



## CLINICO-PROGNOSTIC IMPLICATIONS OF INCREASED LEVELS OF SOLUBLE CD54 IN THE SERUM OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS. RESULTS OF A MULTIVARIATE SURVIVAL ANALYSIS

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### ABSTRACT

**Background and Objective.** Although less specific than sCD23, sCD54 levels have clinico-prognostic relevance in B-cell chronic lymphocytic leukemia (CLL). Since serological markers are now emerging as potentially important in CLL, we tried to verify whether sCD54 might complement clinical stages.

**Methods.** Serum levels of sCD54 were determined at the time of diagnosis in 115 previously untreated CLL patients. Results were correlated with clinico-biological parameters as well as with survival.

**Results.** Life-expectancy was significantly shorter in patients with higher serum levels of sCD54 ( $p < 0.001$ ); however, in a Cox's multivariate survival analysis, the only variables which entered the regression model at a significant level were bone marrow (BM) histology ( $p = 0.03$ ) and lymphocyte doubling time (LDT) ( $p = 0.04$ ). Interestingly, when

LDT was excluded from analysis the only significant variables were clinical stages ( $p < 0.05$ ) and sCD54 ( $p < 0.05$ ). These results suggest that sCD54 and LDT give similar prognostic information.

**Interpretation and Conclusions.** In CLL, sCD54 is a reliable prognostic parameter whose value is independent of clinical stages. When investigated in relation to clinical outcome, serum levels of sCD54 were able to predict progression to a more advanced clinical stage. On the basis of these data, an integrated clinico-biological classification which separates intermediate risk into two prognostic subgroups is proposed.

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*Key words:* chronic lymphocytic leukemia, staging, prognosis, sCD54

Although less specific than sCD23, sCD54 levels have clinico-prognostic implications in chronic lymphocytic leukemia (CLL).<sup>1</sup> Moreover, longitudinal data support the idea that serum levels of sCD54 can be used as an indicator of disease progression.<sup>2,3</sup> Keeping these concepts in mind we analyzed the prognostic impact of sCD54 in an unselected series of 115 previously untreated B-cell CLL patients. Since the usefulness of a novel tumor variable in clinical practice depends largely on whether or not it is independent of existing prognostic parameters,<sup>4</sup> special attention was paid to evaluate this marker through multivariate survival analysis. Furthermore, an integrated clinico-biological classification was devised, thus indicating that serological markers can be successfully incorporated into clinical staging.

### Materials and Methods

#### Patient characteristics

One hundred and fifteen patients diagnosed as having CLL in two different hematological institutions form the basis of this study. The mean age of patients was 66 years (SD, 8.2) and the male to female ratio was 72 to 43. B-CLL was diagnosed according to generally accepted criteria that included peripheral blood lymphocytosis greater than  $5 \times 10^9/L$  and BM lymphocy-

tosis greater than 30%.<sup>5</sup>

As reported in a previous paper dealing with sCD23 levels that included 90 out of the 115 patients belonging to the present study, a complete immunological profile was obtained in each patient at the time of CLL diagnosis.<sup>6</sup> In all cases the predominant leukemic population shared B-cell markers (i.e. CD19, CD20 and CD23) and the CD5 antigen. B-cells were monoclonal with regard to the expression of either  $\kappa$  or  $\lambda$  light chain (LC) surface immunoglobulins. Immunological studies, carried out in flow cytometry, made it possible to diagnose typical B-CLL (i.e. CD5<sup>+</sup>, CD23<sup>+</sup> and weak expression of LC immunoglobulins) in 104 out of 115 cases (90.4%).<sup>7</sup>

According to the Binet clinical staging system,<sup>8</sup> 60 patients were in stage A, 34 in B and 21 in stage C. BM biopsy was performed in 91 patients. A non-diffuse pattern of BM involvement could be recognized in 58 and a diffuse one in 33.<sup>9</sup> Lymphocyte doubling time (LDT) was retrospectively evaluated in 78 patients, all having observation times longer than 1 year.<sup>10</sup>

#### Treatment and follow-up data

Most patients with Binet stage C or symptomatic B at the diagnosis required therapy, which consisted of an alkylating agent-based regimen. Patients in Binet stage A were monitored without therapy until clear evidence of disease progression occurred. As far as survival is concerned, median duration of follow-up for the whole series was 33 months (range, 6-66 months). At the time of the present study 30 patients had died. Death could be related to CLL (i.e. leukemia progression, infections) in 24 patients (80%). Six others died as a consequence of apparently non-CLL related causes (non-lymphoid second neoplasm, 3; cardiovascular disease, 2; car crash, 1). Actuarial median survival was 58 months.

#### Determination of soluble CD54 levels and other assays

Quantitative determinations of both sCD54 and sCD23 levels were carried out by means of an enzyme immunoassay kit (CELL FREE ICAM-1 test kit, CELL FREE CD23 test kit; T cell Diagnostic Inc, Cambridge, MA). All serum samples analyzed were taken at diagnosis of CLL and stored at  $-70^{\circ}$ . The detection limit was 0.30 ng/mL for sCD54 and 15 U/mL for sCD23. sCD54 displayed an interassay coefficient of variance (CV) between 3.4% and 4.5% and an intra-assay CV between 1.6% and 3.0%. The interassay CV for sCD23 was between 5.1% and 9.9%, while the intra-assay CV varied between 2.8% and 9.9%. Details of the immunoenzymatic technique used for sCD54 and sCD23 level determinations have been reported in previously published papers.<sup>2,6</sup> Serum levels of thymidine kinase (TK), determined by means of a radioenzyme (REA) assay (Prolifigen TK-REA, Santagek, Sweden), were based on the transformation of a radioactive substrate into a specific radioactive product. Normal levels of serum TK in our laboratory were less than 5 U/mL. Immunocytochemical staining of proliferating cells was carried out by means of Ki67 Mo Ab (PC-DAKO) on cytological samples obtained from BM aspiration.<sup>11</sup> Either serum levels of TK or BM Ki67 evaluations were available in only 48 patients.

#### Statistical analyses

The Student's t-test and analysis of variance were used to evaluate differences in the means among the various groups. When dealing with discrete variables, statistical analyses were carried out by means of the chi-square test. Survival and disease progression curves were plotted according to the method of Kaplan and Meier<sup>12</sup> and compared with the log-rank test.<sup>13</sup> The Cox<sup>14</sup> proportional hazard regression model was used in multivariate analyses. In addition to sCD54, we included in this analysis those variables which proved to be significant in univariate analysis. The model was tested twice (with and without the inclusion of LDT) by using a series of binary variables. In the case of sCD54 and sCD23, the mean values were utilized as the cutoff (sCD54, 600 ng/mL; sCD23, 2700 U/mL). Histologic BM evaluation revealed both diffuse and non-diffuse patterns of BM involvement. Clinical stages were included in the binary model in this way: A vs B + C. As far as LDT and PB lymphocytosis are concerned, cutoffs were set on the basis of the results of a previous work (LDT, 12 months; PB lymphocytosis,  $40 \times 10^9/L$ ).<sup>10</sup> Out of the 115 patients, only 95 (82.6%) whose follow-up had lasted for 1 year or more were included in the survival analysis. All deaths, whatever the cause, were considered in the survival analyses. As correctly reported by Rozman *et al.*,<sup>15</sup> any effort to classify death events into CLL-related and CLL-unrelated is unrealistic. Death from solid tumor may only apparently be regarded as CLL-unrelated; it is indeed clear that the incidence of non-lymphoid second neoplasms is higher in CLL patients than in the general control population.<sup>16</sup>

#### Results

sCD54 levels in the serum of B-CLL patients were highly elevated in comparison to those of an age-matched control population ( $600.6 \text{ ng/mL} \pm 276.2$  vs  $352 \text{ ng/mL} \pm 162$ ;  $p < 0.0005$ ). As shown in Table 1, the increased levels of sCD54 reflect clinico-biological parameters representative of either tumor mass (clinical stage, pattern of BM histology, absolute peripheral blood lymphocytosis, sCD23 levels) or disease progression (LDT, Ki67 expression on BM lymphocytes, serum TK activity).

To assess the prognostic value of serum sCD54 levels, a survival analysis was performed in the 95 CLL patients (82.6%) whose follow-up times were adequate (more than 1 year). Clear-cut differences in life expectancy were found between patients whose sCD54 serum concentration was less than

Table 1. Distribution of prognostic parameters according to sCD54 serum levels.

sCD54 (ng/mL)	< 600	> 600	p-value
<i>Clinical stage</i>			
A	36 (62%)	24 (42.1%)	0.017
B	17 (29.3%)	17 (29.8%)	
C	5 (8.6%)	16 (28%)	
<i>BM histology</i>			
non diffuse	41 (86.9%)	17 (40.4%)	0.0001
diffuse	8 (16.3%)	25 (59.5%)	
<i>PB lymphocytes (<math>10^9/L</math>)</i>	46.9 $\pm$ 37.8	74.5 $\pm$ 50.2	0.0003
<i>LDT</i>			
> 12 mo	36 (81.8%)	7 (21.8%)	0.0001
< 12 mo	8 (18.1%)	25 (78.1%)	
<i>sCD23 (U/mL)</i>	1708.7 $\pm$ 1280.5	4200.9 $\pm$ 2814	<0.0001
<i>TK (U/L)</i>	12.9 $\pm$ 10.6	36.4 $\pm$ 14	0.0001
<i>Ki67+ BM cells (%)</i>	1.04 $\pm$ 1.08	7.79 $\pm$ 596	0.0001

BP, peripheral blood; BM, bone marrow; LDT, lymphocyte doubling time; TK, thymidine kinase.

600 ng/mL and those with sCD54 equal to or more than 600 ng/mL; the 5-year survival rates were 68% (95% CI: 52% to 84%) and 23% (95% CI: 3% to 43%), respectively ( $p < 0.001$ ; Figure 1). Moreover, the significance of several recognized prognostic factors was tested by univariate analysis. In addition to sCD54 levels, we found that clinical stage ( $p = 0.0001$ ), BM histology ( $p = 0.004$ ), LDT ( $p = 0.00001$ ) and sCD23 levels ( $p = 0.0002$ ) correlated with survival (Table 2).

A multivariate survival analysis was performed using parameters that were statistically significant in the univariate analysis. As shown, BM histology ( $p = 0.03$ ) and LDT ( $p = 0.04$ ) were the only variables which entered the regression model at a significant level (Table 2). Given the correlation between sCD54 and LDT,<sup>1</sup> multivariate survival analysis was again carried out after the exclusion of LDT, whose main drawback is that it cannot be assessed immediately at the time of diagnosis. Interestingly, in this analysis sCD54 ( $p < 0.05$ ) and clinical stage ( $p < 0.05$ ) were the only significant variables (Table 3).

In order to investigate whether sCD54 levels could be a useful variable for isolating subsets of patients within established clinical stages, we analyzed Binet stage B patients whose survival probability did not differ from that of the overall series. Survival was better in stage B patients with sCD54 concentrations under 600 ng/mL [observed/expected (O/E) ratio, 0.36] than in stage B patients with sCD54 above 600 ng/mL (O/E ratio, 1.76; chi-square = 4.86; d.f. = 1;  $p < 0.02$ ). These results prompted us to devise an integrated clinico-biologi-

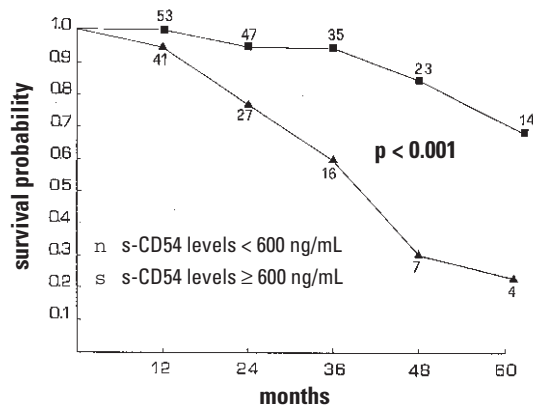


Figure 1. Actuarial survival curves of CLL patients according to sCD54 serum level. The projected survival at 5 years was 68% (95% CI: 52% to 84%) for patients with sCD54 concentrations < 600 ng/mL and 23% (95% CI: 3% to 43%) for patients with sCD54 concentrations  $\geq$  600 ng/mL.

Table 2. Prognostic value of sCD54 and other clinico-biological variables tested in univariate and multivariate proportional hazard regression models.

Variable	Univariate analysis P-value	Multivariate analysis P-value
Clinical stages	0.0001	0.31
BM histology	0.004	0.03
LDT	0.00001	0.04
PB lymphocytosis	0.11	—
sCD23	0.0002	0.9
sCD54	0.00001	0.15

Table 3. Order of the variables entering the regression model and level of significance after exclusion of LDT from analysis.

Variable	Chi-square	P-value
Clinical stages	3.9	<0.05
sCD54 levels	3.8	<0.05
sCD23 levels	1.2	0.26
BM histology	1.1	0.29

cal staging system. For this purpose patients were divided into 4 groups: 1) stage A, 2) stage B with sCD54 levels < 600 ng/mL, 3) stage B with sCD54 levels > 600 ng/mL, 4) stage C. The log-rank test of these survival curves is presented in Table 4. As can be seen, the O/E ratio is either lower or higher than 1. In other words, all groups designated in this way discriminate with respect to the total population.

Due to their partial independence of clinical stages, sCD54 levels were further investigated in relation to clinical outcome in 34 out of the 60

stage A patients followed up for a period ranging from 12 to 66 months without therapy. The 5-year actuarial risk of progression to a more advanced clinical stage was 40% for patients whose sCD54 levels at the time of diagnosis were lower than 600 ng/mL and 87.5% for those with sCD54 levels higher than 600 ng/mL ( $p < 0.001$ ). Furthermore, in 22 patients serum sCD54 concentrations were prospectively analyzed on 2 to 4 occasions. In 8 patients considered in stable disease on the basis of LDT >12 months and the absence of clinical stage progression, sCD54 levels analyzed at intervals ranging from 6 to 38 months (median, 12 months) were not significantly different from those obtained at the time of diagnosis ( $417.7 \pm 154.5$  ng/mL versus  $473 \pm 217.4$  ng/mL;  $P = \text{NS}$ ). Six patients experienced a change in clinical stage (i.e. from A to B or C) after a median time of 18 months (range, 17-30 months). In these patients clinical stage progression paralleled an increase in sCD54 ( $571.4 \pm 281$  ng/mL vs  $1341 \pm 686$  ng/mL;  $p < 0.0005$ ) (Table 4).

sCD54 levels were also measured after response to treatment was assessed in 8 patients. According to NCI-Sponsored Working Group on CLL criteria,<sup>4</sup> 6 partial responses (PR) and 2 non-responses (NR) were observed. Intermittent chlorambucil (CLB) and prednisone (PDN) treatment reduced the values of circulating sCD54 in 6 responder patients ( $845$  ng/mL +  $372.4$  vs  $502$  ng/mL +  $161.5$ ;  $p < 0.005$ ). In contrast, no changes could be observed in non-responding patients after 4 monthly courses of intermittent CLB and PDN. Interestingly, at the time of relapse, 2 previously responding patients again experienced an increase in sCD54 levels.

## Discussion

Besides the well-known clinical variables, there are now some biological parameters in B-CLL that actually reflect the tumor mass, the biology of the neoplasia, and the pace of disease. This is the case for sCD54 whose levels are increased in the serum of B-cell CLL patients.<sup>1-3,17-18</sup> It is not clear whether sCD54 is shed by normal host cells or by tumor cells.<sup>19,20</sup> In a previously published study specifically designed to verify whether CD54 surface expression on leukemic B-CLL cells determined serum levels, we detected a poor correlation between the two parameters.<sup>21</sup> This is in keeping with a recent observation by Schmid *et al.*<sup>22</sup> who demonstrated in an *in vitro* culture assay that sICAM-1 (sCD54) seems to be shed by accessory cells, probably T-cells, rather than by leukemic cells. In contrast, significant amounts of sICAM-3 released by leukemic cells parallel the constitutive membrane expression of this molecule.<sup>23</sup>

To the best of our knowledge only Christiansen *et al.*<sup>1</sup> have addressed the impact of sCD54 on overall survival in B-CLL patients. In a multivariate analysis, sCD54 was the only biological marker of bor-

Table 4. Log-rank analysis of survival curves in CLL according to a modified version of Binet's staging system in which sCD54 levels are used to subclassify stage B.

	Observed (Total)	Expected	O/E	Median survival (months)
Stage A	7 (42)	18.70	0.37	not reached
Stage B with sCD54 levels < 600 ng/mL	2 (16)	3.88	0.51	not reached
Stage B with sCD54 levels ≥ 600 ng/mL	8 (16)	3.04	2.63	37 months
Stage C	12 (21)	3.37	3.56	31.5 months

Chi-square for heterogeneity (3 d.f.) = 38.41;  $p < 0.0005$ ; chi-square for trend (1 d.f.) = 17.03;  $p < 0.0005$ ; O/E = observed/expected ratio.

Table 5. Serial determinations of sCD54 levels.

Disease status	N. pts	Serological* follow-up (months)	sCD54°		p-value
			At diagnosis	At change of disease status (if any)	
Stable	8	12	417±145.3	473.7±217.4	NS
Progressive	6	18	571.4±281	1341±686	< 0.0005
Responsive	6	6	845±372.4	502±161.5	< 0.0005
Non-responsive	2	—	1635±49.4	1880.5±431	—
Relapsing	2	23	571±348	1131±114	—

\*median time; °ng/mL.

derline significance in a regression model which included clinical stage and LDT.<sup>1</sup> According to our experience, only BM histology and LDT entered the regression model at a significant level, thus confirming LDT as an independent prognostic variable in CLL.<sup>10</sup> However, in the Cox proportional hazard model which excluded LDT, sCD54 reached a statistically significant level. These data clearly show that LDT and sCD54 give similar prognostic information.

An additional piece of information coming from the present study is that there is a certain degree of independence between sCD54 and clinical stage. Indeed patients belonging to the intermediate-risk category (Binet stage B) could be subdivided into two different prognostic subgroups on the basis of their sCD54 levels. Finally, taking into account the prognostic value of sCD54 and its partial independence of clinical stage, we devised an integrated clinico-biological classification system, thus indicating that serological markers can be successfully incorporated into clinical staging.<sup>24</sup>

In conclusion, sCD54 levels offer an additional prognostic tool in CLL whose value is independent of clinical stage. Whether increased sCD54 concentrations reflect the host's immune response to malignant cells is still a matter of debate.

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