may act as tumor suppressors[153].

First discoveries of an association between miRNA deregulation and cancer were made studying the region at chromosome 13q14, frequently deleted in CLL. This region contains two microRNA genes, miR-15a and miR-16-1, involved in the regulation of the BCL2 pathway. The deletion of these two miRNAs causes the development of CLL[158].

In normal CD5+ lymphocytes, miR-15a and miR-16-1 genes are ubiquitously and highly expressed while in almost 60% of CLL cases, the mir-15a and mir-16-1 are deleted, altered, or downregulated[159]. In a few cases of familial CLL, these miRs are dysfunctional[160].

Since the discovery of miR-15a and miR-16-1 there are being found several miRNAs who play a role in the pathogenesis of diseases, CLL in particular.

1.6 PROGNOSTIC FACTORS

The clinical course of CLL is extremely heterogeneous. Some patients can live for decades without presenting any symptoms. They may die of old age or other pathology with no correlation with the CLL. Other patients instead rapidly became symptomatic with high-risk disease. These patients require immediate treatment, and the cause of death is often disease-related. Prognostic factors are crucial for the identification of those patients at higher risk of progression. The research of precise markers is still ongoing and until now there have been identified several prognostic factors[161].

1.6.1 CLINICAL

CLL is a disease of the elderly; the median age at the time of the diagnosis is between 65 and 70 years. The 10-year survival expectation is 59% for those under 60 years, compared with only 6% for those older than 80 years[162]. C CLL is more common in males than in females. Moreover, women have better overall survival, suggesting the hypothesis of a major difference between the sexes in the biology of CLL. Several studies effectuated on the mutational status of IGVH have shown that the majority of patients with unmutated CLL were predominantly males[163,164]. In CLL blood lymphocytes count and their variation seem to correlate with the tumor mass[165]. The time it takes for the lymphocyte count to double is called lymphocyte doubling time (LDT). An LDT of less than 12 months is associated with a decreased progression-free survival (PFS) and overall survival (OS)[166]. If the absolute lymphocytes count double in less than 6 months or if there is a 50% increase in lymphocyte counts within 2 months, it is an indicator of active disease. It is required

treatment as outlined by the International Workshop on Chronic Lymphocytic Leukemia (IWCLL)[167]. It should be used only when lymphocyte count is above 30,000 lymphocytes/µl. Another predictor factor of PFS is the serum β2-microglobulin. It is an extracellular protein that is non-covalently bound to the α-chain of the class I MHC[166,168]. A level of β2 microglobulin >3.5 mg/l is correlated with an advanced disease stage, high tumor burden, bone marrow infiltration, and shorter PFS and OS[169].

1.6.2 FLOW CYTOMETRY

Flow cytometry is a rapid technique, relatively simple. It required peripheral blood, an easy material to obtain, to perform a CLL analysis. Until now, only a couple of superficial antibodies are used as an indicator of prognosis. The discovery of markers that could be found using flow cytometry would give a rapid characterization of the disease, allowing the first stratification of patients into groups with different risks of progression.

1.6.2.1 CD38

CD38 is a transmembrane glycoprotein that is regulated by the tumor microenvironment. It can function as a co-receptor and as an ectoenzyme[166,170]. CD38 is differently expressed dur-ing B-cells development. Precursor cells in bone marrow have a higher level of CD38. Its expression is reduced in resting mature B-cells and is then re-expressed at a higher level on plasmocytes[171]. On B-lymphocytes, CD38 maturation is tightly regulated. CD38 is situated near the BCR-CD19 complex and amplifies its signal intensity, activating cell proliferation[172,173]. (fig.21)

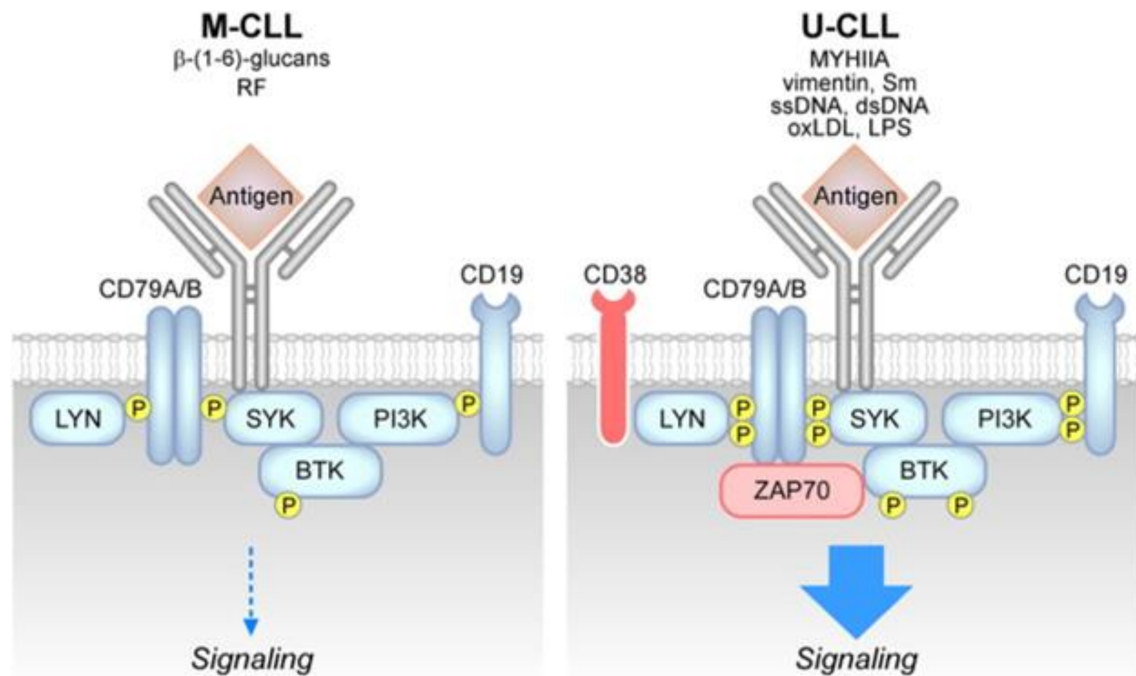

Figure 20. Differences between M-CLL and U-CLL signaling pathways and the role of CD38. (Hacken, E et al, 2016).

CD38 is a marker of activation in naïve B-cells. The percentage of cells, within a CLL clone, that displays CD38 is an indicator of the cellular activation of the clone[91]. In CLL, elevated expression of CD38 is associated with several adverse clinical and biological features, such as advanced disease stage, higher incidence of lymphadenopathy, hepatomegaly, and high-risk cytogenetics[166]. CLL patients with a higher level of CD38 (≥30%) have a shorter time to first treatment. They have a more aggressive clinical course, with inferior survival than patients with less than 30% of CD38+ CLL cells[172]. Several studies have now suggested that even an expression lower than 30% is to be considered a negative prognostic marker[173].

1.6.2.2 CD49d

CD49d expression in CLL is variable. It exhibits a bimodal distribution, with patients showing levels of expression either very high or very low, with rare cases of borderline expression[174]. The percentage of CD49d+ cells, like CD38+ cells, is a strong independent predictor of survival in CLL. Higher expression levels (>30%) are correlated with shorter OS[174]. The CD49d is the alpha4 integrin subunit, associated with the CD29 (beta1) chain, which composes the very late antigen 4 (VLA-4; CD49d/CD29) molecule. CD49d/ CD29 mediates cell-cell- and cell-matrix interaction in CLL. The complex bind to fibronectin and VCAM-1 (VLA-4 ligand), expressed on endothelial cells and bone marrow stromal cells (BMSCs)[175](fig.22).

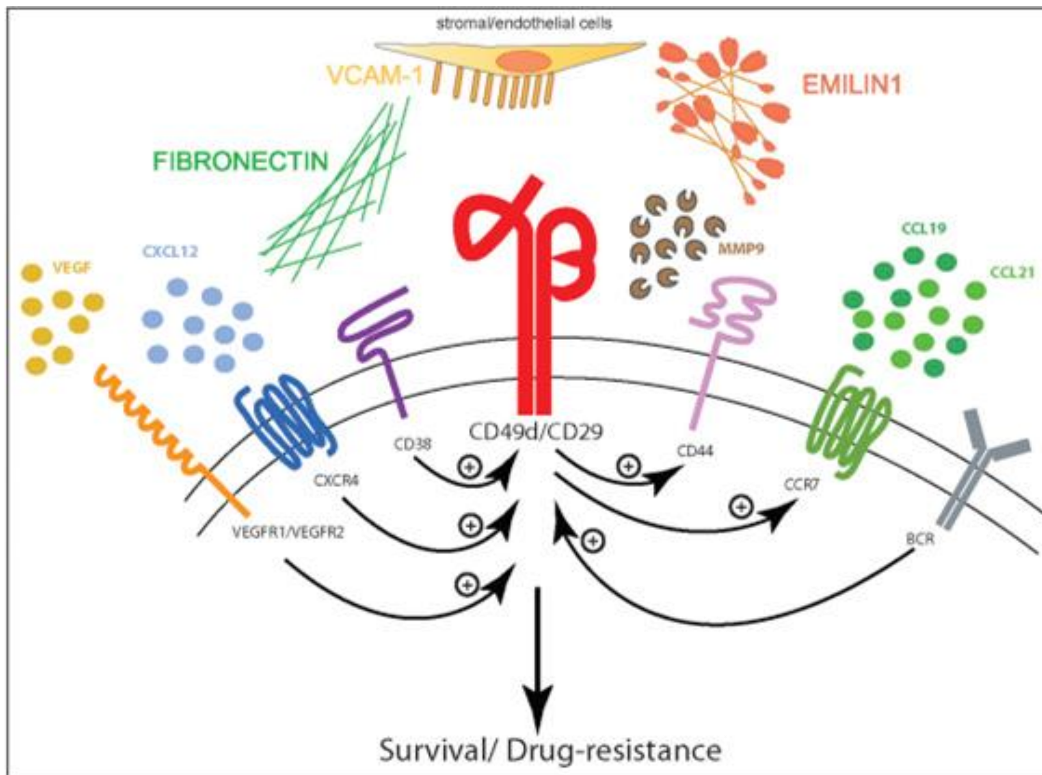

Figure 21. CD49d interactions in a chronic lymphocytic leukemia (CLL) microenvironment. (Dal Bo, M et al. 2014).

CD49d has an important role in leukocyte trafficking and homing, which is confirmed by the presence of lymphadenopathy at diagnosis in patients with a higher level of CD49d[175]. CD49d cooperates with chemokine receptors in CLL cell adhesion to stromal cells, which eventually cross-talk with CD38[95,176].

1.6.3 CYTOGENETIC FACTORS

Conventional cytogenetics is a complex, time-consuming discipline that requires a specialized operator. Conventional cytogenetics though provides a unique view of the complete patient's chromosomal asset. For the analysis of CLL cases are requested both the conventional cytogenetics and the Fluorescence In Situ Hybridization (FISH), which requires only a couple of hours. Chromosomal alterations are mostly the result of independent events. Rarely could happen that from a single catastrophic event derives multiple anomalies. The genetic instability causing cytogenetic abnormalities derives from the degradation of the telomeres that stop working as guardians of the chromosomes. Cytogenetics aberrations are divided into two major categories: numerical and structural. Numerical aberrations include monosomies and trisomies. The former refers to the loss of one of the twin chromosomes. The latter indicates the presence of a third chromosome to one of the chromosome pairs. Structural abnormalities include the loss (deletion) or gain (addition) of genomic material, the presence of an unrecognizable chromosome (marker),

or the exchange of genomic material from one chromosome to another (translocation). Translocation could be of two types: balanced or unbalanced. They are referred as balanced when there is a mutual exchange of chromosomal material. In unbalanced translocation instead, part of the genomic material goes missing. In CLL there are specific alterations that frequently recur: deletion of chromosome 13, trisomy of chromosome 12, deletion of chromosome 17p, and deletion of chromosome 11q.

1.6.3.1 TELOMERES

Telomeres are non-coding nucleoprotein complexes. Their main function is the capping of eukaryotic chromosomal ends to protect them from being recognized as DNA damage. They are associated with aging-related disease and with risks of cancer[177]. The telomere is a sequence of DNA whose function is to act as a guide for the mRNA primer without been replicated[70]. Telomere DNA is a repetitive hexamer of TTAGGGs that is bound by a specialized protein complex known as shelterin. Telomerase synthesized new telomere DNA to balance the shortening that normally occurs during DNA replication, after every cell cycle, there is a physiological shortening of 50-100 kb of the telomere[178]. Therefore, the length of the telomere may indicate the number of replications the cell has done[70]. In specific cell type: germ cells, stem cells, activated lymphocytes, and epidermal cells, where is necessary to keep a high level of proliferation, the telomeres are constantly elongated by telomerase[70].

The telomerase has two essential components: the telomerase reverse transcriptase (TERT) and telomerase RNA template component, TR (also known as TERC). The latter adds telomere repeats onto the 3′ ends of chromosomes. Shelterin proteins prevent chromosome ends from fusing and being recognized as double-strand breaks. They also regulate telomerase access to the telomere and promote telomere repeat elongation[178,179]. When the length of the telomere is under a specific cut off, a signal is sent to arrest cell proliferation and start the process of degradation mediated by the p53 and Rb genes. If protective mechanisms, such as the TP53 tumor-suppressor gene, are inactive, thus allowing continued proliferation, telomeres become extremely short and dysfunctional. End-to-end fusions ultimately cause chromosomal instability[178]. Telomere dysfunction is associated with tumorigenesis across various cancers. Short telomeres may favor genomic instability, checkpoint deregulation, and activation of telomerase, leading to malignant transformation[177]. Telomere length (TL) has been reported to be a prognostic indicator in CLL. (fig.24)

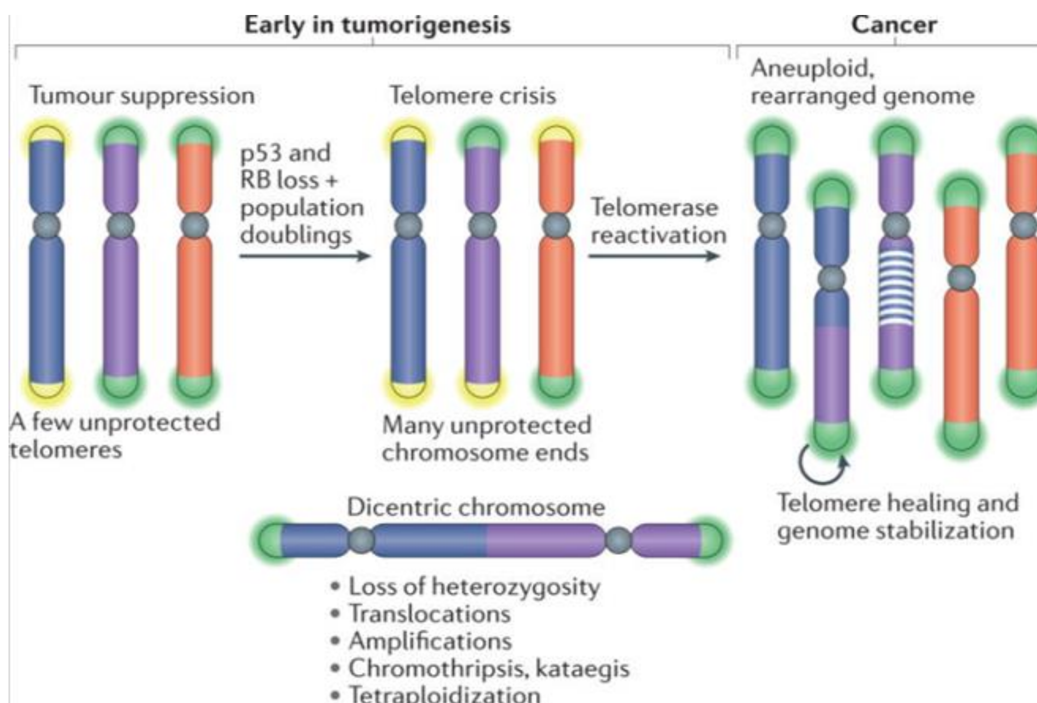

Figure 22. Telomere crisis. (Maciejowski J et al. 2017)

Telomere dysfunction and short telomeres are related to patient survival, treatment requirements, and transformation to Richter syndrome in CLL[180,181]. Early-stage CLL patients exhibited extensive telomere erosion and fusion, indicating that telomere shortening and dysfunction can precede clinical progression[182]. Short telomeres in CLL have been associated with other poor risk factors, such as U-IGHV, presence of 17p-, 11q-, TP53, and SF3B1 mutations. Patients with U-IGVH have shorter telomeres than patients with M-IGVH. This may be because the M-CLL, unlike the U-CLL, derives from lymphocytes that have been through the germinal center, where there is a high telomeric action[70,180]. Cases with mutated ATM or TP53 have defective DNA damage response and repair mechanisms due to impaired function. The presence of short telomeres may contribute to developing CK. Another outcome of genomic instability is chromothripsis. A phenomenon where a chromosome is shattered and rearranged in a single catastrophic genetic event[183].

1.6.3.2 CHROMOTHRIPSIS

The word ‘chromothripsis’ means ‘chromosome shattering’. It is the phenomenon where dozens of clustered chromosomal rearrangements arise because of a single event. Typically, it involves only a single chromosome or a few[184]. Chromosomes that undergo chromothripsis are first shattered into many pieces. Then they get reassembled back

together in a random order by DNA repair processes, most likely nonhomologous end joining[185]. (fig.23)

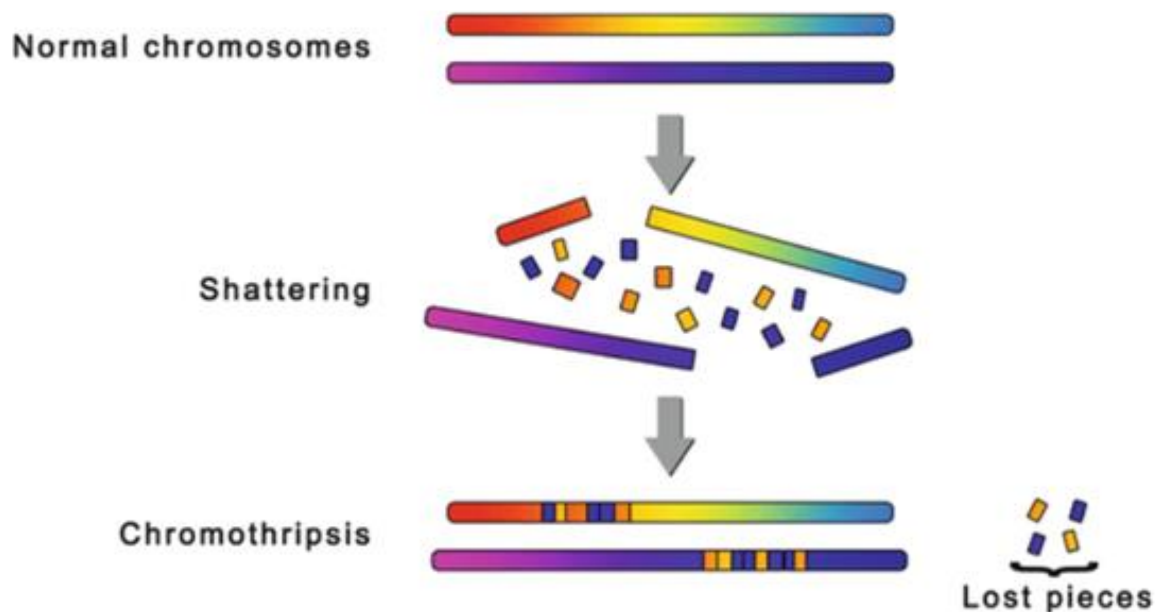

Figure 23. Model of chromothripsis formation. (Marcozzi et al. 2018)

Chromothripsis has been discovered in CLL and is associated with poor prognosis[186]. In tumor cells, chromothripsis causes the loss of tumor suppressors and dysregulation of genes with known cancer links. The shattering can also cause oncogene amplification and the loss of pathways involved in genome stability, such as inactivation of p53[186].

1.6.3.3 CHROMOSOMAL ABERRATIONS IN CLL

1.6.3.3.1 13q DELETION

The deletion on chromosome 13 (13q-) is the most common genetic abnormality in CLL cases, found more often in patients with M-IGVH and it is usually associated with a favorable prognosis (fig.25). In 70-80% of the cases, it has been observed as a heterozygous mutation while in only 20-25% of the cases is a homozygous mutation. Molecular studies have shown that the deletion is located in a 790 kb region between the D13S1150 e D13S25 markers[70]. It was found that the region always missing was a 30kb between the exon 2 and 5 of the DLEU2 gene. This region encodes for two small miRNAs: miR-15a and miR-16-1[187]. These miRNAs regulate the function of several oncogenes, the most important being BCL2, found overexpressed in almost all cases of CLL[188]. Patients with deletion of 13q have a better prognosis with a TTFT and OS superior to those of other CLL cases, even the one with a normal karyotype.

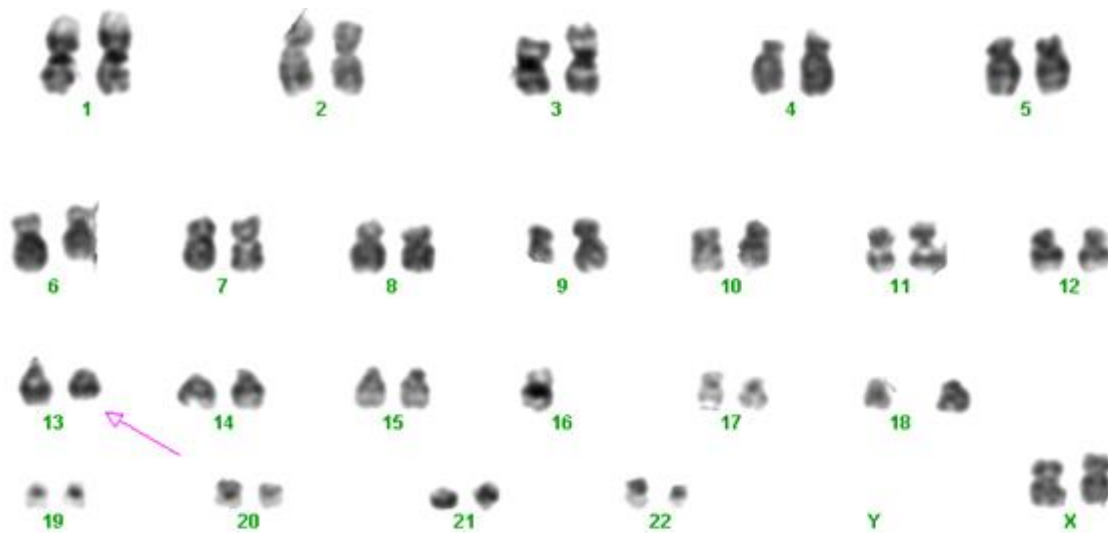

Figure 24. Karyogram with a 13q deletion.

Two main characteristics influence the prognostic value of 13q-: percentage of abnormal nuclei and the size of the deleted region. Having >85% of mutated nuclei is associated with a shorter TTFT, more comparable to those with a normal FISH pattern or trisomy 12[189]. The size of the deleted region is still a matter of debate. Some studies show a different TTFT when the deleted regions included only the minimal deleted region (MDR), composed of DLEU2/MIR-15a/MIR-161, as opposed to when the deletion included also the RB1 gene. Other studies have seen no difference. Also, a matter requiring further investigation is the loss of heterozygosity (LOH), highlighting the fact that the role of the 13q deletion in CLL is not as clear as was thought before.

1.6.3.3.2 TRISOMY 12

Trisomy of chromosome 12 is a cytogenetic abnormality found in about 15% of patients with CLL at diagnosis. As the deletion of chromosome 13q14, trisomy 12 is a precocious abnormality that remains stable during all the disease phases[45,70] (fig.26). It has been associated with an atypical morphology of the lymphocytes. It is also frequent in about 30% of patients with small lymphocytic lymphoma, the tumoral counterpart of CLL[190].

On chromosome 12, in the region 12q13-15 is located in the MDM2 gene. It is involved in the apoptosis of TP53 by mediating the bound between TP53 and the ubiquitin ligase causing TP53 degradation[70,191].

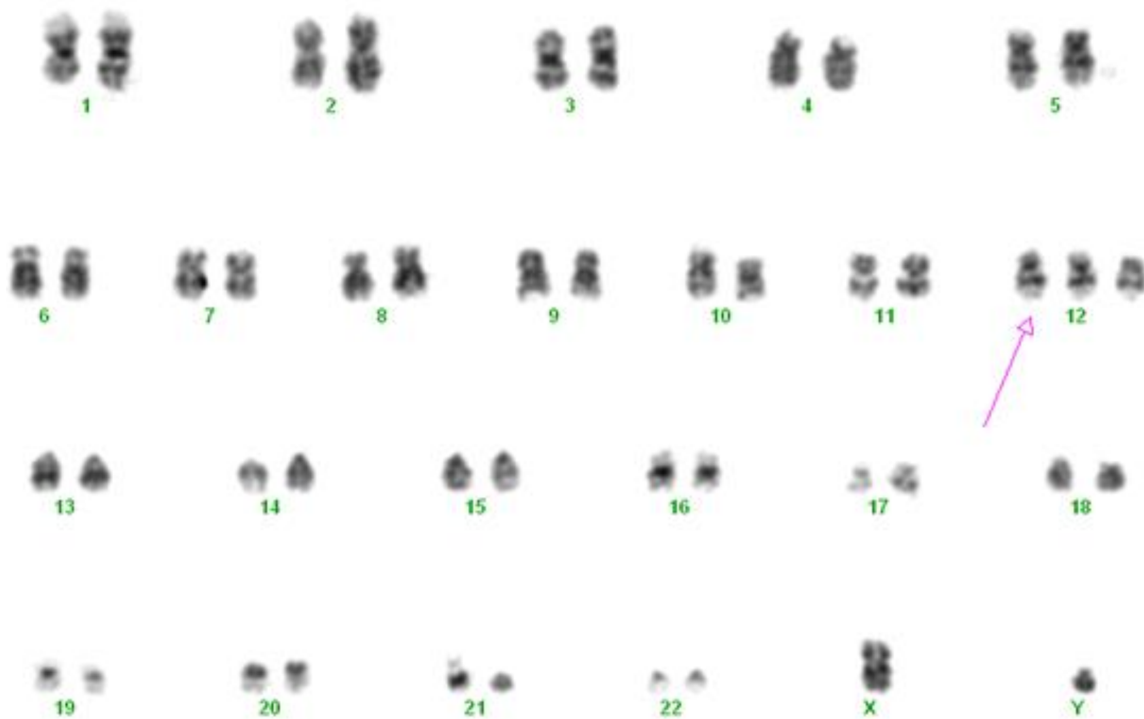

Figure 25. Karyogram with trisomy 12

The higher level of TP53 degradation causes the loss of the TP53 function to regulate the cell cycle, increasing the survival of the leukemia cells. CLLs with trisomy 12 is generally classified as an intermediate-risk group characterized by high rates of cell proliferation as well as clinical and biological heterogeneity since it is linked to additional genomic aberrations such as trisomy 19, high frequency of NOTCH1 mutations, and enrichment in aggressive stereotyped subset 8 and IgG-switched heavy chains1[192,193]. Moreover, trisomy12 was recently identified as an important modulator of response to kinase inhibitors in CLL[193].

Cells from patients with trisomy12 and concurrent NOTCH1 mutations may be more resistant to apoptosis induced cell death, leading to a less favorable course[92].

1.6.3.3.3 11q DELETION

The deletion of 11q (11q-) is found in about 10% of CLL cases but it can reach 25% in patients with more advanced disease[45] (fig.27). This anomaly is associated with a poor prognosis, a shorter TTFT, and OS. Furthermore, this deletion is often associated with a U-CLL, positivity for the CD38+, SF3B1 mutation, and genomic instability. They tend to develop other cytogenetics aberration with the progression of the disease[194]. The minimal region of deletion, on 11q22.3-23.1, often involves the Radixin (RDX) and Ataxia telangiectasia mutated (ATM) genes, involved in the pathway of DNA damage repair.

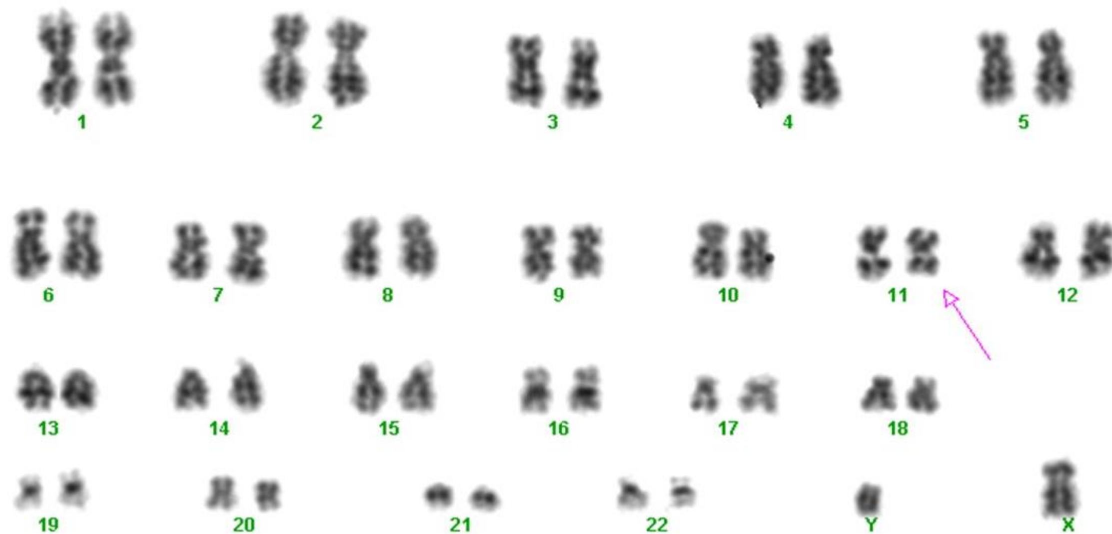

Figure 26. Karyogram with an 11q deletion

In 30% of the cases, 11q deletion is biallelic causing a further shortening of the survival[195]. Included in the deleted region of the 11q is the BIRC3 gene. BIRC3 is involved in the NF-κb pathway, but it does not seem to change the prognostic values of the deletion, unless mutated on the other allele[70]. The prognostic value of 11q- may depend on the number of abnormal nuclei and the presence of other cytogenetic abnormalities. Patients with ATM deletions superior to 25% have a shorter TTFT relative to those with the ATM deletion under 25%[196]. The ATM gene encodes a nuclear serine/threonine kinase whose activity is induced by chromosomal double-strand breaks. The DNA breaking could arise endogenously or after exposure to DNA-damaging agents, including chemotherapeutic drugs[186]. ATM protects the integrity of the genome by regulating the cell-cycle arrest by preventing the processing of damaged DNA. ATM also activates the DNA repair pathways or induces apoptosis if the DNA damage cannot be repaired[125].

1.6.3.3.4 17p DELETION

The deletion of chromosome 17p13 (17p-) is one of the most important prognostic factors for CLL. As in any other type of cancer, patients with this abnormality are associated with more aggressive disease and shorter OS[197]. The 17p deleted region includes the tumor suppressor gene TP53, which encodes for the p53 protein (fig.28). The p53 is fundamental for DNA damage control and is often called the ‘guardian of the genome’, for its critical role in maintaining genomic integrity[198]. Due to the loss of p53-dependent apoptotic function and cell-cycle arrest, cells accumulate mutations on mutations. This may result in malignancies associated with chemo refractoriness. Mutations represent the most frequent form of TP53 inactivation (about 70%) in CLL. TP53 mutations are frequently subclonal

and clinically significant even in heterozygosis. In most CLL cases, del(17p) will harbor a TP53 mutation on the other allele. Most TP53 mutations are missense and localized at the DNA-binding core domain. Despite that some codons are more frequently mutated (hotspots), deleterious variants may occur in any part of the coding sequence[199]. As the disease progresses, the incidence rises to 10–12% at the time of first-line treatment, 40% in fludarabine-refractory CLL, and 50–60% in Richter syndrome[125]. The presence of mutated p53 increases in the pathological clone as a result of the selection of chemoresistant clones during the therapy. The genomic instability due to the deletion of p53 causes the development of other cytogenetics abnormalities. Therefore, patients with 17p- often are associated with a complex karyotype[70].

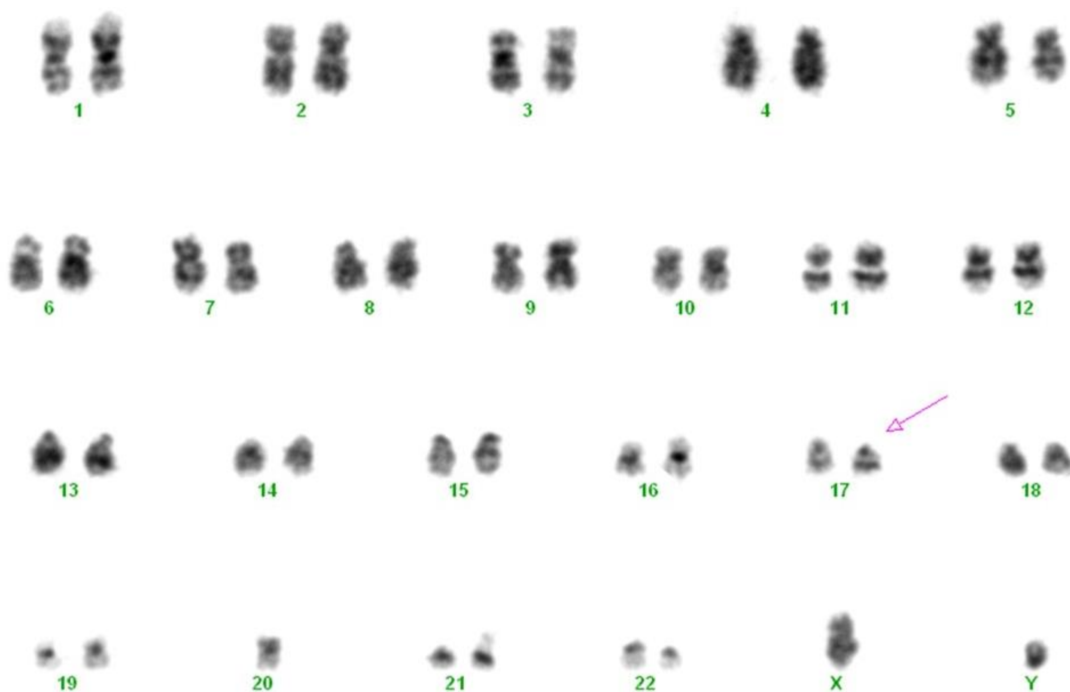

Figure 27. Karyogram with a 17p deletion.

P53 plays a pivotal role in limiting clonal expansion, maintaining genomic stability, and mediating the action of DNA-damaging chemotherapy. It is a transcription factor that is present at low levels under resting conditions but becomes activated in case of DNA damage. Activation, which occurs by phosphorylation, prolongs the half-life of p53 protein and allows it to accumulate in the nucleus. Here it regulates the expression of a wide range of genes that induce apoptosis, cell cycle arrest, and DNA repair[200]. A pivotal role in the activation of p53 is played by ATM, at the same time regulated by serine-threonine kinases CHK2 and CHK1. ATM becomes active when a DNA double-strand break (DSBs) occurs. ATM inhibits MDM2, which main role is to keep a low level of p53 promoting its degradation. P53 binds the DNA as a four components ligand, when even one of its

components is mutated it affects the function of the ligand[201]; regardless of their fitness, patients with CLL carrying genetic aberrations such as del(17p) and/or TP53 mutation, respond poorly to chemo immunotherapy. Therefore, they require different therapeutic approaches. An increasing understanding of the disease biology has led to the development of targeted drugs for the treatment of CLL, such as the BTK inhibitors and PI3K inhibitors[202]. Given the importance of TP53 mutations in driving therapeutic decisions in CLL, international guidelines for molecular biology labs have been established by the ERIC group.

1.6.3.3.5 COMPLEX KARYOTYPE

CK is a heterogeneous quantitative and qualitative cytogenetic category. It includes numerical (i.e. monosomies and trisomies) and structural abnormalities (i.e. balanced and unbalanced translocations, marker chromosomes, isochromosomes, deletions, insertions, and additions)[214]. CK is defined by the presence of at least 3 chromosome aberrations in the same clone, but the precise definition is still a matter of debate[204]. In recent years there has been a debate on whether update the definition of CK from 3 chromosome anomalies to 5. In acute B lymphoblastic leukemia (B-ALL) the presence of at least 5 abnormalities is required for the definition of CK[205]. Moreover, in cases of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), patients with more than or equal to 5 abnormalities had a significantly worse OS than those with 3 or 4 abnormalities[206] (fig. 29). It seems that the number of abnormalities could influences the prognostic value of CK in several types of leukemia.

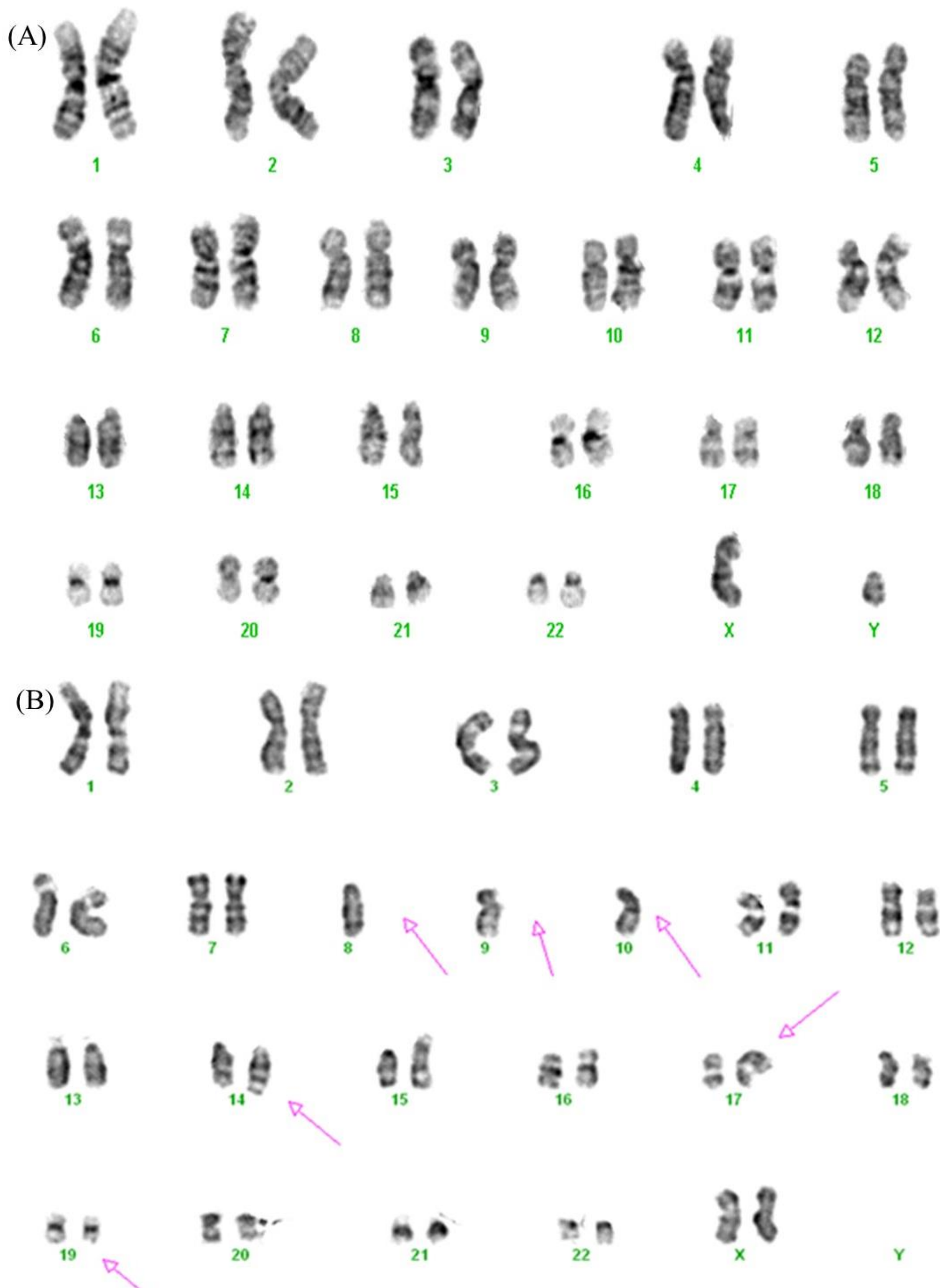

Figure 28. A: Normal karyotype [46, XY]. B: Complex karyotype t(14;19), -8,-9,-10, der(17)t(17;?)(p11 ;?.)

CK is a manifestation of genetic instability. In cancer, it is associated with an adverse outcome and worse response to chemoimmunotherapy. It highlights an unfavorable meaning in patients with relapsed or refractory CLL[207]. CK may be related to the presence of non-functional telomeres that cause defects in the repairing process of damaged DNA. This could lead to apoptosis induced by damaged DNA[208]. Conventional cytogenetic

analysis was not always possible given the difficulty of obtaining metaphases from CLL cells. FISH was and still is, the chosen technique to quickly analyze chromosomal aberration in CLL, but it has its limits. FISH can highlight only specific and marked sequences on the chromosomes. Anomalies involving other chromosomes, or other regions not labeled by the probes would go unseen. Through FISH is almost impossible to detect CK. With the introduction of CpG oligonucleotides and interleukin 2 in the culture medium, it became easier to obtain metaphases from CLL and therefore perform a cytogenetics analysis. The diffusion of cytogenetics analysis in CLL has allowed a better understanding of its chromosomal alteration, redefining the role of some prognostic markers and highlighting the impact of CK. It is a negative prognostic factor, independent from CLL-IPI (International Prognostic Index). The presence of genetic and chromosomal aberration occurs in 82% of patients with CLL, where CK represents 20% of cases[208]. In recent years, the importance of CK has grown, but a lot remains to discover about this dynamic and evolving cytogenetic aberration.

1.7 SLAMF1

Signaling-lymphocytic-activation-molecule-family1 (SLAMF1/CD150) is a multifunctional type I transmembrane glycoprotein. It belongs to the immunoglobulin superfamily of 9 genes (SLAMF1 to SLAMF9). They code for adhesion/costimulatory molecules that initiate signal transduction networks in T and B lymphocytes, natural killer, and antigen-presenting cells[209,210,]. CD150 is actively involved in the regulation of innate and adaptive immunity. It may also be involved in the regulation of CLL B-cell microenvironment and pathobiology[211,212]. Within the T-cell lineage, CD150 is expressed starting on immature CD4−CD8− thymocytes. Its expression increases on CD4+CD8+ thymocytes while dropping on naïve T cells. SLAMF1 is also involved in the regulation of the Gram-negative bacterial phagosome through the ubiquitous cellular autophagy machinery and serves as a receptor for the measles virus[213]. During T-B lymphocyte cross-talk, SLAMF1 works as a self-ligand and mediates a signal transduction pathway that enhances lymphocyte activation[209]. SLAMF1 signaling functions are mediated by two immunoreceptor tyrosine-based switch motifs (ITSMs) in its intracellular domain that directly binds SH2-containing proteins SAP (SLAM-associated protein), SHP-2, SHIP, and regulatory subunit of PI3-kinase[212,214]. This receptor and its adaptor-associated molecule SAP are involved in B and T cell co-stimulation, induction of cytokine synthesis (mainly IFN). SLAMF1 is located at the genomic segment of human chromosome 1q23 and

consists of eight exons that are separated by seven introns[211,215]. Several alternatively spliced isoforms have been reported for CD150: the canonical transmembrane CD150 isoform (mCD150) with two ITSM signaling motifs in the cytoplasmic domain, a secreted CD150 isoform (sCD150) without a transmembrane region, and a novel CD150 isoform (nCD150) with an alternative cytoplasmic tail[211] (fig.30).

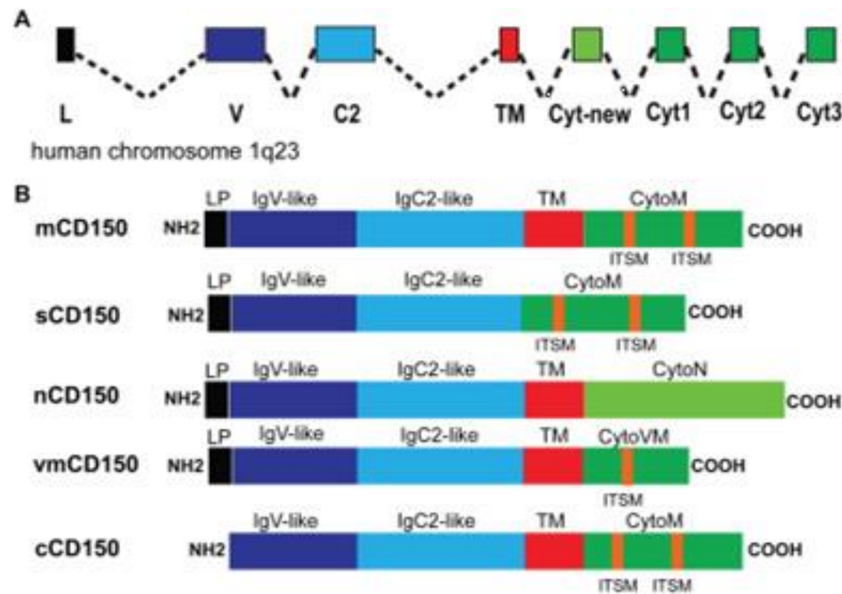

Figure 29. Structure of SLAMF1 gene and alternatively spliced CD150 isoforms. (Gordiienko et al, 2019).

There is a noticeable heterogeneity in CD150 surface expression on the subpopulations of normal B cells. Early CD150 expression is detected on nearly half of B-cell progenitors in the bone marrow. The CD150 expression is regulated during B-cell differentiation with the lowest level on naive human B-cells. It is completely absent on immature B-cells, slightly downregulated on memory B-cells. The expression increase toward plasma cells differentiation and it is higher in activated B-cells[212,214]. Numerous data indicate that CD150 is rather aberrantly expressed on the surface of malignant B-cells. CD150 cell surface expression level is much lower in B-cell malignancies than that in normal B-cell subsets[212]. CLL patients with CD150+ malignant B-cells seem to have a better prognosis compared to patients with CD150- leukemic cells. Cell movement and vesicle trafficking emerged as the 2 main cellular processes affected[213]. SLAMF1 induces the autophagy flux by modulating a signaling circuit that involves ROS generation, activation of JNK1/2 and BCL2 phosphorylation. It is ultimately associated with beclin1 and VPS34 to stabilize the autophagy macro complex. In line with these observations, CD150+ cells are more sensitive to therapeutic agents. SLAMF1-deficient CLL cells are resistant to the action of autophagy-inducing therapies[214]. Decrease of chemokine receptor CXCR4 expression level

in CD150+ CLL B-cells results in reduced chemotaxis of malignant B-cells toward bone marrow or lymph nodes[212] (fig.31). CD150 expression is regulated via numerous cell-type specific extracellular stimuli that lead to activation of key transcription factors essential for SLAMF1 promoter activity. CD150 in malignant B-cells directly binds beclin-1, involved in the autophagosome generation. These signaling events support several CD150 functional properties in B-cells, for example, augmentation of proliferation induced by CD40, induction of proliferation, and Ig synthesis by activated B-cells. SLAMF1 in CLL B-cells can function alone or in cooperation with other cell surface receptors, one of the most important is CD180. CD180 is co-expressed and co-localized with CD150 on the cell surface membrane in around 60% of CLL cases. The ligation of CD150 and CD180 blocks Akt kinase phosphorylation reducing protein synthesis in malignant B cells and attenuates CLL B-cells propagation in this way[214,212].

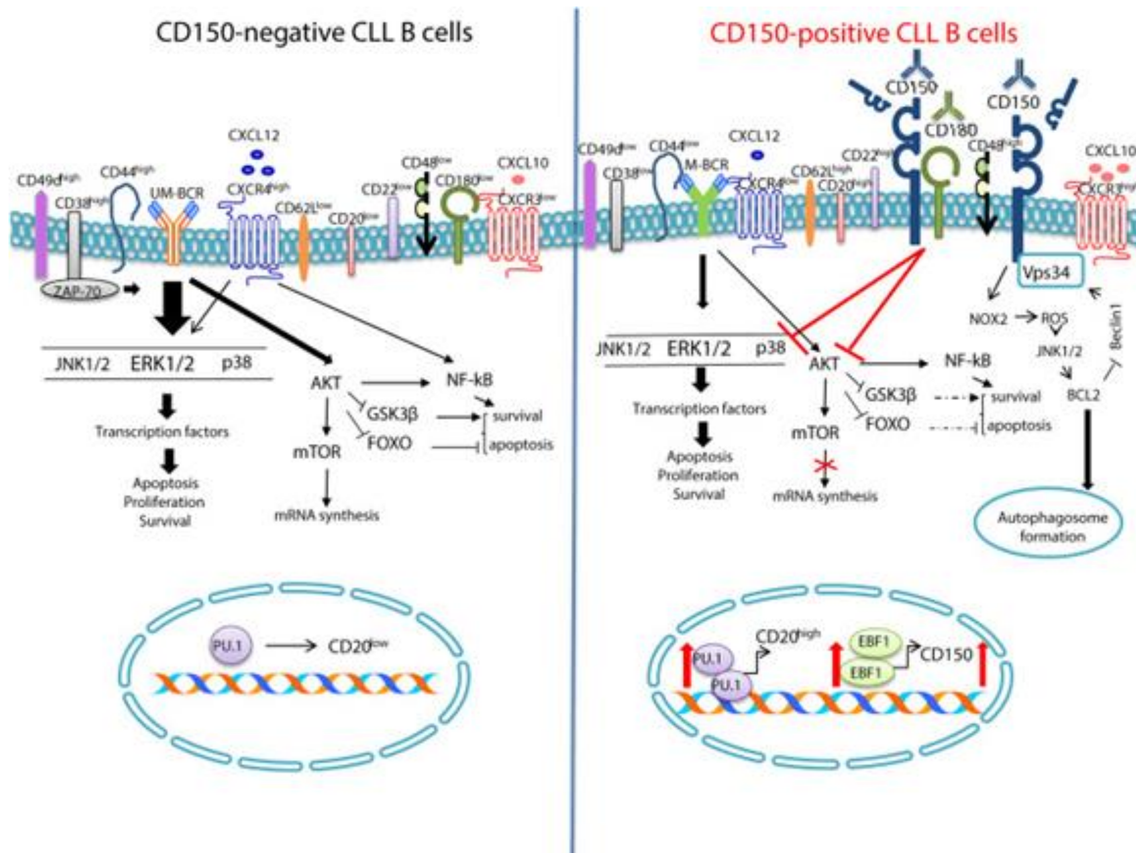

Figure 30. Signaling pathways in CD150-negative and CD150-positive chronic lymphocytic leukemia B cells. (Gordiienko et al, 2019).

The cell surface expression of combined CD150 and CD180 is not profitable for CLL B-cells. This is supported by CD150 and CD180 differential expression on CLLs B-cells with significantly decreased levels compared to normal B-cell analogs. One of the scenarios for

CLL B-cells is to avoid cell surface expression of CD150 and CD180 by retaining these receptors in cytoplasmic compartments.

1.7.1 miRNAs THAT TARGET SLAMF1

As stated before, miRNAs are very important mRNA regulators that can target up to several hundred mRNAs. An aberrant miRNA expression can disturb a multitude of cell signaling pathways and profoundly influence cancer onset and progression[155]. There are several miRNAs that target SLAMF1 and could be the cause of SLAMF1 dis-regulation in patients with unbalanced rearrangements. The family of miR-17-92 is abundantly expressed in B-cells, though their level decreases with the maturation of the cells. *In vivo* experiments have proved that the elimination of miR-17-92, or any of the enzymes involved in their biogenesis, causes an arrest in B-cells developments and overexpression of Bcl2[216]. miR-17-92 are regulated by Myc, who directly initiates the pri-miR-17-92 transcription. In B-malignancies, the up-regulation of Myc causes the overexpression of miR-17-92, which promotes tumor invasiveness and is therefore associated with a poor prognosis[217]. miRNAs may also alter the chemosensitivity of the cell, influencing the expression of the molecular targets of a drug and causing drug resistance The clusters of miR-148a is thought to cause drug-resistant due to their property to alter the activation of p53–responsive genes[218]. Other miRNAs involved in the TP53 signaling pathway are miR-132 and miR-212, the upregulation of these miRNAs showed a proliferative effect on progressive CLL, but not in stable ones[219]. Despise targeting SLAMF1, the effect of these miRNAs on its expression it is still unknown.

2 AIM OF THE STUDY

CLL is a very heterogeneous disease where patients can have very different outcomes. Characterized by many genetic anomalies, CLL's origin remains unknown. In most cases, CLL is an asymptomatic disease with an indolent progression where the most adopted therapy plan is "watch and wait". However, there are other cases where CLL has a rapid progression that can lead to an early death. Many prognostic factors have been found for the identification of patients at high-risk of rapid progression and treatment requirement. Still, the identification of better and more precise markers that could predict the outcome of the disease is an active field of research. In recent years, the importance of CK as a negative prognostic factor has grown. CK includes many different abnormalities. This study aimed to evaluate whether the presence of single abnormalities harbored in a CK could alter the prognostic impact of CK-CLL in terms of TTFT and OS, and to compare the gene expression profile of CLL with and without unbalanced translocations The results of this study led to the identification of a specific set of patients with a different gene expression profile than others with a more indolent condition. These patients presented a dysregulation of several genes involved in the key pathway for cell survival and migration. Among these differentially expressed genes, there was the signaling lymphocytic activation molecule family member 1 (SLAMF1). This gene is involved in the mechanism that regulated lymphocytes activation, modulates responses to chemokines, and is involved in autophagosome formation. Because of the role that plays in the autophagosome pathway, SLAMF1 is an indirect regulator of BCL2, a proto-oncogene, target to many new-generation compounds, such as venetoclax. A dysregulation of SLAMF1 could be an indicator of the patient's resistance to BCL2-inhibitors. The analysis of SLAMF1 could be easily performed using flow cytometry or ddPCR. If these prognostic values would be confirmed, SLAMF1 could be used as a new prognostic factor for faster identification of high-risk patients. In the second part of this study, the goal was to validate the prognostic impact of SLAMF1 expression to identify high risk CLL.

3 MATERIAL AND METHODS

3.1 IDENTIFICATION OF CLL

Patients with a suspected diagnosis of CLL, showing more the 10,000 wbc/µl, and higher than 30% of B lymphocytes in the blood, were analyzed using flow cytometry analysis to identify the presence of pathological clone.

3.2 FLOW CYTOMETRY

Peripheral blood (PB) of the patients were collected to obtain a concentration of 10,000cell/µl for a total volume of 100µl. Single or combinational antibodies were added manually in each tube. The antibodies combination in the tubes for samples evaluated before 2019 and their property are listed below.

Table 1 Representation of the combination of Antibodies in the tubes.

	FITC	**PE**	**PerCP**
TUBE 1:	CD4	CD8	CD3
TUBE 2:	CD3	CD16+CD56	HLA-DR
TUBE 3:	CD19	CD5	CD45
TUBE 4:	SIgκ	SIgλ	CD19
TUBE 5:	CD20	CD23	CD3
TUBE 6:	FMC7	CD38	
TUBE 7:	CD10	CD22	
TUBE 8:	CD103	CD22	
TUBE 9:	CD25	CD19	
TUBE 10:	CD11c	CD19	
TUBE 11:	CD200	CD19	

Table 2 Description of the used Antibodies.

ANTIBODY	FLUOROCHROME	CLONE	BRAND
CD4/CD8/CD3	Tritest ™		BD Biosciences
CD3/CD16+CD56	Simultest™		BD Biosciences
HLA-DR	PerCP	(L243)	BD Biosciences
CD19	FITC	4G7	BD Biosciences
CD5	PE	L17F12	BD Biosciences
CD45	PerCP	2D1	BD Biosciences
CD20	FITC	L27	BD Biosciences
CD23	PE	EBVCS-5	BD Biosciences
CD3	PerCP-Cy™ 5.5	SK7	BD Biosciences
FMC7	FITC		BD Biosciences
CD38	PE	HB-7	BD Biosciences
CD19	PerCP-Cy™ 5.5	SJ25C1	BD Biosciences
K/L/19	Oncomark™		BD Biosciences
CD10	FITC	SS2/36	DAKO mouse anti_human
CD22	PE	S-HCL-1	BD Biosciences
CD103	FITC	Ber-ACT8	BD Biosciences
CD25	PE	2A3	BD Biosciences
CD11c	PE	S-HCL-3	BD Biosciences
CD200	PE		BDPharmingen™ mouse anti-human

All sample analyzed after 2019 were stained using BD Biosciences OneFlow LST, Lyotube 129, Lyotube 130, Lyotube 131, where the antibodies are already present in the tube in a lyophilized form which includes the following combination:

LST

ANTIBODY	FLUOROCHROME	CLONE
CD8	FITC	SK1 (Leu2a)
Anti-Lambda	FITC	1-155-2
CD56	PE	MY31 (Leu-19)
Anti-Kappa	PE	TB28-2
CD5	PerCP-Cy™5.5	L17F12
CD19	PE-Cy™7	SJ25-C1

Anti-TCRγ/δ-1	PE-Cy7	11F2
CD3	APC	SK7
CD38	APC-H7	HB7
CD4	V450	SK3 (Leu3a)
CD20	V450	L27
CD45	V500-C	2D1

Lyotube 129

ANTIBODY	FLUOROCHROME	CLONE
FMC7	FITC	FMC7
CD23	PE	EBVCS-5
CD5	PerCP-Cy™5.5	L17F12
CD19	PE-Cy™7	SJ25-C1
CD200	APC	MRC OX-104
CD38	APC-H7	HB7

Lyotube 130

ANTIBODY	FLUOROCHROME	CLONE
CD103	FITC	Ber-ACT8
CD10	PE	HI10a
CD5	PerCP-Cy™5.5	L17F12
CD19	PE-Cy™7	SJ25-C1
CD25	APC	2A3
CD49d	APC-H7	9F10

Lyotube 131

ANTIBODY	FLUOROCHROME	CLONE
CD81	FITC	JS-81
CD22	PE	S-HCL-1
CD5	PerCP-Cy™5.5	L17F12
CD19	PE-Cy™7	SJ25C1
CD79b	APC	3A2-2E7
CD43	APC-H7	1G10

In both techniques, the blood and the antibodies were incubated for 20 minutes in the dark after a brief shaking. Once the cells are stained, a 1X buffered solution with <1,5% formaldehyde and <50% diethylene glycol (BD Biosciences™ lysing solution) was added to the blood to lyse the red cells. After incubating in the dark for 10 minutes, the cells are washed with PBS to remove the fragment of red cells, and once the supernatant was clear it was acquired at the cytometer.
At least 10,000 lymphocytes were acquired using the Diva software and FACSCanto II flow cytometer (BD Biosciences™) for the samples obtained after 2018 and FCASCalibur (BD Biosciences™) was used instead, for patients' samples obtained before 2018. Lymphocytes were gated using the Foward scatter (FSC) versus the Side scatter (SSC). Dot plots were generated on this population to determine CD5/CD19. The LOQ (limit of quantification) was represented by 30 events in a gate. A pathological clone was identified when the ratio between the superficial immunoglobulin κ (sIg κ) and ʎ (sIg ʎ) was out of the range 1-2,5. If the presence of a pathological clone was found a second step of the analysis was performed, including Matutes score and prognostic antibodies. To determine the expression intensity, quadrant markers were set using the antibody with the SSC. The samples were classified as CLL when they had a Score Matutes > 3/5.

3.3 CYTOGENETICS

3.3.1 BLOOD SEPARATION

From every patient, following informed consent, were collected 20 ml of PB in a sterile syringe containing heparin. The blood was then diluted with PBS (Phosphate-buffered saline) (Sigma-Aldrich™) and carefully divided into four falcons (Thermo Scientific™), containing 3ml of Lympholyte® (Thermo Scientific™), without breaking the surface. After being centrifuged for twenty minutes at 2200 rpm the blood was separated into its different components, where the upper level, composed of plasma, was discharged and the mononuclear cells were collected. The pellet was washed with 14 ml of PBS (Sigma-Aldrich™) and centrifuged at 1500 rpm for 10 minutes. Once that the supernatant was discarded, the pellet was resuspended in about 1ml of PBS, and 200µl of the suspension was taken to count the number of lymphocytes/ul using Advia®2120i (Diatek Srl).

3.3.2 CELL CULTURES

Every sample was divided into four T25 flasks (Thermo Scientific™) and cultured for 72 hours at 37°C with different cytokines:

- 10 million cells were cultured in 5ml RPMI 20% with 100µl of IL2 and 100µl of oligodeoxynucleotide.
- 10 million cells were cultured in 10ml RPMI 20% and Chromo lymph (EuroClone™).
- 20 million cells were cultured in 10 ml RPMI 20% and 50 µl of TPA (12-O-tetradecanoylphorbol 13-acetate).
- 20 million cells were cultured in 10 ml RPMI 20% and 400 µl of LPS (Lipopolysaccharides).

3.3.3 CELL HARVEST

After three days, the cells were blocked in the metaphase state by adding 100 µl of colcemid (Gibco™ KaryoMAX™ Colcemid™ Solution in HBSS) and incubating for at least one hour. The specimens were then transferred in a 15 ml falcon and centrifuges at 2000 rpm for 10 minutes. The supernatant was discarded, and the falcons were filled with a hypotonic solution 0.075M KCl (Sigma-Aldrich™) pre-warmed to 37°C. The samples were mixed and after been incubated for 20 min at 37°C they were centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded, and the pellet was resuspended by vigorous vortexing. A solution of 3:1 methanol (Sigma-Aldrich™) and glacial acetic acid (Sigma-Aldrich™) was added to the sample drop by drop while continuously vortexing. The process was repeated until the supernatant was clear and without a trace of red cells. The specimens were sealed with parafilm and stored at -20°C until the time of use.

3.3.4 FISH

The samples were centrifuged at 2000 rpm for 10 min to remove the methanol: acetic acid solution and to add a fresh one until the samples reach the right concentration for a FISH slide. Every slide meant for FISH (Menzel - Glasen™) was cleaned before use to remove any trace of grease or dust by leaving them in a 12% acetic acid solution over night (O.N) first and then immersed in ethanol 96% until use. The slides were taken directly from the ethanol solution and dried with clean gauze. For every probe 5µl of the specimen were dropped on the slide and examined with a phase-contrast microscope to ensure a good spread. The back of the slide was screeched with a drill to delimitate the area of the drop. The slide was immersed in a 70% acetic acid solution for about 30 seconds to eliminate debris or remaining broken red cells. Afterward, the slides were dehydrated by placing them consecutively for 1 minute each through 70%, 85%, and 100% ethanol. In the area previously delimitated were placed in a dark environment 5 µl of the probes, stored until

use at -20°C. For each specimen 5 different probes were used for detecting 5 specific regions in different chromosomes:

ATM (MetaSystem™): a probe designed to detects the deletion in 11q22.3, it spans from marker D11S2179 to the NPAT region.

CEP 12 (Leica Biosystem™): a centromeric probe to highlight the centromeric region of chromosome 12, from 12p11.1-q11.1.

DLEU/LAMP (MetaSystem™): this probe detects the deletion on chromosome 13q, the DLEU locus located in 13q14.2, including D13S319. LAMP on 13q34.

IGH (MetaSystem™): a probe to label the region of the IGH locus at 14q32.

TP53 (MetaSystem™): this probe detects the deletion in 17p13 and involves the TP53 gene locus.

After each probe was placed on the slide, it was then covered by a square coverslip (10mm, Menzel - Glasen™). To keep the coverslip in place and to avoid evaporation, its edges were sealed with rubber cement. For the denaturation of the sample, the slide was placed in a hotplate at 75°C for 2 minutes and then incubate in a humidified chamber at 37°C ON for the hybridization. The next day the rubber cement and the coverslip were discarded, and the slide was washed first in a pre-warmed solution of 0.4 X SSC (Sigma-Aldrich™) at 73°C for 2 minutes and then immersed in a solution of 2 X SSC (Sigma-Aldrich™) at room temperature (RT) for 1 minute. After the slide was dry, 5 µl of the counterstain DAPI (celbio) (4',6 diamidino-2-phenylindole) were applied and over layered with a coverslip, any bubbles on the specific area were removed and sealed with transparent nail polish. The slides were kept at -20°C in the dark for at least 20 minutes before analyzing, to let the nail polish dry and the DAPI to enter the cell. At least 200 nuclei were read using a fluorescence microscope (Nikon Instruments S.p.a) with the Cytovision® (Leica Biosystems™). After the analysis, the slides were stored at -20°C for a maximum of a week.

3.3.5 KARYOTYPE

After harvesting the cells, each sample was diluted or concentrated to reach the best concentration for a karyotype slide. 100 µl of the specimen were 'dropped from above' on a clean slide (10mm, Menzel - Glasen™) to favorite the spread of the cells. The slides were put on a hot plate to dry for about 15 seconds and then checked at the inverted microscope to control the concentration. For every patient, a karyotype slide was prepared from each culture with a different cytokine. Once the concentration of the sample on the slides was satisfactory they were incubated at 60°C in a warm chamber ON. The next day

the slides were immersed in a solution of SSC 2X at 60°C for twenty minutes, then washed with distilled water and dried on air. The dry slides were positioned on a horizontal surface, covered with about 5 ml for a slide of a 3:4 Write (Sigma-Aldrich™) solution for 6 minutes, then was added distiller water to block the reaction, wait for a minute, and then washed with tap water.

High-resolution microscopy (Nikon Instruments S.p.a) with immersion objectives (100 x) was used for the analysis of twenty metaphases for each patient using two different analysis systems, Cytovision® (Leica Biosystems™) and Genikon® (Nikon Instruments S.p.a) set up for G banding. The majority of metaphases were obtained from the culture with oligonucleotide and IL2. A karyotype was defined as complex by the presence of at least 3 chromosome aberrations in the same clone[174]. The following cytogenetic aberrations that were considered are the following: monosomies, trisomies, deletions, balanced translocations, unbalanced rearrangements (including chromosome additions, derivatives, insertions, duplications, ring, dicentric, and marker chromosomes), and the presence of ≥5 abnormalities.

3.4 MOLECULAR ANALYSIS

3.4.1 SAMPLES IDENTIFICATION

The cohort of specimens in the second part of this study comprised all 349 samples collected from CLL patients at the time of the diagnosis, before any treatment, stored at -80°C at the University of Ferrara. Ethical approval for this study was obtained from the Ethical Committee of Ferrara Hospital.

3.4.2 RNA ISOLATION

The RNA was extracted from every sample using the phenol-chloroform technique. In every specimen was added 250µl chloroform, shaken briefly for 15 seconds and after 5 minutes at RT centrifuge at 10,000 rpm for 5 minutes. Once the specimen is divided into three layers, the aqueous phase was drawn and gently mixed with 550 µl isopropanol, left a RT for 10 minutes, and centrifuged at 14,000 rpm for15 minutes. The samples were then put on ice, the isopropanol poured off and it was added 1ml of 75% ethanol. After centrifuged the samples at 9,500 rpm for 5 minutes, the ethanol was removed, and the pellet was left to air-dry. Once the pellet was dry it was added 50 µl of RNase-free water and the RNA quality was assessed using NanoDrop 2000 (Thermo Scientific™).

For subsequent RT-PCR was used the Thermo Scientific Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific™). Into a sterile tube on ice mix 1µl RNA template with 1µl primer and add 12 µl of water. Consequentially add to the mix 4 µl 5X Reaction Buffer, 1 µl RiboLock RNase Inhibitor (20 U/µL), 2 µl 10 mM dNTP Mix, and RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/µL) for a total of 20 µl. Gently shake and centrifuge the mix before incubating at 42°C for 60 minutes.

3.4.3 DROPLET DIGITAL PCR

Based on the ddPCR Supermix for Probes (No dUTP) (Bio-Rad Laboratories, Hercules, CA) protocol, for the ddPCR was prepared a reaction mixture of 20 µl total volumes, including 10 µl 2 × QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 7 µl RNase/DNase-free water, 1 µl of the primers (Thermofisher TaqMan™Gene Expression Assay (FAM)), 2 µl cDNA. 70 µl of QX200 Droplet Generation Oil for EvaGreen (BioRad Laboratories) was added to the mix, transferred into a QX100/200 DG cartridge (Bio-Rad Laboratories), and was loaded into a QX200 Droplet Generator (Bio-Rad Laboratories). It was created an emulsion made of 40 µl of oil and the solution, distributed into a clean 96-well PCR plate. The plates were covered with pierceable foil heat seals (Bio-Rad Laboratories) and sealed using a PX1 PCR plate sealer (Bio-Rad Laboratories) to avoid evaporation and contamination. 40 cycles of PCR were performed following the ddPCR Supermix for Probes (No dUTP) (Bio-Rad) protocol. After the PCR cycles, the droplets were analyzed using the QX200 droplet reader (Bio-Rad Laboratories). Both data were normalized to β-actin to determine the relative mRNA levels in platelets. The coefficient of variation (CV) of intra-assay and inter-assay of both PCR assays was less than 12%.

3.5 LUCIFERASE ASSAY

Two sets of primers were designed to amplify the target sequences of three different mRNA. One set was used for the target region of miR-17-miR-93, while the other was designed to amplify the target regions of both miR-148 and miR-132-212, located very close to each other.

The Advantage® 2 Polymerase Mix (Clontech Labs, Thermo Fisher Scientific) was used to perform the PCR that was later purified using ExoSap-IT TM (Thermo Ficher Scientific). To examine the correct function of the primers, the samples were sequenced using the

Sanger technique. Once it was verified that the primers bind to the correct sites it proceeded with the digestion of the luciferase vector.
It was prepared a 40 µl of reaction mix with vector psiCHECK™-2 (Promega™ Corporation), and 1 µl each of the restriction enzymes Not1 (New England BioLabs inc.) and Xho1 (New England BioLabs inc.). The reaction was incubated for an hour at 37°C. After the digestion, the mix was loaded on a 1% with TBE Gel Green (Embi tec ®) and run for as long as necessary. Once the segments were adequately separated from each other, they were cut from the gel. The fragments were then purified with the QIAquick Gel Extraction Kit (Qiagen). The results were quantified using the NanoDrop™ 2000c Spectrophotometers (Thermo Scientific™).
The samples were incubated with the SAP polymerases for an hour at 37°C for eliminating the phosphoryl group. It was then used the MinElute PCR Purification Kit (Qiagen) and the results quantified at NanoDrop™ 2000c Spectrophotometers (Thermo Scientific™).
For the ligation, it was used 50ng of the vector, and 50ng of the insert. It was then added 1 µl of the ligase enzyme and 1.5 µl of its buffer. The reaction was left O.N at 16 °C.
The transformation was performed following the protocol of the XL10-Gold Ultracompetent Cells (Aligent). Afterword, each sample were plated in three different LB Agar Plates, Ampicillin-100 (Teknova) at different concentration (10 µl, 100 µl, 250 µl), while the control vector was used with a higher concentration (1 µl, 10 µl). The plates were left O.N. at 37°C.
The next day, five colonies were picked for every miRNAs and cultured in 5 ml of LB medium (Sigma-Aldrich) with ampicillin. The colonies were then left on a shaker at 37°C O.N.
Afterward, the cultures were centrifuged at 3000 rpm for 15 minutes, the surfactants were removed, and the pellet suspended. It has then proceeded with the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen™).
The minipreps were analyzed with Sanger sequencing to control the correct insertion of the miRNAs inside the vector.
If the transformation was a success, the Maxiprep protocol was applied (QIAGEN® Plasmid Plus Maxi Kit).
For every miRNA were also designed primers with a mutated target sequence. The Advantage® 2 Polymerase Mix (Clontech Labs, Thermo Fisher Scientific) was also used to perform a PCR with the mutated primers and the Maxipreps. Then we proceed following the mutagenesis kit (QuikChange II XL Site-Directed Mutagenesis Kit – Agilent). After that, it was prepared the Miniprep using the same protocol as before. The sequences were

analyzed with Sanger sequencing to verify the rightful substitution of the target sequences with the mutated ones.
Once the sequence was confirmed it was performed the transformation and the Maxiprep using the same procedure as cited before. In this case also, the results of the Maxiprep were sent to be analyzed through Sanger sequencing.
Cells from the human embryonic kidney 293 cells (HEK-293) were cultured in 10% Medium. At 70% confluent the cells were transfected in a 24-well.
The next day the cells were transfected using the Lipofectamine™ 3000 protocol (Invitrogen™) and incubated for 24 hours. The next day it was performed the luciferase assay Dual-Luciferase® Reporter Assay System (Promega™ Corporation). For analyzing the samples, it was used GloMax® 96 Microplate Luminometer (Promega™ Corporation).

3.6 STATISTICAL ANALYSIS

The Mann–Whitney test and the Fisher's exact test were applied for quantitative and categorical variables, respectively. TTFT was calculated as the interval between diagnosis and the start of first-line treatment. OS was calculated from the date of diagnosis until death due to any cause or until the last patient follow-up. Proportional hazards regression analysis was used to identify the significant independent prognostic variables on TTFT. Survival curves were compared by the log - rank test. The stability of the Cox model was internally validated using bootstrapping procedures[161]. Statistical analysis was performed using Stata 16,0 (Stata Corp., College Station, TX). The definition of SLAMF1 cut-off was obtained using the recursive partitioning analysis.

4 RESULTS

In this study it was selected a cohort of 90 samples collected in the Hematology Unit of Ferrara. The samples belonged to treatment-naive patients with CLL and CK admitted to the Ferrara Hospital between 2000 and 2017 and stored at -80°C. The median age of these patients was 67.4 years (range 40–94) and the median period of follow-up was 51.3 months. The most significant prognostic factors were evaluated in all patients (Table 3): the mutational status for the genes IGVH, NOTCH1, SF3B1 and BIRC3, the chromosomal abnormalities del(11q) and del(17p) and the expression of CD38 and ZAP70, whose cut-off was established at 30%.

Table 3 List of patients' prognostic factors.

VARIABLE	N. of pts (90)	%
Age <=65/> 65 years	43/47	47.8/52.2
Gender m/f	58/32	64.4/35.6
Binet Stage a/b-c	63/27	70.0/30.0
CD38 neg/pos (cut off 30%)	46/42	52.3/47.7
ZAP-70 neg/pos (cut off 30%)	36/24	60.0/40.0
IGHV mut/unmut	21/39	35.0/65.0
11q deletion yes/no	24/66	26.7/73.3
TP53 aberration yes/no	28/42	40.0/60.0
NOTCH1 mut/WT	5/41	10.9/89.1
SF3B1 mut/WT	6/31	16.2/83.8
BIRC3 mut/WT	2/35	5.4/94.6

For every patient, it was performed a cytogenetics analysis to evaluate their chromosomal abnormalities. All the patients having a CK had numerous abnormalities, but the presence of more than 5 abnormalities was considered a specific subgroup within the CK (Table 4).

Table 4 Patients' cytogenetics abnormalities.

VARIABLE	N. of pts (90)	%
Monosomies yes/no	42/48	46.7/53.3
Trisomies yes/no	34/56	37.8/62.2
Deletions yes/no	68/22	75.6/24.4
Balanced translocations yes/no	30/60	33.3/66.7
Unbalanced rearrangements yes/no	66/24	73.3/26.7
>=5 abnormalities yes/no	35/55	38.9/61.1

For every cytogenetics abnormality was then evaluated their impact on the OS, both in the univariate and multivariate statistical analysis (Table 5).

Table 5 Impact of karyotypic abnormalities on overall survival (OS).

Karyotype category	HR	95% CI	p	HR	95% CI	p
		Univariate			**Multivariate**	
Deletions yes/no	0.811	0.363-1.815	0.612	-	-	-
Unbalanced rearrangements yes/no	2.948	1.237-7.029	0.015	2.513	1.037-6.094	0.041
Monosomies yes/no	1.420	0.742-2.718	0.290	-	-	-
>=5 abnormalities yes/no	2.131	1.120-4.055	0.021	2.786	0.922-3.458	0.085
Trisomies yes/no	0.717	0.366-1.406	0.334	-	-	-
Balanced translocations yes/no	0.881	0.453-1.711	0.708	-	-	-

The results display that what influences the OS besides the CK; is the presence of unbalanced rearrangements and of 5 or more abnormalities. It was then decided to focus our attention on the cases with unbalanced rearrangements. In the table below (Table 6) are reported the more common prognostic marker for CLL and their association with the presence of unbalanced rearrangements.

Table 6 Genetic and biological characteristics of patients with unbalanced rearrangements.

Variable	Unbalanced rearrangements		p
	no	yes	
Age, <=65 / > 65 years	13/11	30/36	0.485
Sex m/f	16/8	42/24	1.000
Binet Stage a/b-c	19/5	44/22	0.306
CD38 neg / pos (cut off 30%)	13/11	33/31	1.000
ZAP-70 neg /pos (cut off 30%)	11/3	25/21	0.130
IGHV mut/unmut	7/8	14/31	0.352
11q deletion yes/no	11/13	13/53	0.029
TP53 aberration yes/no	16/3	26/25	0.014
Monosomies yes/no	5/19	37/29	0.004
Trisomies yes/no	15/9	19/47	0.006
≥5 abnormalities yes/no	3/21	32/34	0.003
NOTCH1 mut/WT	2/12	3/29	0.633
SF3B1 mut/WT	2/11	4/20	1.000
BIRC3 mut/WT	1/12	1/23	1.000

From the results shown in table 6, it appeared that patients with unbalanced rearrangements have a higher rate of 17p deletion, monosomies, and a karyotype with more than 5 abnormalities while displaying a lower incidence for 11q deletion (P = 0.029) and trisomies (P = 0.006).

It was then calculated the prognostic impact of several prognostic factors, using both univariate and multivariate statistical analysis, on the OS (Table 7).

Table 7 Univariate and multivariate analysis for OS.

OS	Univariate analysis		Multivariate analysis (n=90)	
Variable	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
Age	2.553 (1.291-5.049)	0.007	2.392 (1.197-4.780)	0.014
Binet Stage b-c vs a	1.243 (0.900-1717)	0.187	-	-
IGHV unmut vs mut	1.305 (0.530-3.215)	0.652	-	-
TP53 aberrations yes vs no	1.134 (0.533-2.412)	0.744	-	-
CD38 pos vs neg	1.847 (0.923-3.696)	0.083	-	-
ZAP70 pos vs neg	1.388 (0.658-2.929)	0.390	-	-
Del(11q) yes vs no	1.123 (0.564-2.237)	0.741	-	-
Unbalanced rearr. yes/no	2.948 (1.237-7.029)	0.015	2.797 (1.135–6.897)	0.025
Deletions yes/no	0.811 (0.363–1.815)	0.612	-	-
Monosomies yes/no	1.420 (0.742–2.718)	0.290	-	-
≥5 abnormalities yes/no	2.131 (1.120–4.055)	0.021	0.716 (0.364–1.409)	0334
Trisomies yes/no	0.717 (0.366–1.406)	0.334	-	-
Balanced translocations yes/no	0.881 (0.453–1.711)	0.708	-	-

Based on the result of the univariate analysis, the presence of 5 or more abnormalities, unbalanced rearrangements, and age >65 years were associated with an inferior OS. The multivariate analysis was confirmed the negative prognostic impact on the OS of age >65 years and unbalanced rearrangements. (fig.32).

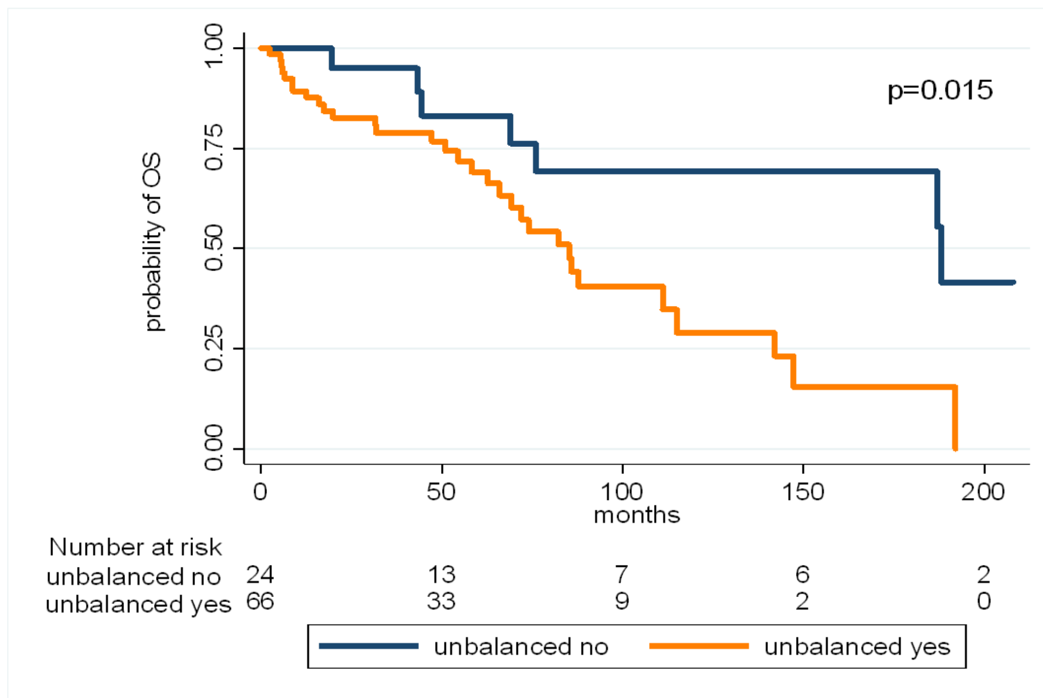

Figure 31.OS according to the presence of unbalanced rearrangements.

The analysis proceeded by measuring the impact of these prognostic factors on the TTFT. As it emerges from the results (Table 8), in this case more factors have a statistical impact on the time to first treatment. The presence of unbalanced rearrangements kept their statistical significance, but in this case, also the mutational status of IGVH and the Binet stage have an impact to determine the TTFT. In multivariate analysis, advanced stage, unmutated IGHV, and unbalanced rearrangements retained their negative prognostic impact on TTFT.

Table 8 Univariate and multivariate analysis for time to first treatment (TTFT).

TTFT	Univariate analysis		Multivariate analysis (n=60)	
Variable	HR (95% CI)	p	HR (95% CI)	p
Age	0.950 (0.579-1.559)	0.841	-	
Binet Stage b-c vs a	1.493 (1.148-1.942)	0.003	1.851 (1.305-2.627)	0.001
IGHV unmut vs mut	2.624 (1.269-5.426)	0.009	2.238 (1.062-4.718)	0.034
TP53 aberrations yes vs no	1.382 (0.800-2.388)	0.246	-	-
CD38 pos vs neg	1.304 (0.789-2.155)	0.300	-	--
ZAP70 pos vs neg	1.128 (0.603-2.113)	0.705	-	-
Del11q yes vs no	1.156 (0.662-2.020)	0.609	-	-
Unbalanced rearr. yes/no	2.007 (1.124-3.582)	0.018	2.375 (1.027-5.492)	0.043

Unbalanced rearrangements result, surprisingly, to have a stronger prognostic impact on CLL than TP53 mutation and the deletion of chromosomal 11q, which are among the worst prognostic factors (fig.33).

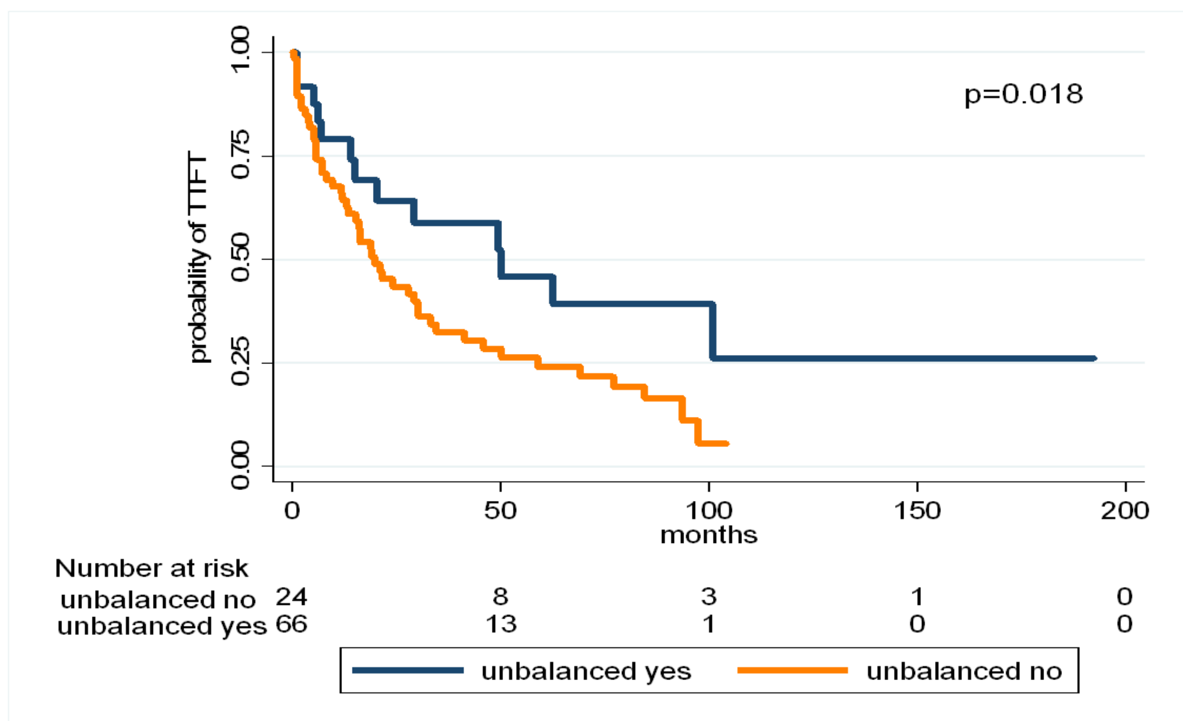

Figure 32. TTFT according to the presence of unbalanced rearrangements.

4.2 GENE EXPRESSIONPROFILING

It was analyzed the mRNA expression profiles of 23 patients, with (n=11) and without (n=12) unbalanced rearrangements. The cases investigated were representative of the entire cohort in terms of age, stage, TTFT, and survival. The analysis identified 160 differentially expressed genes (P<0.1, fold change cut-off >2.0). Among the differentially expressed mRNAs, there were genes involved in the response to DNA damage and cell cycle regulation. The heat-map below (fig. 34) represented the average expression of the 30 most altered genes between patients with and without unbalanced rearrangements.

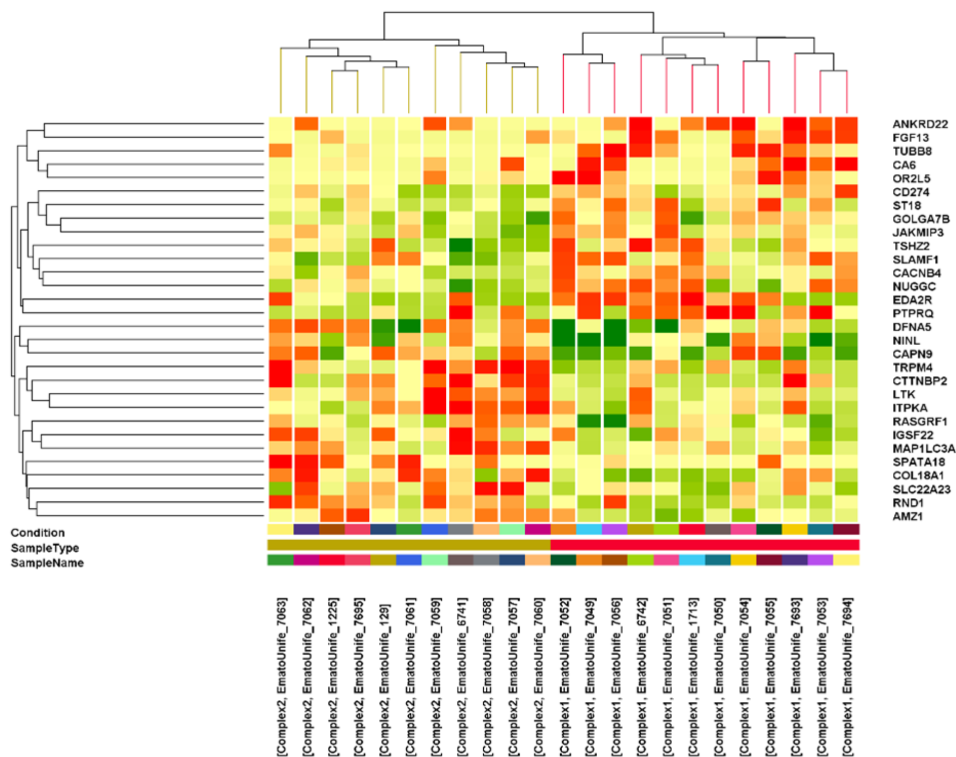

Figure 33.Heat-map representation of the average expression of the 30 most differentially expressed genes between patients with and without unbalanced rearrangements. The colors of the genes represented on the heat map correspond to the expression values normalized on gene mean expression across all samples: green indicates down-regulated; red indicates up-regulated. Legend: Complex1 = without unbalanced rearrangements; Complex2 = with unbalanced rearrangements.

Among the dysregulated genes that emerged from the GEP analysis was SLAMF1, a self-ligand adhesion/co-stimulatory molecule involved in the lymphocytes activation and autophagy pathway.

4.3 SLAMF1 EXRESSION

In the analysis of SLAMF1 expression patients were divided in CK3, CK with ≥ 3 cytogenetic abnormalities; CK5, CK with ≥ 5 cytogenetic abnormalities and MSA (major structural abnormalities) intended as the principal CLL's abnormalities. The 349 patients showed that a CK3 and a CK5 were observed in 37 (11,3%) and 13 patients (4,0%), respectively, while 39 (11,9%) had (MSA). Of these 39 patients with MSA, 25 (64,1%) harbored a CK3 while 11 (28,2%) presented CK5. A lower SLAMF1 expression was observed in patients with CK3 and CK5 as compared to patients without CK3 and CK5. A decreased SLAMF1 expression was also noted in patients with MSA as compared to those without MSA, a bigger difference was noted when MSA was associated with CK5 in comparison to those with MSA without CK5 (Table 9). There was no difference between patients with MSA and CK3 and patients with MSA without CK3.

Table 9 Comparison of SLAMF1 expression in a different set of patients based on their cytogenetic alterations. MSA, major structural abnormalities: CK3, complex karyotype with ≥ 3 cytogenetic abnormalities; CK5, complex karyotype with ≥ 5 cytogenetic abnormalities

	Median value	Range	p
CLL with CK3 (n=37)	1.87	0.24-13.29	
CLL without CK3 (n=291)	4.14	0.08-31.13	<0.001
CLL with CK5 (n=13)	1.05	0.26-9.42	
CLL without CK5 (n=315)	4.05	0.08-31.13	<0.001
CLL with MSA (n=39)	1.63	0.24-21.23	
CLL without MSA (n=289)	4.18	0.08-31.13	<0.001
CLL with MSA and CK3 (n=25)	1.54	0.24-13.29	
CLL with MSA without CK3 (n=14)	1.97	0.84-21.23	0.193
CLL with MSA and CK5 (n=11)	2.36	0.24-21.23	
CLL with MSA without CK5 (n=28)	1.05	0.26-7.72	0.018

To further investigate the clinical and biological impact of SLAMF1 expression, patients were subdivided into three groups based on the level of SLAMF1 expression: patients with a SLAMF1 level ≤2.81 were categorized as low-SLAMF1, those with a level ≥6.24 were considered as high-SLAMF1, all the other cases as int-SLAMF1. The three groups of patients differed significantly according to known relevant prognostic factors, including CD38 positivity, high levels of β2-microglobulin, IGHV unmutated status, 11q deletion, and higher risk CLL International Prognostic Index (IPI) categories (Table 10). No correlation was found with NOTCH1, SF3B1, and BIRC3 mutations. In comparison to the int-SLAMF1 group, patients with low-SLAMF1 levels presented a distinct high-risk genomic and clinical profile with a higher incidence of TP53 disruption, CK3, CK5, and MSA, and need for treatment, while patients with a higher SLAMF1 expression had a lower incidence of advanced Binet Stage. It was also found an association between trisomy 12 and a low level of SLAMF1, supporting the idea that SLAMF1 downregulation might be used to identify these patients.

Table 10 Clinical and biological characteristics and correlations with SLAMF1 levels.L=low: I=intermediate; H=high. Tri=trisomy; del=deletion; mut=mutation.

	N (%)	***SLAMF1* N (%)**				***SLAMF1* I vs H**	***SLAMF1* I vs L**
variable		L	I	H	p	p	p
Age ≤ 65/> 65 years	167/182 (47.8/52.2)	52/64 (44.8/51.2)	56/60 (48.3/51.7)	59/58 (50.4/49.6)	0.685	0.794	0.693
Gender M/F	207/142 (59.3/40.7)	71/45 (61.2/38.8)	69/47 (59.5/40.5)	67/50 (57.3/42.7)	0.847	0.791	0.893
Binet stage A/B-C	284/65 (81.4/18.6)	85/31 (73.3/26.7)	92/24 (79.3/20.7)	107/10 (91.5/8.5)	0.001	0.010	0.354
CD38 neg/pos§	214/134 (61.5/38.5)	44/71 (38.3/61.7)	76/40 (65.5/34.5)	94/23 (80.3/19.7)	<0.001	0.012	<0.001
b2m >3.5 mg/L no/yes	268/79 (77.2/22.8)	73/43 (62.9/37.1)	90/24 (78.9/21.1)	105/12 (89.7/10.3)	<0.001	0.029	0.009
***IGHV* mut/unmut**	203/136 (59.9/40.1)	37/78 (32.2/67.8)	68/41 (62.4/37.6)	98/17 (85.2/14.8)	<0.001	<0.001	<0.001
Normal FISH yes/no	104/245 (29.8/70.2)	26/90 (22.4/77.6)	35/81 (30.2/69.8)	43/74 (36.8/63.2)	0.058	0.332	0.233
13q14 del yes/no	185/164 (53.0/47.0)	52/64 (44.8/55.2)	66/50 (56.9/43.1)	67/50 (57.3/42.7)	0.104	1.000	0.088
Trisomy 12 yes/no	64/285 (18.3/81.7)	37/79 (31.9/68.1)	17/99 (14.7/85.3)	10/107 (8.6/91.4)	<0.001	0.158	0.003
11q22 del yes/no	30/329 (8.6/91.4)	22/94 (19.0/81.0)	8/108 (6.9/93.1)	0/117 (0.0/100.0)	<0.001	0.003	0.010
***TP53* mut yes/no**	21/327 (6.0/94.0)	13/103 (11.2/88.8)	2/114 (1.7/98.3)	6/110 (5.2/94.8)	0.011	0.280	0.006

CK3 yes/no	37/291 (11.3/88.7)	22/90 (19.6/80.4)	9/101 (8.2/91-8)	6/100 (5.7/94.3)	0.002	0.595	0.019
CK5 yes/no	13/315 (4.0/96.0)	11/101 (9.8/90.2)	0/110 (0.0/100.0)	2/104 (1.9/98.1)	<0.001	0.240	0.001
MSA yes/no	39/289 (11.9/88.1)	27/85 (24.1/75.9)	6/104 (5.5/94.5)	6/100 (5.7/94.3)	<0.001	1.000	<0.001
CLL IPI low/int/high very high	168/107/68 (48.9/31.2/19.8)	28/44/43 (24.3/38.3/37.4)	56/42/15 (49.5/37.2/13.3)	84/21/10 (73.0/18.3/8.7)	<0.001	0.001	<0.001
***NOTCH1* mut/wt**	17/211 (7.5/92.5)	7/68 (9.3/90.7)	5/75 (6.3/93.7)	5/68 (6.9/93.1)	0.815	1.000	0.555
***SF3B1* mut/wt**	14/214 (6.1/93.9)	6/69 (8.0/92.0)	6/74 (7.5/92.5)	2/71 (2.7/97.3)	0.330	0.280	1.000
***BIRC3* mut/wt**	9/219 (3.9/96.1)	3/72 (4.0/96.0)	4/76 (5.0/95.0)	2/71 (2.7/97.3)	0.912	0.683	1.000
Treatment yes/no	156/193 (44.7/55.3)	78/38 (67.2/32.8)	45/71 (38.8/61.2)	33/84 (28.2/71.8)	<0.001	0.407	<0.001

Given the distinct genetic features, we considered low-SLAMF1 cases as an independent subgroup in multivariate analysis, while intermediate and high-SLAMF1 cases were merged into one subgroup. Low-SLAMF1 levels confirmed their negative prognostic impact on TTFT and OS in the multivariate analysis, independently of high-risk CLL IPI, while in this model, the CK5 did not retain statistical significance. The negative prognostic impact of lower levels of SLAMF1 on TTFT and OS was confirmed in another model where low- and intermediate-SLAMF1cases were merged. In this second model, the CK5 did not retain statistical significance.

As shown by fig. 35, patients with higher SLAMF1 have a longer TTFT, while patients with a lower ratio of SLAMF1 have a shorter TTFT, therefore associated with a more aggressive disease.

Table 11 TTFT according to slamf1 ratio. U.A.= univariate analysis; M.A.= multivariate analysis.

	U. A.		**M. A. Model I**		**M. A. Model II**	
TTFT	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
CLL IPI hih-very high	2,61 (2,19-3,12)	< 0.001	2,43 (2,01-2,95)	< 0.001	2,51 (2,08-3,04)	< 0.001
CK5	6,52 (3,53-12,06)	< 0.001	1,44 (0,72-2,86)	0,304	1,76 (0,89-3,49)	0.105
Low SLAMF1	3,16 (2,29-4,35)	< 0.001	2,27 (1,61-3,21)	< 0.001	-	-
Low-int SLAMF1	2,96 (2,01-4,37)	< 0.001	-	-	2,50 (1,65-3,77)	< 0.001

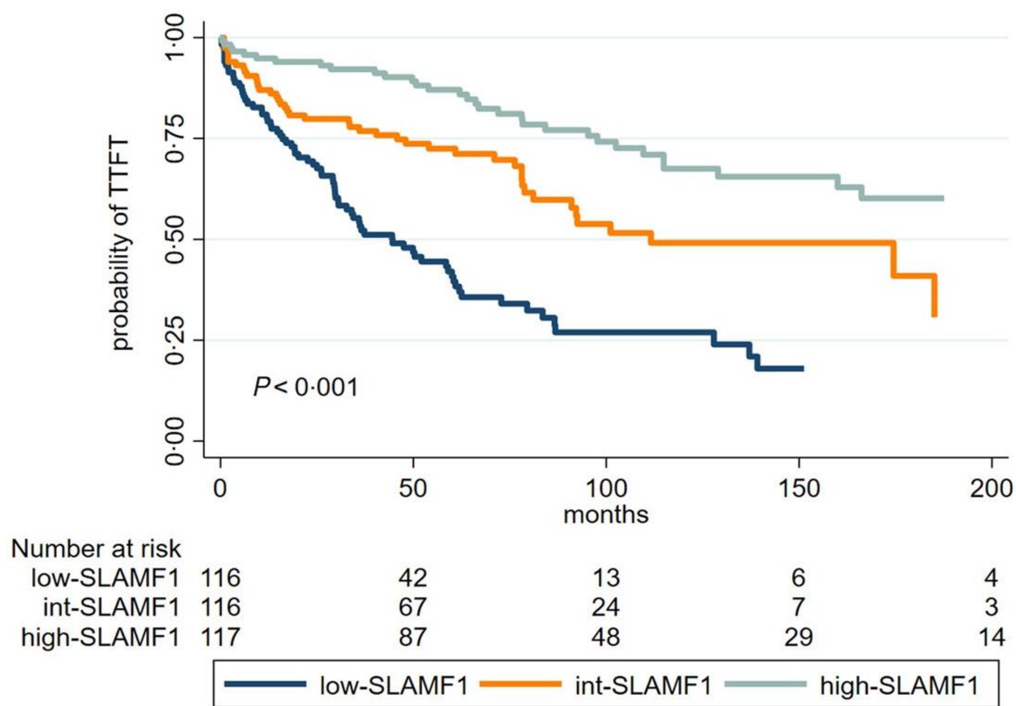

Figure 34. TTFT according to SLAMF1 expression.

The same analysis was made for the impact of SLAMF1 on the OS. (Table 12).

Table 12 OS according to SLAMF1 ratio. U.A.= univariate analysis; M.A.= multivariate analysis.

	U. A.		**M. A. Model I**		**M. A. Model II**	
OS	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
CLL IPI hih-very high	2,23 (1,81-2,75)	< 0.001	2,07 (1,64-2,60)	< 0.001	2,03 (1,62-2,55)	< 0.001
CK5	4,89 (2,34-8,24)	< 0.001	1,15 (0,55-2,43)	0,707	1,29 (0,62-2,68)	0,489
Low SLAMF1	2,58 (1,76-3,80)	< 0.001	1,79 (1,17-2,76)	0.008	-	-
Low-int SLAMF1	3,27 (1,98-5,39)	< 0.001	-	-	2,77 (1,57-4,88)	< 0.001

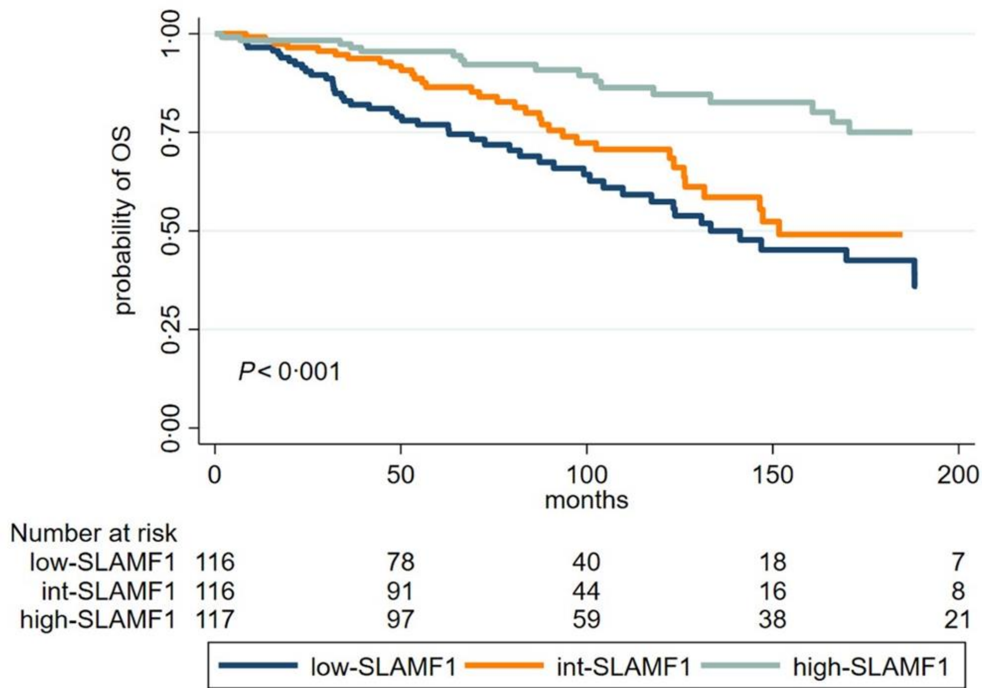

Figure 35. OS according to SLAMF1 expression.

Figure 36 shows the impact of low-SLAMF1 on the OS. In univariate analysis, lower levels of SLAMF1 were significantly associated with a worse TTFT and with a worse OS. An inferior TTFT and OS were also associated with higher-risk CLL IPI prognostic scores and the CK5, which was recently identified as the most relevant independent prognostic adverse cytogenetic biomarker.

To verify if the action of specific miRNAs regulates the expression of SLAMF1 it was performed a luciferase assay. It was evaluated the effect of the miR-17, miR-132 and miR-148, in a vector with a wild type sequence and in a mutated vector where the target sequence was altered. In both cases, it was used a wild type vector without the miRNA as a negative control.

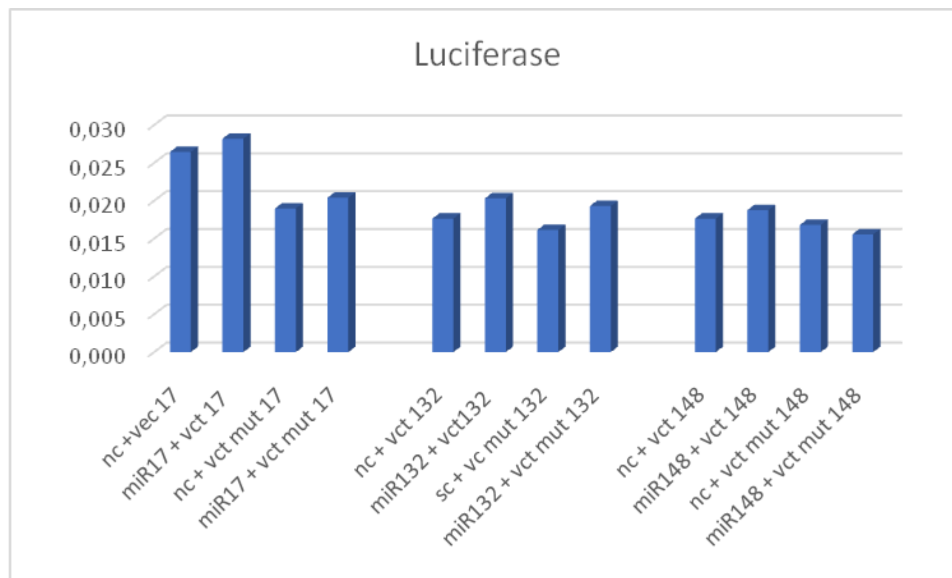

Figure 36. Expression of miR-17, miR-132, miR-148 in a negative control (nc)

The result of the luciferase assay, illustrate in figure 37, show no significant difference between the samples with a functional target sequence and the ones with a mutated one. No difference was noted also between the samples with the miRNAs and the negative controls. This means that the action of these miRNAs is not responsible for the regulation of SLAMF expression.

5 DISCUSSION

CLL is a very heterogeneous disease with different clinical outcomes. Some patients can live years symptoms-free without any therapy needed, while others could present an aggressive disease with a TTFT of only a few months and a short OS. Since CLL is an incurable disease, it is vital for identifying patients at high-risk of rapid progression and treatment requirement. Therefore, it is important to find newer and more reliable prognostic factors to provide the best treatment for the patients. CK has a well-known role as a prognostic factor, independent of the CLL-IPI (International Prognostic Index). CK is a negative prognostic factor associated with an inferior outcome, fastest disease progression, and with relapsed/ refractory patients[207]. CK is defined by the presence, in the same clone, of at least 3 aberrations. Although, in recent years, it has been suggested to update the definition on more than 5 aberrations[204,206]. It has been proved that CK has a stronger prognostic value than TP53 in relapsed CLL treated with novel agents[220,221].
Despite having such a strong prognostic value, CK is still not well explored subject. CK is a heterogeneous cytogenetic category that includes numerical and structural abnormalities. If the number of anomalies could alter the prognostic value of CK it was supposed that the same could happen based on the type of structural aberration harbored in the CK.
In this study, it was decided to further investigate the role that single abnormalities plays in defining the prognostic value of CK.
The samples used belonged to CLL patients with CK, treated at the Ferrara Hospital between 2000 and 2017. In order to avoid bias, only treatment-naive patients were included, since treatment can induce genetic changes. It was obtained a final cohort of 90 samples. For every patient were analyzed cytogenetic abnormalities and the most common prognostic factors, such as immune phenotype, clinical characteristics, and molecular anomalies. It was considered the impact that every single cytogenetics abnormality, harbored in CK, had on the prognosis of CLL. The statistical univariate analysis has shown that unbalanced translocation and the presence of more than five abnormalities have a significant impact on the OS. However, only the unbalanced rearrangement kept the statistical value in the multivariate statistical analysis.[222] Then it was investigated the presence of an association between unbalanced translocation and other prognostic factors. The results show that with the presence of unbalanced translocation there is a lower incidence of 11q deletion and a higher incidence of TP53 aberration. Moreover, as we confirmed in Rigolin et al, 2018[222], the prognostic impact of unbalanced translocation still

resulted as an independent prognostic marker from TP53 aberration. This observation strengthen our hypothesis than unbalanced CK could be used as an independent prognostic marker stand-alone.

Our next step was to confront the value of unbalanced CK as prognostic factor, looking to different predictors associated to the CLL progression.

It was evaluated the impact on the OS of the most common prognostic factors and unbalanced rearrangement. The results display that in patients with a CK, only age and unbalanced translocation represent the discriminating elements to predict a worse prognosis. The same analysis, related to the TTFT, confirmed the prognostic value of unbalanced translocation and the age of the patients. For the TTFT, the Binet stage also seems to be statistically relevant to predict the progression of the disease in patients with CK. It was also decided to perform a GEP analysis to study the possible associations between unbalanced rearrangements and molecular disruption. The analysis was carried out on only 23 patients from the previously cohort of 90 patients. They were selected to represent the entire population in terms of age, gender, and stage of the disease. Half of the cohort (11 patients) presented unbalanced translocation. The other samples (12) were patients without unbalance rearrangements. As a result, patients with unbalanced translocation showed a distinct mRNA expression profile than other patients. From the GEP emerged that more than 160 genes were differentially expressed between the two cohorts. Among those genes, several were involved in cell cycle control or DNA damage regulation. It was decided to investigate the genes that resulted altered in the GEP analysis. Four genes in particular, as we reported in Rigolin et. al.[222], came to attention: RASGRF1, CTTNBP2, TRPM4, and SLAMF1. RASGRF1 is a guanine exchange factor (GEF) involved in BCR signal, generally overexpressed in B cell malignancies. As a part of BCR signaling, it is inhibited by Ibrutinib[223,224]. CTTNBP2 is a Lyn substrate also involved in the BCR pathway and therefore inhibited by Ibrutinib[225]. TRPM4 is largely studied in diffuse large B-cell lymphoma[226]. Then, we decided to focus on SLAMF1. As previously mentioned, SLAMF1 is a key regulator of several pathways involving lymphocytes maturation and activation[209]. It is also involved in the regulation of B-cell microenvironment in CLL[211,212]. To this date, there are only few studies that analyze the role of SLAMF1 in this disease. Therefore, we decided that the possibility of an association between SLAMF1 and CLL deserved further investigation as SLAMF1's role in lymphocytes activation, as stated before. The expression of SLAMF1 was studied in all the patients' sample stored at Ferrara's laboratory. The cohort consisted of 349 samples from untreated CLL patients diagnosed with CLL at Ferrara's Hospital between the 2004

and 2019. Patients were grouped based on prognostic factor, and the levels of SLAMF1 expression were analyzed for each group. In particular, patients with CK were divided into two groups based on the presence of 3 abnormalities (CK3) or more than 5 (CK5). The down-regulation in the SLAMF1 expression was observed in patients with CK3, CK5, and major chromosomal structural abnormalities (MSA). Patients with both CK5 and structural abnormalities displayed a lower level of SLAMF1 compared to the other groups. In patients with structural abnormalities, no differences were found between the ones with CK3 and the ones without it. These results, recently published[227], lead to the hypothesis that the presence of MSA has a stronger prognostic value than CK3.

Based on the level of expression of SLAMF1 it was obtained a division of the patients into three major groups: low-SLAMF1, int- SLAMF1, high-SLAMF1. A lower expression of SLAMF1 shown an association with worst TTFT and OS in a multivariate model. Interestingly, patients with int‐SLAMF1 levels showed an intermediate outcome. It could possibly be correlated with an intermediate level of high‐risk genomic characteristics, including 11q deletion and IGHV mutational status. Lower SLAMF1 levels were associated with loss of response to therapeutic agents based on autophagy induction, like fludarabine or ABT-737[228]. Given the role of SLAMF1 in autophagy and chemotaxis, when downregulated may render CLL cells potentially unresponsive to autophagy‐inducing drugs, including BCL‐2 inhibitors. As we reported in Rigolin et al. 2020[227] the stratification of SLAMF1 expression in different groups identified three groups of patients with a very different outcome. However, further studies are needed to define the best and most relevant cut‐off for clinical applications.

We hypothesized that the dysregulation of SLAMF1 expression could be due to the action of miRNAs. As stated before, miRNAs have an important role in the pathogenesis of CLL and they are involved in the regulation of several genes. To test this theory it was decided to evaluate if the miRNAs have an impact on the expression of SLAMF1 in normal cells before proceeding with testing on the patients' cells. The miRNAs suspected to cause a down-regulation of SLAMF1 expression were the ones that are over-expressed in CLL. Among the miRNAs that target SLAMF1 only three were reported overexpressed in CLL cases: miR-17, miR-132, and miR-148. For each miRNAs, it was compared their action in a wild-type vector, including a functioning sequence, against their action in a mutated vector where the target sequence was altered. From the results of the luciferase assay, no significant difference had emerged between the action of the miRNAs in the wild-type and mutated vectors. This indicates that the analyzed miRNAs are not directly involved in the

regulation of SLAMF1. The causes of SLAMF1 de-regulation in CLL with unbalanced rearrangements have to be revealed yet.

6 CONCLUSION

CLL is characterized by a heterogeneous behavior that manifests in very different ways. Some patients are asymptomatic for years while others manifest an aggressive disease progression. Therefore, it is fundamental to identify the patients who require immediate treatment. The necessity to separate the patients into risk categories to better identify who is in immediate needs of treatment, led to the continuous search of more precise markers that could help to better understand the status of the disease and define a personalized therapeutic strategy. In recent years CK has become an increasingly important prognostic marker in patients with CLL. Patients with CK are associated with a worse prognosis [219] . CK includes many different types of abnormalities. In this study, it has emerged that only unbalanced rearrangements, among all the cytogenetics abnormalities, have a negative prognostic meaning on both the OS and the TTFT. This correlation between unbalanced rearrangement and a bad prognosis is stronger than between a negative outcome and 11q deletion or the mutation of 17p. Until today, CK is considered as a negative prognostic marker no matter what kind of anomalies it harbors. In this study we proven that is not always the case. Patients with a CK should not be considered all in the same way. They could be divided into different risk categories based on the presence of unbalanced rearrangement. This stratification has clinical implications. It could help define a better therapeutic strategy for those patients. This finding could change the prognostic significance of CK. In conclusion patients with unbalance rearrangements have a higher risk of a quick progression and relapse. This study also highlights the importance of the karyotype, as an important tool to predict the development of the disease. It was also found that who harbor unbalanced rearrangements has a different gene expression profile than the rest[222]. Among the differentially expressed genes, it was investigated the role of the SLAMF1 gene as a prognostic factor. It was measured the expression of SLAMF1 in treatment- naive patients. The results showed that the expression of SLAMF1 could be divided in three groups. Patients with negative prognostic markers have a lower expression of SLAMF1. As we reported in Rigolin et al[227], the down-regulation of SLAMF1 displayed a statistically significant association with shorter TTFT and OS . This indicate SLAMF1 as a strong and independent prognostic factor. SLAMF1 is involved in the autophagy pathway and as an indirect modulator of BCL-2. A downregulated SLAMF1 may be used as an indicator of CLL cells unresponsive to autophagy‐inducing drugs, such as venetoclax, a BCL‐2 inhibitor. SLAMF1 is a superficial molecule that could be easily

evaluated, with quicker and relatively simple techniques, including ddPCR and flow cytometry. This could have relevant practical and clinical implications. The strong correlation between low SLAMF1 levels, cytogenetic complexity, and high‑risk genetic features suggest that SLAMF1 could represent an alternative to more complex and time-consuming analysis. The availability of a prognostic marker that could be quickly measured using peripheral blood would simplify the stratification of the patients in a prognostic category and help define a quick therapeutic strategy. The luciferase assay had shown that the SLAMF1 expression is not regulated by the action of the investigated miRNAs. Therefore, our hypothesis is that SLAMF1 could be regulated by other factors those we have not investigated yet. In conclusion, this study support SLAMF1 as a reliable and cost‑effective biomarker, simple to evaluate and with potential prognostic and predictive significance. This research show that more discovers are needed to fully understand the genetic and biological mechanisms that regulate the CLL.

All the results of this work have been published in two articles[222,227] that are attached at the end of this thesis.

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