# *LPL* is the strongest prognostic factor in a comparative analysis of RNA-based markers in early chronic lymphocytic leukemia

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The online version of this article has a Supplementary Appendix.

## ABSTRACT

### Background
The expression levels of *LPL*, *ZAP70*, *TCL1A*, *CLLU1* and *MCL1* have recently been proposed as prognostic factors in chronic lymphocytic leukemia. However, few studies have systematically compared these different RNA-based markers.

### Design and Methods
Using real-time quantitative PCR, we measured the mRNA expression levels of these genes in unsorted samples from 252 newly diagnosed chronic lymphocytic leukemia patients and correlated our data with established prognostic markers (for example Binet stage, CD38, *IGHV* gene mutational status and genomic aberrations) and clinical outcome.

### Results
High expression levels of all RNA-based markers, except *MCL1*, predicted shorter overall survival and time to treatment, with *LPL* being the most significant. In multivariate analysis including the RNA-based markers, *LPL* expression was the only independent prognostic marker for overall survival and time to treatment. When studying *LPL* expression and the established markers, *LPL* expression retained its independent prognostic strength for overall survival. All of the RNA-based markers, albeit with varying ability, added prognostic information to established markers, with *LPL* expression giving the most significant results. Notably, high *LPL* expression predicted a worse outcome in good-prognosis subgroups, such as patients with mutated *IGHV* genes, Binet stage A, CD38 negativity or favorable cytogenetics. In particular, the combination of *LPL* expression and CD38 could further stratify Binet stage A patients.

### Conclusions
*LPL* expression is the strongest RNA-based prognostic marker in chronic lymphocytic leukemia that could potentially be applied to predict outcome in the clinical setting, particularly in the large group of patients with favorable prognosis.

Key words: LPL, RNA-based markers, chronic lymphocytic leukemia, prognosis.

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## Introduction

Chronic lymphocytic leukemia (CLL) accounts for roughly 30% of all leukemias in Western countries and is characterized by a heterogeneous clinical course.[1,2] Recognizing this, the Rai and Binet staging systems were developed approximately three decades ago to stratify patients according to disease burden and the degree of cytopenia.[3-5] However, these staging systems have a limited capacity to predict clinical outcome at an early stage of the disease. In the past decade, several biomarkers have been suggested as potential prognostic factors in CLL. These include the mutational status of the immunoglobulin heavy variable (*IGHV*) genes[6,7] and certain recurrent genomic aberrations.[8] Additionally, flow-cytometry analysis of CD38 and Zeta-chain-associated protein kinase 70 (ZAP70) are considered to be independent prognostic markers in CLL.[9,10] Given the difficulties in standardization of flow cytometry methods for ZAP70 measurement, analysis of mRNA expression levels has been proposed as a promising alternative.[11,12]

In recent years, several additional markers with prognostic potential in CLL have emerged. Lipoprotein lipase (*LPL*), initially identified in gene expression profiling of CLL, is one of the most differentially expressed genes in *IGHV* mutated *versus* unmutated CLL, where it is significantly higher expressed in unmutated cases.[13-15] In addition, T-cell leukemia/lymphoma 1 (*TCL1A*),[16,17] CLL-upregulated gene-1 (*CLLU1*)[18,19] and myeloid cell factor-1 (*MCL1*)[20] have also been found to display higher expression in unmutated CLL patients. Moreover, high expression of these markers at the protein and/or mRNA transcription level has been associated with inferior treatment-free and overall survival (OS) in a number of independent CLL cohorts.[19-26]

The current study aimed to validate and further investigate the prognostic strength of *LPL*, *ZAP70*, *TCL1A*, *CLLU1* and *MCL1* mRNA expression in CLL prognosis, either as single markers or in combination with established markers. Herein, we measured the mRNA expression levels in non-purified tumor samples from 252 newly diagnosed CLL patients from a Scandinavian population-based cohort. In summary, we found *LPL* expression to be the strongest RNA-based prognostic marker in CLL. In addition, we noted that high *LPL* expression was associated with poor outcome in favorable prognosis subgroups, including patients with Binet stage A, mutated *IGHV* genes, CD38 negativity or favorable cytogenetics.

## Design and Methods

### Patients

Two hundred and fifty-two CLL patients were included from the Swedish cohort of the Scandinavian Lymphoma Etiology (SCALE) study, a population-based case-control study including patients aged 18-74 years.[27] All cases were classified according to recently revised criteria and displayed the typical CLL immunophenotype ($CD5^+/CD19^+/CD23^+$).[28,29] The CLL samples, collected over the period from 1999 to 2002,[27] were obtained from peripheral blood and contained 70% or more tumor cells. Median time for sample collection was three months from the date of diagnosis. The study included 160 men and 92 women with a median age at diagnosis of 64 years (quartile range 57-69 years). The median follow-up time for the cohort was 102 months (quartile range 77-113 months). Survival data was available for all patients, while treatment data was obtained in 223 cases (88%) of whom 114 were treated. Binet stage was available in 239 cases (95%): stage A n=188, stage B n=39, and stage C n=12. Informed consent was obtained according to the Helsinki declaration and the study was approved by the local Ethics Review Committees.

### IGHV gene mutational analysis and cell surface CD38 expression

*IGHV* subgroup-specific polymerase chain reaction (PCR) and sequence analysis was performed on genomic DNA as previously described.[30] Sequences were aligned using the IMGT/V-QUEST tool in the IMGT database.[31,32] *IGHV* sequences with less than 98% identity to germline were classified as mutated, whereas cases with 98% or more identity were considered unmutated. Immunophenotyping for CD38-positivity was performed as previously described.[33] A 7% cut off was applied to delineate CD38 positive from negative samples.

### Analysis of recurrent genomic aberration

High-resolution genomic screening to detect del(17)(p13), +12, del(11)(q22), and del(13)(q14) was performed on 244 cases (97%) using Affymetrix 250K SNP-arrays from which data on known recurrent genomic aberrations were extracted, as described in a previous study.[34]

### Expression analysis of RNA-based markers

RNA was isolated from peripheral blood mononuclear cells (PBMCs) using spin-column technology (Qiagen, Hilden, Germany). RNA integrity and quality was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized from 400 ng total RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The transcription levels of *LPL*, *TCL1A*, *ZAP70*, *CLLU1* and *MCL1* were quantified using real-time quantitative PCR (RQ-PCR) and gene specific Taqman MGB® probes (Applied Biosystems, Foster City, CA, USA) (*Online Supplementary Table S1*) and the data were normalized against internal beta-2-microglobulin (β2M) expression levels using the comparative Ct method. The reactions were run on a Stratagene Mx 3005 instrument (La Jolla, CA, USA).

### Statistical analysis

Receiver operating characteristics (ROC) curve analysis was applied to calculate the expression cut-off value for each RNA-based marker. The cut-off value predicting survival as above or below cohort median with the highest sensitivity and specificity was used for further analysis. Shapiro-Wilk's test was applied to assess normal distribution, the Mann-Whitney U test was used to compare data in subgroups while the $\chi^2$ test was utilized to investigate the association between the RNA-based markers and other prognostic markers. Kaplan-Meier analysis was performed to construct survival curves. OS was measured from the date of diagnosis to either the last follow-up date (defined as censored) or death (all deaths included), whereas time to first treatment (TTT) was defined as date of diagnosis until the starting date of initial treatment or last follow up. A multivariate log rank test was used to assess differences. Cox's proportional hazards model was applied to evaluate independent associations between single risk factors and OS as well as TTT. All statistical analyses were carried out using Statistica version 9.1 (Stat Soft, Tulsa, OK, USA).

## Results

### Prognostic markers and clinical outcome

As expected, Binet staging, *IGHV* gene mutational status, recurrent genomic aberrations and CD38 expression were all significant prognostic factors of clinical outcome (Table 1).[4-8] For the RNA-based markers, mRNA expression analysis of *LPL*, *ZAP70*, *CLLU1* and *TCL1A* was performed in all 252 cases, while *MCL1* expression was measured in 248 of the cases included in this study. The expression cut off for each RNA-based marker was defined using ROC curve analysis as follows: $6.90\times10^{-5}$ for *LPL*, $1.25\times10^{-2}$ for *ZAP70*, $6.97\times10^{-3}$ for *TCL1A*, $1.75\times10^{-3}$ for *CLLU1* and $3.02\times10^{-1}$ for *MCL1*. High expression of *LPL*, *ZAP70*, *TCL1A* and *CLLU1* was associated with shorter OS and TTT, while no significant difference in outcome was observed for *MCL1* expression (Table 1, Figures 1 and 2). *LPL* was most significant in delineating OS and TTT between low and high expressing groups ($P<0.00001$). In sub-analyses excluding patients treated within six months of diagnosis to distinguish between progressive disease and advanced disease at diagnosis, *LPL*, *ZAP70*, *CLLU1* and *TCL1A* all remained prognostic of time to first treatment (*Online Supplementary Figure S1*).

### Multivariate analysis of prognostic markers

In multivariate analysis including only the RNA-based markers, *LPL* expression was the only significant independent prognostic factor for OS as well as TTT (Table 2). This finding also held true when studying Binet stage A patients only (*Online Supplementary Table S2*). In a multivariate model including *LPL* expression and the established markers, *LPL* expression remained a strong marker for OS, but was not formally significantly associated with TTT (Table 3A). *LPL* expression is strongly associated with *IGHV* mutational status (Figure 3), and if *IGHV* mutational status was excluded from the analysis, *LPL* was highly predictive also for TTT (*Online Supplementary Table S3*). Similar associations were observed when only Binet stage A patients were considered (Tables 3B and 3C).

### RNA-based markers in relation to other prognostic markers

We investigated the expression distribution of the RNA-based markers in relation to *IGHV* gene mutational status,

**Table 1. Overall survival and time to treatment in a Swedish cohort of CLL patients according to established and RNA-based prognostic markers.**

| Variable | Overall survival | | | Time to treatment | | |
|---|---|---|---|---|---|---|
| | N | Median (months) | P | N | Median (months) | P |
| Binet stage | 239 | | <0.0001* | 220 | | <0.0001* |
| A | 188 | NR | | 173 | NR | |
| B | 39 | 81 | | 37 | 2 | |
| C | 12 | 73 | | 10 | 1 | |
| *IGHV* mutational status | 244 | | <0.0001 | 215 | | <0.0001 |
| Mutated | 158 | NR | | 142 | NR | |
| Unmutated | 86 | 83 | | 73 | 14 | |
| Chromosomal aberrations | 244 | | <0.0001* | 215 | | <0.0001* |
| del(13q) | 113 | NR | | 98 | NR | |
| No aberration | 73 | NR | | 63 | NR | |
| Trisomy 12 | 20 | 85 | | 18 | 18 | |
| del(11q) | 28 | 91 | | 26 | 6 | |
| del(17p) | 10 | 49 | | 10 | 1 | |
| *CD38* | 252 | | <0.0001 | 223 | | <0.0001 |
| <7% | 169 | NR | | 148 | NR | |
| >7% | 83 | 87 | | 75 | 14 | |
| *LPL* | 252 | | <0.0001 | 223 | | <0.0001 |
| Low | 145 | NR | | 129 | NR | |
| High | 107 | 87 | | 94 | 18 | |
| *ZAP70* | 252 | | <0.01 | 223 | | <0.01 |
| Low | 136 | NR | | 117 | NR | |
| High | 116 | 104 | | 106 | 39 | |
| *TCL1A* | 252 | | <0.01 | 223 | | <0.01 |
| Low | 127 | NR | | 115 | NR | |
| High | 125 | 109 | | 108 | 41 | |
| *CLLU1* | 252 | | <0.01 | 223 | | <0.001 |
| Low | 136 | NR | | 120 | NR | |
| High | 116 | 108 | | 103 | 26 | |
| *MCL1* | 248 | | 0.44 | 220 | | 0.92 |
| Low | 126 | NR | | 111 | 53 | |
| High | 122 | NR | | 109 | 57 | |

*NR: not reached. NS: not significant. *The P value represents a combined P value for the analysis and indicates that at least one group differs significantly from the rest.*

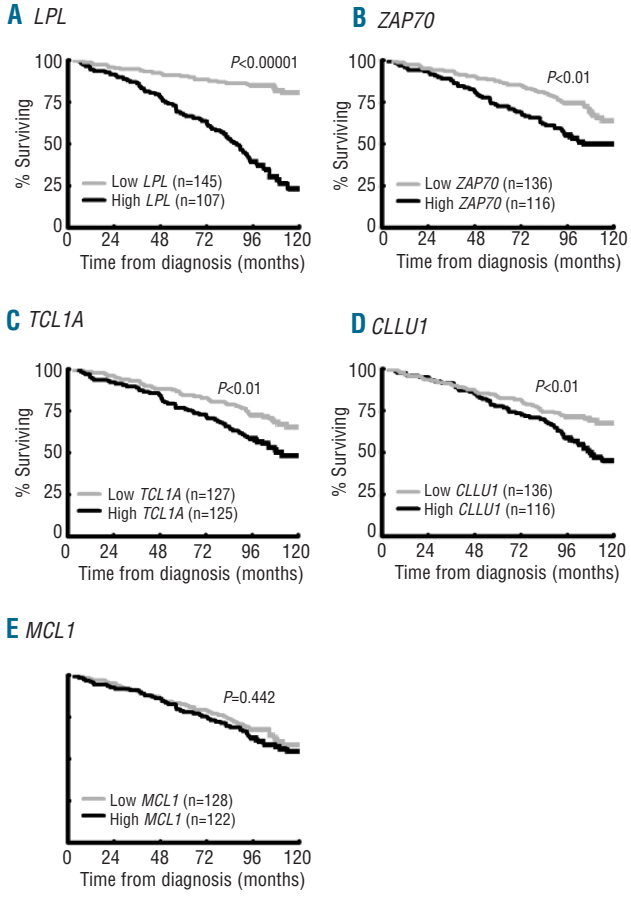



**Figure 1. Expression status of RNA-based markers and overall survival. Kaplan-Meier analysis of overall survival of CLL cases according to the expression status of (A) *LPL*, (B) *ZAP70*, (C) *TCL1A*, (D) *CLLU1* and (E) *MCL1*.**

genomic aberrations, Binet stage and other prognostic markers (*Online Supplementary Table S4*). Briefly, all markers except *MCL1* were differentially expressed when comparing *IGHV* mutated and unmutated cases (Figure 3), whereas high expression of all markers except *TCL1A* was associated with high CD38 expression. Furthermore, *LPL* and *CLLU1* displayed differential expression with regard to Binet stage and recurrent genomic aberrations, while no significant differences in expression were found for any marker when studying patient gender and age at diagnosis.

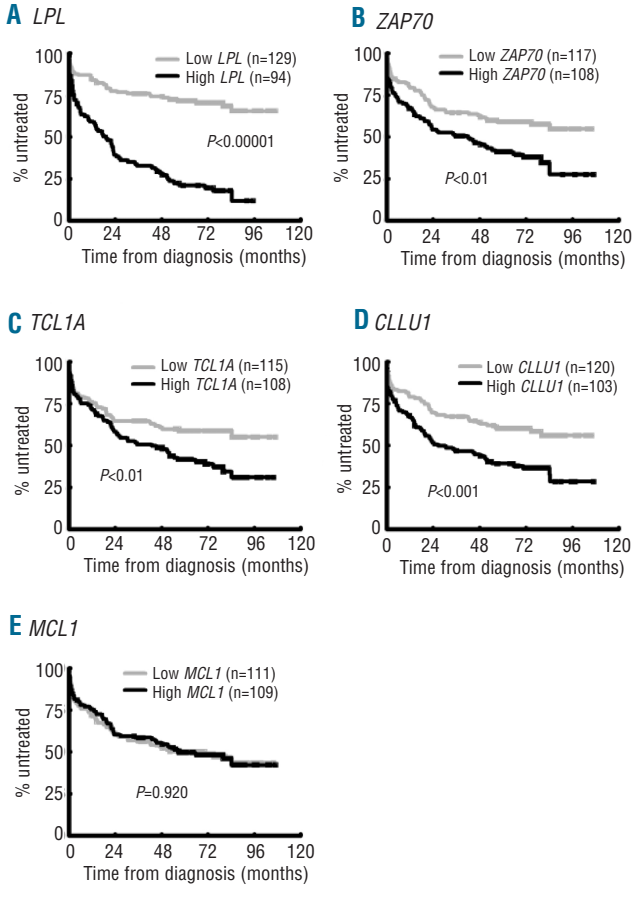



**Figure 2.** Expression status of the RNA-based markers and time to treatment. Kaplan-Meier analysis of time to treatment of CLL cases according to the expression status of (A) *LPL*, (B) *ZAP70*, (C) *TCL1A*, (D) *CLLU1* and (E) *MCL1*.

**Table 2.** Multivariate Cox regression analysis of RNA-based markers.

| Variable | Overall survival (N=252) | | | Time to treatment (N=223) | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | P | HR | 95% CI | P |
| *LPL* | 5.85 | 3.52-9.71 | <0.00001 | 3.67 | 2.37-5.68 | <0.00001 |
| *ZAP70* | 1.27 | 0.84-1.93 | 0.25 | 1.47 | 0.99-2.19 | 0.06 |
| *TCL1A* | 1.25 | 0.82-1.91 | 0.29 | 1.12 | 0.75-1.66 | 0.58 |
| *CLLU1* | 0.80 | 0.52-1.24 | 0.32 | 1.09 | 0.73-1.63 | 0.68 |
| *MCL1* | 1.12 | 0.74-1.70 | 0.59 | 0.85 | 0.58-1.26 | 0.42 |

*The threshold values used in the analysis were determined based on ROC curve analysis. HR: Hazard ratio. CI: Confidence interval.*

**Table 3A.** Multivariate Cox regression analysis of *LPL* and established markers.

| Variable | Overall survival (N=208) | | | Time to treatment (N=208) | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | P | HR | 95% CI | P |
| Age at diagnosis | 2.36 | 1.49-3.76 | <0.001 | 0.97 | 0.66-1.43 | 0.89 |
| Gender | 1.58 | 0.96-2.61 | 0.07 | 1.13 | 0.75-1.71 | 0.54 |
| Binet stage | 3.18 | 1.98-5.11 | <0.0001 | | | |
| *IGHV* mutational status | 1.94 | 1.06-3.56 | 0.03 | 2.43 | 1.35-4.38 | <0.01 |
| Trisomy 12 | 1.19 | 0.58-2.43 | 0.63 | 1.27 | 0.68-2.40 | 0.45 |
| del(11q) | 0.91 | 0.51-1.64 | 0.76 | 1.38 | 0.79-2.42 | 0.26 |
| del(17p) | 4.62 | 2.02-10.61 | <0.001 | 2.02 | 0.89-4.58 | 0.09 |
| CD38 | 1.24 | 0.75-2.06 | 0.40 | 1.63 | 1.01-2.64 | 0.04 |
| *LPL* | 2.77 | 1.46-5.33 | <0.01 | 1.61 | 0.91-2.89 | 0.10 |

*HR: Hazard ratio, CI: Confidence interval. The threshold values used in the analysis were as follows; age at diagnosis: above vs. below median (63.9 yrs); Binet stage: A vs. B/C;* IGHV *mutation status: mutated (<98% germline identity homology) vs. unmutated; CD38: 7%; recurrent genomic aberrations: HR is given in comparison to cases with no detected aberrations/del(13q);* LPL *threshold value based on ROC curve analysis. For analysis of TTT, Binet stage was removed since most Binet stage B/C patients receive treatment at or shortly following diagnosis.*

**Table 3B.** Multivariate Cox's regression analysis of *LPL* and established markers within Binet A subgroup.

| Variable | Overall survival (N=174) | | | Time to treatment (N=174) | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | P | HR | 95% CI | P |
| Age at diagnosis | 2.54 | 1.36-4.73 | < 0.01 | 1.05 | 0.63-1.78 | 0.84 |
| Gender | 1.66 | 0.90-3.06 | 0.10 | 0.83 | 0.49-1.42 | 0.50 |
| *IGHV* mutational status | 2.90 | 1.36-6.15 | < 0.01 | 4.19 | 1.98-8.88 | < 0.001 |
| Trisomy 12 | 1.07 | 0.42-2.75 | 0.89 | 1.80 | 0.78-4.17 | 0.17 |
| del(11q) | 0.49 | 0.21-1.17 | 0.11 | 1.25 | 0.59-2.64 | 0.57 |
| del(17p) | 2.94 | 0.96-9.05 | 0.06 | 1.41 | 0.39-5.09 | 0.60 |
| CD38 | 1.08 | 0.55-2.14 | 0.82 | 1.24 | 0.65-2.37 | 0.51 |
| *LPL* | 4.34 | 2.03-9.27 | < 0.001 | 1.69 | 0.79-3.60 | 0.17 |

*The threshold values used in the analysis are as follows; age at diagnosis: median (63.9); IGHV mutational status: mutated vs. unmutated; recurrent genomic aberrations: HR is given in comparison to cases with no detected aberrations/del(13q); CD38: 7%; LPL: threshold value based on ROC curve analysis. HR: Hazard ratio, CI: Confidence interval.*

**Table 3C.** Multivariate Cox regression analysis of *LPL* and established markers excluding *IGHV* mutational status within Binet A subgroup.

| Variable | Overall survival (N=180) | | | Time to treatment (N=165) | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | P | HR | 95% CI | P |
| Age at diagnosis | 2.68 | 1.44-4.96 | < 0.01 | 1.03 | 0.62-1.71 | 0.91 |
| Gender | 1.60 | 0.88-2.91 | 0.12 | 0.86 | 0.51-1.44 | 0.56 |
| Trisomy 12 | 1.04 | 0.42-2.63 | 0.93 | 1.95 | 0.87-4.40 | 0.11 |
| del(11q) | 0.58 | 0.25-1.36 | 0.21 | 1.64 | 0.79-3.39 | 0.19 |
| del(17p) | 3.20 | 1.05-9.75 | 0.04 | 1.85 | 0.52-6.53 | 0.34 |
| CD38 | 1.77 | 0.97-3.23 | 0.06 | 1.94 | 1.08-3.50 | 0.03 |
| *LPL* | 6.88 | 3.51-13.50 | < 0.0001 | 3.34 | 1.81-6.17 | < 0.001 |

*The threshold values used in the analysis are as follows; age at diagnosis: median (63.9); recurrent genomic aberrations: HR is given in comparison to cases with no detected aberrations/del(13q); CD38: 7%;* LPL*: threshold value based on ROC curve analysis. HR: Hazard ratio, CI: Confidence interval.*

When studying Binet stage A patients, high expression of *LPL*, *ZAP70* and *CLLU1* was associated with shorter OS, while high expression of *LPL*, *ZAP70*, *CLLU1* and *TCL1A* was found to add prognostic information in the analysis of TTT (Figure 4, *Online Supplementary Figure S2*). The additive prognostic information of the different RNA-based markers in subgroups of the established markers is summarized in Table 4. Notably, high *LPL* expression also added significant prognostic information to favorable-prognostic subgroups such as patients with mutated *IGHV* genes, CD38 negativity or favorable recurrent genomic aberrations.

Further analysis of Binet stage A patients showed that LPL expression stratified $CD38^+$ cases in terms of OS as well as TTT (Figure 5). Within the $CD38^-$ group of patients, *LPL* expression was again found to further subdivide clinical outcome (Figure 5). Cox's regression analysis revealed the highest risk of death and highest risk of treatment initiation for the group with high *LPL* expression and CD38 positivity (Figure 5).

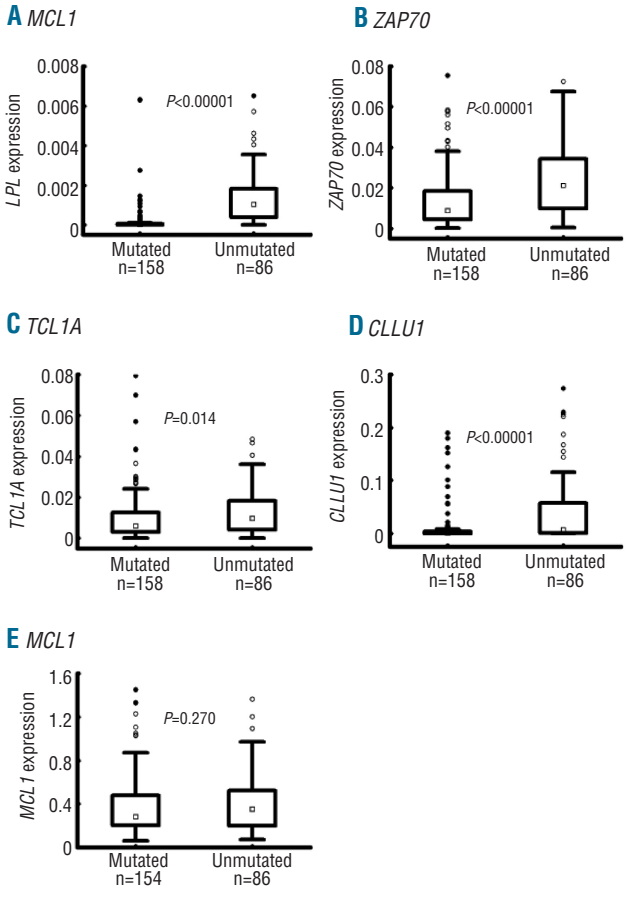



**Figure 3. The expression levels of RNA-based markers in relation to *IGHV* mutation status. Box plots depict the expression levels of (A) *LPL*, (B) *ZAP70*, (C) *TCL1A*, (D) *CLLU1* and (E) *MCL1* within CLL cases carrying mutated and unmutated *IGHV* genes. The box plots show median, 25 and 75 percentile values, non-outlier ranges, outliers and extremes. *P* values are derived from Student's t-test.**

## Discussion

This study set out to identify the most reliable prognostic factor among recently proposed RNA-based markers. In addition, we aimed to investigate the suitability of RNA-based markers in the clinical setting. Since sorting of tumor cells may not be feasible in routine diagnostic laboratories, we chose to analyze unsorted samples. There are several lines of evidence that provide further support for our approach. For example, *LPL* is not expressed in normal B cells and found in very low levels in CD19 negative cells in CLL patients.[15,22] Moreover, differences found in *TCL1A* expression were negligible when comparing sorted *versus* unsorted CLL samples from the same patients.[17] Finally, *CLLU1* expression has previously been reported to be restricted to CLL tumor cells.[18]

In this study, the expression levels of *LPL*, *ZAP70*, *TCL1A* and *CLLU1* predicted OS and time to first treatment in the current cohort of CLL, where high expression of these markers was significantly associated with unfavorable outcome. Particularly, *LPL* expression was found to be the most significant marker for analysis of OS and time to first treatment. In contrast to earlier reports on smaller cohorts,[24,25] the prognostic potential of *MCL1* expression could not be confirmed in this study. In addition, we analyzed TTT excluding patients treated within six months of diagnosis to evaluate disease progression rather than advanced disease at diagnosis. Using this approach, *LPL*, *ZAP70*, *TCL1A* and *CLLU1* all remained significant for the analysis of TTT, where *LPL* appeared to be most informative. Our findings are in contrast to a recent study in which *ZAP70* expression in sorted tumor cells was shown to be a powerful prognostic factor.[12] Since we have performed our analysis on unsorted CLL samples, the expression of *ZAP70* by other immune cells, including T cells, NK cells and normal B cells, may interfere with *ZAP70* quantification in the tumor population,[35] thus *ZAP70* provides less information than *LPL* in non-purified cells.[36]

Among the RNA-based markers, *LPL* expression was the only significant independent prognostic factor for OS and TTT in multivariate analysis. In subsequent analysis, when including *LPL* and the established markers, *LPL* remained an independent prognostic factor for overall survival, also among Binet stage A patients only. When studying TTT, *LPL* lost its significance as an independent factor, probably due to its close relation to the *IGHV* mutational status,[12,36] but regained its significance when the mutational status was excluded. Accordingly, a 73-fold higher median *LPL* expression was found in unmutated *versus* mutated cases (Figure 3), making a clear distinction between the two groups of patients. Therefore, *LPL* expression could be a useful surrogate marker for *IGHV* gene mutational status.

All RNA-based markers, albeit to varying degrees, were found to add prognostic information to the established markers. Here, *LPL* expression out-performed the other markers being able to significantly stratify patients with mutated *IGHV* genes, Binet stage A, favorable recurrent genomic aberrations and CD38 expression for both OS and TTT. Furthermore, *CLLU1* expression successfully stratified patients with Binet stage A while *MCL1* expression was the only significant marker for the analysis of OS and TTT in Binet stage B/C patients (Table 4). This latter finding, however, needs to be confirmed in a larger cohort of patients.

**Table 4.** The prognostic information of RNA-based markers in subgroups of established markers

| Variable | N | | Log-rank *P* values | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *LPL* | | *ZAP70* | | *TCL1A* | | *CLLU1* | | *MCL1** | |
| | OS | TTT | OS | TTT | OS | TTT | OS | TTT | OS | TTT | OS | TTT |
| Binet Stage | | | | | | | | | | | | |
| A | 175 | 160 | < 0.001 | < 0.001 | < 0.01 | 0.03 | 0.03 | < 0.001 | 0.001 | 0.01 | NS | NS |
| B/C | 47 | 43 | NS | NS | NS | NS | 0.02 | NS | NS | NS | 0.04 | 0.05 |
| IGHV mutational status | | | | | | | | | | | | |
| Mutated | 158 | 142 | < 0.001 | 0.03 | NS | NS | NS | 0.03 | 0.02 | NS | NS | NS |
| Unmutated | 86 | 73 | < 0.01 | NS | NS | NS | NS | NS | NS | NS | NS | NS |
| Genomic aberrations | | | | | | | | | | | | |
| del(13q) | 113 | 98 | < 0.001 | < 0.001 | NS | 0.04 | 0.03 | 0.03 | 0.008 | NS | 0.02 | < 0.01 |
| No aberration | 73 | 63 | < 0.001 | < 0.01 | NS | NS | NS | NS | NS | NS | 0.05 | NS |
| Trisomy 12 | 20 | 18 | 0.02 | NS | 0.02 | NS | NS | NS | NS | NS | NS | NS |
| del(11q) | 28 | 26 | NS | NS | NS | NS | NS | NS | NS | 0.03 | < 0.01 | 0.02 |
| del(17p) | 10 | 10 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| CD38 | | | | | | | | | | | | |
| < 7% | 169 | 148 | < 0.001 | < 0.001 | NS | NS | NS | 0.04 | 0.03 | 0.04 | NS | NS |
| ≥ 7% | 83 | 75 | < 0.001 | < 0.01 | 0.04 | NS | NS | NS | NS | NS | NS | NS |

*NS: not significant. NA: not available; log rank test could not be performed due to low number of cases. *The number of cases included in the analysis of OS and TTT for MCL1 are as follows: Binet stage A (n=172 and n=157), Binet stage B/C (n=47 and n=43), mutated* IGHV *genes (n=154 and n=139), unmutated* IGHV *genes (n=86 and n=73), del(13q) (n=111 and n=96), no aberration (n=71 and n=62), trisomy 12 (n=20 and n=18), del(11q) (n=28 and n=26), del(17p) (n=10 and n=10), CD38 negative cases (n=165 and n=145) and CD38 positive cases (n=83 and n=75).*

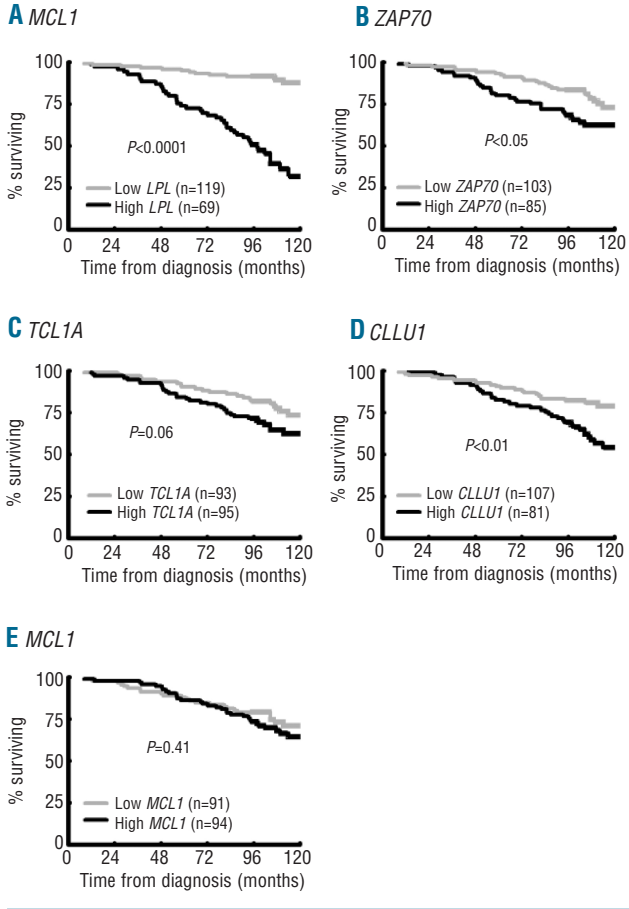
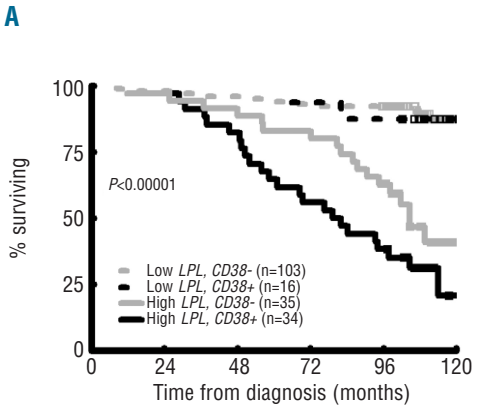



**Figure 4.** Overall survival of patients within Binet A subgroup. Kaplan-Meier analysis of overall survival of CLL cases according to the expression status of (A) ***LPL***, (B) ***ZAP70***, (C) ***TCL1A***, (D) ***CLLU1*** and (E) ***MCL1***.

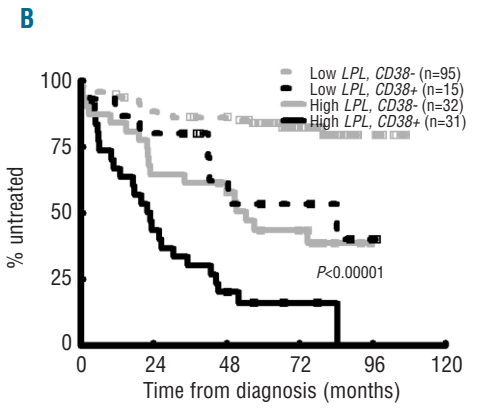
Although several reports have investigated individual RNA-based markers in CLL prognosis, few studies have systematically compared the relative prognostic strength between multiple RNA-based markers. van't Veer *et al.* demonstrated that *LPL* expression was the best predictive marker for survival among ten different RNA-based markers in unsorted CLL samples, equaling *IGHV* gene mutational status in strength and out-performing *ZAP70* expression.[36] Furthermore, in two recent studies, *LPL* expression in CD19 sorted CLL samples was again shown to be a strong predictor of clinical outcome in different panels of RNA-based markers.[37,38] Interestingly, our results agree with these findings, even though there is little overlap in genes analyzed between the studies, and further support the prognostic strength of *LPL* expression in CLL.

The vast majority of CLL patients have indolent disease at diagnosis but may still experience progression. Thus, prognostic markers in Binet stage A patients are particularly useful. Analysis of the *IGHV* gene mutational status is quite laborious and our identification of *LPL* expression as a surrogate marker has a potential clinical role. We also found that *LPL* expression in combination with CD38 expression could further subdivide CLL patients. Binet A patients with high expression of both *LPL* and CD38 showed the worst clinical outcome, while those with low expression of both markers appeared to have the most favorable clinical outcome; patients with mixed pattern showed an intermediate outcome for time to first treatment (Figure 5). Similarly, *ZAP70* and CD38$^+$ Binet stage A patients recently showed the highest risk for needing treatment.[39] These data provide prognostic stratification for good-prognosis patients which might be useful in a clinical setting.

In summary, our current study verifies the prognostic value of the transcriptional status of *LPL*, *ZAP70*, *TCL1A* and *CLLU1* with respect to OS and TTT. Furthermore, we




| Risk category | Univariate analysis OS, n=188 |
|---|---|
| | HR (95% CI, *P* value) |
| Low *LPL*, *CD38-* | 1 |
| Low *LPL*, *CD38+* | 0.34 (0.08-1.40, *P*=0.14) |
| High *LPL*, *CD38-* | 2.47 (1.40-4.36, *P*=0.002) |
| High *LPL*, *CD38+* | 5.49 (3.19-9.41, *P*=0.0001) |




| Risk category | Univariate analysis TTT, n=173 |
|---|---|
| | HR (95% CI, *P* value) |
| Low *LPL*, *CD38-* | 1 |
| Low *LPL*, *CD38+* | 1.22 (0.56-2.66, *P*=0.63) |
| High *LPL*, *CD38-* | 1.85 (1.08-3.18, *P*=0.03) |
| High *LPL*, *CD38+* | 5.21 (3.15-8.64, *P*<0.0001) |

**Figure 5. *LPL* and CD38 expression status subdivides overall survival and time to treatment in the Binet A subgroup.** Kaplan-Meier curves analysis of overall survival (A) and time to treatment (B) within the Binet A subgroup of patients according to *LPL* and CD38 expression status. Cox's univariate analysis indicates hazard ratios (HR) for different marker combinations for overall survival and time to treatment analysis. The threshold values used in the analysis are as follows; LPL threshold value based on ROC curve analysis; CD38: 7%.

report that the *LPL* expression status is the strongest RNA-based marker for clinical outcome using unsorted CLL cells. We also demonstrate that *LPL* expression in combination with CD38 expression can further subdivide Binet stage A patient outcome. Our data thus imply that RQ-PCR analysis of *LPL* could be used as an important prognostic marker in clinical routine practice, especially for indolent cases of CLL. The fact that no cell sorting appears necessary and the distinct differences in expression between poor *versus* good prognosis patients are additional advantages which may facilitate the analysis in diagnostic laboratories. RQ-PCR is routinely being applied for diagnostic and prognostic purposes in leukemia, e.g. in chronic myelogeneous leukemia.[40] In spite of this, further efforts are needed to standardize the *LPL* measurement techniques. In addition, *LPL* expression during the course of disease still needs to be investigated. Large collaborative efforts to address these issues are, therefore, required.

## Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.*

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