

# Clinical significance of LAIR1 (CD305) as assessed by flow cytometry in a prospective series of patients with chronic lymphocytic leukemia

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## ABSTRACT

Most patients affected by chronic lymphocytic leukemia are diagnosed by flow cytometry. Several immunophenotypic markers have been identified as significant and independent prognostic variables, especially from retrospective cohorts. However, while attractive because their detection is inexpensive and feasible in most laboratories, only few have been validated by independent series. The expression of leukocyte-associated immunoglobulin-like receptor-1 (also known as LAIR1, LAIR-1 or CD305), an inhibitor of B-cell receptor-mediated signaling, has been reported to be lacking in high-risk chronic lymphocytic leukemia. However, its correlation with biological variables and its prognostic significance remain unknown. We investigated 311 consecutive patients, prospectively enrolled since 2007. Methods for studying patients were standardized and included clinical assessment, immunophenotype, fluorescence *in situ* hybridization, and status of immunoglobulin heavy chain variable region genes. Overall, 22.1% of patients had Binet stage B or C disease, 38.5% had unmutated immunoglobulin genes, 15.1% had high-risk cytogenetic abnormalities, 23.4% were CD38<sup>+</sup>, 37.8% CD49d<sup>+</sup>, and 59.8% LAIR1<sup>+</sup>. Expression of LAIR1 was inversely related to that of CD38 ( $P=0.0005$ ), but was not associated with CD49d expression ( $P=0.96$ ). A significantly lower expression of LAIR1 was observed in patients with Binet stage B or C disease ( $P=0.023$ ), and in the presence of high-risk cytogenetic abnormalities ( $P=0.048$ ) or unmutated immunoglobulin heavy chain variable region genes ( $P<0.0001$ ). At univariate analysis LAIR1<sup>+</sup> was significantly associated with longer time to first treatment ( $P=0.0002$ ). This favorable effect of LAIR1<sup>+</sup> was confirmed by multivariate analysis (hazard ratio=2.1,  $P=0.03$  for LAIR1). Our results indicate that LAIR1 expression is a reliable and inexpensive marker capable of independently predicting time to first treatment in newly diagnosed unselected patients with chronic lymphocytic leukemia.

## Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with a highly variable clinical course. Some patients have a life expectancy which resembles that of the age-matched general population, while others progress and need treatment within a few months of diagnosis. Several clinical and biological variables, some of which validated in prospective studies,<sup>1</sup> have been reported to predict the outcome of CLL patients when assessed at presentation of the leukemia. Among them, the old-fashioned but still widely used clinical staging systems initially proposed by Rai and/or Binet,<sup>2,3</sup> or the more demanding mutational status of the variable region of the heavy-chain locus of the immunoglobulin genes (*IGHV*) and fluorescent *in situ* hybridization are the hallmarks for discriminating patients with an aggressive or indolent clinical course.<sup>4,5</sup> Although the latter methods have been standardized, tests are still expensive and cannot be provided by all laboratories. For this reason, the search for new cytoflu-

orimetric markers is still of great interest, especially after CD38 and ZAP-70 have been shown to have major limitations, the former because of its low prognostic power and the latter because of well-recognized technical problems.<sup>1,6,7</sup> Recently, CD49d and other markers, such as CD25, CD26 and CD69, have been advocated as being predictive and reliable in identifying patients with peculiar molecular characteristics of disease and different prognoses.<sup>8-12</sup>

Leukocyte-associated immunoglobulin-like receptor-1 (LAIR1), also known as CD305, is a transmembrane glycoprotein that acts as an inhibitory receptor and is expressed by most immune cells. The known LAIR1 ligands are extracellular matrix collagen and C1q, the first component of the complement.<sup>13,14</sup> LAIR1 expression varies during B-cell differentiation and has recently been demonstrated in patients with CLL.<sup>15,16</sup> The *in-vivo* role of LAIR1 in B cells consists in its inhibiting B-cell receptor (BCR)-mediated signaling<sup>15</sup> and in controlling kinase pathways involved in cell proliferation.<sup>15</sup> Recently, two studies performed in CLL patients showed that LAIR1 is more

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expressed in early stages of CLL than in advanced CLL<sup>17</sup> and that its expression is lower in patients with high-risk CLL.<sup>16</sup>

The association of LAIR1 expression with commonly recognized clinical and biological variables, and its prognostic role in patients with CLL is still unknown. In this study we analyzed LAIR1 expression in a prospective cohort of 311 unselected CLL patients consecutively enrolled in the “CLL Veneto” registry, showing that the expression of this molecule has a relevant impact on disease progression.

## Methods

### Patients, follow-up and end-points

Patients with a new diagnosis of CLL presenting to three major Institutions of the Veneto region were enrolled in a prospective, regional registry since 2007, which formed the basis for biological and clinical investigations (CLL Veneto project). Three hundred and eleven patients affected by CLL were enrolled in the present study from the Hematology Departments of Vicenza, Verona and Padua. The registry and this study were approved by the Institutional Review Boards of each participating Institution and all patients signed informed consent before any study procedure. All patients met the International Workshop CLL (IWCLL) criteria for the diagnosis of CLL and were treated following standard criteria for therapy onset in CLL.<sup>18</sup>

All samples for immunophenotypic, cytogenetic and molecular analyses were obtained from peripheral blood or bone marrow specimens at presentation of the patients and, therefore, before the administration of any cytotoxic treatment.

Patients were prospectively accrued between February 2007 and April 2013. The median follow-up from the time of CLL diagnosis was 32 months (range, 1-75.1). Time-to-first-treatment (TTFT) was chosen as main parameter to ascertain tumor aggressiveness. Indeed, only 15 of the 311 patients have died so far, which prevented the use of overall survival as the study's end-point. Furthermore, TTFT was a reliable end-point for assessing tumor progression because the reasons for and timing of starting cytotoxic treatment were standardized among centers by the “CLL Veneto” project, resembling previously reported international guidelines.<sup>18</sup>

### Immunophenotypic analysis

Anticoagulated peripheral blood samples were used for immunophenotypic analysis. The samples were analyzed within 24 hours of collection at the flow-cytometry laboratories of the three hematology centers. Immunophenotyping was performed according to well-established techniques,<sup>19</sup> and is briefly described in the *Online Supplementary Methods*.

Besides standard immunophenotypic markers required for the diagnosis of CLL,<sup>18</sup> all patients were analyzed for the expression of CD38 (HB7 clone), CD49d (9F10 clone), and CD305 (DX26 clone). The cut-off value for LAIR1 positivity was 30%, as reported in *Online Supplementary Figure S1* and the *Online Supplementary Methods*.

### Fluorescence in situ hybridization and IGHV mutation analysis

Cytogenetic abnormalities involving deletions in chromosomes 11q23, 13q14, 17p12, and trisomy 12 were evaluated by interphase fluorescence *in situ* hybridization as previously described.<sup>20,21</sup> A complex karyotype was defined as the presence of three or more abnormalities.

Total RNA was obtained from peripheral blood specimens and

the *IGHV* mutation analysis was performed as previously described.<sup>20,21</sup>

### Statistical methods

Data are presented as relative frequencies or mean/median values and their standard deviation, as well as range, for each categorical or continuous variable under study. The differences between groups, the correlations between variables, and the survival statistics were calculated using the SPSS 20 software (IBM Corp., Armonk, NY, USA) as described in the *Online Supplementary Methods*.

## Results

### Clinical and biological features according to LAIR1 expression

Overall, LAIR1 was positive in 186 (59.8%) of the 311 CLL patients. There were significantly different distributions of some clinical and biological features between the LAIR1 positive (LAIR1<sup>+</sup>) and negative (LAIR1<sup>-</sup>) patients, as reported in Table 1 and Figure 1. In particular, LAIR1<sup>+</sup> patients presented with significantly lower clinical stage, had a lower rate of *IGHV*-unmutated cases, and more favorable cytogenetic lesions as compared to LAIR1<sup>-</sup> patients. Moreover, a positive direct antiglobulin test was detected more frequently in LAIR1<sup>-</sup> patients, which was consistent with the finding of a significantly higher occurrence of autoimmune hemolytic anemia at CLL presentation in LAIR1<sup>-</sup> patients.

### LAIR1 expression and other immunophenotypic prognostic markers

Overall, CD38 and CD49d were positive in 23.4% and in 37.8% of patients, respectively (Table 1).

When we analyzed the different expression of CD38 and CD49d in LAIR1<sup>+</sup> and LAIR1<sup>-</sup> groups, we observed a significant association between LAIR1<sup>+</sup> and CD38<sup>+</sup> patients ( $P=0.019$ ), while no significant difference in CD49d expression was observed between the two groups ( $P=0.13$ ), although CD49d was slightly less expressed in LAIR1<sup>+</sup> patients. Similarly, when immunophenotypic expression was computed as a continuous variable, the expression of LAIR1 was inversely related to CD38 but not to CD49d expression ( $P=0.0005$  and  $P=0.96$ , respectively), as shown in Figure 1. Conversely, CD49d expression was strongly associated with CD38 expression ( $P<0.0001$ ; Figure 1C). The analysis of median fluorescence intensity of LAIR1, CD38, and CD49d confirmed the strong relation between CD38 and CD49d ( $P<0.0001$ ), while no relation emerged between LAIR1 and CD38 or CD49d using values of median fluorescence intensity (*Online Supplementary Figure S3*).

Nineteen patients were analyzed for LAIR1 expression over time. As shown in *Online Supplementary Figure S2*, no significant variation ( $P=0.64$  by Wilcoxon test) was observed among either initially positive or negative cases. The median time from diagnosis to the second analysis was 48 months (range, 9-71) with half of the patients having received immunochemotherapy during this period.

### Prognostic relevance of LAIR1 expression and univariate analysis

A significantly lower proportion of LAIR1<sup>+</sup> patients ini-

tiated cytotoxic treatment during follow-up (42 of 183, 22.9%) compared to LAIR1<sup>+</sup> patients (53 of 124, 42.7%;  $P<0.001$ ). This translated into a significant difference in terms of TTFT according to LAIR1 expression ( $P=0.0002$ ), as shown in Figure 2. Univariate analysis also identified high expression of CD38 ( $P=0.00003$ ), high expression of CD49d ( $P=0.00002$ ), unmutated *IGHV* ( $P<0.00001$ ), high-risk cytogenetic lesions, defined as del17p, del11q or complex karyotype ( $P=0.0003$ ), and Binet stage ( $P<0.00001$ ) as statistically significant predictors of a shorter TTFT, as shown in Figure 2.

### Multivariate analysis

Since the three phenotypic markers showed significant correlations in their expression and were all predictive for TTFT, LAIR1, CD38, and CD49d were included in a Cox proportional hazard regression model to test their strength as independent prognostic factors in terms of TTFT in our cohort of CLL patients. As shown in Table 2A, LAIR1<sup>+</sup> (HR 2.269,  $P=0.002$ ) and CD49d<sup>+</sup> (HR 2.232,  $P=0.008$ ) maintained independent significant associations with shorter TTFT, while CD38 did not.

The multivariate analysis was then extended to other clinical and biological variables that were significant in univariate analysis. As shown in Table 2B, LAIR1 expression was again significantly associated with TTFT (HR

2.047,  $P=0.037$ ) together with *IGHV* status (HR 2.881,  $P=0.011$ ) and Binet stage (HR 6.457,  $P<0.0001$ ), while CD49d expression lost its predictive value (HR 1.668,  $P=0.178$ ).

In order to visualize their additive prognostic value in terms of TTFT, LAIR1 expression, *IGHV* status and Binet stage were then computed together in a Kaplan-Meier curve, as shown in Figure 3.

### Discussion

Here we report for the first time that LAIR1 expression, although related to commonly recognized risk factors, has a significant and independent impact on time to tumor progression in patients with CLL, supporting LAIR1 as an easily applicable and inexpensive marker to predict TTFT in patients presenting with CLL.

Importantly, our results were obtained from a prospective cohort of newly diagnosed unselected patients with CLL who were standardized, in terms of phenotypic, biological and cytogenetic characterization, as part of the "CLL Veneto" project. The short follow-up of recruited patients prevented us from investigating the role of LAIR1 expression in terms of survival. However, when addressing the clinical behavior and aggressiveness of a disease,

**Table 1.** Clinical and biological characteristics of 311 patients with chronic lymphocytic leukemia at disease presentation, then divided according to LAIR1 expression.

	All patients (n. 311)	LAIR1 <sup>+</sup> (n. 186)	LAIR1 <sup>-</sup> (n. 125)	P value <sup>o</sup>
Median age, years (range)	66 (30.6-90)	66 (36-90)	67 (30.6-87)	0.59 <sup>s</sup>
Female gender	121/311 (38.9%)	69/186 (37.1%)	52/125 (41.6%)	0.48
Median lymphocyte count, x10 <sup>6</sup> /mmc (range)	9.7 (2.1-656)	9.4 (2.1-270)	11 (2.1-656)	0.13*
Autoimmune hemolytic anemia	11/311 (3.5%)	2/186 (1.1%)	9/125 (7.2%)	<b>0.008</b>
Immune thrombocytopenic purpura	7/311 (2.2%)	5/186 (2.7%)	2/125 (1.6%)	0.70
Direct antiglobulin test	13/145 (8.9%)	3/86 (3.5%)	10/59 (16.9)	<b>0.007</b>
Binet stage				
A	233/299 (77.9%)	147/178 (82.6%)	86/121 (71.1%)	<b>0.023</b>
B	47/299 (15.7%)	23/178 (12.9%)	24/121 (19.8%)	0.145
C	19/299 (6.4%)	8/178 (4.5%)	11/121 (9.1%)	0.146
Fluorescence <i>in situ</i> hybridization				
Normal	77/211 (36.5%)	39/124 (31.4%)	38/87 (43.7%)	0.081
del13q	84/211 (39.8%)	57/124 (46.0%)	27/87 (31.0%)	<b>0.033</b>
12+	18/211 (8.5%)	14/124 (11.3%)	4/87 (4.6%)	0.131
del11q	17/211 (8.1%)	10/124 (8.1%)	7/87 (8.0%)	1.00
del17p	9/211 (4.3%)	1/124 (0.8%)	8/87 (9.2%)	<b>0.004</b>
3 or more alterations	6/211 (2.8%)	3/124 (2.4%)	3/87 (3.5%)	0.692
<i>IGHV</i> mutational status				
Unmutated <i>IGHV</i>	77/200 (38.5%)	34/126 (27.0%)	43/74 (58.1%)	<b>&lt;0.001</b>
Immunophenotype				
CD38	64/274 (23.4%)	30/165 (18.2%)	34/109 (31.2%)	<b>0.019</b>
CD49d	90/238 (37.8%)	51/150 (34.7%)	39/88 (44.3%)	0.129
Treatment, survival and follow-up				
Had cytotoxic treatment	95/307 (30.9%)	42/183 (22.9%)	53/124 (42.7%)	<b>&lt;0.001</b>
Median TTFT, months (range)	23.5 (0-75)	25.1 (0-75.1)	18.9 (0-74.7)	<b>0.023*</b>
Dead patients	15/311 (4.8%)	7/186 (3.8%)	8/125 (6.4%)	0.295
Median overall survival, months (range)	30.7 (0.5-75.1)	29.5 (0.5-75.1)	33.9 (1-75)	0.44

del13q: deletion in chromosome 13q14; del11q: deletion in chromosome 11q23; del17p: deletion in chromosome 17p12; +12: trisomy 12; *IGHV*: immunoglobulin heavy chain variable region genes; TTFT: time to first treatment; Statistical tests: <sup>o</sup>the differences between the categorical variables were computed by Fisher exact test; <sup>s</sup>t-test; \*Mann-Whitney U test. Statistically significant results are shown in bold.

TTFT is a reliable prognostic marker strictly related to tumor progression, especially in a prospective cohort in which criteria for treatment initiation were standardized between centers before the patients' enrollment. A merit of our study is that our population reflects real-life, unselected patients with CLL who routinely present to our Institutions, making our results readily useful for the clinician.

Immunophenotypic analysis was performed by multi-color flow cytometry evaluating the percentage of CD19<sup>+</sup>CD5<sup>+</sup> cells expressing LAIR1, CD38 or CD49d. We observed a significant inverse correlation between the expression of LAIR1 and CD38 and we confirmed the strong correlation of expression between CD38 and CD49d already observed by others.<sup>22</sup> Further specific studies will need to confirm these findings and analyze cell biology according to patterns of expression of single molecules. Furthermore, consistently with our findings, Poggi *et al.* recently observed that the expression of LAIR1 is higher in patients with low-risk CLL.<sup>16</sup> In our series, LAIR1 expression allowed us to discriminate different set of patients with significant biological and clinical differences, confirming that LAIR1<sup>-</sup> patients were more likely to have a worse clinical stage at diagnosis and adverse biological factors, such as unmutated *IGHV* genes, CD38<sup>+</sup>, or high-risk cytogenetic lesions.<sup>16,17</sup>

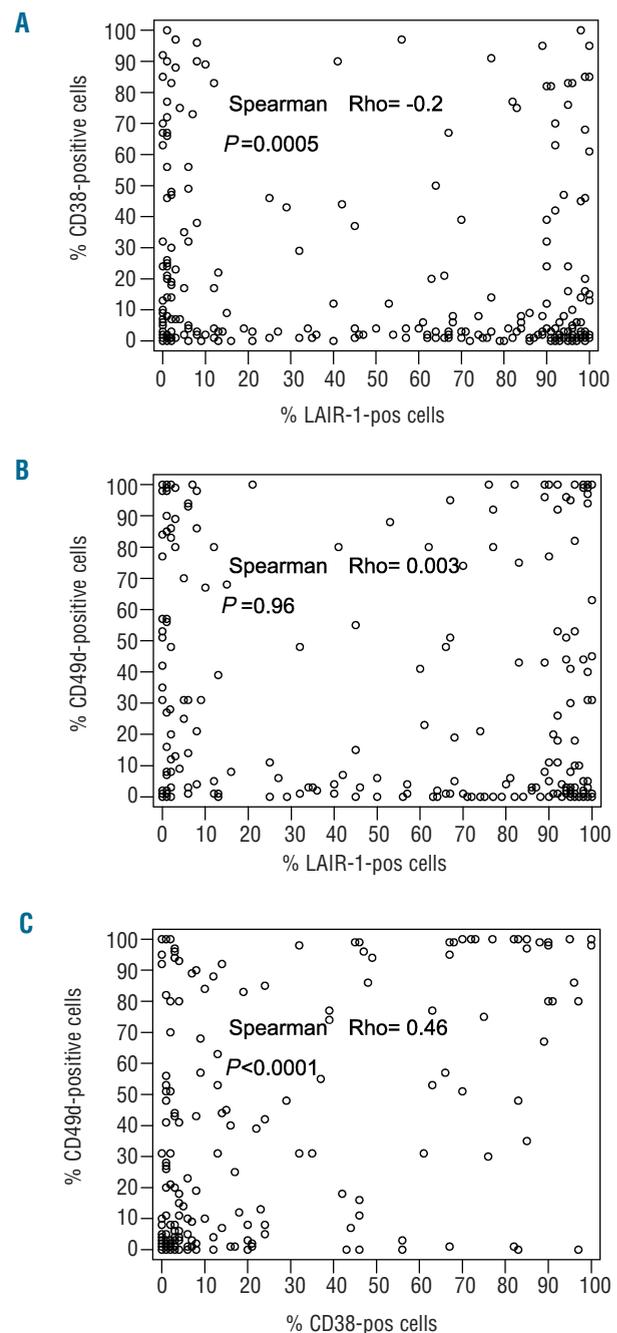
In the last years several studies have focused on the identification of biological markers that could be easily used to foresee the prognosis of CLL patients. Many new immunophenotypic markers have been proposed for identifying high-risk patients,<sup>11</sup> in addition to historical ones, such as CD38 and ZAP-70. Indeed, the prognostic power of CD38 expression has been questioned by several studies.<sup>1,6</sup> On the other hand, many experts have described technical problems with the study of ZAP-70 expression.<sup>1,7</sup> Our previous results confirmed these technical issues showing high discordance in the quantification of ZAP-70 expression using either different monoclonal antibodies or different approaches for the analysis.<sup>23</sup> For these reasons we did not consider ZAP-70 expression in our study. Recently, based on a retrospective cohort, CD49d has been advocated as an independent prognostic marker.<sup>8-10</sup> However, the expression of LAIR1 in our series seemed to overcome the prognostic power of CD49d, at least in terms of TTFT. In line with our findings, Del Poeta *et al.* recently reported a reduced prognostic power of CD49d when all significant prognostic factors for CLL were included in the multivariate analysis.<sup>12</sup>

The cut-off for positivity of LAIR1 was set at 30%. This choice of cut-off was made considering the distribution of frequencies of positive cells in our cohort of patients, as previously done by Damle *et al.* for CD38<sup>+</sup> (Online Supplementary Methods and Figure 1). Differently from others, this unsupervised procedure did not consider any clinical parameter to set the cut-off, obviating selection bias.<sup>8,9,12</sup>

Interestingly, LAIR1 expression was related to the occurrence of autoimmune phenomena. Both a direct antiglobulin test and autoimmune hemolytic anemia were more frequently observed in LAIR1<sup>-</sup> patients, with a quite high percentage of LAIR1<sup>-</sup> patients having autoimmune hemolytic anemia (7.2%) at presentation with CLL. Since it is well known that both the direct antiglobulin test and autoimmune hemolytic anemia are associated with *IGHV* status in patients with CLL,<sup>24</sup> our findings might be a con-

sequence of the association between unmutated *IGHV* and lower LAIR1 expression, or, more intriguingly, might reflect the biological activity of LAIR1 and its role in switching-off B cells and their potential antibody-producing activity. A larger number of patients and specific studies will be needed to address this point.

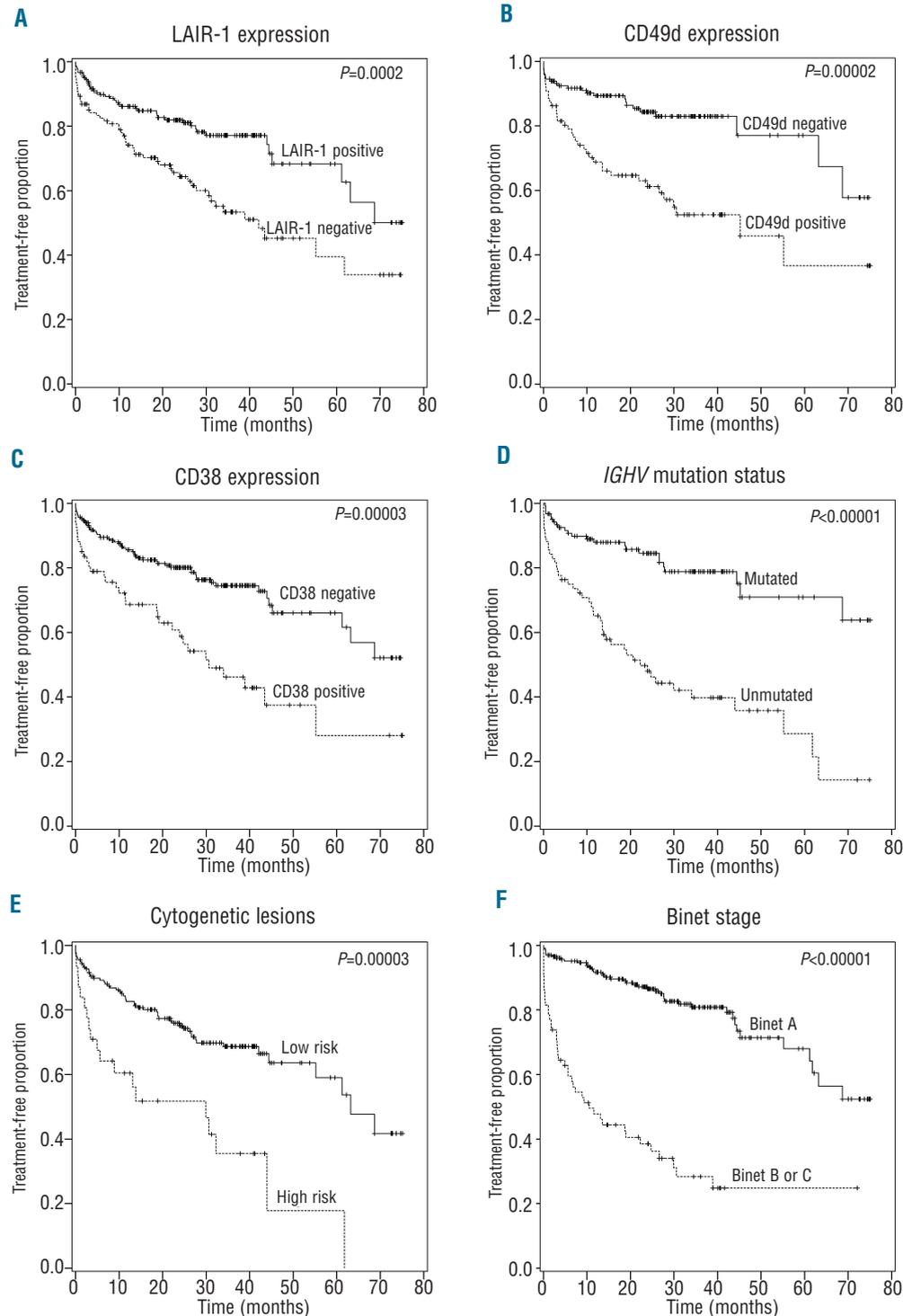
The independent prognostic power of LAIR1 expression may be connected to its peculiar biological characteristics



**Figure 1.** Correlation between LAIR1 and other immunophenotypic prognostic markers. Scatter plots for percentage of positive cells for LAIR1 and CD38 (A), LAIR1 and CD49d (B), and for CD49d and CD38 (C). The two-tail Spearman test for non-parametric data was performed. P value < 0.05 was considered to indicate a statistically significant correlation.

and function. Its activity on the BCR activation pathway makes LAIR1 very attractive, since drugs targeting essential components of BCR signaling (e.g., Bruton tyrosine kinase inhibitors) have recently shown impressive activity in patients with CLL.<sup>25</sup> LAIR1, now designated as CD305, is an inhibitory receptor expressed on almost all hematopoietic cells, particularly on immune system cells. After the binding of its known ligands,<sup>13,14</sup> LAIR1 inhibits the activation of immune cells using two immunoreceptor tyrosine-based inhibitory motifs located in the cytoplas-

mic tail of the receptor. LAIR1 is expressed during B-cell ontogenesis, but is lost on a subset of memory B cells, in all germinal center B cells, in plasmablasts, and in plasma cells.<sup>15</sup> From a functional point of view, LAIR1 cross-linking results in inhibition of  $Ca^{2+}$  mobilization induced by BCR-triggering. On the other hand, prolonged BCR- or CD40-stimulation induces down-regulation of LAIR1 on naïve B cells *in vitro*,<sup>15</sup> suggesting an inhibitory role for LAIR1 on BCR signaling, as for other B-cell inhibitory receptors, such as CD22 and FcγRIIb.<sup>15</sup> Poggi *et al.* demon-



**Figure 2.** Time to first treatment (TTFT) according to identified prognostic variables. Kaplan-Meier plot for TTFT according to: phenotypic expression of LAIR1 (A), CD49d (B), and CD38 (C); IGHV status (D); cytogenetic lesions (E); and Binet stage (F). A log-rank test was performed to compare the curves.  $P$  values  $<0.05$  are considered statistically significant.

**Table 2.** Multivariate Cox regression analysis for time-to-first treatment (TTFT).

A		
Parameter	HR (CI 95%)	P value
LAIR1 <sup>+</sup>	2.269 (1.355-3.800)	0.002
CD49d <sup>+</sup>	2.232 (1.234-4.037)	0.008
CD38 <sup>+</sup>	1.646 (0.909-2.981)	0.100
B		
Parameter	HR (CI 95%)	P value
LAIR1 <sup>+</sup>	2.077 (1.057-4.081)	0.034
CD49d <sup>+</sup>	1.575 (0.771-3.128)	0.213
Unmutated <i>IGHV</i> status	2.675 (1.232-5.804)	0.0013
High risk FISH (del11q/del17p/complex)	2.041 (0.943-4.416)	0.070
Binet B or C	6.285 (3.071-12.816)	<0.0001

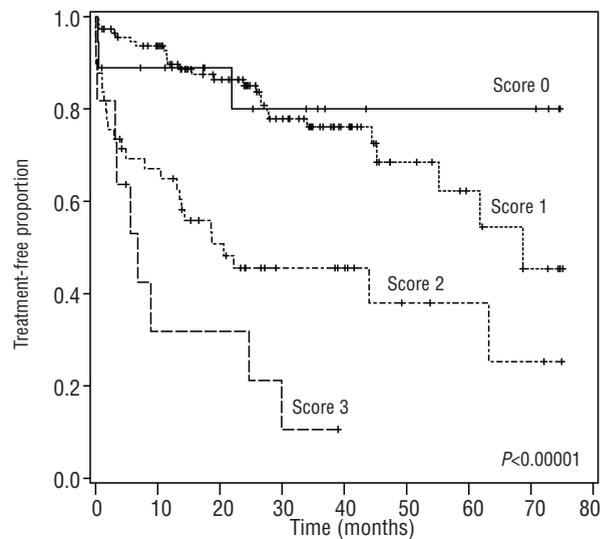
HR: hazard ratio; CI: confidence interval; *IGHV*: immunoglobulin heavy chain variable region genes; FISH: fluorescence in situ hybridization; del11q: deletion in chromosome 11q23; del17p: deletion in chromosome 17p12.

strated the role of LAIR1 in the modulation of BCR signaling pathways implicated in CLL-cell activation, with its inhibitor capacity being completely lost or significantly reduced when CLL cells did not express LAIR1.<sup>16</sup> Furthermore, *in vitro* studies confirmed that collagen produced by lymph node-derived mesenchymal stromal cells was able to inhibit B-cell functions through LAIR1 engagement.<sup>26</sup> Altogether these data strengthen and increase the appeal of LAIR1 having an active role in the pathophysiology of CLL with special emphasis on microenvironmental interactions inducing BCR activation.<sup>25,27</sup> The relationship between LAIR1 expression and therapy with BCR signaling inhibitors certainly deserves investigation in the future.

The information about LAIR1 expression and function on neoplastic B cells is scanty so far, but for several reasons CLL represents the right setting for characterizing this molecule. Our data show that LAIR1 has an independent role in predicting TTFT of patients with CLL, while longer follow-up is needed to establish its ability to predict survival. Its role *in vivo* in these patients is also substantiated by the association between its low expression on the surface of B cells and aggressive clinical presentation of the disease, which is consistent among different studies. Finally, the influence of LAIR1 on B-cell activation pathways requires further studies that will establish the role of this molecule in the context of new targeted therapies.

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**Figure 3.** Time-to-first treatment (TTFT) curves based on the combination of LAIR1, *IGHV* status and Binet stage. Kaplan Meier plot for TTFT showing the additive prognostic value of LAIR1, unmutated *IGHV* status, and Binet stage B or C. Attributing a score of 1 to each variable, the curves represent the sum of adverse variables (0, 1, 2, or 3). A log-rank test was performed to compare different curves. P values are as follows:  $P=0.471$  between 0 and 1;  $P<0.0001$  between 1 and 2;  $P=0.06$  between 2 and 3.

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## Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at [www.haematologica.org](http://www.haematologica.org).

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