# Comparison of ZAP-70/Syk mRNA levels with immunoglobulin heavy-chain gene mutation status and disease progression in chronic lymphocytic leukemia

Luca Laurenti
Aleksandar Petlickovski
Carlo Rumi
Stefania Gobessi
Paola Piccioni
Michela Tarnani
Pierluigi Puggioni
Sara Marietti
Simona Sica
Giuseppe Leone
Dimitar G. Efremov

Background and Objectives. The protein tyrosine kinase ZAP-70 has recently emerged as a major prognostic indicator in chronic lymphocytic leukemia (CLL). ZAP-70 is structurally and functionally homologous to Syk, a key mediator of B-cell receptor signaling. We therefore evaluated ZAP-70 expression in CLL B cells using Syk as an intracellular standard.

Design and Methods. The relative amounts of ZAP-70 and Syk were determined in purified B cells from 92 CLL patients using a novel reverse transcriptase/polymerase chain reaction (RT-PCR) procedure that co-amplifies both transcripts with equal efficiency. The ZAP-70/Syk mRNA ratio was correlated with $V_H$ gene mutation status, median treatment-free survival and FACS analysis of ZAP-70 expression.

Results. ZAP-70 was expressed in the majority of cases with unmutated $V_H$ genes (88%), but also at lower levels in a substantial fraction of cases with mutated $V_H$ genes (44%). High levels of ZAP-70, defined as ZAP-70/Syk mRNA ratios above 0.25, were observed mainly in cases with unmutated $V_H$ genes and correlated with short treatment-free survival. In contrast, no difference was observed in the median treatment-free survival between patients with low ZAP-70/Syk ratios (0.05-0.25) and patients with no or negligible ZAP-70 expression (ZAP-70/Syk<0.05). In 73 cases ZAP-70 expression was investigated by RT/PCR and FACS analysis; concordance with $V_H$ gene mutation status was 86% and 71%, respectively.

Interpretation and Conclusions. ZAP-70 is frequently expressed in CLL B cells, but only high levels correlate with unmutated $V_H$ gene status and progressive disease. Expression of ZAP-70 can be accurately assessed by analysis of the ZAP-70/Syk mRNA ratio, thus providing an alternative to FACS analysis.

Key words: chronic lymphocytic leukemia, immunoglobulin $V_H$ gene mutation status, ZAP-70, Syk, prognosis.

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*From the Department of Hematology, Catholic University Hospital "A. Gemelli", Rome, Italy (LL, CR, PP, MT, PP, SS, GL); ICGEB Hematology Group, Monterotondo-Outstation, CNR Campus "A. Buzzati-Traverso", Rome, Italy (AP, SG, SM, DGE).*

*Correspondence:*
*Dimitar Efremov, M.D., Ph.D., ICGEB Outstation, Monterotondo CNR Campus "Adriano Buzzati-Traverso", Via E. Ramarini 32 I-00016 Monterotondo Scalo, Rome, Italy.*
*E-mail: efremov@icgeb.org*

*Luca Laurenti, M.D.*
*Department of Hematology Catholic University Medical School Largo A.Gemelli 8, I-00168 Rome, Italy. E-mail: laurenti@rm.unicatt.it*

The clinical course and outcome of chronic lymphocytic leukemia (CLL) is highly variable. Some patients have rapidly progressive disease and die early in spite of therapy, whereas others exhibit a stable disease and have a normal life span.[1] Prognosis in CLL is classically assessed using the Rai and Binet staging systems, which are based on clinical and hematologic parameters.[2,3] Although effective in classifying CLL patients into broad prognostic subgroups, these staging systems cannot predict the course of the disease in individual patients, in particular those with early stage disease. Therefore, novel genetic and biological parameters, such as immunoglobulin $V_H$ gene mutation status, cytogenetic abnormalities and expression of CD38 and ZAP-70, are increasingly being used to predict the risk of disease progression.[4-13] Among these, the absence of somatic mutations in the $V_H$ genes appears to be a particularly strong prognostic indicator of aggressive disease. However, sequencing of rearranged $V_H$ genes is rather cumbersome and expensive, and therefore has not been adopted as a routine laboratory investigation.

ZAP-70 is a protein tyrosine kinase which plays a key role in proximal T-cell receptor signaling. Until recently, it was believed that ZAP-70 is expressed only in T-cells and natural killer (NK) cells. However, gene expression profiling with cDNA microarrays revealed that ZAP-70 is expressed also in CLL B cells, particularly in those with unmutated $V_H$ genes.[14] Subsequently, expression of ZAP-70 was evaluated by several groups using different flow-cytometry procedures.[11-13,15] These studies confirmed the association between ZAP-70 expression and $V_H$ gene mutation status and showed that CLL patients with a high percentage of ZAP-70 positive leukemic cells have signifi-

cantly shorter treatment-free and overall survival. However, the flow-cytometry analysis also revealed that expression of ZAP-70 is a continuous variable, thus requiring an arbitrary cut-off value to define positive and negative cases.[11-13] Moreover, in the two largest series the percentage of ZAP-70-positive CLL cells was close to the cut-off value in a significant fraction of cases.[13,15] Considering that the flow-cytometry procedures are inherently prone to small imprecisions, it is likely that precise assignment of ZAP-70 positivity may not be possible in all cases. Therefore, alternative approaches based on different methodological and standardization principles would be useful in validating the flow-cytometry data, especially in cases with equivocal results.

The possible role for ZAP-70 in the pathogenesis of CLL is also a matter of substantial interest. The significant homology with Syk indicates that ZAP-70 may also play a role in CLL B-cell receptor (BCR) signaling. Furthermore, ZAP-70 can reconstitute some aspects of BCR function in Syk-deficient lymphoma B cells, indicating that the two protein tyrosine kinases are functionally homologous.[16] More recently, ZAP-70 was shown to associate directly with the BCR complex in CLL B cells and to undergo tyrosine phosphorylation following stimulation by antigen.[17] In these cells stronger activation of other BCR signaling molecules was also observed, indicating that ZAP-70 can enhance BCR signal transduction.[18] These findings are intriguing, considering that ZAP-70 was considered inferior to Syk as an immunoreceptor signaling molecule, due to its lower intrinsic enzymatic activity, its lower capacity to associate with certain tyrosine phosphorylated immunoreceptor tyrosine-based activation notifs (ITAM) and its requirement for activation by Src family kinases.[19-21] Therefore, in order to understand better how ZAP-70 can affect BCR signaling, it would be useful to evaluate the relative quantities of these two related protein tyrosine kinases in CLL B cells.

In the present study, we took advantage of the considerable homology between the coding sequences of ZAP-70 and Syk to develop an RT-PCR procedure that allows accurate measurement of the relative levels of the two transcripts. We used this procedure to determine the relative expression of ZAP-70 and Syk in purified CLL B cells from 92 patients. The findings were correlated with the $V_H$ gene mutation status, percentage of ZAP-70 positive cells and clinical course of the patients.

## Design and Methods

### Patients and cell samples

Blood samples were collected from 92 patients whose morphologic and immunophenotypic features satisfied standard criteria for B-cell CLL.[22] Informed consent was obtained from all patients according to the Declaration of Helsinki and approval for the study was obtained from the institutional human research committee at the Catholic University Medical School in Rome. Sixty-two patients (67%) were male and the median age of all the patients was 63 years (range 33 to 84). At diagnosis 72 patients (78%) were in Binet stage A, 15 patients (16%) in stage B and 5 patients (6%) in stage C. The median duration of follow-up from diagnosis was 48 months. Fifty-seven (62%) patients had never been treated and 35 (38%) had received treatment at some stage during the course of the disease. Treatment was initiated for symptomatic or progressive disease, according to National Cancer Institute Working Group criteria.[22] The patients who had been treated had not received chemotherapy or steroids for at least 3 months prior to the sampling.

Peripheral blood mononuclear cells were separated by Ficoll gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). B cells were isolated with the use of magnetic beads coupled to monoclonal antibodies specific for CD19 (Dynal Biotech, Oslo, Norway). CD19 selection typically resulted in more than 98% purity as assessed by flow cytometry. In some experiments purification was also performed with the B-cell negative isolation kit (Dynal Biotech) or using Dynabeads M-450 (Dynal Biotech) coated with goat anti-human IgM (Southern Biotechnology Associates, Inc., USA).[23]

### Quantification of the ZAP-70/Syk mRNA levels

Measurement of ZAP-70 and Syk mRNA was based upon a similar procedure used for the quantification of the relative amounts of $^{A}\gamma$ and $^{G}\gamma$ globin mRNA.[24] Briefly, total cellular RNA was isolated from the CD19-selected B cells using Trizol reagent (Invitrogen), according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed using random hexamers and then PCR amplified with primers ZSf 5' TCATGCAC(G)CAGCTGGACAACC 3' and ZSr 5' CCAG(A)GTACTTCATC(G)CCCATGGA 3'. The ZSf primer was 5' end labeled with the fluorescent dye TET. The PCR reactions were performed in 100 μL for 30 cycles. A 2 μL aliquot of the PCR was digested for 2 hours with 6 U of the restriction enzyme BstNI (New England BioLabs, Beverly, MA, USA). One tenth of the reaction volume was denatured and the PCR products were size-separated and quantified on an ABI PRISM 310 capillary electrophoresis system using the GeneScan Analysis 3.1.2 software (Applied Biosystems, Forster City, CA, USA).

ZAP-70 and Syk plasmid DNA standards were prepared by RT-PCR of mRNA from the Jurkat T-cell lymphoma and BJAB B-cell lymphoma cell lines, respectively. The PCR fragments were cloned in the

pDrive cloning vector using the PCR cloning$^{plus}$ kit (Qiagen, Valencia, CA, USA). Plasmids containing the ZAP-70 and Syk fragments were linearized by restriction enzyme digestion and mixed in equimolar amounts.

### *Ig $V_H$ gene sequence analysis*

The PCR amplification, cloning, and sequencing of $V_H$ region genes have been described in detail elsewhere.[25] Briefly, RNA was reverse transcribed using random hexamers and then PCR amplified with a degenerate $V_H$ FWR1 primer in combination with Cμ, Cγ and Cα reverse primers. Samples that failed to amplify with these combinations were amplified with a mixture of forward primers complementary to leader sequences of $V_H$ families 1 to 6. PCR products were purified with the QIAquick PCR purification kit (Qiagen) and either sequenced directly or cloned with the PCR cloning$^{plus}$ kit (Qiagen). Sequencing was done with the BigDye Terminator v3.1 Cycle Sequencing kit and ABI 3100 genetic analyzer (Applied Biosystems). Candidate germline genes were assigned by searching the VBASE directory. Percentage homology was calculated by counting the number of mutations between the 5' end of FR1 and the 3' end of FR3. Sequences with less than 2% differences from germline $V_H$ sequences were considered unmutated.

### *Immunoblot analysis*

Cell pellets were lysed in ice-cold 1% Nonidet P-40 (NP-40) lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium deoxycholate) containing 1:40 dilution of a protease inhibitor cocktail for mammalian cells (Sigma-Aldrich). The protein concentration of each cell lysate was determined with the RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein samples (20 μg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on Immobilon-P polyvinilidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blotted at 4°C with antibodies against ZAP-70, Syk (both from Cell Signaling Technology) and β-actin (Sigma, Saint Louis, MO, USA). Immunodetection was done with anti-rabbit IgG horse-radish peroxidase-linked or anti-mouse IgG horse-radish peroxidase-linked antibodies (Cell Signaling Technology) and the ECL Plus enhanced-chemiluminiscence detection system (Amersham Biosciences, Buckinghamshire, UK) with BioMax MR films (Eastman Kodak, Rochester, NY, USA).

### *Flow cytometry analysis of ZAP-70*

Fifty microliters of whole blood in EDTA containing approximately $5\times10^5$ cells were incubated at room temperature in the dark for 20 minutes with CD3 PE-Cy5, CD56 PE-Cy5, CD5 FITC and CD19 PE-Cy7 to identify membrane antigens. After incubation, samples were centrifuged at 1500 rpm for 5 minutes. Cell pellets were resuspended in 100 μL of fixation medium A (Caltag Laboratories, Burlingame, CA, USA) and incubated at room temperature in the dark for 15 minutes. Then, cells were pelleted again at 1500 rpm for 5 minutes, resuspended in 100 μL of permeabilization medium B (Caltag Laboratories) and incubated with the ZAP-70 monoclonal antibody at room temperature in the dark for one hour. The ZAP-70 antibody was R-phycoerythrin conjugated (clone 1E 7.2, Caltag Laboratories). Samples were washed twice by centrifugation at 1500 rpm for 5 minutes and resuspended in 500 μL of phosphate-buffered saline. Finally, $1\times10^4$ fixed cells were analyzed by flow cytometry (BD FACSCanto, Becton Dickinson) using the gating strategy described by Crespo *et al.*[11]

### *Statistical analysis*

Correlations between ZAP-70 expression and $V_H$ gene mutational status were analyzed using Wilcoxon's rank-sum test. The cut-off ZAP-70/Syk value that best discriminated mutated from unmutated cases was determined using a receiver operating characteristic plot. The median treatment-free survival, calculated from diagnosis to initial therapy, was estimated by the method of Kaplan and Meier and assessed by the log-rank test. Data for patients who had not received treatment were regarded as censored. Statistical analyses were performed using the SigmaStat 3.1 program (Systat Software Inc., Richmond, CA, USA).

## Results

Human ZAP-70 and Syk have approximately 60% homology at the mRNA level. We therefore searched for short stretches of sequence identity to design PCR primers expected to co-amplify both transcripts with equal efficiency. Two such sequences were identified, separated by a 150 nt region that contained BstNI restriction enzyme sites at different locations in ZAP-70 and Syk (Figure 1A). These restriction enzyme sites were subsequently used to distinguish between the ZAP-70 and Syk PCR fragments.

To verify that ZAP-70 and Syk can be co-amplified with equal efficiency we first tested cloned ZAP-70 and Syk cDNA. Serial dilutions of an equimolar mixture of the two fragments were subjected to PCR amplification for various numbers of cycles. The PCR products were digested with BstNI and the obtained fragments of 51 and 62 bp, corresponding to ZAP-70 and Syk, respectively, were separated and quantified by capillary electrophoresis on a genetic analyzer. The ZAP-70/Syk ratio obtained in all instances was identical to the input plasmid DNA (Figure 1B).

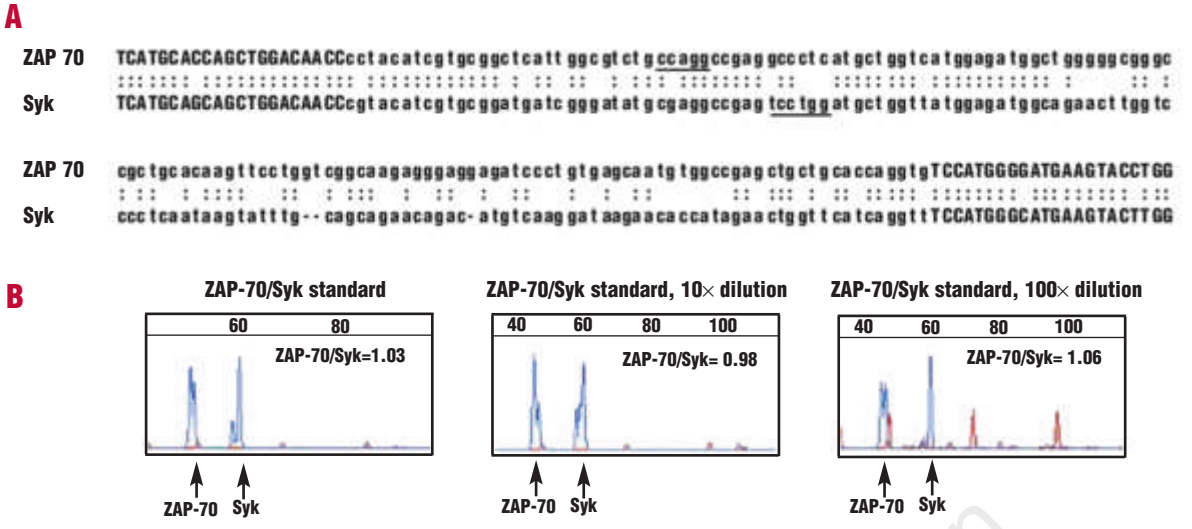


Figure 1. Analysis of the ZAP-70/Syk mRNA ratio by quantitative RT-PCR A. Alignment of the homologous ZAP-70 and Syk nucleotide sequences. The sequences used to design the PCR primers are shown in bold upper case letters. The *BstN*I site in each fragment is underlined. Following restriction enzyme digestion, 51 bp and 62 bp TET-labeled fragments are obtained for ZAP-70 and Syk, respectively. B. An equimolar mixture of plasmids with cloned ZAP-70 and Syk cDNA fragments was diluted 10 and 100 fold and the three samples were subjected to PCR, *BstN*I restriction enzyme digestion and capillary electrophoresis on a genetic analyzer. The ZAP-70/Syk ratios obtained and the position of the ZAP-70 and Syk fragments are indicated in each diagram. The analysis shown is after 30 cycles of PCR. Identical results were obtained when the same mixtures were subjected to 40 PCR cycles (*data not shown*).

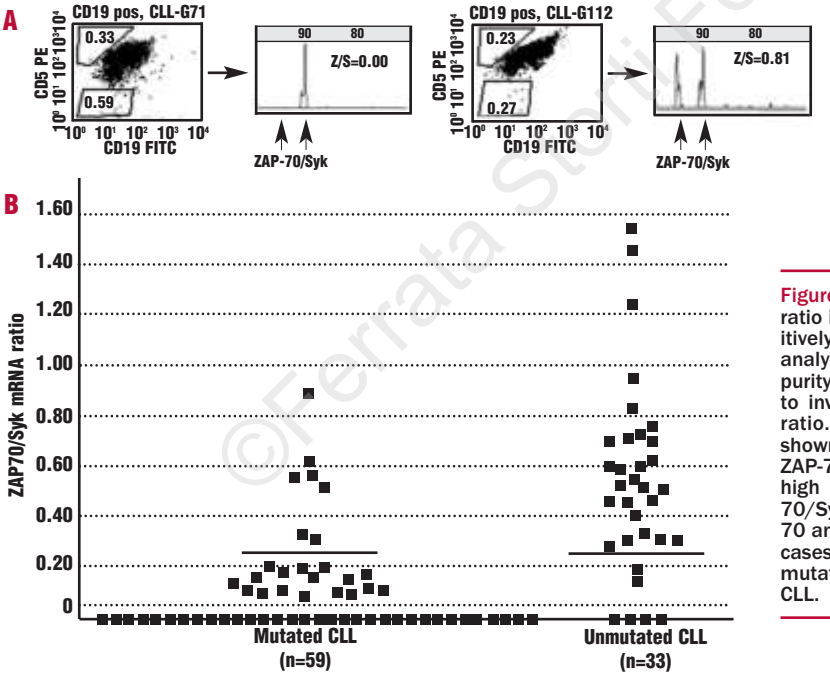


Figure 2. Analysis of the ZAP-70/Syk mRNA ratio in CLL B cells. A. CLL B cells were positively selected with CD19 Dynabeads and analyzed by flow cytometry to confirm the purity of the leukemic cell population, prior to investigation of the ZAP-70/Syk mRNA ratio. Two representative analyses are shown (sample CLL-G71 without detectable ZAP-70 mRNA and sample CLL-G112 with high levels of ZAP-70 mRNA). The ZAP-70/Syk ratios and the position of the ZAP-70 and Syk fragments are indicated in both cases. B. ZAP-70/Syk mRNA ratios in 59 $V_H$ mutated and 33 $V_H$ unmutated cases with CLL.

### *ZAP-70/Syk mRNA levels and $V_H$ gene mutation status*

We next tested the procedure in leukemic B-cells purified by CD19 positive selection. The purity of the leukemic cell population was confirmed by flow cytometry using antibodies specific for CD5 and CD19. Two typical results are shown in Figure 2A. In initial experiments the CLL B cells were also purified by positive selection with anti-IgM coated Dynabeads or by negative selection with a mixture of antibodies specific for CD2, CD3, CD7, CD14, CD16, and CD56. No differences were observed in the ZAP-70/Syk mRNA ratios with the use of the different purification procedures (*data not shown*).

The ZAP-70/Syk mRNA ratio was then determined in CD19-purified leukemic B cells from 92 CLL patients. In 37 cases (40%) ZAP-70 was either not

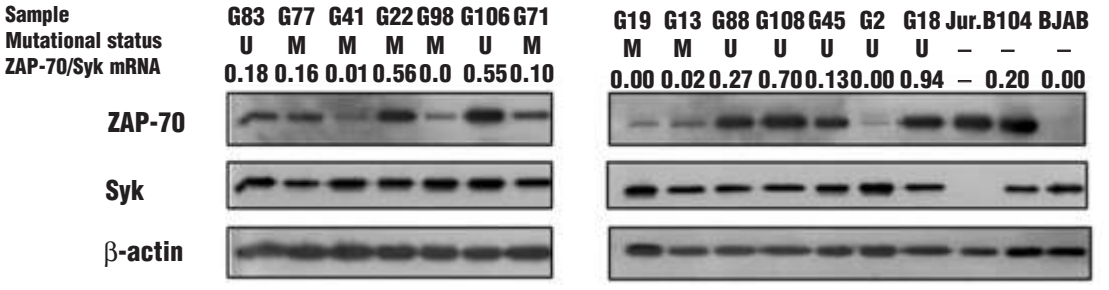


**Figure 3.** Expression of ZAP-70 and Syk protein in CLL B cells and lymphoma cell lines. Immunoblotting was performed with antibodies specific for ZAP-70, Syk and β actin on lysates from purified CD19-positive CLL cells. An analysis of 14 representative cases is shown. The T-cell lymphoma cell line Jurkat and the B-cell lymphoma cell line BJAB were included as a positive and negative control for ZAP-70, respectively. B104 is a B-cell lymphoma cell line that co-expresses ZAP-70 and Syk. The $V_H$ gene mutational status and the ZAP-70/Syk mRNA ratio for each sample is shown at the top of the figure.

detected or the amount was considered negligible (ZAP-70/Syk<0.05). ZAP-70 mRNA was present in the remaining 55 samples (60%), with ZAP-70/Syk mRNA values ranging from 0.07 to 1.58. The levels of ZAP-70 rarely exceeded the levels of Syk (only three cases had a ZAP-70/Syk ratio >1.0), indicating that Syk is predominantly expressed in CLL B cells.

To investigate the association with $V_H$ gene mutation status, we compared the ZAP-70/Syk mRNA ratios in CLL cells with mutated and unmutated $V_H$ genes (Figure 2B). ZAP-70 mRNA was detected in 26 of the 59 (44%) $V_H$ mutated and 29 of the 33 (88%) $V_H$ unmutated samples. However, the levels of ZAP-70 were on average significantly lower among the ZAP-70 positive cases with mutated $V_H$ genes (median ZAP-70/Syk mRNA ratio 0.15, range 0.07 to 0.90) than among those with unmutated $V_H$ genes (median 0.55, range 0.13 to 1.58, $p<0.001$, Mann-Whitney rank sum test). Therefore, we investigated whether a particular ZAP-70/Syk mRNA ratio can be used to discriminate between the $V_H$ mutated and $V_H$ unmutated cases. Using receiver operating characteristic plot analysis we observed that a ZAP-70/Syk ratio of 0.25 could correctly assign the $V_H$ gene mutation status in 86% of the cases. Based on this threshold 82% of the $V_H$ unmutated and only 12% of the $V_H$ mutated samples were classified as cases with high ZAP-70 expression.

Nineteen $V_H$ mutated and two $V_H$ unmutated cases had detectable ZAP-70 mRNA that was below the 0.25 threshold. To verify that these cases express ZAP-70 protein, we performed western blotting analysis with CLL cells purified by CD19-positive selection from 20 representative cases. The cell lines Jurkat and BJAB were used as a positive and negative control, respectively. The samples were probed with antibodies against ZAP-70, Syk and β-actin. CLL B cells with ZAP-70/Syk values between 0.05 and 0.25 had readily detectable ZAP-70 protein, although considerably less than the amount detected in the samples with ZAP-70/Syk ratios above 0.25 (Figure 3). Only a very faint band was observed in samples with ZAP-70/Syk ratios below 0.05, which again validated the data of the RT-PCR assay.

### *Correlation between ZAP-70/Syk mRNA levels and time to initial therapy*

We next compared the clinical course in patients with different levels of ZAP-70 expression (Figure 4A). The patients were stratified into three groups according to the ZAP-70/Syk mRNA ratio: (i) patients with negligible/undetectable ZAP-70 (ZAP-70/Syk<0.05), (ii) patients with low ZAP-70 expression (ZAP-70/Syk=0.05-0.25), and (iii) patients with high ZAP-70 expression (ZAP-70/Syk>0.25). The median treatment-free survival was significantly shorter in the group of patients with high ZAP-70 expression (30 months) compared to the groups with low or negligible/undetectable ZAP-70 (not reached in both groups, $p=0.0005$ and $p=0.00001$, respectively) (Figure 4A). In contrast, the median treatment-free survival of the group with low ZAP-70 expression was not significantly different from that of the group with negligible/undetectable ZAP-70 mRNA ($p=0.92$), indicating that only high levels of ZAP-70 are associated with an unfavorable clinical course. The survival curves of cases with low or negligible/undetectable ZAP-70 levels were similar to the survival curve of patients with mutated $V_H$ genes (Figure 4B). Likewise, the median treatment-free survival in patients with high ZAP-70 expression was similar to that in patients with unmutated $V_H$ genes (18 months).

We also examined in detail the discordant cases for ZAP-70 expression and $V_H$ gene mutation status. The seven $V_H$ mutated cases with high ZAP-70 expression (>0.25) had 89 to 96% homology with a known germline $V_H$ gene. Two of these patients had received first treatment at 51 and 60 months after diagnosis, where-

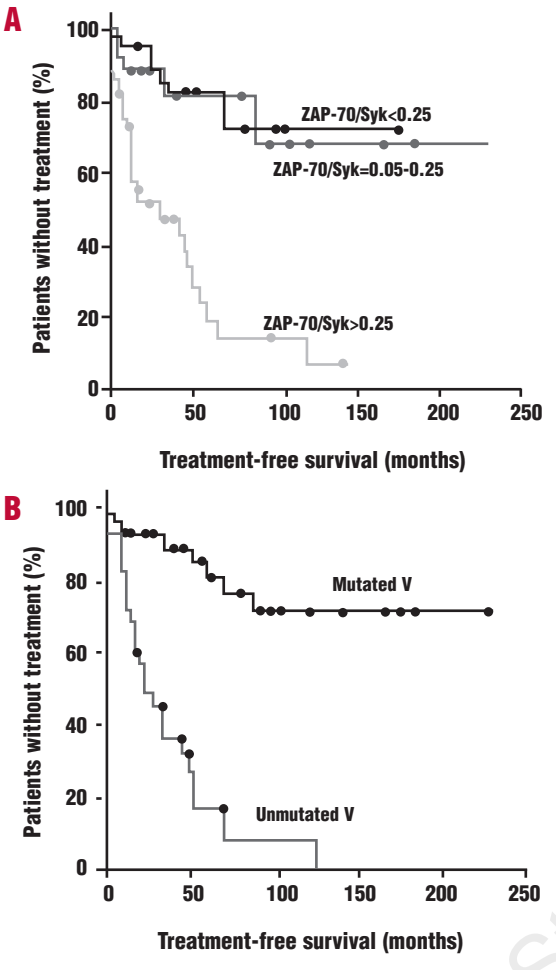


Figure 4. Time from diagnosis to initial therapy in CLL patients stratified according to ZAP-70/Syk mRNA ratio and $V_H$ gene mutation status. A. Comparison of treatment-free survival in patients with high (ZAP-70/Syk>0.25), low (ZAP-70/Syk=0.05-0.25) and negligible/undetectable ZAP-70 expression (ZAP-70/Syk<0.05). B. Comparison of treatment-free survival in patients with mutated and unmutated $V_H$ genes.

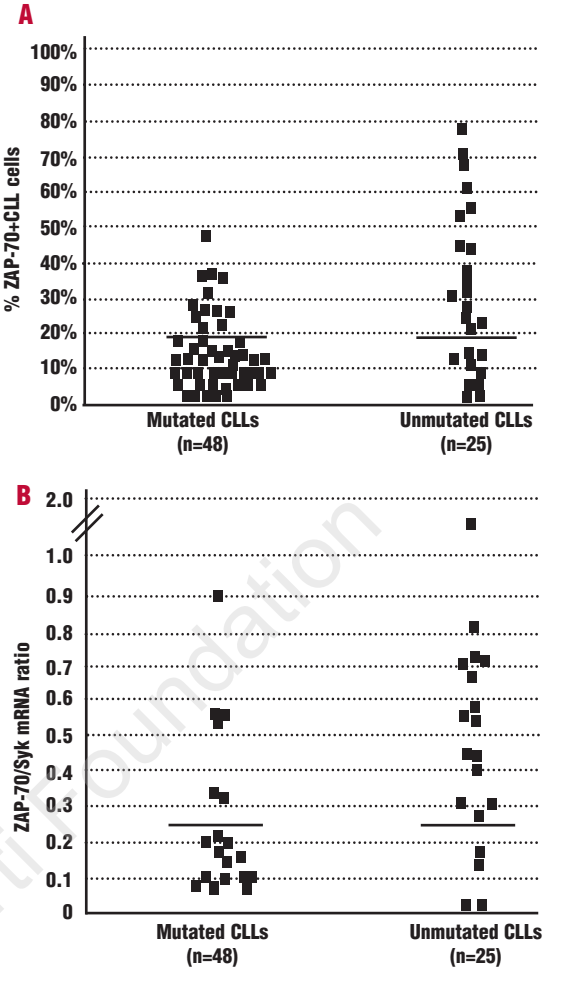


Figure 5. Expression of ZAP-70 evaluated by flow cytometry and RT-PCR. A. ZAP-70 expression was investigated in 73 CLL cases by both procedures. The percentage of ZAP-70-positive CLL cells is shown in panel A and the ZAP-70/Syk mRNA ratios are shown in panel B.

as the remaining 5 patients were still untreated after 4, 11, 40, 97, and 141 months of observation. Only one of these cases expressed the V3-21 gene, which is associated with poor prognosis and ZAP-70 expression regardless of its mutational status.[10,26] Among the six $V_H$ unmutated cases with low or undetectable ZAP-70 mRNA, three had received treatment between 8 and 31 months after diagnosis. The remaining three patients have remained stable after 18, 44 and 49 months of follow-up.

### *Analysis of ZAP-70 expression by RT-PCR and flow cytometry*

Expression of ZAP-70 was investigated by flow cytometry in 73 cases from this series. Samples were considered positive when ZAP-70 was detected in at least 20% of the CLL B cells. Using this cut-off value, 12 of the 48 $V_H$ mutated (25%) and 16 of the 25 $V_H$ unmutated cases (64%) were regarded as ZAP-70 positive (Figure 5A). The concordance between $V_H$ gene mutation status and ZAP-70 expression determined by FACS was 71%. A better concordance (86%) was observed when assignment of ZAP-70 positivity in the same group of patients was based on the ZAP-70/Syk ratio ($p$=0.043 by $\chi^2$ analysis). Using this approach 84% of the $V_H$ unmutated and only 12.5% of the $V_H$ mutated cases expressed ZAP-70 above the 0.25 cut-off value (Figure 5B).

## Discussion

Several important studies have recently shown that analysis of ZAP-70 expression by flow cytometry can

predict $V_H$ gene mutation status and prognosis in CLL.[11–13,15] However, the available flow cytometry tests are not easy to perform and still require standardization and validation before they can be generally adopted in clinical laboratories. The three published methods use two different anti-ZAP-70 antibodies, different fluorochromes and different gating strategies to define ZAP-70 positivity in the CLL clone.[11–13] Moreover, they use two different cut-off values (10% and 20%) to discriminate between ZAP-70 positive and negative cases.[11–13] Considering that a significant fraction of cases in the two largest series had a percentage of ZAP-70 positive CLL cells that was close to the cut-off value, it is likely that precise assignment of ZAP-70 positivity may not always be possible, unless the results are verified by an independent procedure. We therefore investigated whether an alternative approach, based on a comparison of the levels of ZAP-70 with an internal cellular standard, can be useful in predicting $V_H$ gene mutation status and disease progression. We reasoned that Syk could be a good candidate for an internal standard, because it is constitutively expressed in all CLL B cells and is structurally and functionally homologous to ZAP-70.[16,27]

The procedure that we developed compares the relative levels of ZAP-70 and Syk mRNA in purified CLL B cells. We found that the assay is highly reproducible in several sets of experiments, including analysis of equimolar mixtures of cloned ZAP-70 and Syk DNA fragments, repetitive analysis of the same mRNA samples, and analysis of samples from the same patients whose cells were separately purified with CD19 Dynabeads. Moreover, in experiments evaluating different purification procedures, identical ZAP-70/Syk mRNA ratios were obtained after purification of the CLL B cells with CD19-positive selection, B-cell negative isolation and IgM-positive selection. Finally, immunoblotting analysis of selected samples showed a strong correlation between the protein and mRNA levels.

We next examined the relative levels of ZAP-70 and Syk mRNA in CD19-purified CLL B cells from 92 patients. We detected considerable levels of ZAP-70 mRNA (ZAP-70/Syk>0.05) in almost 60% of the samples. The majority of cases with unmutated $V_H$ genes expressed ZAP-70, in line with previously reported data obtained by DNA microarray gene expression profiling.[10] However, we also detected ZAP-70 mRNA in a substantial fraction of cases with mutated $V_H$ genes (44%). Interestingly, most of these cases had significantly lower ZAP-70/Syk mRNA ratios than did the ZAP-70-positive cases with unmutated $V_H$ genes. This difference in ZAP-70 expression was confirmed at the protein level by immunobloting analysis, which closely followed the mRNA data. We therefore investigated whether a particular ZAP-70/Syk cut-off value can allow better discrimination between the $V_H$ mutated and unmutated cases. A 0.25 ratio correctly assigned the $V_H$ gene mutation status in 86% of the cases, with 82% of the $V_H$ unmutated and only 12% of the $V_H$ mutated cases showing ZAP-70/Syk ratios above this threshold. Thus, these results suggest that ZAP-70 is frequently expressed in CLL B cells with mutated $V_H$ genes, but the levels of expression are usually significantly lower than in cases with unmutated $V_H$ genes.

We next classified the CLL patients into three groups based on the levels of ZAP-70 mRNA: i) patients with negligible or undetectable ZAP-70 expression (ZAP-70/Syk<0.05), ii) patients with low ZAP-70 expression (ZAP-70/Syk=0.05-0.25) and iii) patients with high ZAP-70 expression (ZAP-70/Syk>0.25). Comparison of the last group with the groups that had low or undetectable ZAP-70 expression showed a significant difference in the time to initial treatment, confirming that ZAP-70 is an important determinant of prognosis in CLL. In contrast, no difference was observed in the treatment-free survival between cases with low ZAP-70 and cases with undetectable ZAP-70. These comparisons suggest that the 0.25 cut-off value for the ZAP-70/Syk mRNA ratio is also optimal for predicting the requirement for therapy. More importantly, they show that only high ZAP-70 expression is associated with an increased risk of rapid disease progression.

Recent studies have indicated that ZAP-70 may play an important role in signal transduction through the BCR in CLL B cells.[17,18] It is therefore interesting that Syk was found to be the predominantly expressed protein tyrosine kinase in the large majority of cases from our series. In most cases with high ZAP-70 expression the levels of Syk were still approximately two-fold higher, making unlikely the possibility that ZAP-70 merely serves to substitute for eventual inadequate expression of its functional B-cell homologue. A more likely explanation could be that ZAP-70 can promote BCR signaling indirectly, possibly by augmenting the activation of Syk, as observed in CLL B cells in which ZAP-70 was introduced by an adenoviral vector.[18] Alternatively, ZAP-70 and Syk may differentially activate certain signaling pathways, analogous to what has been seen in studies investigating T-cell receptor signaling.[28–30] It is to be hoped that further studies with B-cell lines engineered to co-express the two protein tyrosine kinases at levels similar to those observed in CLL B cells will clarify this issue.

In 73 cases from our series we also evaluated the percentage of ZAP-70 positive cells by flow-cytometry. In our hands analysis of the ZAP-70/Syk mRNA ratio predicted the $V_H$ gene mutation status more accurately than the percentage of ZAP-70-positive CLL cells. However, it should be noted that the concordance between the percentage of ZAP-70-positive cells and the $V_H$ gene mutation status in our series was only 71%, which is lower than in other published series in which it ranged from 77% to 95%.[11–13] The reasons for this discrepancy

are unclear, but may include the use of different antibodies (clone 1E7.2 was used in our study and in the study by Rassenti *et al.*,[13] whereas clone 2F3.2 was used in the studies by Crespo *et al.*[11] and Orchard *et al.*),[12] different fluorochromes, gating strategies or patient populations.[31] Nevertheless, the greater discordance of our flow cytometry results further underlines the need to validate the data with an independent procedure such as the RT-PCR approach described in this paper.

In conclusion, this study shows that expression of ZAP-70 in CLL B cells can be accurately assessed by using Syk as an internal cellular standard. However, it should be noted that the procedure employed to quantify the relative levels of ZAP-70 and Syk requires several steps which make it less practical than flow cytometry procedures. We are currently trying to simplify the procedure by adapting it in a real-time RT-PCR format, using internal ZAP-70 and Syk probes to eliminate the restriction enzyme digestion and capillary electrophoresis steps. However, separation of the CLL B cells and assessment of their purity will still be required. Nevertheless, we believe that these additional steps are worth the effort at least for cases with ZAP-70 positive cells near the cut-off value, for which validation of the flow cytometry results will be essential in order to assess prognosis properly.

*All authors (LL, AP, CR, SG, PP, MT, PP, SM, SS, GL and DE) gave substantial contributions to the conception and design of the study, analysis and interpretation of data, drafting and revising the article, and gave the final approval of the present version of the manuscript. In particular, AP, SG, SM and DE performed the quantification of the ZAP-70/Syk ratios and the analysis of the $V_H$ gene mutation status. CR and PP performed the FACS analysis. LL, PP, MT, SS and GL collected and analyzed the patients' data. DE, LL and SS performed the statistical analysis. DE and LL supervised the study. The authors declare that they have no potential conflict of interest.*

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