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Development of a new flow cytometric method to study chronic lymphocytic leukemia.

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INDICE

ABSTRACT

Background: B-cell chronic lymphocytic leukaemia (B-CLL) is a B-cell neoplasm that in many patients shows an indolent course and survive for a prolonged period without therapy, but in others, CLL progresses rapidly and they die of the disease. Because of the difficulty in identifying such patients at diagnosis, only patients with progressive or symptomatic disease currently are recommended for therapy. Although easily characterized by diagnostic markers, the evaluation of an individual CLL patient's prognosis remains a problematic issue. .

Methods: We have performed a simple six colours flow cytometry assay for ZAP-70 in peripheral blood of 5 healthy donors and 20 B-CLL patients. Samples were incubated with the following monoclonal antibodies: anti CD3-PerCP, anti CD5 FITC anti CD38-APC, anti CD45 APC Cy-7, anti CD19 PE Cy-7, treated with the Intrasure kit and finally stained with the anti-human/mouse ZAP-70-PE conjugate. Samples were acquired on a FACSCanto flow cytometry and analyzed with FacsDiva software (Becton-Dickinson). Quantification of CLLU1 expression in CD19+ cells of donors and buffy coat of patients was performed with the use of CLLU-1 ProfileQuant Kit (Ipsogen) by RQ-PCR.

Conclusion: About ZAP-70, the ratio between B-MFI/ T-MFI could be used to discriminate positive and negative patients instead of T-cells residual method. About CLLU-1 expression we have analyzed 5 healthy donors and 20 patients, donors showed very low levels of expression of the gene while CLL patients showed variable levels of expression.

1 INTRODUCTION

1.1 ***B-cell chronic lymphocytic leukemia***

B-cell chronic lymphocytic leukemia (B-CLL) is a B-cell neoplasm characterized by an indolent course with progressive splenic and lymph node enlargement associated with chronic lymphocytosis. This disease is related to the accumulation of monoclonal B cells with the morphology of small mature lymphocytes.

With an age-adjusted incidence of 4.1/100,000 inhabitants, chronic lymphocytic leukemia (CLL) is the most common type of leukemia in western countries. More than 15,000 newly diagnosed cases and 4,500 deaths are currently estimated[1]. The median age at diagnosis lies between 67 and 72 years. More male than female patients (1.7:1) are affected [2].

As the incidence rate rises with age, the prevalence and mortality of CLL are likely to increase further due to the demographic changes in society in the forthcoming decades. Moreover,the proportion of younger patients with early stage CLL and minimal symptoms seems to increase due to more frequent blood testing[3]. CLL is characterized by the clonal proliferation and accumulation of mature, typically CD5-positive B-cells within the blood, bone marrow, lymph nodes, and spleen[4]. Very recently, it has been reported that in CLL the capacity to generate clonal B cells might be acquired at the hematopoietic stem cell (HSC) stage, suggesting that the primary leukemogenic event in CLL might involve multipotent, self-renewing HSCs. The leukemic transformation is initiated by specific genomic alterations causing the deletion of specific micro-RNA genes and increasing the resistance of B cells towards apoptosis. Deletions on the long arm of chromosome 13, specifically involving band 13q14 (del(13q14)) represent the single most frequently observed cytogenetic aberration in CLL, occurring in approx. 55% of all cases. An isolated del(13q14) is typically characterized by a benign course of the disease. The miRNAs, miR-15a and 16-1, were recently identified to be located in the critical region of del(13q14)[5].

The pathophysiologic role of these miRNAs is further underscored by the phenotype of genetically engineered mice carrying a targeted deletion of the mir- 15a/16-1 locus in combination with a deletion of the noncoding RNA gene DLEU2. These animals develop a monoclonal B-cell lymphocytosis-like disorder, CLL, and lymphoma, suggesting that the miRNAs 15a and 16-1 indeed play a role in CLL leukemogenesis[6]. Deletions of the long arm of chromosome 11 (del(11q)) can be found in 25% of chemotherapy-naıve patients with advanced disease stages and 10% of patients with early stage disease[7]. These deletions frequently encompass band 11q23 harbouring the gene ATM, which encodes for the proximal DNA damage response kinase ATM. In addition, patients carrying a del(11q) clone typically show a bulky lymphadenopathy, rapid progression, and reduced overall survival[8]. Interestingly, some of the poor prognostic features of del(11q) seem to be overcome by the use of chemoimmunotherapy[9]. Trisomy 12 is observed in 10–20% of CLL patients. However, the genes involved in the pathogenesis of CLLs carrying a trisomy 12 are largely unknown. Furthermore, the prognostic relevance of trisomy 12 remains a matter of debate[10]. Deletions of the short arm of chromosome 17 (del(17p)) are found in 5–8% of chemotherapy-naıve patients. These deletions almost always include band 17p13, where the prominent tumor suppressor gene TP53 is located. CLL patients carrying a del(17p) clone show marked resistance against genotoxic chemotherapies that cannot be overcome by the addition of anti-CD20 antibodies in the context of state of the art chemo-immunotherapy[9]. Mutations of TP53 are found in 4–37% of patients with CLL, and have been associated with very poor prognosis[11]. Among cases with confirmed del(17p), the majority show mutations in the remaining TP53 allele (>80%). In cases without del(17p), TP53 mutations are much rarer, but have a similarly detrimental effect on chemotherapy response and overall survival[10]. TP53 mutations are also associated with higher genomic complexity in CLL, indicating that a crippled DDR promotes a “mutator phenotype” in CLL[10]. The recently reported whole genome sequencing projects in CLL have revealed a

number of recurrent somatic gene mutations that occur in parallel to the abovementioned structural genomic aberrations. These include the genes NOTCH1, MYD88, TP53, ATM, SF3B1, FBXW7, POT1, CHD2, and others[7]. Of note, TP53, ATM, POT1, and CHD2 encode for proteins critically involved in DNA damage signaling and DNA repair[12]. Intriguingly, both del(17p) and del(11q), as well as inactivating somatic mutations in TP53 and ATM are enriched in patients with secondary resistance to DNA-damaging chemotherapy[7]. This observation underscores the critical importance of the ATM-Chk2-p53 signaling axis in mediating apoptosis in response to DNA damage in CLL. Survival of CLL cells strictly depends on a permissive microenvironment composed of cellular components like macrophages, T cells, or stromal follicular dendritic cells providing stimuli for activation of crucial survival and proproliferative signaling pathways in transformed cells[13]. This microenvironment produces various essential proteins (chemokines, cytokines, and angiogenic factors) that interact with leukemic cells via appropriate surface receptors or adhesion molecules to support the survival of CLL cells[14]. In light of these important advances it is not surprising that the management of this leukemia is constantly undergoing important changes that have started 20 years ago and still gain in dynamics [15]. Several new drugs have been approved (fludarabine, bendamustine as well as three monoclonal antibodies, alemtuzumab, rituximab, and ofatumumab). Chemoimmunotherapies composed of fludarabine and rituximab (with our without cyclophosphamide), or of fludarabine and alemtuzumab have shown to improve overall survival when used as therapy for CLL patients. In addition, several specific inhibitors interrupting.

Typically, B-CLL cells exhibit a characteristic immunophenotype, co-expressing CD19, CD5 and CD23, in the absence or low expression of surface CD22, CD79b and FMC7.Surface immunoglobulins are clonal but weakly expressed or undetectable. Patients with B-CLL have heterogeneous courses. Some survive for a long time without therapy,

while others die rapidly despite aggressive treatment. Recently, breakthroughs have been made in the identification of molecular and cellular markers that may predict disease progression

1.1.1 Diagnosis

In most cases the diagnosis of CLL is established by blood counts, differential counts, a blood smear, and immunophenotyping. The World Health Organization (WHO) classification of hematopoietic neoplasias describes CLL as leukemic, lymphocytic lymphoma, being only distinguishable from SLL (small lymphocytic lymphoma) by its leukemic appearance[16].

The diagnosis of CLL requires the presence of 5,000 B-lymphocytes/mL in the peripheral blood for the duration of at least 3 months. The clonality of the circulating B-lymphocytes needs to be confirmed by flow cytometry. The 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. These cells may be found admixed with larger or atypical cells, cleaved cells, or prolymphocytes, which may comprise up to 55% of the blood lymphocytes[17]. Finding prolymphocytes in excess of this percentage would favor a diagnosis of prolymphocytic leukemia (B-cell PLL). Gumprecht nuclear shadows, or smudge cells, found as cell debris, are other characteristic morphologic features found in CLL.

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 5,000 B-lymphocytes per µL blood is defined as "monoclonal B-lymphocytosis" (MBL)[18]. The presence of a cytopenia caused by a typical marrow infiltrate defines the diagnosis of CLL regardless of the number of peripheral blood B-lymphocytes or of the lymphnode involvement. MBL seems to progress to frank CLL at a rate of 1–2% per year[19]

.The definition of SLL requires the presence of lymphadenopathy and the absence of cytopenias caused by a clonal marrow infiltrate. Moreover, the number of B-lymphocytes in the peripheral blood should not exceed 5,000/μL. In SLL, the diagnosis should be confirmed by histopathological evaluation of a lymph node biopsy whenever possible.

1.1.2 Treatment

Two widely accepted staging methods co-exist, the Rai[20] and the Binet system[21]. The original Rai classification was modified to reduce the number of prognostic groups from five to three[20]. Both systems describe three major prognostic groups with discrete clinical outcomes. These two staging systems are simple, inexpensive, and solely rely on a physical examination and standard laboratory tests. They do not require ultrasound, computed tomography, or magnetic resonance imaging.

The modified Rai staging system defines:

- Low-risk disease as patients who have lymphocytosis with leukemia cells in the blood and/or marrow (lymphoid cells >30%) (former Rai stage 0).
- Patients with lymphocytosis, enlarged nodes in any site, and splenomegaly and/or hepatomegaly annual clinical updates in hematological malignancies (lymph nodes being palpable or not) are defined as having intermediate risk disease (formerly considered Rai Stages I or II).
- High risk disease includes patients with disease-related anemia (as defined by a hemoglobin (Hb) level less than 11 g/dL) (formerly Stage III) or thrombocytopenia (as defined by a platelet count of less than 100 x 10^9/L) (formerly Stage IV).

  The Binet staging system is based on the number of involved areas, as defined by the presence of enlarged lymph nodes of greater than 1 cm in diameter or organomegaly, and on whether there is anemia or thrombocytopenia. The areas of

involvement considered are (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 femoral (involvement of both groins counts as one area). (4) Palpable spleen. (5) Palpable liver (clinically enlarged). Binet stages are defined as follows:

- Stage A. Hb > 10 g/dL and platelets >100 x 10^9/L and up to two of the above involved.
- Stage B. Hb > 10 g/dL and platelets >100 x 10^9/L and organomegaly greater than that defined for Stage A (i.e.,three or more areas of nodal or organ enlargement).
- Stage C. All patients who have Hb of less than 10 g/dL and/or a platelet count of less than 100 x 10^9/L irrespective of organomegaly.

Criteria for initiating treatment may vary depending on whether or not the patient is treated in a clinical trial. 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. Studies from both the French Cooperative Group on CLL[22], the Cancer and Leukemia Group B (CALGB)[23], and the Spanish Group Pethema[24] in patients with earlystage disease showed that the use of alkylating agents did not prolong survival in this specific situation. This result was confirmed by a meta-analysis[25]. In one study, treated patients with early-stage disease had an increased frequency of fatal epithelial cancers compared with untreated patients[22]. Therefore, the potential benefit of an early-intervention therapy with anti-leukemia drugs remains to be proven. 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, some of these patients (in particular Rai intermediate risk or Binet stage B) can be monitored without therapy until they have evidence for progressive or symptomatic/active disease as defined by CLL guidelines:

- evidence of progressive marrow failure (anemia and/or thrombocytopenia);
- massive (i.e.,6 cm below the left costal margin) or progressive or symptomatic splenomegaly;
- massive nodes (i.e.,10 cm in longest diameter) or progressive or symptomatic lymphadenopathy;
- progressive lymphocytosis with an increase of>50% over a 2-month period;
- lymphocyte doubling time (LDT) of less than 6 months;
- autoimmune anemia and/or thrombocytopenia that is poorly responsive to corticosteroids or other standard therapy;
- disease-related symptoms such as unintentional weight loss 10% within the previous 6 months, significant fatigue, fevers of greater than 38.0°C for 2 or more weeks without other evidence of infection;
- or night sweats for more than 1 month without evidence of infection.

Patients with initial blood lymphocyte counts of less than 30.000/µL may require a longer observation period to determine the LDT. Also, factors contributing to lymphocytosis or lymphadenopathy other than CLL (e.g., infections) should be excluded.

Patients with CLL may present with a markedly elevated leukocyte count; however, the symptoms associated with leukocyte aggregates that develop in patients with acute leukemia rarely occur in patients with CLL. Therefore, the absolute lymphocyte count should not be used as the sole indicator for treatment.

Several genetic markers, in particular some of the above defined genetic and chromosomal aberrations, have been described to add prognostic information to these two staging systems, but so far there is no consensus or evidence that the use of additional markers is needed in general practice to define a treatment indication[26]. It is anticipated that the prognostic classification might change in the near future (Bahlo et al., submitted).

Response Assessment

In essence the following response categories can be separated:

complete remission, partial remission, stable disease, and progression, as well as refractory disease. In addition, the assessment of minimal residual disease (MRD) has been introduced as an additional and increasingly important category of response assessment.

1.1.3 Treatment Components

1.1.3.1 Single Agents

Cytostatic agents. Monotherapy with alkylating agents has served as initial, front-line therapy for CLL, and chlorambucil has been considered the "gold standard" for several decades[25]. Even today, this drug remains an appropriate option, particularly in frail elderly or unfit patients. The advantages of chlorambucil are its low toxicity, low cost, and convenience as an oral drug; the major disadvantages are its low to nonexistent CR rate and some side effects that occur after extended use (prolonged cytopenia, myelodysplasia, and secondary acute leukemia).

Novel results indicate that chlorambucil monotherapy may be used less frequently, since the combination with anti-CD20 antibodies has proven more effective.

Three purine analogues are currently used in CLL: fludarabine, pentostatin, and cladribine (2-CdA). Fludarabine monotherapy produces superior overall response (OR) rates compared with other treatment regimens containing alkylating agents or corticosteroids[27]. Fludarabine induced more remissions and more complete remissions (CR) (7–40%) than other conventional chemotherapies, like CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone), CAP (cyclophosphamide, doxorubicin, prednisone), or chlorambucil, but did not improve overall survival when used as single agent[27]. Similarly, cladribine monotherapy was shown to produce a higher CR rate than chlorambucil plus prednisone (47% vs.12%) without resulting in a longer survival[28]. More recently,

bendamustine, 4-[5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid, which has been used in Germany for more than 30 years, was compared with chlorambucil in a randomized trial. Bendamustine produced improved responses but greater toxicity[29].

1.1.3.2 Monoclonal Antibodies

Anti-CD20 antibodies

CD20 is an activated, glycosylated phosphoprotein expressed on the surface of mature Bcells. The protein has no known natural ligand[30] and its function is not yet discovered. It is suspected to act as a calcium channel in the cell membrane. As CD20 is expressed on most B-cell malignancies, the introduction of the anti-CD20 antibody Rituximab in 1998 improved the treatment of most CD20-positive non-Hodgkin lymphomas including CLL [31]. In CLL, Rituximab is less active as a single agent than in follicular lymphoma, unless very high doses are used [32]. In contrast, combinations of rituximab with chemotherapy have proven to be very efficacious therapies for CLL. Ofatumumab is a fully humanized antibody targeting a unique epitope on the CD20 molecule expressed on human B-cells, resulting in increased binding affinity to CD20, prolonged dissociation rate, and increased cell kill due to greater CDC activity and similar ADCC activity compared with Rituximab, especially in cells expressing low levels of CD20[33].

The humanized and glycoengineered monoclonal antibody Obinutuzumab showed impressive results in vitro with higher rates of apoptosis in B-cells in comparison to Rituximab[34]. The humanization of the parental B-Ly1 mouse antibody and subsequent glycol-engineering lead to higher affinity binding to CD20 type II epitope, increased antibody-dependent cellular cytotoxicity (ADCC), low complement-dependent cytotoxicity (CDC) activity, and increased direct cell death induction[35]. Major side effects included infections, neutropenia, thrombocytopenia, and tumor lysis syndrome, which all resolved. There were no dose-limiting toxicities.

1.1.1.3 Other Monoclonal Antibodies

Alemtuzumab is a recombinant, fully humanized, monoclonal antibody against the CD52 antigen.Alemtuzumab has proven effective in patients with high-risk genetic markers such as deletions of chromosome 11 or 17 (del(11q) and del(17p)) and TP53 mutations[36]. Therefore, alemtuzumab is considered a reasonable therapeutic option for patients with these poor prognostic features.

1.1.3.4 New Drugs in Clinical Development

In addition to the approved drugs described in the previous section, there are an increasing number of very hopeful new compounds in clinical development[37]. Agents targeting B-cell receptor signaling. B-cell receptor signaling seems to play an important role for the survival of CLL cells[38]. Different aspects of the B-cell-receptor have been recognized as a prognostic marker in chronic lymphocytic leukemia, such as immunoglobulin heavy chain variable gene (IGHV) or stereotypy.

Continuous or repetitive BCR signaling supports CLL cell survival. This might explain why inhibition of BCR signaling is a new and potent strategy to treat CLL. The B-cell receptor signaling in CLL cells is supported by different tyrosine kinases, such as Bruton's tyrosine kinase (BTK), Spleen tyrosine kinase (Syk), ZAP70, Src family kinases (in particular Lyn kinase) as well as PI3K[37].

Spleen tyrosine kinase, so called Syk, transfers and enhances the signal of the B-cell receptor. Activation of Syk results in cell survival through activation of phosphatidylinositol 3-kinases and AKT [38]. The expression of Syk is upregulated in chronic lymphocytic leukemia cells turning it into a promising target[39].

Fostamatinib disodium, the first clinically available oral Syk inhibitor, induces apoptosis through disruption of B-cell receptor (BCL) signaling.

Class I phosphatidylinositol 3-kinases (PI3Ks) regulate cellular functions relevant to oncogenesis[40]. Expression of the PI3K p110 d isoform (PI3K- d) is restricted to cells of hematopoietic origin where it plays a key role in B cell proliferation and survival. In CLL the PI3K pathway is constitutively activated and dependent on PI3K d[41]. CAL-101 is an oral PI3Kd-isoformselective inhibitor, which promotes apoptosis in primary CLL cells in a time- and dose-dependent manner without inducing apoptosis in normal T cells or natural killer cells and without diminishing antibody-dependent cellular cytotoxicity.

CAL-101 inhibits CLL cell chemotaxis toward CXCL12 and CXCL13 and migration beneath stromal cells (pseudoemperipolesis). CAL-101 also down-regulates secretion of chemokines in stromal cocultures and after BCR triggering[41]. CAL-101 reduces survival signals derived from the BCR or from nurse-like cells, and inhibits BCR- and chemokine-receptor-induced AKT and MAP kinase (ERK) activation[41].

Bruton tyrosine kinase (Btk) leads to downstream activation of cell survival pathways such as NF-jB and MAP kinases via Src family kinases[42]. Ibrutinib (formerly called PCI-32765) is an orally active small molecule inhibiting Bruton's tyrosine kinase (BTK) that plays a role in the signal transduction of the B-cell receptor (BCR). Inhibition of BTK might induce apoptosis in B-cell lymphomas and CLL-cells[42].

Dasatinib is a Src- and Abl- kinase inhibitor that induces apoptosis in primary CLL cells[43]. In addition Dasatinib seems to increase the apoptotic effects of various agents like fludarabine, chlorambucil, sorafenib, the HSP90 inhibitor 17-DMAG, dexamethasone, or the BH3- mimetic ABT-737[43]. In summary, dasatinib seems effective in reduction of nodular tumor masses, but seems to lack efficacy on peripheral blood lymphocytes.

Proteins in the B cell CLL/lymphoma 2 (Bcl-2) family are key regulators of the apoptotic process[44]. The Bcl-2 family comprises proapoptotic and prosurvival proteins. Shifting the balance toward the latter is an established mechanism whereby cancer cells evade apoptosis. Bcl-2, the founding member of this protein family, is encoded by the BCL2

gene which was initially described in follicular lymphoma as a protein in translocations involving chromosomes 14 and 18[45].

The Bcl-2 inhibitor ABT-263 (Navitoclax) and ABT-199. ABT-263 is a small molecule Bcl-2 family protein inhibitor that binds with high affinity (Ki_1 nM) to multiple anti-apoptotic Bcl-2 family proteins including Bcl-XL, Bcl-2, Bcl-w, as well as Bcl-B and has a high oral bioavailability[46]. Initial studies showed very promising results for this drug as a single agent. However, its therapeutic use seemed somewhat limited by severe thrombocytopenias being a prominent side effect. Therefore, the compound was re-engineered to create a highly potent, orally bioavailable and Bcl-2-selective inhibitor, ABT-199.

AT101 is an orally active BH3-mimetic, which inhibits the anti-apoptotic activity of Bcl-2, Bcl-XL and Mcl-1 and might be an active agent for the treatment of CLL, as the resistance to apoptosis in CLL cells is associated with high levels of Bcl-2 protein expression. AT101 was found to induce apoptosis in CLL cells in vitro and to overcome drug resistance mediated by the microenvironment[47]. It showed a good tolerability and satisfactory efficacy in combination with weekly infusions of rituximab in previously treated CLL patients[48].

1.1.3.5 Immunomodulatory drugs

Lenalidomide is a second generation thalidomide analogue and an immunomodulatory agent with antiangiogenic properties that is used in treatment of myelodysplastic syndrome and multiple myeloma and is currently investigated in the treatment of CLL. It showed encouraging results in the treatment of high risk patients including carriers of a del(17p)[49]. In 58% of the patients lenalidomide causes a so called tumor flare reaction, which leads to a sensation of heat and burning in the lymph nodes and occurs only in CLL patients[50].

The combination of lenalidomide and rituximab seems to increase the response rate without a higher risk of toxicity, even in patients with del(17p) and/or unmutated IGHVstatus.

Everolimus has shown good efficacy in hematological malignancies[51], in particular T-cell lymphoma, and was therefore tested in CLL as well. So far, the results in CLL have been disappointing with low response rates, and the enthusiasm for this drug has been further reduced by severe infectious complications.

1.1.3.6 Combination chemotherapy

A major advance in CLL treatment was achieved by the combined use of different treatment modalities, in particular for patients that had a good fitness.

Since purine analogs and alkylating agents have different mechanisms of action and partially non-overlapping toxicity profiles, it seemed logical to combine the two modalities for achieving synergistic effects. Preclinical studies demonstrated that exposure of CLL cells to fludarabine and cyclophosphamide resulted in synergistic cytotoxicity[52].

Fludarabine has been evaluated in a variety of combination regimens. The combination of fludarabine with another purine analog, cytarabine, appeared to be less effective than fludarabine alone, while the combination of fludarabine with chlorambucil or prednisone increased hematological toxicity without improving the response rate compared with fludarabine alone[53].

1.1.3.7 Chemoimmunotherapy

Since preclinical studies showed evidence for a synergy between rituximab and fludarabine[54], rituximab combinations with fludarabine or fludarabine-based regimens were investigated in Phase II trials. The synergistic activity of fludarabine and alemtuzumab was initially suggested by the induction of responses, including one CR, in 5 of 6 patients who were refractory to each agent alone[55].

The combination of alemtuzumab with rituximab has also been studied in patients with lymphoid malignancies, including those with refractory/relapsed CLL, producing an ORR of 52% (8% CR; 4% nodular PR, nPR; 40% PR)[56]. These results need to be confirmed by larger trials.

1.1.3.8 Selecting of the Right Treatment:

Parameters to be considered

Given the impressive choice of options, the right choice of treatment of a given CLL a patient becomes a task that requires experience, a good clinical judgment and an appropriate use of diagnostic tools. The following parameters should be considered before recommending a treatment for CLL[26]:

1. The clinical stage of disease.

2. The fitness of the patient.

3. The genetic risk of the leukemia.

4. The treatment situation (first versus second line, response versus non-response of the last treatment).

1.1.4Immunophenotyping

CLL cells co-express the T-cell antigen CD5 and B-cell surface antigens CD19, CD20, and CD23[57]. The levels of surface immunoglobulin (Ig), CD20, and CD79b are characteristically low compared to those found on normal B cells[170]. Each clone of leukemia cells is restricted to expression of either kappa or lambda immunoglobulin light chains. In contrast, B-cell PLL cells do not express CD5 in half of the cases, and typically express high levels of CD20 and surface Ig . Also, the leukemia cells of mantle cell lymphoma, despite also expressing B cell surface antigens and CD5, generally do not express CD23. The chronic lymphoid leukemias comprise a number of biologically distinct

neoplasms of mature lymphocytes[57]. The availability of specific therapies for certain of these tumors makes accurate diagnosis imperative. In most cases, detailed immunophenotypic analysis of the chronic lymphoid leukemias permits specific classification and initiation of the most appropriate therapy. For example, the characteristic immunophenotype of B-cell chronic lymphocytic leukemia (CLL): CD5+, CD23+, FMC7-, CD20 dim+, clonal surface immunoglobulin (sIg) dim+, distinguishes it from another CD5+ B-cell lymphoproliferative disorder, mantle cell lymphoma, which typically displays the composite phenotype: CD5+, CD23-, FMC7+, CD20 bright+, clonal sIg bright+. Likewise, hairy cell leukemia has a characteristic immunophenotype: CD5-, CD11c bright+, CD25+, CD103+, which distinguishes it from other CD5- B-cell lymphoproliferative disorders, including the morphologically similar splenic lymphoma with circulating villous lymphocytes. Immunophenotypic analysis by flow cytometry may also identify clinically relevant subsets of patients within a diagnostic category of leukemia. Thus, in patients with CLL, deviation from the typical immunophenotype is associated with trisomy 12 and mixed-cell morphology. In summary, careful immunophenotyping by flow cytometry facilitates accurate diagnosis in the chronic lymphoid leukemias, and in some settings, may also offer additional therapeutically relevant information. Several studies have shown that immunophenotypic analysis of neoplastic cells is of great help for the investigation of minimal residual disease (MRD). This is mainly due to the fact that flow cytometry immunophenotyping is more sensitive than morphology in detecting residual leukemic cells. Additionally, immunophenotyping has proved to be of utility for evaluating the effectiveness of high-dose chemotherapy and for predicting relapses.

1.2 ZAP-70

Chronic lymphocytic leukemia (CLL) is a small mature B-cell neoplasm that usually expresses CD5 along with CD23 and displays a variable clinical course. A well-established prognostic marker identified over 10 years ago that can help distinguish patients with CLL with aggressive disease from those that have more indolent clinical courses is the mutational status of the expressed immunoglobulin heavy chain variable (VH) gene[58]. Patients with CLL that use mutated VH gene (less than 98% homology to the germline counterparts) may not require treatment for 20 years or more following diagnosis. In contrast, patients with CLL that express unmutated VH genes (98% or more homology to the germline counterparts) often require treatment after a few years following diagnosis and have shorter survival. Since these findings, information regarding the mutational status of IgVH has become of considerable value in the prognostic assessment of B-CLL patients. However, IgVH mutational analysis is still a complex and time-consuming procedure, not yet routinely performed in most laboratories.[60] Therefore, many subsequent studies focused on the identification of alternative markers with similar prognosticvalue to that of IgVh mutations, and whose expression could be investigated by flow cytometry. The recently introduced techniques of gene expression profiling(GEP) have been also used for the identification of additional molecules to be employed as potential prognosticators in B-CLL. Amog them, the gene encoding for the T cell specific zeta-associated protein 70 (ZAP-70), firstly identified by GEP, has been demonstrated to have both prognostic relevance and predictive power as surrogate for IgVH gene mutations, when its expression is investigated by flow cytometry. Subsequent gene expression profiling studies indicated that expression of ZAP-70 was very good at differentiating CLL with mutated VH genes from CLL cases expressing unmutated VH genes. These findings opened up the possibility that ZAP-70 expression analysis of CLL could be

used as a surrogate marker of VH mutational status and be developed as a relatively simple clinical test. [59]

There are two different approaches to define if a given B-CLL population has to be considered positive for ZAP-70 expression. The first one , in analogy to standard procedures of flow cytometry staining, is based on the signal obtained by using a irrelevant isotype-matched control Mab; according to this method, B-CLL cells are defined as ZAP-70 positive when the expression levels of the protein exceed those of its corresponding isotypic control. The other method is based on the expression level of ZAP-70 in normal T and NK cells of the same sample, which are known to constitutively express the protein, as a sort of internal positive control; according to this latter strategy, B-CLL cells are defined as ZAP-70 positive when they express ZAP-70 protein at levels comparable to those found in ZAP-70 expressing T and NK cells. The cut-off level used to define the ZAP-70 category (positive or negative) of a given B-CLL sample differs in the various studies, usually ranges fron 10% to 20% of positive cells.[61]

In theory, flow cytometry is particularly well suited for clinical evaluation of ZAP-70 in part because CLL cells and normal T-cells, which are often present in peripheral blood specimens and express high levels of ZAP-70, can be separately analyzed. However, flow cytometric analysis of ZAP-70 has proven to be problematic as differences in antibodies, permeabilization techniques, negative controls, and/or other procedures can lead to discordant results between different laboratories and variable degrees of correlation with VH mutational status or clinical data . Many of these problems are likely related to suboptimized detection of ZAP-70 and only weak positive ZAP-70 staining has been achievable using currently available antibodies. Moreover, because of weak ZAP-70 related staining, the method used to set a threshold to remove background staining is critical and can have a major impact on the reported ZAP-70 value. In addition, because well-established methods generate a continuum of ZAP-70 levels among CLL cases, the significance intermediate ZAP-70 values may have

to prognosis is difficult to interpret without individual laboratories also performing clinical correlation and/or VH mutation studies. A key feature is the use of isotypic controls at concentrations that differ from those recommended by the suppliers but selected to insure normal peripheral blood B-cells are ZAP-70 negative. Although ZAP-70 appears to be expressed in early normal B-cell development, and in activated B-cells by Western blot analysis of sorted cells, mature peripheral blood B-cells are ZAP-70 negative using this sensitive method.[62]

Among the available prognostic immunophenotypic markers in CLL, zeta-chain-associated protein kinase 70 (ZAP-70) is one of the most promising markers because of its strong correlation with IGHV mutational status. Flow cytometric analysis of ZAP-70 gives an advantage of the simultaneous assessment of its levels in the clonal B-cell as well as the residual T- and NK-cells (internal positive control), and normal remaining (NR) B-cell (internal negative control). However, the detection of this intracellular protein needs to be robust and reproducible in order to reduce intra-laboratory variations. This means that the intrinsic variability of blind replicate must be strictly controlled. An early step in this direction was the introduction of the use of a normal donor sample as an external control for ZAP-70 assessment. This was previously described by Rassenti et al. using normal donor T-cells as a reference for ‘‘percent of positive cells’’. More recently, a normalization step of adding B-cells from a pool of normal donor peripheral blood mononuclear cells constitutes a second step toward standardization. A study previously reported the advantage of using two clones for ZAP-70 expression analysis and utilizing normal donor blood as a reference control. Flow cytometric detection of ZAP-70 in CLL has proven to be technically challenging and generates variable results depending on the particular methods used for staining and analysis.[63] As the intensity of ZAP-70 staining in positive CLL cases is typically low, the choice of controls and/or method to set a threshold to distinguish positive staining from nonspecific background staining is critically important. Controls used for ZAP-70

analysis include internal CLL specimen T-cell positive controls, internal CLL specimen normal B-cells negative controls, and external B-cells from a normal donor negative controls. However, all of these have potential problems in that T-cells in patients with CLL may not be normal and can show variable ZAP-70 expression levels. Similarly, CD5 negative B-cells in patients with CLL may also be abnormal and possibly activated due to interactions with the CLL or T-cell populations, which could affect ZAP-70 levels, and are often not present in sufficient numbers to adequately characterize in many specimens. Setting a negative threshold based on ZAP-70 staining of external B-cells from a normal donor peripheral blood specimen, although attractive, does not take into account possible differences in nonspecific staining properties of CLL cells relative to B-cells due to CLL cell size or other membrane/cytologic properties that could be present in many cases. The use of isotypic control antibodies to set a negative threshold differs from prior flow cytometry studies of ZAP-70 expression in CLL that also used isotypic controls and also eliminates problems that can be associated the use of isotypic controls. It is well known that monoclonal isotype controls are often selected for low binding activity and have different nonspecific binding properties relative to the monoclonal antibodies they are parried with so are not necessarily good negative controls, even when used at the same concentrations. In addition, differences between isotypic controls and test antibodies with respect to fluorochrome to protein labeling or manufacturing can also contribute to the unreliability of isotypic controls. Moreover, all of these issues are potentially more problematic when staining cytoplasmic antigens such as ZAP-70 where the effects of fixation, permeabilization, and antibody trapping can come into play and further affect nonspecific antibody binding. Insuring normal B-cells appear ZAP-70 negative relative to an isotypic control effectively compensates for any differences in nonspecific binding properties and/or F:P ratios between isotype and the ZAP-70 antibody. The only assumption using our ‘‘experimentally matched’’ isotypic control method is that the physical or cellular properties affecting nonspecific binding of antibody to CLL cells are the same as those affecting

nonspecific binding to normal B-cells. Interestingly, Hassanein et al. also recently reported finding a bimodal distribution of ZAP-70 expression among 125 CLL cases analyzed using the same antibody we employ with a trough around 40% which is similar to the gap in our distribution that occurred between values of 30–60%.[64] A bimodal as opposed to continuous distribution of ZAP-70 values may be related to greater precision and/or sensitivity of our assay due in part to previous optimization of the staining procedure by use of a noncommercial saponin fixation step and performing Scatchard analysis of binding curves to select antibody concentrations that give the highest signals relative to background staining. Moreover, many laboratories run a single external B-cell control, which could also diminish the precision of the resultant ZAP-70 values because of variable B-cell staining among different individuals. Using an optimized isotypic control threshold appears to be necessary to obtain a bimodal distribution, because use of a ZAP-70 stained external B-cell threshold generated a continuous distribution of ZAP-70 values similar to that reported by Rassenti et al. with the same staining procedure. In summary, use of our experimentally optimized isotypic control method for assessing ZAP-70 expression in CLL cases has the potential to improve interpretation by placing the resultant ZAP-70 values in one of two groups, and generating better correlation with VH mutational status compared with other methods [65].

1.3 CLLU-1

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with a highly variable outcome depending on clinical and biological characteristics. Clinical staging is a relatively simple method of assessing the outcome of CLL patients based on clinical parameters. In the last two decades, multiple biological markers have been shown to have prognostic relevance in CLL, most notably chromosomal aberrations, IGHV mutations and expression of surface markers such as ZAP-70 and CD38.

Gene expression analyses have also revealed the heterogeneity of CLL, with distinct gene expression signatures being associated with particular genetic subgroups of CLL In particular, the expression of genes such as CLLU1, and LPL have recently been associated with different outcomes in CLL.[67]

CLLU1 located at chromosome 12q22, encodes a novel transcript, which has been identified as a marker specific to CLL.In particular, high CLLU1 expression (CLLU1-H) is significantly more frequent in unmutated IGHV and CD38+ CLL. CLLU1-Hh as been shown to be associated with shorter overall survival and shorter time to first treatment in patients with early stage CLL, especially those younger than 70 years. In addition, CLLU1 expression has been shown to be a specific and stable marker in CLL cells, making it a promising marker not only for prognosis but also for minimal residual disease analysis. However, the effect of CLLU1 expression on response to therapy is currently unknown and, to date, there are no data on the significance of CLLU1 expression with regards to progression-free survival and overall survival in patients receiving first-line therapy. We assessed the value of CLLU1 expression as a prognostic marker in the LRF CLL4 randomized controlled trial. CLLU1, located at chromosome 12q22, encodes a transcript specific to chronic lymphocytic leukemia and has potential prognostic value.[68] However, CLLU1 expression was not an independent predictor of overall survival in a multivariate model including TP53 aberrations, beta-2 microglobulin

level, age and IGHV mutation status. Nor did it predict response to treatment. CLLU1 expression analysis helps to refine the prognosis of patients with chronic lymphocytic leukemia who have mutated IGHV genes. The accurate diagnosis of B-cell lymphocytosis has important prognostic and therapeutic implications. The distinction of chronic lymphocytic leukemia (CLL) from other B-cell neoplasms, however, can be difficult since leukemic manifestations of mantle cell lymphoma (MCL) and splenic marginal zone lymphoma (SMZL) can be morphologically indistinguishable and can immunophenotypically overlap with CLL. The diagnostic scoring system for the specific immunophenotype of CLL proposed by Matutes et al. comprises a set of five markers which have proven to be useful in many cases . In cases with 2 or 3 of five markers, additional parameters are needed to establish the correct diagnosis. A number of surface and molecular markers have been investigated and proposed to be diagnostically useful and/or to have prognostic significance in B-cell lymphocytosis, five of which have recently been combined into a new molecular scoring system for risk stratification for CLL, including ZAP70, LPL (lipoprotein lipase), CLLU1, microRNA-29c and microRNA-223. The chronic lymphocytic leukemia upregulated gene-1 (CLLU1) has been shown to be the first highly specific CLL gene . Six tran-scripts have been identified, two of which contain a putative coding sequence with low similarity to human interleukin-4. Overexpres-sion of the transcript cDNA1 appears to be specific for CLL, is of prognostic significance and can be used for MRD detection. High levels of CLLU1 expression in patients with CLL have been correlated with unfavorable prognostic markers, i.e. unmutated IgVH, high CD38 expression, high ZAP70 expression and unfavorable cytogenetics (17p, 11q).[69]

2 THE AIM OF WORK

The aim of work it was to apply a single tube to analyze expression of ZAP-70 by multiparametric flow cytometry assay in healthy donors and in B-CLL patients and to analyze expression of CLLU-1 in the same samples

Our goal is to show that a poor clinical outcome and a bad response to therapy is observed in patients with ZAP-70+, and high CLLU1 expression levels and to show a possible correlation of the above topics with other aberrations (atypical morphology, atypical immunophenotype).

3MATERIALS AND METHODS

3.1 B-CLL Patients and controls

The study includes peripheral blood samples, collected after informed consent for diagnostic purposes, from healthy donors and 20 patients affected by B-CLL. PB mononuclear cells were isolated by centrifugation on Ficoll-hypaque gradient, suspended in PBS 10% dimethylsulphoxide, and stored in liquid nitrogen until use.

3.2Analysis for ZAP-70

We have performed a simple flow cytometry assay for ZAP-70. Peripheral blood (PB) samples from 5 healthy donor and 20 B-CLL patients was processed and analyzed by flow cytometry within 24 hours. 50 µl or approximately 10 x 10^6 cells of each samples were incubated for 15 minutes in the dark at room temperature with the following monoclonal antibodies: anti CD3-PerCP, anti CD5 Fitc, anti CD45 APC Cy-7, anti CD19 PE Cy-7, anti-CD38-APC. Then they were treated with 100 µl of reagent A from BD Intrasure Kit for 5 minutes, then treated with lysing solution for 10 minutes and finally stained with the anti-human/mouse ZAP-70 R-PE conjugated. All samples were analyzed on a BD FACSCANTO II using FACS-DIVA software. Up to 100000 events were acquired per tube.

3.3 Samples preparation

CD19+ cells of donors was magnetically labelled with Whole Blood CD19 MicroBeads. Then, the cell suspension was applied onto a MACS Column, which was placed in the magnetic field of MACS separator. The magnetically labelled CD19+ cells were retained within the column. After removing of the column from the magnetic field, the magnetically retained CD19+ cells were eluted as positively selected cell fraction.

3.4 Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCRs)

Quantification of CLLU1 expression in CD19+ cells of donors and buffy coat of patients was performed with the use of CLLU-1 ProfileQuant Kit (Ipsogen) by RQ-PCR. An endogenous control (beta2-microglobulin transcript) is amplified from the sample as well as CLLU-1 transcript. Standard curve of known amounts of both the endogenous B2 M control and the CLLU-1 cDNA allow the calculation of the ratio of CLLU1 signal to endogenous B2M signal in each sample. Normalised copy number is given of the ratio of CLLU1 CN/ B2M CN.

3.5Statistical analysis

Relation between B MFI/T MFI of ZAP-70 positive patients versus healthy donors, ZAP-70 negative patients versus healthy donors and expression of CLLU-1 between B-CLL patients and healthy donors was assessed using *t*-test.

4 RESULTS AND DISCUSSION

Among immunophenotypic markers also associated with prognosis of B-CLL , ZAP-70 expression is one of the most promising because of its strong correlation with IgH mutational status. ZAP-70, a member of the Syk–ZAP-70 protein tyrosine kinase family, is normally expressed in T and natural killer cells and has a critical role in initiation of T-cell signaling. Recent studies have found that ZAP-70 is associated with enhanced signaling by the cell surface immunoglobulin receptor in CLL B cells and that measurement of ZAP-70 can serve as a surrogate for mutational status of IgVH. ZAP-70 is an independent negative prognostic marker in chronic lymphocytic leukemia (CLL). Usually, its expression is investigated by flow cytometric protocols in which the percentage of ZAP-70 positive CLL cells is determined in respect to isotypic control (ISO-method) or residual ZAP-70 positive T cells (T-method). The first approach is based on the signal obtained using an isotype-matched antibody as negative control. Accordingly, a CLL sample is defined as ZAP-70 positive when at least 20% of CLL cells have a signal exceeding that of isotypic control. The second approach is based on the expression of ZAP-70 on normal T cells, which constitutively express the protein and hence are utilized as an internal positive control. Following this strategy, a CLL sample is defined as ZAP-70 positive when at least 20% of CLL cells express ZAP-70 at levels comparable to those found in the residual T cell component.

Also CD38 positivity as well as the other biological variables were able to segregate intermediate risk patients who experienced a shorter survival. The prognostic significance of CD38 expression on clinical outcome of B-CLL patients was also corroborated by the results of the multivariate analysis. Therefore, some studies proposes CD38 expression as a novel and significant prognostic indicator that delineates similar overlapping groups of B-CLL patients. In addition, this parameter can be determined easily and rapidly by flow

cytometry in most hematologic laboratories, and, consequently, it may be a very useful addition to the current staging systems. Actually, this simple test may enable physicians to accurately predict a favourable or an unfavourable clinical course. Experience suggests that CD38 positive B-CLL patients have a progressive and an unfavourable disease.

Despite these important clinical observations, the reason why Ig VH gene mutation status and expression of ZAP-70 and CD38 influence or correlate with disease progression are still undefinited. Cells of patients with CLL may express surface IgM with polispecific (natural) antibody activity and bind to a variety of different antigens, including self-antigens. This specificity, however, is detected rarely for surface IgM from unmutated CLL cases. This findings led to hypothesis that the reaction between surface IgM of unmutated CLL cases and self antigens induces cell activation and that continuous stimulation would favour expansion of unmutated CLL clones in vivo. In contrast, cells from mutated cases would not be stimulated via their surface IgM , and their expansion would be more indolent. Tentatively, it could also be proposed that activation through surface IgM induces the expression of both ZAP-70 and CD-38, inasmuch as both these structures are activation markers for normal B cells. Moreover, they could be involved in the IgM-dependent signal trasduction pathway, although this issue is still controversial. CLLU1 is a novel gene, located at chromosome 12q22, up regulated selectively in chronic lymphocytic leukaemia (CLL) cells and highly expressed in patients with adverse prognostic factors such as high ZAP-70 and CD38 expression or unmutated genes encoding variable regions of the immunoglobulin gene (IgVH). High levels of CLLU1 expression were associated with shorter overall survival ($P < .001$), with a 7% increase in risk of early death by each doubling of the CLLU1 expression level. Stratification for age at diagnosis demonstrated a strong prognostic significance of CLLU1 expression in patients younger than 70 years ($P < .001$). Quantification of CLLU1 expression was performed with the use of TaqMan One-Step RT-PCR

The lymphocyte population was gated on side-scatter versus CD45 plot. ZAP-70 expression was determinated on B and T. CD3 cells were displayed in a side scatter versus fluorescence (CD3 Per-CP) gated on lymphocyte population. CD19 cells were displayed in a fluorescence (CD19 PE-Cy7) versus fluorescence (CD5 APC) dot-plot gated on lymphocyte population. To quantify ZAP-70 expression , we used a method in which the identification of ZAP-70+ population was driven by an internal positive control represented by the population of normal T cells (Fig.1). Following this method, a CLL sample was defined as ZAP-70 positive when at least 30% of CLL cells express ZAP-70 at levels comparable to those found in the residual T cell.

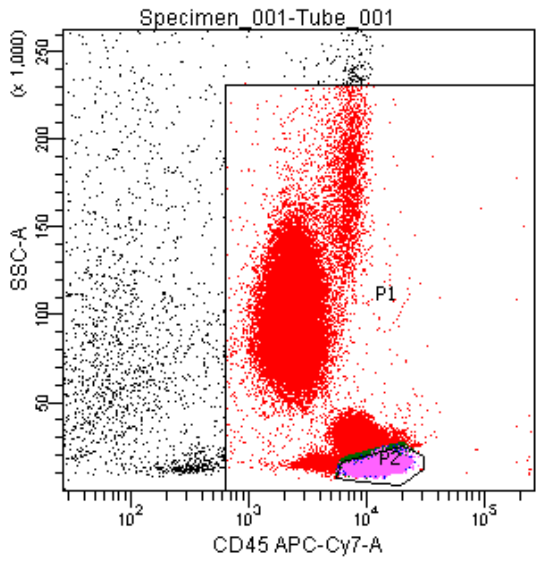
Analysis of ZAP-70 expression in B and T cells was performed by calculating the mean fluorescence intensity (MFI) of each populations and the ratio (MFIR) between the MFI of B-cells and MFI of T cells. (Tab. 1).

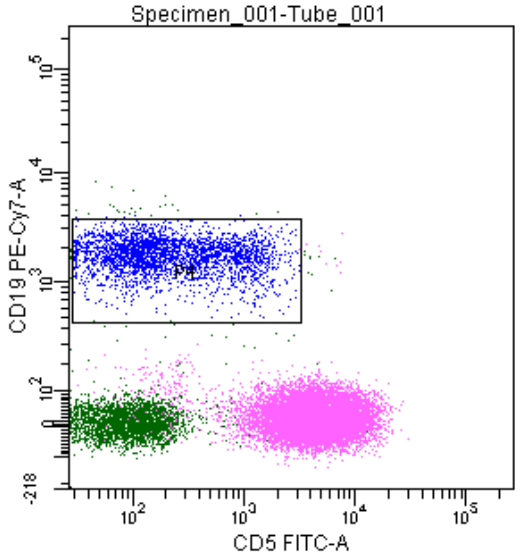
About ZAP-70 expression we have analyzed 5 healthy donors and 20 B-CLL patients and we measured the same parameters and determinate the same statistic indexes (means and standard deviation for each parameters). All healthy donors showed negative values about ZAP-70 expression, while seven patients showed positive values about the same parameter.

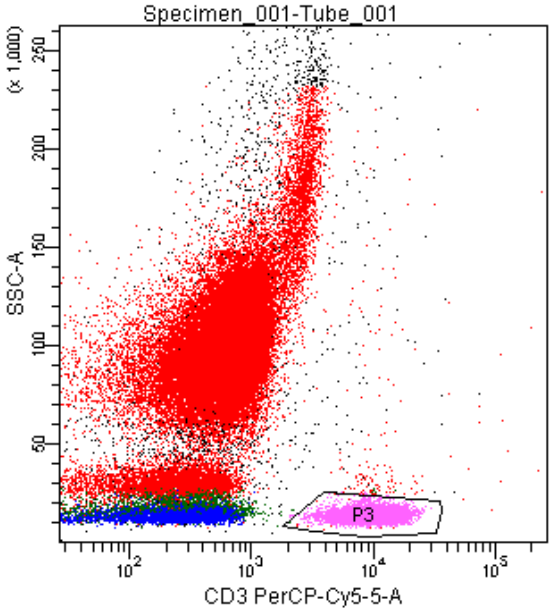
Comparing values of the ratio of B MFI and T MFI between ZAP-70 positive patients and ZAP-70 negative patients we have found these populations were significantly different with a p-value = 0,0034 (Fig.3), instead the difference B MFI/ TMF T between negative patients and controls was non-significant with p-value = 0,2134 (Fig.4).

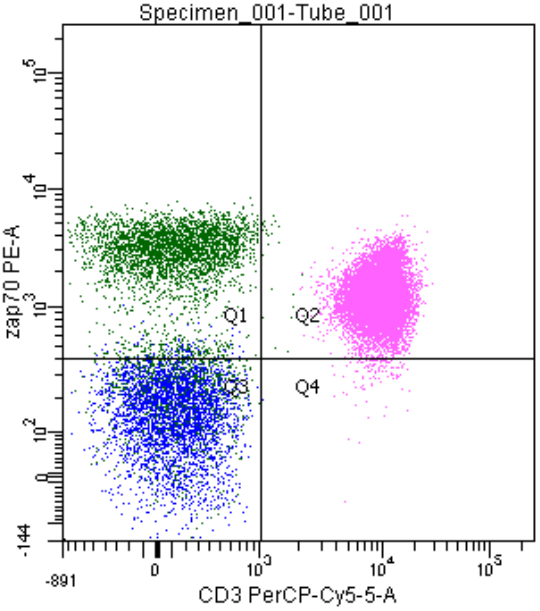
About CLLU-1 the difference between CLLU-1 expression levels of donors versus B-CLL patients was significant with p value = 0,0366. (Fig.5)

5 FIGURES:





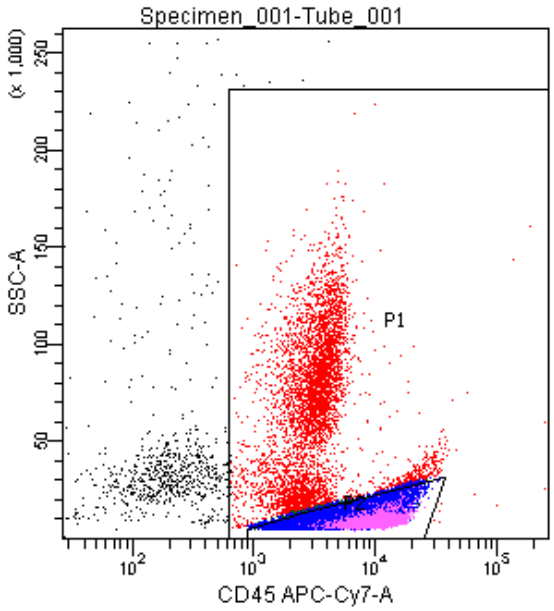


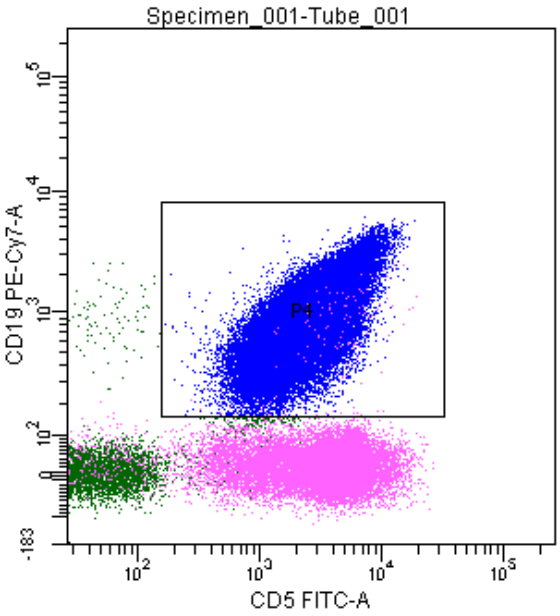


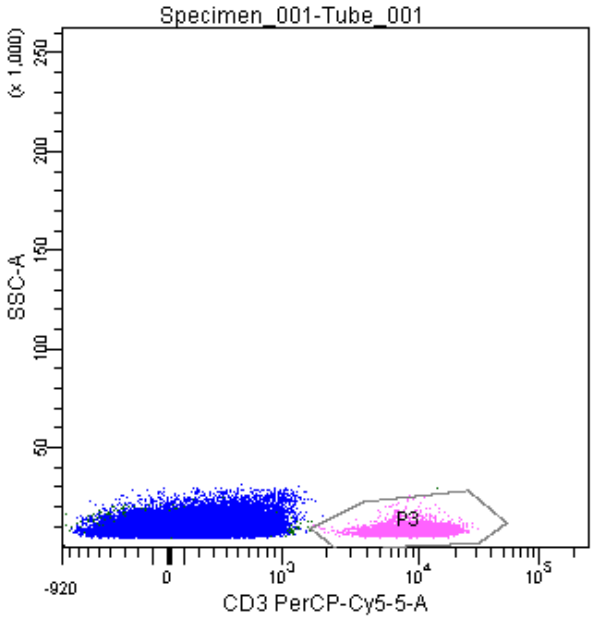
Tube: Tube_001

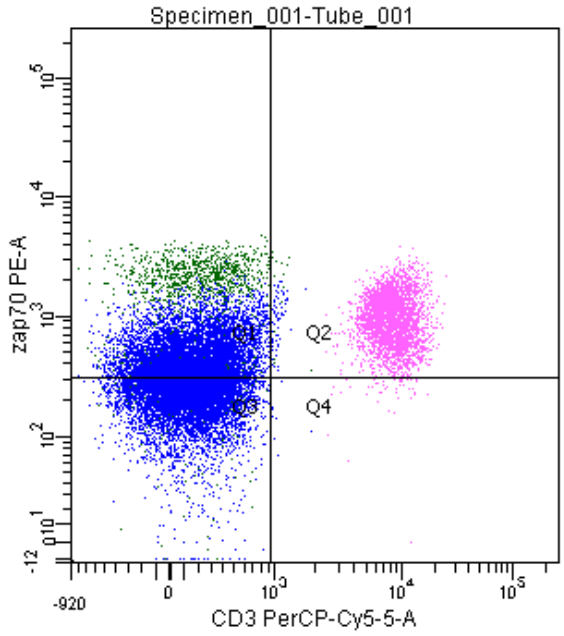
Population	#Events	%Parent	%Total
All Events	76,707	####	100.0
P1	72,505	94.5	94.5
P2	21,346	29.4	27.8
P4	2,976	13.9	3.9
Q1	69	2.3	0.1
Q2	3	0.1	0.0
Q3	2,902	97.5	3.8
Q4	2	0.1	0.0
P3	13,492	63.2	17.6

Fig1: Evaluation of ZAP-70 expression in a healthy donor. Dot plots A, B, C show the gating regions to select lymphocytes, B-cells and T-cells. D shows the method of analysis used to quantify expression of ZAP-70 by B-cells.









Tube: Tube_001

Population	#Events	%Parent	%Total
All Events	100,000	####	100.0
P1	99,075	99.1	99.1
P2	94,170	95.0	94.2
P4	63,935	67.9	63.9
Q1	29,540	46.2	29.5
Q2	493	0.8	0.5
Q3	33,847	52.9	33.8
Q4	55	0.1	0.1
P3	18,160	19.3	18.2

Fig2: Evaluation of ZAP-70 expression in a B-CLL patient Dot plots A, B, C show the gating regions to select lymphocytes, B-cells and T-cells. D shows the method of analysis used to quantify expression of ZAP-70 by B-cells.

6 TABLES:

donors	Ly %	T%	B %	%ZAP 70 positive cells	MFI-T	MFI-B	B/T	CLLU-1/B2M*100
C1	29,4	63,2	13,9	2,3	1304	149	0,114264	0,002
	39,3	76,7	12,6	28,1	1089	189	0,173554	0,005
C3	29,2	84	2	17,6	1249	296	0,23699	
C4	40,3	71,2	8,5	6,3	949	336	0,354057	0,007
C5	32,1	69,4	5,7	2	1011	123	0,121662	0,004
mean	34,06	72,9	8,54	11,26	1120,4	218,6	0,200105	0,0045
ds	5,375221	7,856208	4,900306	11,34032627	152,1342	93,04999	0,088644	0,00212132

Tab 1: Percentage of total, B and T lymphocytes, ZAP-70 positive cells, T-MFI, B-MFI, B-MFI/TMFI and CLLU-1 expression in donors.

Patients	Ly %	T%	B %	%ZAP 70 positive cells	MFI-T	MFI-B	B/T	CLLU-1/B2M*100
P1	96,9	2,5	95,3	0,1	1367	283	0,207023	0,495
P2	91,6	36,2	63,3	60	1341	881	0,656972	
P3	94	2,1	95,8	53,7	2310	871	0,377056	
P4	90,3	3,9	94,5	28	2517	702	0,278903	
P5	88,5	4,2	92,8	21,9	1148	322	0,280488	0,253
P6	95	19,3	67,9	46,2	998	363	0,363727	0,345
P7	84,3	94,7	2,9	0,6	1795	178	0,099164	
P8	75,5	13,6	76,4	11,8	1390	765	0,55036	0,08
P9	86,6	5	85,5	4,9	2249	322	0,143175	
P10	62,1	21,7	65,9	6,8	542	117	0,215867	0,031
P11	82,6	10,9	81,5	11,4	2007	619	0,308421	
P12	88,6	10,5	85,7	30,6	1078	471	0,43692	0,24
P13	69,5	30,1	58,5	70,7	1587	635	0,400126	0,03
P14	85,4	30,1	64,4	25,3	2266	521	0,229921	0,01
P15	93,9	2,3	92,8	18,1	2473	982	0,397089	0,462
P16	67,1	13,7	75,7	1,6	1069	219	0,204864	
P17	79,5	6,4	87,5	24,4	1659	490	0,295359	0,025
P18	88	3,5	92,9	34,1	1824	693	0,379934	0,253
P19	94,6	3,1	95,2	13,6	1617	559	0,345702	0,275
P20	53,9	31,9	48,2	95,3	2720	1977	0,726838	
mean	83,395	17,285	76,135	27,955	1697,85	598,5	0,344895	0,212785714
SD	11,98699	21,41623	22,42931	25,67227616	593,764	406,959	0,159637	0,172496706

Tab 2: Percentage of total, B and T lymphocytes, ZAP-70 positive cells, T-MFI, B-MFI, B-MFI/TMFI and CLLU-1 expression in B-CLL patients.

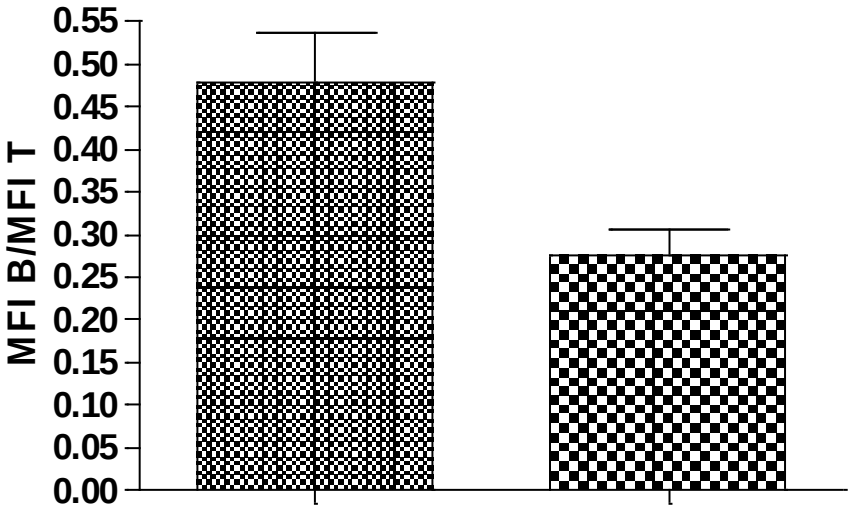


Fig 3: B MFI/ T MFI in ZAP-70 positive patients and in ZAP-70 negative patients

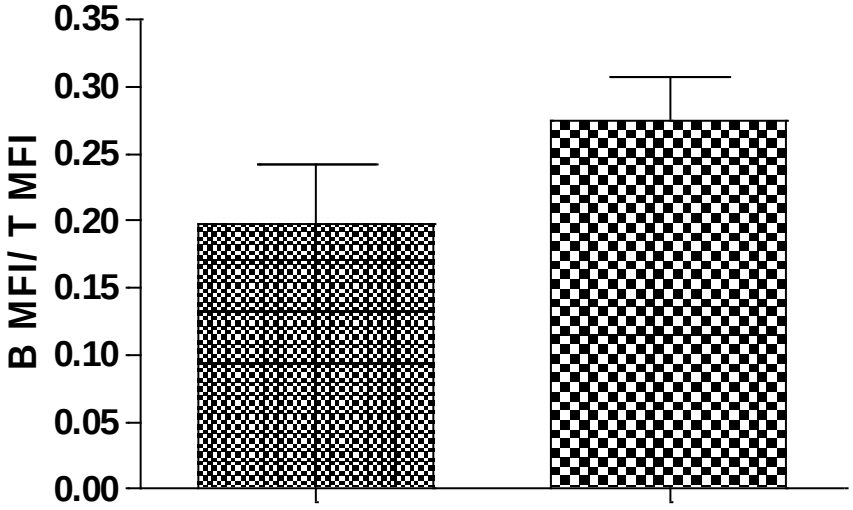


Fig 4: B MFI/ T MFI in healthy donors and in ZAP-70 negative patients

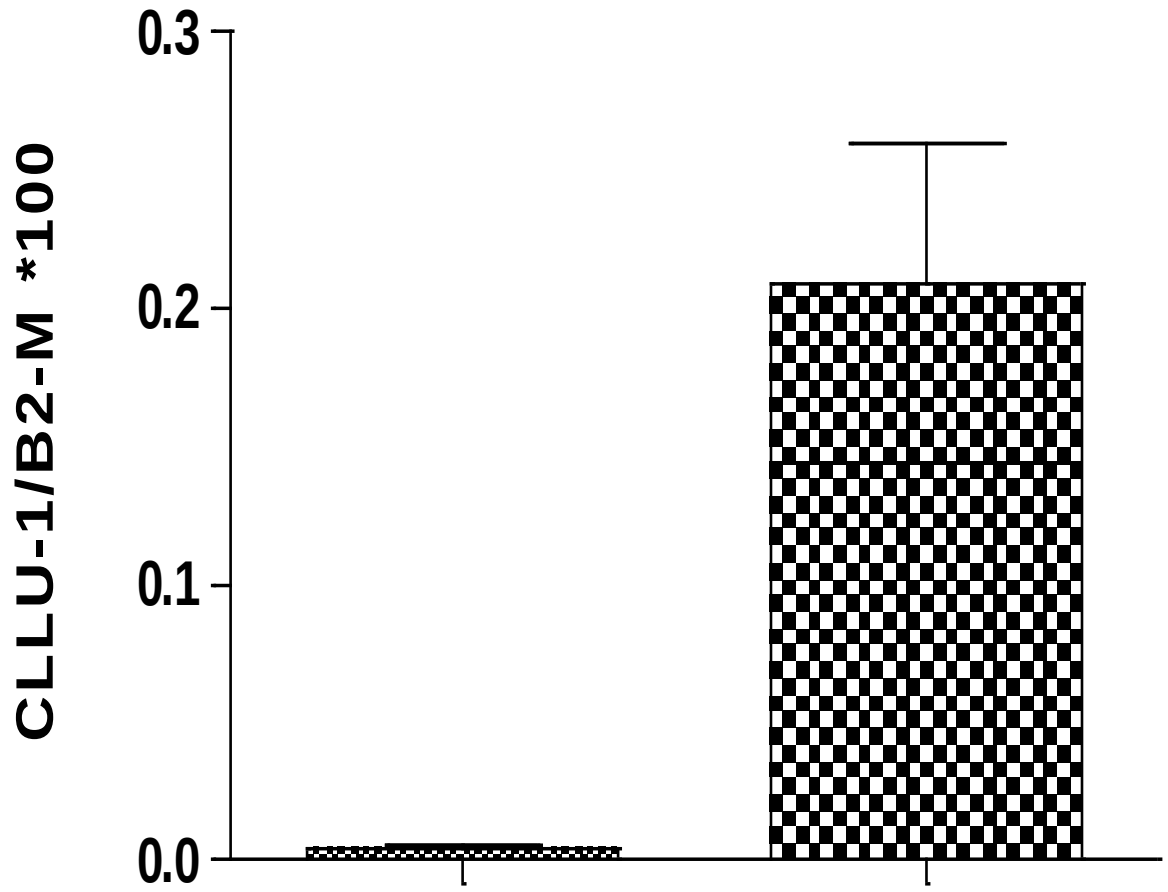
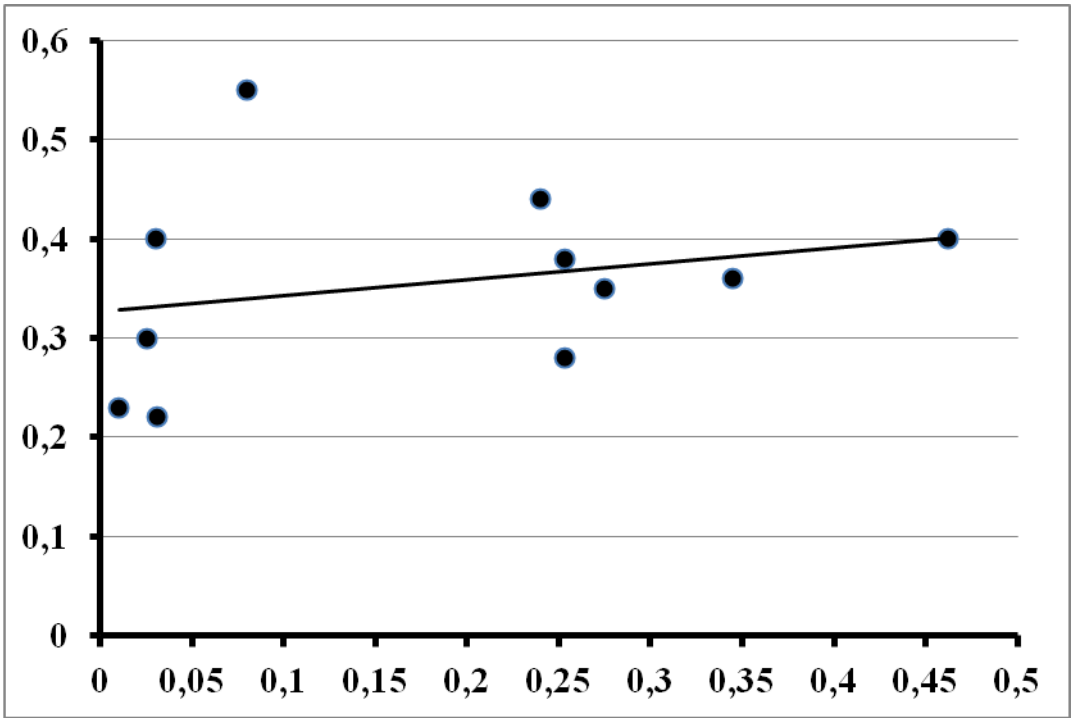


Fig. 5: Normalised copy number of CLLU-1 gene in healthy donors and in B-CLL patients.

Patients	Time diagnosis	Stage Disease at diagnosis	Therapy	Genetic delections	disease course	CLLU-1/B2*100	B/T
P1	2003	II di Binet	yes	11q22.3	progress	0,495	0,21
P5	2006	II Binet	yes	normal	progress	0,253	0,28
P6	2011	I di Rai	no	normal	no progress	0,345	0,36
P8	2010	0 di Rai	no	13q 14.3	no progress	0,08	0,55
P10	2010	II di Rai	no	normal	no progress	0,031	0,22
P12	2008	0 di Rai	yes	normal	progress	0,24	0,44
P13	2010	0 di Rai	no	normal	no progress	0,03	0,40
P14	2012	I di Rai	no	normal	no progress	0,01	0,23
P15	2009	0 di Rai	no	13 q 14.3	NA	0,462	0,40
P17	2007	I di Rai	NA	NA	NA	0,025	0,30
P18	2008	I di Rai	no	normal	progress	0,253	0,38
P19	2010	I di Rai	no	13q 14.3	no progress	0,275	0,35

Fig 6: Patient characteristics



r = -0,019
t = -0,059
p = 0,954 NS

Fig 7: B MFI/ T MFI and normalised copy number of CLLU-1 gene

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