Lipoprotein Lipase mRNA Level in Whole Blood Is a Prognostic Marker in B Cell Chronic Lymphocytic Leukemia

Femke Van Bockstaele,† Valerie Pede,† Ann Janssens, Filip Callewaert, Fritz Offner, Bruno Verhasselt, and Jan Philippe*

Background: Chronic lymphocytic leukemia (CLL) is characterized by high individual variability in clinical course and the need for therapy. Differentiation of prognostic subgroups is based primarily on the mutation status of the genes for the variable region of immunoglobulin heavy chain (IGHV). The time- and labor-intensive nature of this analysis necessitates the use of easily applicable surrogate markers.

Methods: We developed a quantitative PCR (qPCR) method for determining lipoprotein lipase gene (LPL) mRNA levels and analyzed samples of lysed whole blood and CD19-selected cells from 50 CLL patients. Associations of LPL and ZAP70 (ζ-chain (TCR) associated protein kinase 70 kDa) expression with IGHV mutation status, overall survival (OS) and treatment-free survival (TFS) were investigated.

Results: Lysed samples of whole blood and CD19-selected cells were similar with respect to LPL expression (R = 0.88; P <0.0001). LPL expression was significantly associated with IGHV mutation status (χ²(1) = 15.3; P <0.0001) and showed an 89.3% specificity, a 68.2% sensitivity, an 83.3% positive predictive value, and a 78.1% negative predictive value for IGHV mutation status. LPL expression was significantly associated with both OS and TFS in log-rank tests (both P values = 0.002). LPL-positive patients had a significantly shorter median TFS time (23 months) than LPL-negative patients (88 months) (P = 0.002).

Conclusions: LPL mRNA level is a valuable prognostic marker in CLL. The method does not require cell purification, and its applicability with archived samples facilitates its use in the clinical routine and other studies.

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Chronic lymphocytic leukemia (CLL) is heterogeneous with a continuous spectrum of disease (1). At one extreme are patients who have an almost normal life expectancy with no need for treatment; at the other are patients who die of drug-resistant disease as early as 2 years after initial diagnosis (2). The appearance of new therapies has shifted the therapeutic goal from control of the leukocytosis to the achievement of a molecular remission, especially in younger patients, for whom the CLL diagnosis has a significant impact on life expectancy. Therefore, reliable prognostic factors are of utmost importance in the design of randomized clinical trials for determining if early treatment is meaningful for all younger patients or at least for a high-risk subgroup of such patients. The current clinical consensus recommends against relying exclusively on clinical staging systems such as the Rai (3) or Binet (4) score for prognostic assessment of CLL patients. An assessment of biological and genetic markers at diagnosis has provided more accurate predictions of disease outcome, and it is important, therefore, that such markers be evaluated in clinical trials. Markers recently demonstrated to be of value are the mutation status of the genes of the variable region of the immunoglobulin heavy chain (IGHV) and the levels of CD38 and ZAP70 (ζ-chain (TCR) associated protein kinase 70kDa). The IGHV muta-

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3 Nonstandard abbreviations: CLL, chronic lymphocytic leukemia; IGHV, variable region of the immunoglobulin heavy chain; qPCR, quantitative PCR; TFS, treatment-free survival; Ct, threshold cycle; OS, overall survival; LDT, lymphocyte doubling time.
tion status was introduced as a new prognostic marker in 1999 (5, 6). The difficulty of performing IGHV mutation analysis in the routine diagnostic laboratory prompted a search for surrogate markers, and studies have investigated the relationship between IGHV mutation status and gene expression profile in CLL cells (7). Wiestner et al. (8) found that ZAP70 best distinguished CLL subtypes. Further clinical studies confirmed the clinical usefulness of the ZAP70 protein and mRNA levels as prognostic markers (9–16). Another study of gene expression profiling defined a limited set of genes that were expressed differentially in progressive unmutated and stable mutated CLL cases. Two other identified genes were LPL (lipoprotein lipase) and ADAM29 (ADAM metalloproteinase domain 29) (17). Interestingly, Rosenwald et al. (7) and Klein et al. (18) had mentioned LPL as among the most differentially expressed genes. van ’t Veer et al. (19) and the German CLL Study Group (20) further confirmed the predictive value of LPL. LPL expression was a more reliable marker than ZAP70 in real-time quantitative PCR (qPCR) assays of CLL samples, even with unselected peripheral blood mononuclear cells (19).

We describe the validation of a new qPCR analysis to measure LPL expression in whole blood and its power as a prognostic marker in CLL.

Materials and Methods

Patients and Sample Collection

Fifty-seven patients diagnosed with CLL at our institution were included in the present study, which was approved by the Ethical Committee of the Ghent University Hospital, and all patients provided informed consent. We confirmed the diagnosis and clinical stage for all patients [Moreau–Matutes–Catovsky score of at least 4 on a 5-point scale (21)]. patients were untreated at the time of blood collection. Cytogenetic characteristics were evaluated for 53 patients. The median age at diagnosis was 59 years (range, 33–76 years), and the median duration of follow-up was 80 months (range, 15–464 months). Sixty percent of the patients needed therapy during follow-up. Treatment was started when patients developed massive lymphadenopathy, progressive anemia, thrombocythemia, or splenomegaly, or when the lymphocyte count exceeded 10^11 cells/L. We recorded 5 deaths during the course of this study, all of which were CLL-related. Treatment-free survival (TFS) and disease-related mortality were calculated from the time of diagnosis. Inclusion of patients was based on the availability of biological samples: CD19-selected cells from peripheral blood mononuclear cells were obtained from all patients, and unselected lysed whole blood samples were available for 50 patients. Peripheral blood mononuclear cells were isolated on a lymphoprep gradient (Nycomed), and CD19^+ cells were selected by means of the EasySep technology (Stem Cell Technologies) according to the manufacturer’s instructions. Purity was at least 98% by flow cytometry. Unselected cells from whole blood were obtained after erythrocytes were lysed with erythrocyte lysis buffer (Qiagen).

RNA Isolation and cDNA Synthesis

All analyses were carried out with frozen cells. Total cellular RNA was extracted with the Trizol method (Invitrogen) or the RNeasy Kit (Qiagen) according to the suppliers’ instructions. We determined mutation status by synthesizing cDNA from 1 μg RNA by means of the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). For gene expression analysis, cDNA was synthesized from 1 μg RNA with the Reverse Transcriptase Core kit (Eurogentec) according to the manufacturer’s instructions.

Determination of IGHV Mutation Status

Six IGHV family specific primers that anneal to sequences in the leader region and a consensus primer for the heavy-chain joining region were used to amplify cDNA (22). We used an alternative set of primers specific for the FR1 region when amplification with these primers failed (23). Clonal PCR products were purified with the QiAquick PCR Purification Kit (Qiagen), and both strands were sequenced by fluorescence dye chain termination with an ABI Prism 310 Genetic Analyzer (Applied Biosystems). IGHV sequences were considered mutated if the homology with the closest germ line counterpart was <98%.

Flow Cytometric Analysis of ZAP70 Expression

We analyzed intracellular ZAP70 expression with flow cytometry according to Crespo et al. (9). In brief, cells were fixed and permeabilized with the Fix and Perm kit (ImTec Diagnostics) according to the manufacturer’s instructions, incubated with ZAP70 antibody (clone 2F3.2; Upstate Biotechnology), washed, and incubated with phycoerythrin-labeled goat antimouse antibody (Caltag Laboratories). After washing the cells, we followed one of 2 procedures: We stained the cells with CD3 conjugated with peridinin chlorophyll protein and a cyanine dye (Cy5.5), with allophycocyanin-conjugated CD56 + CD5, and with fluorescein isothiocyanate-conjugated CD19 (BD Biosciences) and performed a 4-color flow cytometric analysis with a FACSsort flow cytometer (BD Medical Systems). Alternatively, cells were stained with fluorescein isothiocyanate-conjugated CD3 (BD Biosciences), CD19 tandemly conjugated with phycoerythrin and Texas Red (Beckman Coulter), and CD5 tandemly conjugated with

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4 Human genes: LPL, lipoprotein lipase; ZAP70, ζ-chain (TCR) associated protein kinase 70kDa; ADAM29, ADAM metalloproteinase domain 29; HMBS, hydroxymethylbilane synthase; ABL1, v-abl Abelson murine leukemia viral oncogene homolog 1; IGHV1–69, immunoglobulin heavy variable 1–69; IGHV3–21, immunoglobulin heavy variable 3–21; IGHV3–23, immunoglobulin heavy variable 3–23; IGHV5–7, immunoglobulin heavy variable 3–7; IGHV3–30, immunoglobulin heavy variable 3–30; IGHV4–34, immunoglobulin heavy variable 4–34; CD38, CD38 molecule.
phycoerythrin and Cy7 (Beckman Coulter), and allo-
phyocyanin-conjugated CD56 (Beckman Coulter) and
analyzed on a FC500 instrument with CXP software
(Beckman Coulter). The cutoff for ZAP70 expression on
CLL cells was set so that 95% of the T cells were positive
for the ZAP70 protein.

QUANTITATIVE REVERSE TRANSCRIPTION–PCR
We analyzed ZAP70 and LPL expression by measuring
mRNA with qPCR on an ABI Prism 7700 Sequence
Detector (Applied Biosystems) or a Bio-Rad IQ5 Real
Time Detection System (Bio-Rad). Wiestner et al. (8)
have previously described the primers and the probe we used
for ZAP70; those for LPL were designed with Primer
Express V2.0 software (Applied Biosystems) and are as
follows: forward (exon 2), 5’-CAG CAG CAA AAC CT
CAT GGT-3’; probe (boundary of exons 2 and 3), 5’-FAM-
CCA TGG CTG GAC GGT AAC AGG AAT GT-TAMRA-
3’; reverse (exon 3), 5’-AGT TTT GGC ACC CCA CTC
TCA-3’. For both LPL and ZAP70, 150 nmol/L of probe
and 600 nmol/L of each primer were used. PCR reactions
were performed with the qPCR Core kit (Eurogentec)
under the following cycling conditions: 50 °C for 2 min,
an initial denaturation step of 95 °C for 10 min, and 50 cycles
of 95 °C for 30 s and 60 °C for 1 min. All reactions were
done in duplicate, and each PCR run included controls
and either calibrator samples [cDNA from the HL-60 cell
line (ATCC)] or, in the case of ZAP70, a calibration curve
of 6, 10-fold dilutions of Jurkat (ATCC) cDNA. Two
so-called housekeeping genes, HMBS (hydroxymethylbi-
lane synthase; primers and probe courtesy Dr. E. Mensink
and Dr. L. van de Locht, Nijmegen, The Netherlands) (24)
and ABL1 (v-abl Abelson murine leukemia viral oncogene
homolog 1) (25) were used to normalize ZAP70 and LPL
expression. We performed 2 validation experiments to
demonstrate that the threshold cycle (ΔCt) method was
capable of quantifying LPL expression. We compared the
ΔCt values of LPL and HMBS and of LPL and ABL1 for an
HL-60 cDNA dilution series. The logarithm of the input
amount was plotted vs the ΔCt value. The absolute value
of the slope of the trend line was 0.075 for HMBS and
0.021 for ABL1. Because these values are <0.1, the effi-
ciencies of the target and reference gene PCRs can be
considered approximately equal, and the ΔCt method
can be used (ABI Prism 7700 Sequence Detection System,
User Bulletin #2, 2001; Applied Biosystems). We used the
calibration-curve method to quantify ZAP70 expression
and calculated the geometric mean of the results for both
reference genes to obtain final quantitative results.

STATISTICAL ANALYSIS
ROC curve analyses were performed with MedCalc sta-
tistical software (MedCalc Software) to determine the
ZAP70 and LPL expression cutoff values that best distin-
guished between mutated and unmutated cases. Associa-
tions between different clinical markers were described
with Pearson χ² statistics (with the Yates continuity cor-
rection for 2 × 2 tables), Spearman correlation coefficients,
or odd ratios and 95% confidence intervals. We used the
Kaplan–Meier method to analyze overall survival (OS)
and TFS. The log-rank statistic was used to determine
significant associations between individual clinical mark-
ers and OS or TFS. P values <0.05 were considered
statistically significant. All analyses were performed with
the SPSS software package, version 13.0 (SPSS).

Results
PATIENT CHARACTERISTICS AND STANDARD
PROGNOSTIC MARKERS
We summarize the biological and clinical characteristics
of the 57 CLL patients included in this study in Table 1.
Twenty-six patients (54%) had unmutated IGHV genes. In
11 patients (19%), the CLL clone expressed an IGHV gene
segment associated with a bad prognosis (IGHV1–69,
IGHV3–21, or IGHV3–23) (26), and 14 patients (25%) had
an IGHV gene segment associated with a good prognosis
(IGHV3–7, IGHV3–30, or IGHV4–34) (27). Deletion 11q
was the most frequently observed cytogenetic aberration
(n = 12), followed by trisomy 12 (n = 7) and deletion 17p
(n = 1).

Cross-tabulations showed a significant association only
between mutation status and lymphocyte doubling time
(LDT) (χ²(1) = 8.4; P = 0.004) and between mutation
status and cytogenetic factors (χ²(1) = 5.4; P = 0.02).

UNSELECTED, LYSED WHOLE BLOOD AND CD19-
SELECTED CELLS SHOW SIMILAR LPL EXPRESSION
LPL expression in lysed samples of whole blood and
expression in CD19-selected B cells for the 50 evaluated
patients were strongly correlated (Fig. 1) (R = 0.88; P
<0.0001). The cutoff values calculated by ROC curve
analysis revealed 6 patients with discordant results. Al-
though the samples of lysed whole blood were LPL-
negative for these patients, the results for the selected
B-cell samples were positive. This discrepancy did not
appreciably affect the association with mutation status or
prognosis; therefore, only results obtained with whole
blood are discussed further.

To evaluate changes in LPL expression over time, we
sampled blood from 11 patients at a median interval of 12
months and evaluated the lysed samples for LPL expres-
sion. LPL expression did not change over time except for
1 patient; this single discrepant result was close to the
threshold value, however.

STRONG ASSOCIATION BETWEEN LPL AND ZAP70
EXPRESSION AND MUTATION STATUS
Cross-tabulations for IGHV mutation status and ZAP70
and LPL expression are summarized in Table 1. Mutation
status was significantly associated with the ZAP70 flow
cytometric result (χ²(1) = 16.5; P <0.0001), with the
ZAP70 qPCR result (χ²(1) = 25.9; P <0.0001), and with
LPL expression (χ²(1) = 15.3; P <0.0001) (Fig. 2). Concor-
dance rates were 79%, 84%, and 80%, for the ZAP70 flow cytometric analysis, the ZAP70 qPCR result, and the LPL qPCR result, respectively.

We also assessed the distinguishing power of ZAP70 and LPL expression with performance variables such as sensitivity, specificity and positive and negative predictive values (Table 2). LPL expression showed a specificity of 89.3% and a positive predictive value of 83.3%—a performance at least equal to the 2 ZAP70 methods.

LPL EXPRESSION AND OTHER PROGNOSTIC MARKERS
The scatter plot of the percentage of ZAP70-positive cells vs the LPL qPCR result for 50 patients (Fig. 3A) revealed a significant association between LPL and ZAP70 expression (68%; \( \chi^2(1) = 0.3939; P = 0.047 \)). When only the 22 unmutated cases were considered, this association was not statistically significant (odds ratio, 1.100; confidence interval, 0.1489–8.1254). Eleven patients (50%) with unmutated IGHV genes were positive for LPL and ZAP70 expression, 2 patients (9%) were negative for both tests, and 9 patients (41%) showed discordant results (4 positive for LPL expression and negative for ZAP70 expression; 5 LPL-negative and ZAP70-positive). LPL expression was also significantly associated with ZAP70 expression determined by qPCR analysis (70% concordance: \( \chi^2(1) = 9.1; P = 0.003 \)). With qPCR analysis, all 22 unmutated cases were positive for ZAP70 expression, and 15 were also positive for LPL expression.

Table 1. Cross-tabulations of prognostic markers vs IGHV gene mutation status.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>IGHV-U, %</th>
<th>IGHV-M, %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>57</td>
<td>26 (54)</td>
<td>31 (46)</td>
<td>NS</td>
</tr>
<tr>
<td>Male</td>
<td>34</td>
<td>12 (35)</td>
<td>22 (65)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>23</td>
<td>14 (61)</td>
<td>9 (39)</td>
<td></td>
</tr>
<tr>
<td>Binet A</td>
<td>50</td>
<td>21 (42)</td>
<td>29 (58)</td>
<td>NS</td>
</tr>
<tr>
<td>Binet B</td>
<td>5</td>
<td>4 (80)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>Binet C</td>
<td>2</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td></td>
</tr>
<tr>
<td>IGHV1-69, IGHV3-21, or IGHV3-23</td>
<td>11</td>
<td>7 (64)</td>
<td>4 (36)</td>
<td>NS</td>
</tr>
<tr>
<td>IGHV3-7, IGHV3-30, or IGHV4-34</td>
<td>14</td>
<td>4 (29)</td>
<td>10 (71)</td>
<td></td>
</tr>
<tr>
<td>Other IGHV gene segment</td>
<td>32</td>
<td>15 (47)</td>
<td>17 (53)</td>
<td></td>
</tr>
<tr>
<td>CD38 expression, ( \geq 30% )</td>
<td>9</td>
<td>5 (56)</td>
<td>4 (44)</td>
<td>NS</td>
</tr>
<tr>
<td>CD38 expression, ( &lt; 30% )</td>
<td>43</td>
<td>19 (44)</td>
<td>24 (56)</td>
<td></td>
</tr>
<tr>
<td>LDT ( \leq 12 ) months</td>
<td>13</td>
<td>11 (85)</td>
<td>2 (15)</td>
<td>0.004</td>
</tr>
<tr>
<td>LDT ( &gt; 12 ) months</td>
<td>44</td>
<td>15 (34)</td>
<td>29 (66)</td>
<td></td>
</tr>
<tr>
<td>del11q, del17p, or trisomy 12</td>
<td>18</td>
<td>13 (72)</td>
<td>5 (28)</td>
<td>0.02</td>
</tr>
<tr>
<td>No adverse cytogenetic aberrations</td>
<td>35</td>
<td>12 (34)</td>
<td>23 (66)</td>
<td></td>
</tr>
<tr>
<td>ZAP70 (flow cytometry)(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>24</td>
<td>19 (79)</td>
<td>5 (21)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Negative</td>
<td>33</td>
<td>7 (21)</td>
<td>26 (79)</td>
<td></td>
</tr>
<tr>
<td>ZAP70 (qPCR)(^b)</td>
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<td></td>
</tr>
<tr>
<td>Positive</td>
<td>33</td>
<td>25 (76)</td>
<td>8 (24)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Negative</td>
<td>24</td>
<td>1 (4)</td>
<td>23 (96)</td>
<td></td>
</tr>
<tr>
<td>LPL (qPCR)(^b, c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>18</td>
<td>15 (83)</td>
<td>3 (17)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Negative</td>
<td>32</td>
<td>7 (22)</td>
<td>25 (78)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Mutation status is based on a 98% cutoff value. IGHV-U indicates unmutated IGHV gene; IGHV-M, mutated IGHV gene.

\(^b\) Differentiation of positive and negative cases for ZAP70 and LPL expression was based on optimal cutoff values determined by ROC curve analysis.

\(^c\) LPL results are shown for lysed samples of whole blood.
We also calculated cross-tabulations of LPL expression with respect to other known prognostic markers. Patients positive for LPL expression were more likely to have one or more cytogenetic aberrations than patients negative for LPL expression ($\chi^2(1) = 4.9; P = 0.027$). LDT was also significantly associated with LPL expression (odds ratio, 0.07; confidence interval, 0.01–0.37).

A scatter plot of the percentage of cells positive for ZAP70 expression vs the ZAP70 qPCR result for 57 patients (Fig. 3B) revealed 42 patients (74%) with concordant results for both methods, 12 patients (21%, 6 unmutated and 6 mutated) negative for ZAP70 expression by flow cytometry but ZAP70-positive in the qPCR analysis, and 3 unmutated patients (5%) with a positive flow cytometric result and a negative result for ZAP70 expression by qPCR analysis. The association between the 2 methods was statistically significant ($\chi^2(1) = 12.881; P < 0.0001$).

**PROGNOSTIC VALUE OF LPL EXPRESSION AND OTHER MARKERS**

All 5 CLL-related deaths occurred in the patient subgroup characterized by unmutated IGHV genes and by ZAP70 and LPL expression positivity. Log-rank tests demonstrated a significant association between OS and IGHV mutation status ($P = 0.017$), ZAP70 protein production ($P = 0.006$), and LPL expression ($P = 0.002$). High CD38 levels ($P = 0.003$), short LDT ($P = 0.01$), and the presence of adverse cytogenetic aberrations ($P = 0.006$) were also significantly associated with survival. ZAP70 expression as determined by qPCR analysis did not have a predictive value with regard to OS. Similar results were obtained when the analysis was restricted to patients with stage A disease (results not shown). Of interest is that LPL expression appeared to be a strong predictor of not only OS but also TFS. LPL-positive patients had a significantly shorter TFS time (median, 23 months) than LPL-negative patients (median, 88 months; $P = 0.002$), whereas no significant difference in TFS could be demonstrated between ZAP70 expression–positive and –negative cases (flow cytometry, $P = 0.35$; qPCR, $P = 0.26$). Log-rank tests for the Kaplan–Meier curves for TFS (Fig. 4) also demonstrated a significant difference in TFS for patients differing in IGHV mutation status ($P = 0.03$), LDT ($P = 0.0008$), or the presence of cytogenetic abnormalities ($P = 0.018$). When only patients with stage A disease were considered, LPL expression, LDT, and the presence of cytogenetic abnormalities remained significant predictors of TFS ($P = 0.021, 0.0001$, and 0.034, respectively).

![Fig. 2. Correlation between LPL (A) or ZAP70 expression (B) and IGHV gene mutation status.](image)

LPL expression was determined by qPCR analysis of lysed samples of whole blood from 50 CLL patients. Blood samples from 57 CLL patients were analyzed by flow cytometry for ZAP70 expression. Optimal cutoff values for LPL and ZAP70 expression were determined by ROC curve analysis and are indicated by a horizontal line. Closed circles represent CLL-related deaths.

We also calculated cross-tabulations of LPL expression with respect to other known prognostic markers. Patients positive for LPL expression were more likely to have one or more cytogenetic aberrations than patients negative for LPL expression ($\chi^2(1) = 4.9; P = 0.027$). LDT was also significantly associated with LPL expression (odds ratio, 0.07; confidence interval, 0.01–0.37).

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**Table 2. Performance indices for ZAP70 and LPL expression with respect to the unmutated IGHV gene status.**

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>PPV, %</th>
<th>NPV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZAP70 (flow cytometry)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.1</td>
<td>83.9</td>
<td>79.2</td>
<td>78.8</td>
</tr>
<tr>
<td>ZAP70 (qPCR)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>96.2</td>
<td>74.2</td>
<td>75.8</td>
<td>95.8</td>
</tr>
<tr>
<td>LPL (qPCR)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>68.2</td>
<td>89.3</td>
<td>83.3</td>
<td>78.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> IGHV gene mutation status was based on a 98% cutoff value. PPV indicates positive predictive value; NPV, negative predictive value.
<sup>b</sup> Flow cytometric analysis of ZAP70 was performed with fresh blood samples ($n = 57$).
<sup>c</sup> ZAP70 expression was determined in CD19-selected cells ($n = 57$).
<sup>d</sup> LPL expression was determined in lysed samples of whole blood ($n = 50$).
has primarily been evaluated by flow cytometry and because we used qPCR analysis to quantify LPL expression, we evaluated both techniques. The 74% concordance rate for the 2 methods reflected the similarity in performance. Although ZAP70 expression is a valuable surrogate marker for IGHV mutation status, flow cytometry has some drawbacks. ZAP70 staining requires cytoplasmic permeabilization, which increases the complexity of the procedure. The use of different commercially available monoclonal antibodies, either directly or indirectly labeled with different fluorochromes, can lead to conflicting results in an individual patient. If present, ZAP70 expression in CLL-cells is weak compared with the strong but variable expression in T cells, which has been proposed as an internal control. Consequently, this variability can lead to false-positive results in an analysis based on the percentage of ZAP70-positive T cells (9). We recently demonstrated that this difficulty can be partially overcome by use of the more robust Kolmogorov–Smirnov statistic for the analysis of flow cytometric results (30). Finally, in vitro changes in ZAP70 expression necessitate prompt analysis after blood sampling. There has been no consensus on the best methodology until now, and there is a complete lack of assay standardization. We believe that flow cytometric measurement of ZAP70 is feasible in the context of larger clinical trials if well-calibrated results can be produced in one or a few laboratories. The molecular approach to measuring ZAP70 expression demands not only preparation of a mononuclear cell suspension but also isolation of B cells with high purity, because ZAP70 is strongly expressed in T and natural killer cells. Therefore, to look for other markers is tempting. LPL expression appears to be one of the better markers for distinguishing mutated and unmutated CLL cases (7, 17, 18). LPL expression can be measured in unpurified peripheral blood mononuclear cells, and expression in normal hematopoietic subsets is below or just above the detection cutoff point, thus limiting a potential confounding effect in the measurement of LPL in CLL cells (19). The procedure can be further simplified, because we observed no clinically significant difference in LPL expression between purified B cells and lysed whole blood. Thus, the absence of a cell-separation procedure allows working with blood-sampling tubes with RNA-stabilization reagents (31) or with stored samples of lysed blood. The 2 housekeeping genes used in our study can be reduced to one without a loss of prognostic power (data not shown). With IGHV mutation status as the comparison method, LPL expression showed a positive predictive value of 83% and a negative predictive value of 78%. Oppenzo et al. (17) obtained higher percentages (91% and 86%, respectively), but they used the LPL/ADAM29 ratio to distinguish between the 2 subgroups. Because ADAM29 expression is high in other blood cell types, such as monocytes, T cells, natural killer cells, and granulocytes (19), this approach is impossible to compare with ours, which is based on lysed samples of whole blood. From

![Fig. 3. Correlation between flow cytometric results for ZAP70 expression and (A) LPL qPCR results (n = 50) and (B) ZAP70 qPCR results (n = 57).](image)

LPL expression was determined by qPCR in lysed samples of whole blood, the percentage of ZAP70-positive CLL cells was determined by flow cytometry, and CD19-selected cells were analyzed by qPCR analysis for ZAP70 expression. Optimal cutoff values were obtained by ROC curve analysis and are indicated by quadrant borders. Mutated cases, unmutated cases, and CLL-related deaths are indicated by x’s, open circles, and closed circles, respectively.

**Discussion**

The search for reliable prognostic markers early in CLL disease began many years ago with the clinical staging systems of Rai et al. (3) and Binet et al. (4), but interest in such markers has renewed with the discovery of the prognostic value of IGHV mutation status in CLL B cells (5, 6). Several independent studies have confirmed the initial results (28, 29), but this technique is too laborious for a general diagnostic clinical laboratory. Gene expression studies have suggested a number of alternatives, with ZAP70 expression being among the most promising (7, 8). ZAP70 expression has been evaluated by different techniques, such as immunocytochemistry, qPCR analysis, and flow cytometry (9). Because ZAP70 expression

![Diagram A](image)

**Diagram A**

![Diagram B](image)

**Diagram B**

**Fig. 3. Correlation between flow cytometric results for ZAP70 expression and (A) LPL qPCR results (n = 50) and (B) ZAP70 qPCR results (n = 57).**
our data, we conclude that LPL expression is at least as powerful as ZAP70 expression for indicating IGHV mutation status in CLL.

Mutation status was, as expected, a reliable prognostic clinical marker for TFS, as well as for disease-related mortality. ZAP70 expression measured by flow cytometry or by qPCR appears to be a potential prognostic marker for TFS, but a demonstration of statistical significance requires more patients. The same was true for CD38 expression. LPL expression, however, is a highly statistically significant predictor of an emerging requirement for treatment ($P = 0.002$) and survival ($P = 0.002$). The presence of adverse cytogenetic aberrations and a short LDT also confirmed their prognostic value.

The strong prognostic value of LPL expression agrees with the results of others (17, 19, 20). Heintel et al. also
found an association between IGHV mutation status and LPL protein production. However, the magnitude of the difference in LPL mRNA levels between the 2 groups of patients (mutated vs unmutated) as assessed by qPCR analysis was greater than the difference in the intracellular LPL staining intensities (20).

Neither the function of LPL in CLL nor the molecular mechanisms regulating its synthesis are known. That CLL B cells express heparan sulfate (Peter Van Landschoot, personal communication), which can stabilize LPL expression on the cellular membrane, invites the speculation that membrane-associated LPL affects the biological behavior of CLL cells, such as cell spreading, migration, and intracellular signaling (32).

In conclusion, we have shown that measurement of LPL mRNA levels in whole blood correlates with IGHV mutation status, TFS and survival, and requires less time and labor than the determination of ZAP70 expression. Therefore, this method deserves to be incorporated into new or ongoing clinical trials that evaluate different treatment strategies in CLL, stratified by different prognostic profiles.

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