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The relationship between the polymorphism (rs6449182) of CD38 and the prognosis of patients with chronic lymphocytic leukemia treated with fludarabine, cyclophosphamide and rituximab (FCR)

A 4-year prospective cohort study

FINAL DEGREE PROJECT

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"We look for medicine to be an orderly field of knowledge and procedure. But it is not. It is an imperfect science, an enterprise of constantly changing knowledge, uncertain information, fallible individuals, and at the same time lives on the line. There is science in what we do, yes, but also habit, intuition, and sometimes plain old guessing. The gap between what we know and what we aim for persists. And this gap complicates everything we do."

Atul Gawande

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1. LIST OF ABBREBIATIONS

ADP	Adenosine diphosphate
Ag	Antigen
BCR	B-cell Receptor
β2m	Beta 2 microglobuline
BTK	Bruton tyrosine kinase
cADPR	cyclic ADP ribose
CIT	Chemoimmunotherapy
CLL	Chronic lymphocytic leukemia
DLBCL	Diffuse large B-cell Lymphoma
FCR	Fluradabine, cyclophosphamide, rituximab
FDA	Food and drug administration
FISH	Fluorescence in situ hybridation
GC	Germinal center
IgVH	Immunoglobulin heavy chain variable region
IL	Interleukin
LDH	Lactate dehydrogenase
LDT	Lymphocyte doubling time
mAb	Monoclonal antibody
MBL	Monoclonal B cell lymphocytosis
MRD	Minimal residual disease
NAD+	Nicotinamide adenine dinucleotide
PCR	Polymerase chain reaction
PECAM-1	Platelet endothelial cell adhesion molecule-1
PS	Performance Status
SNP	Single nucleotid polymorphisms
SLL	Small lymphocytic lymphoma
RS	Richter syndrome
TTP	Time to progression
TTT	Time to next treatment
UM	Unmutated
ZAP-70	Z-associated protein of 70 kD

2. ABSTRACT

Background: Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults in Europe and in North America. It is caused by the neoplastic transformation of a population of B-lymphocytes coexpressing CD5, CD19 and CD23. Although the etiology is not well known there is evidence of the existence of a genetical base. CLL is not only caused by the accumulation of B-lymphocytes due to a failure in the apoptosis. It is also caused by the active proliferation of the B-cells in response to the signs sent by the environment. Its clinical course is variable. One third of CLL patients are affected by an indolent form that does not require treatment. Another third of patients with leukemia will require iterative therapies and a small fraction patients develop Richter syndrome that reduces its overall survival to 5 to 8 months. This variability is due to the heterogeneity of the molecules that participate in the pathogenesis. One of these molecules is CD38. Some studies have demonstrated that the presence of this marker confers a worse prognostic, specially the presence of one polymorphism of CD38, rs6449182. Fluradabine, clyclophosphamide and rituximab (FCR) is considered the gold standard therapy for young patients under 65 years with CLL because achieves high percentage of minimal residual disease (MRD). But it has not been studied if in CLL patients with this polymorphism, FCR continues offering these good results.

Objective: To analyze the relationship between the polymorphisms of CD38 and treatment outcomes in CLL patients treated with FCR.

Methods: The design is a multicenter prospective cohort study. 338 patients will be recruited during 4 years. We will analyze the presence of the polymorphism and evaluate the minimal residual disease (MRD) after the treatment with FCR.

Key words: *chronic lymphocytic leukemia, CD38 antigen, single nucleotide polymorphism, minimal residual disease.*

3. INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by the clonal expansion of mature lymphocytes coexpressing CD5, CD19 and CD23 in blood, bone marrow and secondary lymph tissues(1,2).

From a biological point chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) are different manifestations of the same disease, which are managed in the same way(3). A diagnosis of small lymphocytic lymphoma (SLL) is made when lymphadenopathy or splenomegaly because of infiltrating CLL cells is found, with $< 5 \times 10^9$ CLL-type cells in the blood(4)

Its clinical course is variable. One third of CLL patients are affected by an indolent form that does not require treatment. Another third of patients present with leukemia will require iterative therapies, profoundly affecting their quality and length of life. A small fraction (2-8%) of CLL patients develop Richter syndrome (RS), a transformation of the original CLL clone into a diffuse large B-cell Lymphoma (DLBCL). RS is a highly aggressive syndrome with a median overall survival of 5 to 8 months.(5)

The prediction of this highly variable clinical course has been facilitated by the analysis of molecular prognostic factors, including CD38 expression, ZAP70 expression, IgVH mutational status, and common genomic aberrations assessed by fluorescence in situ hybridization (FISH) assay.

3.1 Epidemiology

CLL is the most common leukemia in adults with a median age at diagnosis of 72 years. It is the most frequent form of leukemia in Spain. According to the "Registro Español de leucemias" CLL represents the 25,6% of all leukemia diagnoses. The incidence is 4,2 (IC95%: 3,3-5,1) and 3,1 (IC 95%: 2,3-3,8) cases per 100.000 inhabitants per years, in men and women respectively.(6). CLL is the most common leukemia in Europe and North America and is less common among people of African or Asian origin(7)

Most cases of CLL are preceded by monoclonal B cell lymphocytosis (MBL), a very indolent cell expansion defined by less than 5000 monoclonal B cells in the peripheral blood. MBL is

detectable in approximately 5% of elderly population, and carries a risk of evolving into CLL that approximates 1% per year(8,9).

3.2 Pathophysiology

The etiology of B-CLL is not well-known, although there is evidence of family aggregation as well as racial differences in its incidence that suggest the existence of a genetical base. Moreover, in some types of B-CLL some continuous antigenic stimulus (infectious or autoimmune) could play an important role(10).

Traditionally considered a disease of failed apoptosis, CLL is now revealing itself to be an environment-dependent hematological malignancy.(11) The basic mechanism of proliferation of CLL-cells is not only an intrinsic apoptotic defect as it was thought. CLL is caused by an increased growth rate by active proliferating cells(8).

The B-CLL results from the neoplasic transformation of a population of B-lymphocytes that are not competent and have a failure in their apoptosis mechanisms. These lymphocytes express less surface immunoglobulins than normal lymphocytes. Approximately 50-70% of these lymphocytes have immunoglobulin heavy chain variable region gene (IgVH) mutations and the other ones are unmutated. Mutation of the IgVH implies those lymphocytes need an antigenic stimulus to be activated and to start its proliferation while the unmutated B-cells are antigen-independent and have a highest lymphocyte doubling time (LDT). Unmutated lymphocytes have two important molecules, ZAP70 and CD38, which play a role in the pathogenetic mechanisms and also in the prognosis of B-CLL.

**How is the normal development of B lymphocytes?**
(7,8,12)

Normal B lymphocytes develop in the bone marrow where they mature by recombining their immunoglobulin genes and expressing different surface molecules. These cells express the B-cell receptor (BCR) which is composed of an antigen-binding subunit (the membrane immunoglobulin), which is composed of two immunoglobulin heavy chains and two

(32)
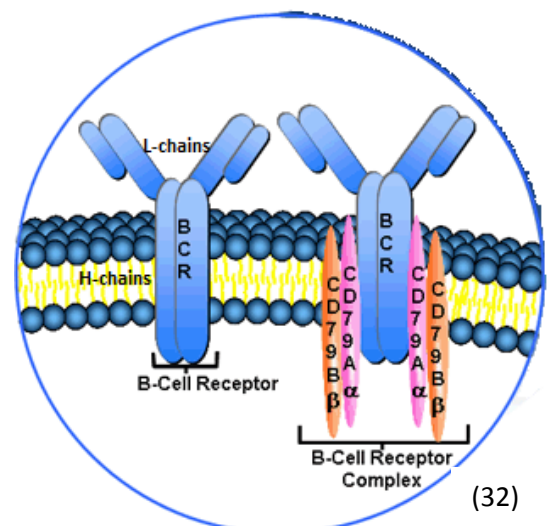
immunoglobulin light chains (variable and constant regions). When an antigen (Ag) engages the BCR, the cell enters in a germinal center (GC) in lymphoid follicles where it rapidly divides and is accompanied by the activation of somatic hypermutation (SHM), which introduces mutations into the immunoglobulin (Ig) heavy variable region genes (IgVH). The mutated GC B-cells then interact with CD4+ T helper (TH) cells and follicular dendritic cells. By interacting with these cells, GC B-cells that have acquired BCR affinity-increasing mutations are selected. GC B-cells that have unfavorable mutations undergo apoptosis.

However the process can proceed without the help of T-lymphocytes and outside germinal centers, in the marginal zone around lymphoid follicles, most often in response to carbohydrates of encapsulated bacteria or viruses, without experimenting somatic hypermutation.

Both processes lead to the development of plasma cells or memory cells.
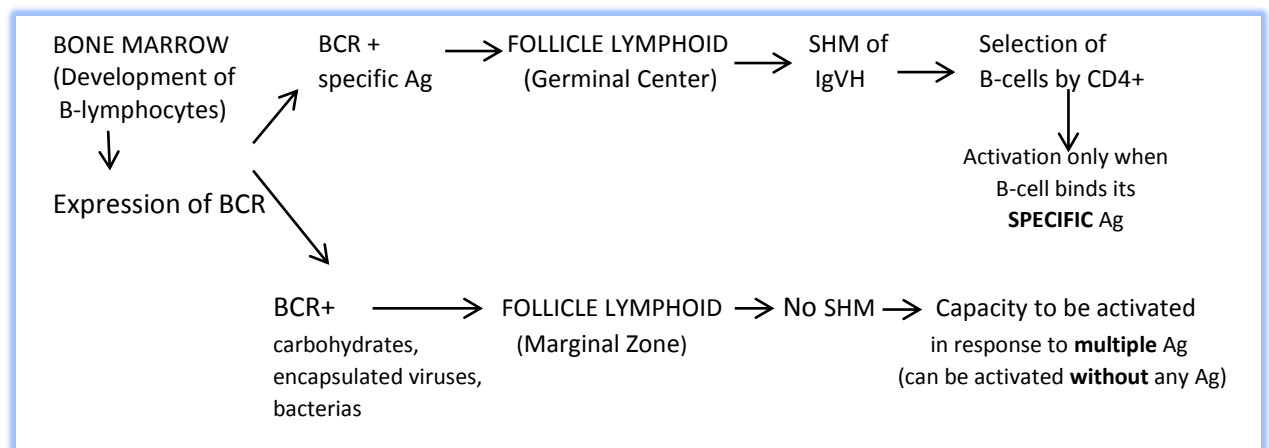
What are the role of BCR and the IgVH mutational status?(8,12)

Many normal B lymphocytes with unmutated IgVH genes produce antibodies capable of binding multiple antigens (in contrast of mutated lymphocytes that only have one specific antigen).

Signals received through BCR are transferred to the nucleus. Certain molecules as CD38 augment signaling of BCR. Stimulatory and growth signals from the environment of CLL cells allow them to avoid apoptosis and proliferate. The major growth effects appear to occur in cases in which the receptor permits binding of autoantigens and maintains the capacity to transmit stimulatory signals to the cell nucleus (i.e. unmutated B-cells)

B-cells with such unmutated polyreactive BCR could expand and convert to CLL cells with repetitive exposure to microbes and to autoantigens if they acquired a genetic abnormality that allows them to resist restraint on clonal size and acquired a growth advantage.

(8)
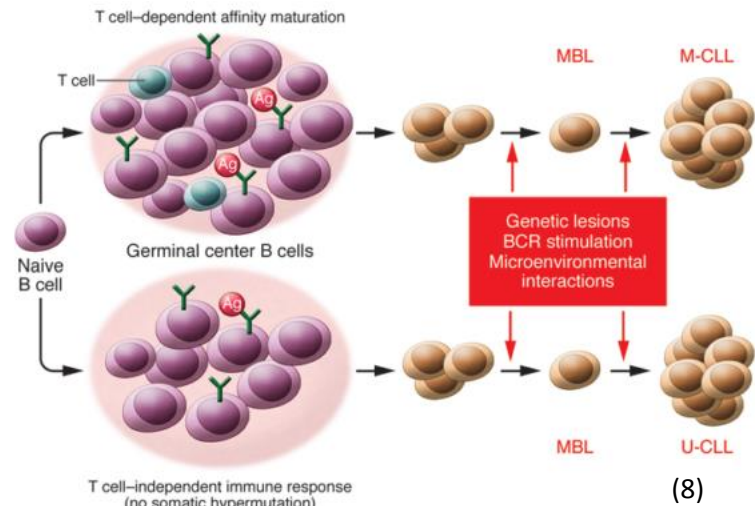
3.3 Common cytogenetic abnormalities found in CLL cells

Nearly a 80% of patients have cytogenetic abnormalities detected by conventional cytogenetic (karyotype) analysis and fluorescence in situ hybridation (FISH)(1).

Deletion of the long arm of chromosome 13 (del[13q]) is the most frequent alteration and occurs in 50%-60% of cases(1,8,12,13). Del(13q) is associated with a favorable clinical outcome, found more often in patients with mutated IgVH genes(13). This lesion is found at a similar frequency in MBL and it may represent an early event in the disease. The B-cells in MBL must acquire additional genetic or epigenetic changes before becoming CLL cells(8).

In contrast, deletion of the short arm of the chromosome 17 (del[17p]), found in 5% to 10% of patients with CLL, is the strongest predictor of poor prognosis (less overall survival and shorter time to progression). Del(17p) disrupts the TP53 tumor suppressor gene and that confers resistance to chemotherapy(12).

Deletion of the long arm of chromosome 11 (del[11q]), found in 5%-20% of patients, is associated with extensive lymphadenopathy and aggressive clinical course with shorter survival. It is because affects the ataxia-teleangiectasia mutated (ATM) gene, the deficiency of which causes genomic instability and is also related with TP53 gene. It is often associated with unmutated IgVH genes (13).

Trisomy 12 occurs in approximately 15% of CLL cases(1,8,13). These patients have a relatively long overall survival and time to progression.

3.4 New markers: CD38 and its role in CLL

CD38 is a transmembrane glycoprotein expressed in immature hematopoietic cells, down-regulated by mature cells, and re-expressed at high levels by activated B cells, T cells, natural killer cells and dendritic cells. It is involved in the regulation of numerous physiologic processes(14). CD38 functions as a leukocyte plasma membrane signaling receptor, interacting with its ligand CD31/PECAM-1 (platelet endothelial cell adhesion molecule-1) molecule, expressed by various endothelial and stromal components. This interaction results in robust proliferation/survival signals(5).

CD38 expression ranges from negative to highly positive. Further, it is dependent on body district, being higher in the lymph nodes and spleen compared to peripheral blood. Percentage of CD38 can apparently vary over time in the same individual(5). CD38 surface levels are sensitive to changes in environment (ie.IL-2), stage of disease and therapy administered. It is useful as a disease marker for leukemia and myeloma, but it might also be relevant in the pathogenesis and evolution of chronic lymphocytic leukemia (CLL)(14)

Enzymatic functions

CD38 is one of the adenosine diphosphate (ADP) ribosylcyclases, a family of multifunctional enzymes. ADP ribosylcyclases play a key role in several physiological processes, including cell proliferation, muscle contraction, stem cell regeneration and hormone secretion.

CD38 has multiple enzymatic activities, which include the degradation of cyclic ADP ribose (cADPR) and the hydrolysis of NAD+. The products of these reactions (mainly ADPR and cADPR) are used inside the cells to open different Ca^{2+} stores, leading to an increase in cytoplasmic Ca^{2+} concentrations, independently of the conventional IP3 pathway. CD38 regulates extracellular NAD^+ levels, thereby limiting the availability of this substrate to a larger family of ectoenzymes, including ADP ribosyl transferases (11,15,16).

Receptorial properties(14,15)

- CD38 ligation is followed by a signaling cascade typical of antigen receptors, including tyrosine phosphorylation of a sequential number of intracellular signal transducers, nuclear events and long-term effects dependent on active protein synthesis. Common players in the CD38 pathway are the z-associated protein of 70 kD (ZAP-70) and the proto-oncogene c-abl. Both substrates are phosphorylated in T, B and NK cells.

- CD38 ligation is followed by an increase in intracellular Ca^{2+} .The contribution of the enzymatic activities to Ca^{2+} currents induced by CD38 ligation is still unknown.

- CD31 (PECAM-1) is a non-substrate ligand for CD38. CD38–CD31 interactions recapitulate all of the signals recorded using agonistic mAbs, including mobilization of Ca^{2+} signaling, as well as more structured events, such as proliferation and cytokine induction differentiation and environment.

- The long-term events resulting from interaction between CD38 and CD31 are functions of cell lineage and differentiation status.

- CD38 as a receptor of T-cell, releasing signals that inhibit apoptosis and activate cell proliferation in CLL lymphocytes.

- CD38 as a receptor of B-cell. Its union with CD31 in lymph nodes (where the expression of CD38 is higher) inhibits apoptosis. In contrast, in bone marrow lead to apoptosis.
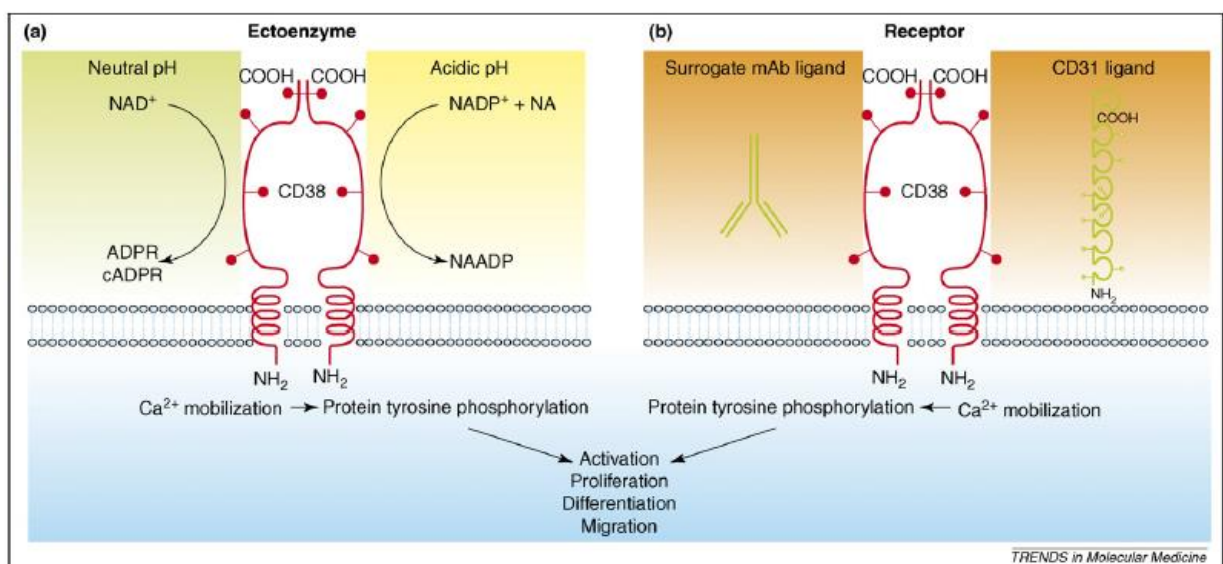
Figure 1. Schematic representation of the pleiotropism attributed to human CD38. The molecule works as an ectoenzyme (a), transforming NAD^+ and $NADP^+$ into cADPR, ADPR and NAADP. The balance between the reactions is influenced by extracellular pH. The enzymatic products are powerful Ca^{2+}-mobilizing compounds inside the cell. CD38 also acts as a receptor (b) interacting with the non-substrate ligand CD31 or with surrogate agonistic mAbs. The resulting intracellular events include Ca^{2+} mobilization, cell activation, proliferation, differentiation and migration. Abbreviations: ADPR, adenosine diphosphate ribose; cADPR, cyclic adenosine diphosphate ribose; NA, nicotinic acid; NAADP, nicotinic acid adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate.

(14)

3.5 CD38 and B-CLL(11,14,15)

CD38 expression is effective in identifying patients with unfavorable prognoses.

Patients with unmutated IgVH genes present with a more aggressive disease from diagnosis, have a shorter time to therapy and shorter overall survival. These patients also display higher percentages of CD38+ cells, indicating that CD38 might be useful as a surrogate marker for the absence of IgVH mutations.

CD38 is currently viewed as an independent risk factor that can be used together with IgVH mutational status and clinical staging to identify CLL patients with a poor prognosis. Further, CD38 expression positively correlates with all of the other negative prognostic markers for CLL examined, including ZAP-70, cytogenetic abnormalities, soluble CD23, soluble β2m, p53 function and cell size.

Its surface expression might affect expansion and proliferation of the neoplastic clone. CD38 ligation by agonistic mAbs is followed by proliferation and blast transformation of a subset of CLL cells. The in vitro signaling properties mediated by CD38 are significantly enhanced by the simultaneous presence of interleukin (IL)-2, which acts through strong up-regulation of CD38 expression.

CD38 and ZAP70 are normal cell components, but when they are both express simultaneously in a B-lymphocyte (due to a neoplasic event), results in a synergy that activates proliferation pathway mediated by CD38. CD38/CD31 induces the activation of processes ZAP70-dependent. Consequently the cells do not respond to the treatment and act as a tumoral reservoir.

Lymph nodes provide the ideal microenvironment and the appropriated signals and receptors to stimulate the proliferation. CD38 is activated after the union to CD31, which leads to overexpression of CD100. These signals require ZAP70. The binding of CD100 to Plexin B1 results in secondary signals that contributes to the proliferation and survival of the tumoral clone.

Additionally, cytokines produced by the stroma of proliferative center (located in the lymph nodes) attract CD38 to CD31 due to the presence of the CXCR4 receptor (that needs ZAP70). Thereby B-lymphocytes are carried to lymph nodes, where they have the best conditions to proliferate. The union of CD38/CD31 causes the release of more cytokines. So it produces a loop that enhances proliferation signals. It leads to the disease progression and in some cases their transformation to Richter syndrome.
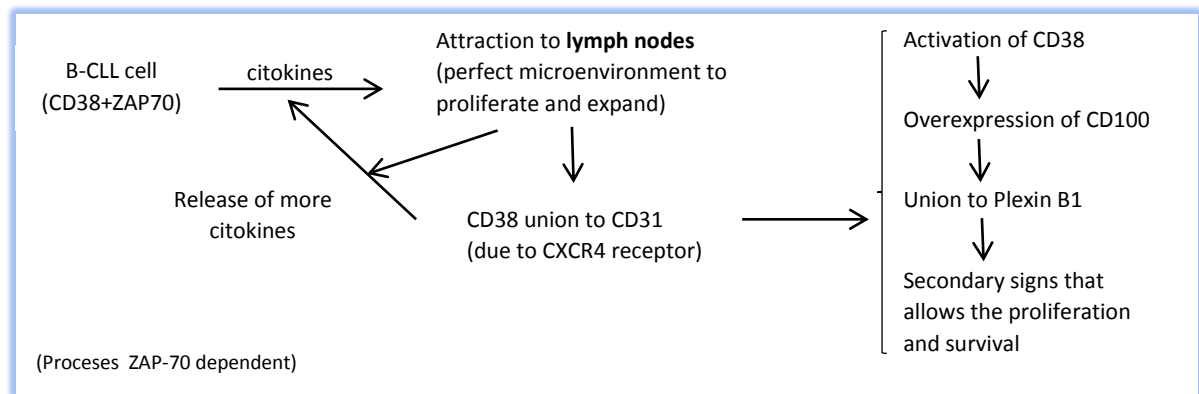
3.6 CD38 genetics and polymorphisms (5,17)

Expression of a normal surface molecule, like CD38, can be independent from neoplastic transformation but detrimental for the clinical outcome of the disease. Modulation of CD38 expression appears to be a crucial issue. Environmental signals play a predominant role; however, genetic differences must also be taken into account.

The CD38 gene is located in the short arm of chromosome 4 (4p15). Its coding sequence is organized in eight exons and more than 98% of the gene represented by introns. The 5' end region of intron 1 contains regulatory elements and potential binding sites for several transcription factors.

CD38 presents different single nucleotid polymorphisms (SNP) that have been correlated with the clinical heterogeneity. Two polymorphisms has been studied, the SNP rs6449182 CD38 *Pvu*II or 184C>G (that leads to the presence, or absence, of a Pvu II restriction site) and the 418C>T, located at the 5'-end of intron 1and in exon 3 respectively(5,17).

The SNP rs6449182 is located in an intronic hotspot, which is part of a CpG island located at the 5' end of the gene and which also contains the functional CD38 retinoic acid responsive element (RARE) responsible for the dramatic upregulation of CD38 expression induced by all-trans retinoic acid (ATRA). In addition, evidence based on a novel truncated CD38 mRNA transcript suggests that one means of controlling CD38 gene expression involves control of transcriptional elongation, where a stop-or-go decision is taken at the 5' end of intron 1.

The frequency of the 3 genotypes and the allele of CD38*Pvu*II and its correlation according to distinct molecular features and clinical parameters have been studied(5,18,19). The C allele

frequency is 0.73-0.79 and the G allele is 0.21-0.27 with a genotype distribution of CC **62%**, GC **34%** and GG **4%**.

The G allele is associated with CLL patients carrying molecular markers of poor prognosis (CD38+, ZAP70+, unmutation, del11/17, CD38+/ZAP70+, UM/CD38+/ZAP70+). GG homozygotes genotype is significant increase in CD38+ patients. Combination of molecular markers can identify patients with unfavorable prognosis more accurately than the use of one marker alone. The more unfavorable prognosis factors, the higher G allele frequency (overall GC heterozygotes). CD38 and ZAP70 are strongly correlated with the absence of mutation (UM).

The G allele is also associated with CLL patients showing clinical and laboratory markers of high tumor mass (age, sex, LDH, Binet stage, affected lymph node areas, spleen size, lines of therapy). Male, Binet Stage B, and patients with more than 2 lymph nodes, enlarged spleen or elevated LDH patients have a significantly higher G allele frequency and higher number of GG homozygotes.

CLL patients with a high tumor mass have a significant risk of developing RS. The transformation is strongly associated with the presence of the G allele. Compared with CC homozygotes, GG patients had a 30.6% increase in the relative risk of developing RS, whereas GC heterozygotes showed a probability of 12.4%.(5)

Concerning the second studied polymorphism 418 C>T, the genotype distribution is CC 99.2% and CT 0.8%. Heterozygous CT genotype was related to elevated risk of B-CLL (OR 6.57). It has been found significantly elevated CD38 expression levels in patients carrying T allele.(17)

3.7 SIGNS AND SYMPTOMS

Approximately in 80% of cases CLL is detected in a routine clinical analysis or by chance, because patients do not have any symptoms(10,20).

Those who do develop signs and symptoms may experience enlarged, but painless, lymph nodes, fatigue, pain in the upper left portion of the abdomen, which may be caused by an enlarged spleen and frequent infections (due to the hypogammaglobulinemia and the malfunctioning of B-CLL cells). Night sweats, weight loss and fever only appear in 10% of cases. During the course of the disease the infections can be incremented also because of the treatment.

In some cases a hemolytic anemia can appear (15-30%).

The most terribly complication of CLL is the transformation to Richter Syndrome a transformation of the original CLL clone into a diffuse large B-cell Lymphoma (DLBCL).

3.8 DIAGNOSIS OF CLL

The diagnose can be done according to "Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute–Working Group 1996 guidelines"(9)

- <u>Blood analyses</u>: The diagnosis of CLL requires the presence of at least $5x10^9$ B lymphocytes/L (5000/µL) in the peripheral blood. The clonality of the circulating B lymphocytes needs to be confirmed by flow cytometry.
  - o CLL or SLL might be suspected in otherwise healthy adults who have an absolute increase in the clonal B lymphocytes but who have less than $5x 10^9$/L B lymphocytes in the blood. However, in the absence of lymphadenopathy or organomegaly (as defined by physical examination or CT scans), cytopenias, or disease-related symptoms, the presence of fewer than $5x10^9$ B lymphocytes per liter of blood is defined as "monoclonal B-lymphocytosis."
  - o The definition of SLL requires the presence of lymphadenopathy and/or splenomegaly. Moreover, the number of B lymphocytes in the peripheral blood should not exceed $5x 10^9$/L. In SLL, the diagnosis should be confirmed by histopathologic evaluation of a lymph node biopsy whenever possible.

- Immunophenotype: CLL cells coexpress the T-cell antigen CD5 and B-cell surface antigens CD19, CD20, and CD23. Each clone of leukemia cells is restricted to expression of either kappa or lambda immunoglobulin light chains.

- Other tests: These tests are not needed to establish the diagnosis of CLL but may help predict the prognosis or to assess the tumor burden.

  o Molecular cytogenetics. Using interphase FISH, cytogenetic lesions can be identified

  o Mutational status of IgVH, and expression of ZAP-70 or CD38

  o Serum markers: Several studies have found that serum markers CD23, thymidine kinase, and β2-microglobulin may predict survival or progression-free survival.

  o Marrow examination: A marrow aspirate and biopsy generally are not required for the diagnosis of CLL. However, a marrow biopsy and aspirate can help evaluate for factors that might contribute to cytopenias (anemia, thrombocytopenia) that may or may not be directly related to leukemia-cell infiltration of the marrow. Because such factors could influence the susceptibility to drug-induced cytopenias, a marrow biopsy is recommended before initiating therapy.

3.9 CLINICAL STAGING AND PROGNOSIS FACTORS

The staging of the disease is done using de Rai and Binet criteria(21–23) (See annex I) The life expectancy of the patients with CLL is 10 years (6).

The prognosis is based on the clinical stages. A part from Rai and Binet stages, other markers can help to establish a most accurate prognostic. (See annex II).

3.10 TREATMENT (24–27)

The 2008 International Workshop on Chronic Lymphocytic Leukemia (IWCLL) criteria are the standard criteria that should be used to identify patients who need first-line treatment of CLL (see annex IV).

In contrast to most other leukemia, CLL is not necessarily treated at diagnosis, but rather after symptomatic disease. This strategy is mainly based on the results of randomized trials that compared early and late treatment that showed no benefit of early treatment(7).

It has been demonstrate by some studies that achieving negative minimal residual disease (MRD) is associated with superior time to progression (TTP), complete remission and survival.
MRD can be analyzed by multicolor flow cytometry. Patients will be defined as having a clinical remission in the absence of MRD when they have blood or marrow with less than one CLL cell per 10 000 leukocytes(28).

Fludarabine is a purine analog, which interferes with DNA synthesis. It also interacts with the microenvironment of CLL cells through the inactivation of CD4+ cells.
Cyclophosphamide is an alkylating agent which adds an alkyl group to DNA. This interferes with DNA replication.
Rituximab is a monoclonal antibody (mAb) anti-CD20. Although in CLL the expression of CD20 is low, rituximab induces the opsonization by activating the complement.
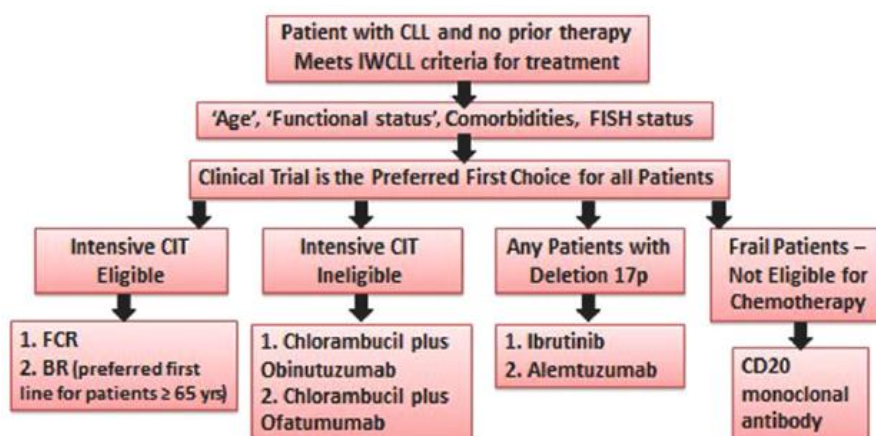

Figure 1. Treatment algorithm for first-line therapy of CLL. FISH, fluorescence in situ hybridization.

(25)

Intensive chemoimmunotherapy (CIT)-eligible patients (non-del[17p])

Fludarabine, cyclophosphamide, and rituximab (FCR) is the standard first-line CIT regimen for patients with CLL who are ≤65 years. The FCR regimen has the highest CR rate, longest remission duration, and most favorable survival of frontline regimens for the treatment of CLL reported to date.

However older age (≥70 years) and impaired renal function (creatinine clearance 30-70mL/min) is associated with lower rate of achieving complete response (overall due to the dose reduction). Unmutated IgVH status and presence of del(17p) have also been identified to be associated with a worse TTP in patients receiving first line FCR. 60% of patients with mutated IGHV who received first-line FCR have remained free of disease progression beyond 10 years (compared with only 10% for patients with unmutated IGHV).

With the exception of neutropenia and leucocytopenia, the frequency of grade 3 or 4 adverse events, including severe or opportunistic infections, is not increased with fludarabine, cyclophosphamide and rituximab.

Intensive chemoimmunotherapy (CIT)-ineligible patients (non-del[17p])

The current standard first-line treatment of older patients who have comorbidities is CIT with chlorambucil and an anti-CD20 antibody (obinutuzumab or ofatumumab).

Combination of chlorambucil and obinutuzumab compared with rituximab results in higher blood and bone marrow negative-MRD and longer progression free survival.

Patients with del(17p)

Patients with del(17p) have the shortest times from diagnosis to first treatment, the lowest response rates to front-line and salvage therapy, and short remission durations(29)

Patients with del(17p) or TP53 gene mutation have poor outcomes with conventional CIT regimens such as FCR, in part due to lack of wildtype p53 function, an important pathway for mediating cytotoxicity of purine analogs.

Alemtuzumab, a humanized anti-CD52 mAb, acts via a p53-independent pathway and was thought to be an attractive strategy for patients with del(17p). Consolidation with an allogeneic stem cell transplant may have contributed to the improved outcomes for this group of patients.

B-cell receptor (BCR) activation plays a crucial role in the pathogenesis of CLL. BTK is a non–receptor tyrosine kinase and plays a crucial role in BCR signaling. Ibrutinib is an oral, selective,

and irreversible inhibitor of BTK (which is highly activated in del17p). Ibrutinib has been extensively studied in patients with relapsed or refractory CLL with a very high overall response rate and improved PFS and OS. Ibrutinib is FDA approved for patients with relapsed/refractory CLL and for patients with del(17p).

Patients who are frail and have significant comorbidities, making them ineligible for CIT

Comorbid conditions and poor performance status can limit the ability of patients older than 70 years to receive CIT.

For these patients, an anti-CD20 mAb therapy could be considered. All patients should be screened for del(17p), and if del(17p) is detected, treatment with alemtuzumab/ibrutinib should be offered, irrespective of age and comorbidities.

4. JUSTIFICATION

Why are important all this molecular features?

As it can be seen there are a lot of interactions and molecules that play an important role in the transformation and in the maintenance of the CLL cells. This explains the heterogeneity in the clinical presentation and in the prognosis.

The Guidelines for the diagnosis and treatment of CLL of the International Workshop on CLL says "the application of these tests (analysis of CD38, ZAP-70 or IgVH mutational status) should not be used in routine practice to influence therapy and is not generally recommended" and that "further clinical trials are needed to standardize the assessment of these parameters and to determine whether they should affect the management of patients with CLL" although multiple studies have demonstrated its paper on the pathogenesis. It has also been demonstrated that its presence confer a worse prognostic.

CD38 analysis is easier to obtain than ZAP-70 and the IgVH mutational status. As the three molecules are related, the presence of CD38 also indicates the presence of ZAP-70 and IgVH unmutated genes. Consequently CD38 is a marker of poor prognosis per se and also is a surrogate marker of the presence of ZAP-70 and IgVH mutational status.

FCR is considered the gold standard therapy for young patients under 65 years with CLL without del(17p). It has been studied that FCR achieves higher rates of MRD and, consequently, higher time to progression, complete remission and survival. Nevertheless those treatment outcomes are not as good in patients with unmutated IgVH. Therefore, taking in account the relation between unmutated IgVH and the presence of CD38, it would be advisable to assess the impact of CD38 on treatment outcome.

As can be appreciated CD38 has different polymorphisms but not all of them confers the same detrimental outcomes. It has been shown that G and T alleles lead to worse prognosis.

What would be the innovation of this study?

There is no information about how these polymorphisms modify the global survival or the disease free-survival in patients that have been treated with FCR or that are candidates to be treated with it.

For all these reasons, and given the fact that every CLL patient is different due to its molecular heterogeneity, it should be investigated the impact of these polymorphisms on treatment outcomes to know if FCR is really a good therapy for them.

5. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

- The expression of the allele G of CD38 reduce the probability of achieving MRD, and as a consequence, overall survival, time to progression

OBJECTIVES

- To analyze the relationship between the polymorphisms of CD38 and treatment outcomes in CLL patients treated with FCR.

6. METHODOLOGY

6.1 Study design

This study is designed as a multicenter prospective cohort study. The hospitals that will participate will be *Hospital Universitario Dr JosepTrueta* (Girona)*, Hospital Duran I* Reynals (Barcelona)*, Hospital Trias I Pujol* (Barcelona)*, Hospital Joan XXIII* (Tarragona)*, Hospital verge de la Cinta* (Tarragona).

Patients with CLL will be treated and analyzed. We will subdivide the group of CLL patients depending on the polymorphism of CD38 they express. Next we will evaluate different treatment outcomes as overall survival, time to progression using a surrogated marker as is MDR.

6.2 Study population

The target populatio are those patients with CLL who will receive chemoimmunotherapy with FCR scheme.

> **Inclusion criteria**

1. Age between 18 and 65 years old

2. Diagnosis of CLL demonstrated according to The 2008 International Workshop on Chronic Lymphocytic Leukemia (IWCLL) criteria

3. Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≤2 (SEE ANNEX III)

4. Life expectancy ≥12 weeks in investigator's judgment.

5. Aspartate aminotransferase (AST; also called serum glutamic-oxaloacetic transaminase [SGOT]) and alanine aminotransferase (ALT; also called serum glutamic-pyruvic transaminase [SGPT]) ≤2 × upper limits of normal (ULN),

6. Serum creatinine≤2 × ULN

7. Willingness and ability to comply with the visit schedule and assessments required by the study protocol

Exclusion Criteria

The study's exclusion criteria include the following:

1. Patients that are not candidates of receiving FCR regimen according to The 2008 International Workshop on Chronic Lymphocytic Leukemia (IWCLL) criteria (i.e: Rai0/BinetA)

2. Patients who received any chemotherapy for CLL

3. Active National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE)(30) grade 3/4 infection

4. Major surgery ≤28 days prior to randomization

5. Known seropositivity for human immunodeficiency virus (HIV)

6. Other malignancy within the last 5 years. Exceptions are:

        a. Curatively treated basal cell/squamous cell skin cancer

        b. Carcinoma in situ of the cervix

        c. Superficial transitional cell bladder carcinoma

        d. In situ ductal carcinoma of the breast after complete resection

7. Any contraindication, known allergy or hypersensitivity to any study drugs

8. Pregnant or lactating

9. Concomitant therapy with any anticancer agents, immunosuppressive agents, other investigational anticancer therapies. Low-dose corticosteroids for the treatment of non-cancer-related illnesses are permitted

10. Any psychological, familial, sociological, or geographical condition potentially hampering compliance with the study procedures or follow-up schedules

11. Severe and/or uncontrolled medical disease that could compromise participation in the study, or any medical or psychiatric condition that, in the opinion of the investigator, would make study drug administration hazardous or obscure the interpretation of data.

6.3 Sample size

Power calculator GRANMO, with the POISSON approximation was used. Accepting an alpha risk of 0.05 and a beta risk of 0.2, with an incidence of positive MRD in the general population with CLL of 18%, in a two-sided test, **112** exposed subjects and **226** in the non-exposed are necessary to recognize as statistically significant a relative risk greater than or equal to 1.83, with a reason between the samples of 2 and an anticipated drop-out rate of 5%.

6.4 Sample selection

This is a consecutive non-probabilistic sampling.

All patients that comes to Institut Català d´oncologia with the diagnose of CLL and meet the criteria will be informed about the study and invited to participate voluntarily by the signature of the informed consent.

6.5 VARIABLES

Dependent:

The polymorphism (rs6449182) of CD38. It will be measured as a qualitative variable.

Independent:

The percentage of MRD (Minimal Residual Disease). It will be measured as a qualitative variable.

It has been demonstrate by some studies that achieving negative minimal residual disease (MRD) is associated with superior time to progression (TTP), time to next treatment (TTT) and overall survival. Therefore in our study MRD will be used as surrogated marker due to the fact that analyze overall survival or time to progression could take a long time and this would increase the costs and also the loss to follow up.

Covariables

As it has been explained above there are different factors which confer a worse prognosis than others. Consequently we will analyze all these variables that can affect and confuse our results.

- Age: It will be measured as a quantitative variable.

  The more age the less tolerance to high doses of chemoimmunotherapy and more possibilities to develop more toxicity. Therefore this fact could decrease the possibility of achieving negative MDR

- Clinical stage: We will use the clinical stages of Rai and Binet. It will be divided on low risk (Rai0, Binet A), intermediated risk (Rai I- II, Binet B) and high risk (Rai II-IV, Binet C).

- Molecular cytogenetics. Using interphase FISH, cytogenetic lesions can be identified. Del(13q), del(17p), del(11q) and trisomy of 12 will be analyzed.

- Expression of ZAP-70 and CD38.

- Serum markers as β2-microglobulin or LDH.

- Toxicities defined according to the Active National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events.

- Number of cycles.

6.6 Measure instruments

CD38 POLYMORPHISM: To isolate genomic DNA a polymerase chain reaction (PCR) amplification will be performed using the primers 5''-CCGGGTGGTGCTGAGTAGGGAGTC-3´ and 5´- CTACGCAGCAGAGCCACCGAGCAG-3´. The reaction will be performed in a 25-µL volume with 1.5mM MgCl2, 200 nMdNTPs, 1.5 pmol of each primer, and 2 U Taq polymerase. Amplification conditions will be as follows: denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 63°C for 1 minute, and 72°C for 1 minute, followed by an extension step of 72°C for 10 minutes on an Authorized Thermal Cycler. The 128-bp amplicon will be digested with 1 U PvuII according to the manufacturer's instructions. The presence of the C allele resulted in digestion of the amplicon to 63- and 65-bp products. CD38 genotypes will be identified following electrophoresis in a 4% NuSieve gel.

MINIMAL RESIDUAL DISEASE: MRD can be analyzed by multicolor flow cytometry. MRD levels are characterized as negative (MRD negativity $<10^{-4}$) or positive ($\geq 10^{-2}$)

Patients will be defined as having a clinical remission in the absence of MRD when they have blood or marrow with less than one CLL cell per 10 000 leukocytes. The blood generally can be used for making this assessment except during the period within 3 months of completing therapy. In such cases, it is essential to assess the marrow for MRD.

7. DATA COLLECTION/study intervention

FIRST EVALUATION. Assessments of clinical disease will include:

- Physical examination (the bidimensional diameters of the largest palpable lymph nodes in each of the following sites should be recorded: cervical, axillary, supraclavicular, inguinal, and femoral). The size of the liver and spleen, as assessed by palpation, will also be recorded.
- ECOG performance status.
- Immunophenotyping of circulating lymphocytes.
- Analyses of genomic aberrations by use of fluorescent in-situ hybridisation (FISH) and the polymorphism of CD38.
- A complete blood cell count.
- Serum chemistry (i.e. creatinine, bilirubin, lactic dehydrogenase).
- Others: Chest radiograph, serologies for human immunodeficiency virus (HIV), cytomegalovirus (CMV), hepatitis (HBV, HCB).

TREATMENT: The treatment will be done following the conventional management of CLL´s patients. Treatment consisted of six 28-day courses of intravenous fludarabine (25 mg/m^2 per day) and cyclophosphamide (250 mg/m^2 per day) for the first 3 days of each treatment course with rituximab at a dose of 375 mg/m^2 on day 0 of the first course, and 500 mg/m2 on day 1 of the second to sixth courses(27).

FOLLOW UP: Assessment of MRD 3 months after the treatment (peripheral blood).

All data obtained will be collected in a common database between all centers in the ICO intranet.

8. STATISTICAL ANALYSES

In the univariate analysis, we will define variables as categorical or continuous. Categorical variable will be described as percentages and proportions. Quantitative variables will be described with means ± standard deviation (the variables with normal distribution) and with median and interquartile range (25-75) (the variables without normal distribution).

In the bivariate analysis we will compare the independent variable with the dependent one. Proportions will be compared with the chi-square (χ^2). The t-test (normal distribution) or Mann-Whitney (without normal distribution) and ANOVA/Kruskal-Walis tests will be used to compare 2 groups or ≥ 3 groups, respectively.

Finally we will do a multivariate analysis by stages such a logistic regression (multinomial logistic regression).

All statistics analyses will be carried out with the Statistical Package for Social Science (SPSS). To manage computed data, Microsoft Excel tool will be used. P value of <0.05 will be considered to indicate statistical significance.

9. LIMITATIONS

The first limitations that may be considered are the typical of a cohort study, including the loss of participants, especially due to toxicities or mortality

Confusion bias: because of the heterogeneity of the pathophysiology there could be a lot of molecules that can affect our results. We have chosen the most important one to make the multivariate analysis.

The needed sample in order to obtain an adequate statistical difference is quite big, which will be solved by the collaboration of 5 hospitals, as a multicenter cohort study.

Information bias: All personal will be informed and trained in order to obtain the samples that we need in a correct way. Moreover all the instruments we will use will be calibrated and validated before beginning the study.

We have chosen a surrogate variable as a way to measure overall survival, time to progression or time to next treatment. In such manner we reduce the time of our study. Although a surrogate variable is an indirect way to measure our outcomes, there are several studies which confirm that is MRD is completely associated with these outcomes(26,28,31).

10. ETHICAL ASPECTS

This protocol will be submitted to the *"Comité de Ética de investigaciónclínica (CEIC) del Hospital Universitario DrJosepTrueta, Hospital Duran I Reynals, Hospital Trias I Pujol, Hospital Joan XXIII, Hospital verge de la Cinta"*

All patients will be provided all needed information documents; the investigator will ascertain that all information is understood by the patient before signing an informed consent approved by *Ministerio de Sanidad, ServiciosSociales e Igualdad* (see Annex IV and V)

This protocol will follow:

1. The *World Medical Association Declaration of Helsinki of Ethical Principles for Medical Research Involving Human Subjects*, last revision in the 64th WMA General Assembly, Fortaleza, Brazil, October 2013.

2. The *"Ley 14/2007, de 3 de Julio, de investigación biomédica"* because we will need different sample of peripheral blood and in some cases of bone marrow.

3. The law *"LEY 41/2002, de 14 de noviembre, básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica"* regulates all the rights and clinical information related with the patient, as well as the informed consent.

4. The "Ley 29/2006, de 26 de julio, de garantías y uso racional de los medicamentos y productos sanitarios (last update of 25th july 2015)" and the "Orden sas/3470/2009, de 16 de diciembre, por la que se publican las directrices sobre estudios posautorización de tipo observacional para medicamentos de uso humano" due to the fact that we will use different drugs for the treatment.

5. All the data collected from each patient will be treated and used anonymously, preserving the confidentiality of the patient according to the "Ley Orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal" and the "Real Decreto 1720/2007, de 21 de diciembre, por el que se aprueba el Reglamento de desarrollo de la Ley Orgánica 15/1999, de 13 de diciembre, de protección de datos de carácter personal."

11. WORK PLAN AND CHRONOGRAM

The study has been designed in 6 phases:

1. Coordination phase (**3 months**): the timeline of the study will be planned, request permission to the hospital research services to access to the statistical data and the protocol will be sent to the CEICs of each hospital in order to be accepted. Videoconference with all the centers that participate will be done, in order to explain the project.

2. Recruitment and treatment (**4 years approximately**): ICO cover 5 million people per year. According to the epidemiology explain above, the incidence of CLL in Spain is approximately 4 cases per 100.000 people year. ICO assist 200 cases per year. Given the fact that not all the patients will fulfill the inclusion and exclusion criteria and that not everyone will participate in our study we calculate that we will recruit 90 patients per year. Therefore we will need nearly 4 years to recruit the 338 patients for our study.

SIMULTANEOUSLY:

3. Data organization: collect the samples and variables. To manage computer data, the Microsoft Office Access database and Microsoft Excel tool will be used.

4. Data extraction and processing database: A relational database will be compiled containing many fields as variables had been chosen. It will be a common database between all the ICO hospitals.

5. Statistical analysis (**1 month**):Using the SPSS software the database will be analyzed according proceedings detailed before (statistical analysis paragraph*)

6. Interpretation of results, writing articles and dissemination of research findings. (**3 months**).

The relationship between the polymorphism (rs6449182) of CD38 and the prognosis of patients with chronic lymphocytic leukemia treated with fludarabine, cyclophosphamide and rituximab (FCR)

TASKS	O	N	D									S	O	N	D	PERSONNEL
	2015			2016		2017		2018			2019					
TASK 1: COORDINATION PHASE. THEORETICAL FRAMEWORK																
1. Setting-up	█	█	█													All research team
2. Literature review	█	█	█	█												Main researcher
3. Study research proposal design	█	█	█	█	█											Main researcher
4. Protocol elaboration and evaluation				█	█											Main researcher + CEICs
5. Coordination with all the hospitals					█											Main researcher
TASK 2: RECRUITMENT AND TREATMENT																
5. Recruitment						█	█	█	█	█	█					Clinical researchers
6. Treatment						█	█	█	█	█	█					
TASK 3: DATA ORGANIZATION																
7. Collection of samples						█	█	█	█	█	█					Clinical researchers
TASK 4: DATA EXTRACTION AND PROCESSING DATABASE																
8. Enter data in the common database						█	█	█	█	█	█					Main researcher and clinical researchers
9. Evaluation of correct data collection						█	█	█	█	█	█					
TASK 5: STATISTICAL ANALYSIS																
10. Data entry in SPSS statistics												█				Statistical consulting
11. Statistical analysis												█				
TASK 6: FINALIZATION AND RESULTS PUBLICATION																
12. Analysis of the results and discussion													█	█	█	All research team
13. Conclusion													█	█	█	
14. Final report elaboration													█	█	█	Main researcher
15. Scientific publications													█	█	█	Publication Department

12. BUDGET

All the variables analyzed are usual as a part of the management of CLL´s patients, except the analysis of the polymorphism.

The necessary hardware and software (PCs, SPSS, Access, Excel) are available as a part of the hospital equipment.

The costs of this study are limited to personnel costs (statistical consulting) and the analysis of polymorphism.

STUDY BUDGET	COST (euros)
1.Staff costs: Statistical Specialist	**1.500,00€**
2. Analysis of CD38 polymorphism	
-QIAamp DNA Blood Mini Kit	**2.000,00€**
-Ficoll-Paque	**980,00 €**
-Taq DNA polimerasa	**3.200,00 €**
-Primers synthesis	**1.020,00 €**
-Plastics (tubes, tips with filters, racks PCR, …)	**800,00 €**
Restriction enzymes	**1.500,00 €**
-Different reactives (agarose, molecular weight marker, ethidium bromide…)	**1.500,00 €**
3.Travel and meetings: The coordination between all hospitals will be done through video conference	**0€**
4. Publication and dissemination:	
- Cost of publication	**2,500€**
- National journey to disseminate the findings	**1,000€**
- International journey to disseminate the findings	**2,000€**
TOTAL COST	**18,000.00€**

13. FEASIBILITY

The study will be proposed to perform in 5 hospitals from Spain. All the procedures that we evaluate will be done routinely, except the analysis of the polymorphism of CD38.

We have estimated that the duration of the data collection will be 4 years approximately. This will be possible with the participation of the 5 hospitals of the ICO, in order to ensure the attainment of the sample size.

We will have a videoconference with all the investigators of each center in order to explain the project design and execution plan. Details will be discussed in order to ensure homogeneity in all centers.

We will select the investigators and provide them the appropriate information to ensure that this protocol is adequately conducted. The system and procedures of recruitment, dispensation of therapy, data management and central data monitoring will be explained. During the study, regular feedback will be provided to each hospital participating and adequate methods of communication will be established. We will ensure that the investigators of each center have previous experience on research and that have all the instruments and personal to carry out the project.

If it would be necessary, some training session will be done in order to ensure that all centers will recollect the samples and will do the procedures in the same way.

Given the fact that ICO have a common database in its intranet, the collection on the data will be easy to do and to analyze.

14. IMPACT ON THE NATIONAL HEALTH SERVICE

The information obtained from this study would be useful to determine if therapy with FCR is really a good option for those patients with CD38 or if it is time to contemplate new therapies, just as it is done in del(17p). In addition the new information could be useful to modify future revisions of the guidelines of the International Workshop on CLL, and include the measurement of CD38 as a recommended analysis that would help to individualize the therapy in order to achieve optimal results.

If the results of this study are positive, showing that FCR does not offer good treatment outcomes in patients with this polymorphism, it could be the beginning of new investigations in order to obtain new drugs focused on this specific target or try to prove if other drugs as is lenalidomide, bendamustine, alentuzumab or ibrutinib could work.

Patients that do not respond to first line treatments have worse prognostic and the response to next treatments will be poorer as the disease progresses. Moreover patients that do not respond to first line treatment will represent more cost to the national health service (because we would be giving them a treatment that does not offer the better results and that costs 205,58€ approximately each session(6)) and also will decrease QALYs (quality-adjusted life year).

Taking in account that nowadays we do not have any alternative therapy for the patients with this polymorphism what we could do if the results are positive is to predict beforehand which patients would have poorer outcomes. Therefore they could be monitored closer and give them the best therapies to try to avoid the complications related with the progression of the disease.

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16. ANNEXES

Annex I: Rai and Binet staging

Rai(21,22)		
Low risk	0	Isolated lymphocytosis with leukemia cells in blood and/or marrow
Intermediate risk	I	Lymphocytosis, enlarged nodes in any site
	II	Lymphocytosis + splenomegaly and/or hepatomegaly
High risk	III	Lymphocytosis + hemoglobin [Hb] level <110 g/L
	IV	Lymphocytosis+ thrombocytopenia (platelet count <100x10^9/L)

Binet(23)		
Low risk	A	No anemia nor thrombocytopenia ≤2 lymph areas affected
Intermediate risk	B	No anemia nor thrombocytopenia ≥3 lymph areas affected
High risk	C	Hb less than 100 g/L and/or a platelet count less than 100x10^9/L, irrespective of organomegaly.

Areas of involvement considered for staging

1. Head and neck, including the Waldeyer ring (this counts as one area, even if more than one group of nodes is enlarged).

2. Axillae (involvement of both axillae counts as one area).

3. Groins, including superficial femorals (involvement of both groins counts as one area).

4. Palpable spleen.

5. Palpable liver (clinically enlarged)

Annex II: Prognosis factors

	Good prognosis Overall survival >10-15 years	Bad prognosis Overall survival <5-7years
Clinical stage	Rai 0, Binet A (Low risk)	Rai III, IV, Binet C (High risk)
Lymphocyte doubling time	>12 months	<12 months
Bone marrow infiltration	Low or moderated	Massive (>70%)
Serum markers (CD23, thymidine kinase and β2-microglobulin)	Normal	Increased
CD38	Low	High
Cytogenetic (FISH)	Normal, isolated del(13q)	Complex karyotype, del(17p), del(11q)
IgVH	Mutated	Unmutated
ZAP-70	Low	High

Annex III. ECOG Performance Status

Developed by the Eastern Cooperative Oncology Group, Robert L. Comis, MD, Group Chair.*

GRADE	ECOG PERFORMANCE STATUS
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all self-care but unable to carry out any work activities; up and about more than 50% of waking hours
3	Capable of only limited self-care; confined to bed or chair more than 50% of waking hours
4	Completely disabled; cannot carry on any self-care; totally confined to bed or chair
5	Dead

*Oken M, Creech R, Tormey D, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J ClinOncol.* 1982;5:649-655.

Annex IV. Indications of treatment

In general practice, newly diagnosed patients with asymptomatic early-stage disease (Rai 0, Binet A) should be monitored without therapy unless they have evidence of disease progression. Some studies in patients with early-stage disease confirm that the use of alkylating agents in patients with early-stage disease does not prolong survival. Whereas patients at intermediate (stages I and II) and high risk (stages III and IV) according to the modified Rai classification or at Binet stage B or C usually benefit from the initiation of treatment.

At least one of the following criteria should be met:

1. Evidence of progressive marrow failure as manifested by the development of, or worsening of, anemia and/or thrombocytopenia

2. Massive (i.e., at least 6 cm below the left costal margin) or progressive or symptomatic splenomegaly

3. Massive nodes (i.e., at least 10 cm in longest diameter) or progressive or symptomatic lymphadenopathy.

4. Progressive lymphocytosis with an increase of more than 50% over a 2-month period or lymphocyte doubling time (LDT) of less than 6 months. LDT can be obtained by linear regression extrapolation of absolute lymphocyte counts obtained at intervals of 2 weeks over an observation period of 2 to 3 months. In patients with initial blood lymphocyte counts of less than $30\text{-}10^9$/L (30 000/_L), LDT should not be used as a single parameter to define a treatment indication. In addition, factors contributing to lymphocytosis or lymphadenopathy other than CLL (i.e., infections) should be excluded.

5. Autoimmune anemia and/or thrombocytopenia that is poorly responsive to corticosteroids or other standard therapy

6. Constitutional symptoms, defined as any one or more of the following disease-related symptoms or signs:

   a. Unintentional weight loss of 10% or more within the previous 6 months;

   b. significant fatigue (ie, ECOG PS 2 or worse; inability to work or perform usual activities);

   c. fevers higher than 38.0°C for 2 or more weeks without other evidence of infection;

   d. Night sweats for more than 1 month without evidence of infection.

The absolute lymphocyte count, hypogammaglobulinemia monoclonal or oligoclonal paraproteinemia do not constitute a basis for initiating therapy.

Patients with CLL who need first-line treatment can be categorized into several groups based on age, comorbidities (i.e. creatinine clearance) and performance status.

Annex V. Information Sheet (available in Spanish-example-, catalan and English).

<u>TÍTULO DEL ESTUDIO</u>: Relación entre la presencia del polimorfismo (rs6449182) de la molécula CD38 en pacientes con leucemia linfática crónica en tratamiento con fluradabina, ciclofosfamida y rituximab.

<u>INVESTIGADORES:</u> Dr. Jose María Roncero Vidal y estudiante Alicia Delgado Garcia.

<u>CENTRO:</u> Hospital Universitari de Girona Doctor Josep Trueta.

INTRODUCCIÓN

Su médico le ha invitado a participar en un estudio de investigación. Este formulario le informa de todo lo que necesita saber sobre dicho estudio y si acepta participar en él será necesario que lo firme. Su firma significa que se le ha informado sobre el estudio y sobre cuáles son sus riesgos, y también que desea participar en él voluntariamente. Lea la información que se detalla a continuación y haga todas las preguntas necesarias sobre cualquier cuestión que no entienda.

PARTICIPACIÓN VOLUNTARIA

La participación en el estudio es voluntaria. Puede elegir no participar y podrá abandonar el estudio en cualquier momento, sin que por ello se altere la relación con su médico ni éste se asegure de que recibe el mejor tratamiento posible para su enfermedad.

¿POR QUÉ SE ESTÁ REALIZANDO ESTE ESTUDIO?

Se le ha pedido participar en este estudio porque tiene una Leucemia Linfática Crónica (LLC), con criterios de tratamiento con fluradabina, ciclofosfamida y rituximab.

Esta investigación se está llevando a cabo con el objetivo de aumentar nuestros conocimientos sobre su enfermedad. Como su médico le habrá explicado, los pacientes diagnosticados de LLC pueden requerir tratamiento durante su evolución y unos pacientes responden mejor que otros al tratamiento. Incluso los que no han respondido suelen necesitar otro tipo de tratamiento. Las razones por las cuales se dan estas diferencias no son bien conocidas. Por ello

se pretende analizar datos que han sido poco estudiados, pero para los cuales hay abundante justificación científica.

¿CUÁNTA GENTE PARTICIPARÁ EN ESTE ESTUDIO?

Participarán aproximadamente 338 pacientes.

¿QUÉ PRUEBAS TENDRÁN QUE REALIZARME SI DECIDO PARTICIPAR EN ESTE ESTUDIO?

Las pruebas que van a realizarse en este estudio son las habituales en el tratamiento y seguimiento de su enfermedad más el análisis de un polimorfismo de la molécula CD38. Necesitaremos la analítica sanguínea previa a empezar el tratamiento de la enfermedad.

¿HAY ALGÚN BENEFICIO POR PARTICIPAR EN EL ESTUDIO?

Si usted acepta participar en este estudio cabe la posibilidad de que no obtenga ningún beneficio médico directo. Su médico cree que analizando los datos de este estudio pueden obtenerse avances en el conocimiento de su enfermedad, pero es imposible predecir si usted se beneficiará de estos conocimientos.

¿QUÉ OCURRIRÁ CON LA CONFIDENCIALIDAD DE MIS DATOS?

Sus registros del estudio y toda la información recogida sobre usted se mantendrá confidencial, de acuerdo a la ley orgánica 15/1999 de 13 de diciembre, de protección de datos de carácter personal.

¿CON QUIÉN PUEDO PONERME EN CONTACTO PARA HACER PREGUNTAS O SI TENGO ALGÚN PROBLEMA?

Si desea hacer alguna pregunta sobre el estudio, o en caso de daño o efectos perjudiciales en relación con la investigación, deberá ponerse en contacto con el Comité Ético de Investigación Clínica del hospital Josep Trueta, en el número 972940282.

Annex VI. Informed Consent

<u>TÍTULO DEL ESTUDIO</u>: Relación entre la presencia del polimorfismo (rs6449182) de la molécula CD38 en pacientes con leucemia linfática crónica en tratamiento con fluradabina, ciclofosfamida y rituximab.

<u>INVESTIGADORES</u>: Dr. José María Roncero Vidal y estudiante Alicia Delgado García.

<u>CENTRO:</u> Hospital Universitari de Girona Doctor Josep Trueta.

---me ha explicado el objetivo de este estudio de investigación, los procedimientos a los que me someteré y los posibles riesgos y beneficios asociados a la participación en este estudio.

Me han hablado de las alternativas a participar en el estudio. He leído y entiendo este formulario de consentimiento. He tenido la oportunidad de hacer preguntas sobre mi enfermedad, los procedimientos implicados en el estudio, los riesgos que conllevan y las alternativas a participar en el mismo. Creo que tengo suficiente información para dar mi consentimiento para participar en este estudio.

Se me facilitará una copia firmada de este formulario de consentimiento informado para conservarla

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Nombre y firma del paciente o representante legal   Fecha

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Nombre y firma de la persona que explica   Fecha

al paciente la información sobre el consentimiento

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Nombre y firma del investigador   Fecha