

# CD31 density is a novel risk factor for patients with B-cell chronic lymphocytic leukaemia

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**Abstract.** CD31 is the physiological ligand for CD38. CD38 expression in a high percentage of malignant cells is a risk factor for patients with B-cell chronic lymphocytic leukaemia (B-CLL). A previous investigation demonstrated that quantification of CD38 improves upon the prognostic value of the percentage expression. A recent study states that the percentage of CD31 expression is not predictive in B-CLL. We reassessed the predictive power of CD31 in a cohort of 120 patients with B-CLL. Peripheral blood cells were stained with PCP-labelled anti ( $\alpha$ )-CD19, FITC- $\alpha$ -CD5 and PE- $\alpha$ -CD31 antibodies. CD31 expression was quantified using beads of specific antibody binding capacity and the density was correlated with clinical outcome. End points were disease-specific survival and time to treatment (TTT). We report that CD31 density was significantly lower in the group of patients with Binet stage B and C of disease progression (P=0.0003). There was an inverse, significant correlation between CD31 and CD38 densities (R=-0.281, P=0.002). All CLL-related deaths occurred in patients with low CD31 density. Low CD31 predicted for poor disease outcome (survival, P=0.0087; TTT, P=0.0064) and identified Binet stage A patients (survival, P=0.0350; TTT, P=0.0716) and those with low CD38 (survival: all patients, P<0.0001; stage A, P=0.003) who followed a more aggressive clinical course. Disease-specific survival of patients with low CD31 and high CD38 densities was significantly shorter than all other groups. In addition, low CD31 density was a poor risk factor irrespective of patient age (survival: all patients, P=0.045; stage A, P=0.021) and identified patients with Binet stage B/C as the highest risk group (P<0.0001).

In conclusion, low CD31 density is an adverse prognostic indicator in B-CLL. Also, low CD31 density enhances the prognostic power of CD38 density. The interaction between CD31 and CD38 and its clinical significance in B-CLL requires further investigation.

## Introduction

A large number of prognostic factors are available to help predict outcome in patients with B-CLL (1,2). Recently described, significant and independent prognostic factors include the mutation status of the immunoglobulin (Ig) heavy chain variable region (VH) (2), p53 mutations (2) and high expression of ZAP-70 (3,4) and CD38 (5).

CD38 acts as a receptor involved in the transduction of signals essential for cell activation and proliferation (6), survival (7) and apoptosis (8). Activation of these pathways is achieved after interaction of CD38 with its ligand which has been identified as the platelet endothelial cell adhesion molecule-1 or CD31 (9). The CD31 molecule, a member of the immunoglobulin (Ig) superfamily, is expressed on normal naive B-cells with unmutated IgVH genes and it is down-regulated upon differentiation to memory cells (10). Cross-linking of CD31 may lead to either cell survival (11,12) or apoptosis (13).

The CD38 (14) and CD31 (15) antigens are expressed in different types of normal vascular cells. Co-expression of these factors has been reported in malignant cells (16-19) and the CD38/CD31 phenotype has been implicated in the aggressiveness of some malignancies (16). In multiple myeloma (MM), CD38 and CD31 were expressed at high density in all patients while CD31 but not CD38 was absent in most patients with plasmablastic MM or plasma cell leukaemia (16). In patients with B-CLL, a higher ratio of CD38/CD31 in the lymph nodes compared with that of the peripheral blood (PB) or bone marrow (BM) correlates significantly with clinical parameters indicative of disease aggression including stage, lymphoma-like tumour distribution and a diffuse pattern of BM infiltration (19). By contrast, although CD38 and CD31 were co-expressed by PB B-CLL B-cells, no significant association between Rai stage and CD38/CD31 phenotype was reported (18). A study carried out by Ibrahim *et al* showed that the percentage of CD31 expression alone did not predict for disease outcome in

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B-CLL (17). However, a low percentage of co-expression of CD31 and CD38 identified a group of patients with a better prognosis than all the other groups (17).

Since the density of CD38 is a significantly better prognostic marker for patients with B-CLL than the percentage expression (20), we performed a retrospective study to reassess the predictive power of CD31 in B-CLL. We quantified CD31 expression using flow cytometry and beads of specific antibody binding capacity and correlated the density of CD31 with clinical outcome. We also examined the impact of CD31 expression on the predictive power of CD38, both factors expressed as density rather than as the percentage of cells expressing each antigen.

## Materials and methods

**Patient population.** A cohort of 120 patients was studied. All patients were diagnosed as having B-CLL on the basis of clinical examination, peripheral blood count, absolute lymphocyte count, immunophenotyping and monoclonality of the  $\kappa$  or  $\lambda$  light immunoglobulin chain expression. The clinical outcomes for all patients were obtained from the patients' records. Patient selection was based on the availability of clinical histories, cryopreserved cells and laboratory findings, including CD38 density (20). Patient informed consent was obtained and ethical approval was granted by the Local Research Ethics Committee.

**Cells.** Peripheral blood mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradient (Nycomed, Sheldon, Birmingham, UK) and cryopreserved in tissue culture medium (RPMI-1640, Invitrogen Life Technologies, Paisley, UK) containing 10% v/v dimethylsulphoxide (NBS Biologicals, Huntington, UK) and 20% (v/v) heat-inactivated fetal calf serum (Invitrogen Life Technologies). Cells were stored at  $-140^{\circ}\text{C}$ .

**Expression of CD31 (percentage).** Peripheral blood lymphocytes ( $1 \times 10^6$ ) were stained for 45 min with 10  $\mu\text{l}$  of peridinin chlorophyll protein-labelled anti ( $\alpha$ )-CD19 (Becton Dickinson, Cowley, Oxford, England), 10  $\mu\text{l}$  of fluorescein isothiocyanate-labelled  $\alpha$ -CD5 (Becton Dickinson) and 15  $\mu\text{l}$  of phycoerythrin-conjugated  $\alpha$ -CD31 (Pharmingen, supplied by Becton Dickinson) monoclonal antibodies, or with PE-labelled control of the same isotype (and amount) as the CD31 antibody (Ab) (IgG1, Pharmingen). Cell-fluorescence was measured on a FACScan flow cytometer as described elsewhere (20).

The period of cell storage at  $-140^{\circ}\text{C}$  did not affect CD31 expression. Dual samples from four patients were thawed at different time intervals and cells were stained for CD31 expression as described above. Student's paired t-test showed that the pairing was effective: correlation coefficient (R)=0.9370; P=0.1079 (two-tailed).

**Quantification of CD31 expression (density).** The expression of CD31 was quantified by flow cytometry using microbeads of specific antibody binding capacity (ABC) (Quantum Simply Cellular kit; Sigma-Aldrich, Poole, Dorset, England) as described elsewhere (20). Briefly, 20  $\mu\text{l}$  of beads were

labelled under the same conditions and with an equal amount of CD31 Ab as the experimental samples. Using incremental quantities of beads and the same amount of the test Ab, it was revealed that the beads at 20  $\mu\text{l}$  were saturated with the Ab. A calibration curve was constructed as described elsewhere (20) and was used to obtain ABC values for the test samples. Storage at  $-140^{\circ}\text{C}$  did not affect ABC values; using dual samples thawed at different time intervals as described previously gave a P-value of 0.3796; R=0.6536. The variation between the different calibration curves was insignificant (n=4; mean of R  $\pm$  SD,  $0.9997 \pm 9.13 \times 10^{-5}$ ).

**Statistical analyses.** Fisher's exact and Spearman rank correlation tests were used to identify the cutoff point for disease outcome and the association between CD38 and CD31 expression, respectively. The Mann-Whitney U test was employed to examine any significant differences in the expression of CD31 between male and female patients or between patients with Binet stage A and stage B/C. Kaplan-Meier survival curves were plotted using SPSS for Windows, version 11. P-values  $<0.05$  were regarded as significant and between 0.05 and 0.1 as suggestive of a trend.

## Results

**Patient population.** The cohort of 120 patients consisted of 70 males and 50 females, median age 70 years (range, 47-91; Table I). At diagnosis, 103 were in Binet stage A and 17 in stage B or C (Table I). Median follow up was 6.9 years (range: 7 months to 22 years 2 months). There were 32 deaths in the patient cohort, 22 from CLL (12 in stage A patients), four from ischaemic heart disease, two from sepsis and four from cancer. Fifty-seven patients had received treatment for CLL before entry to this study. The patient demographics are given in Table I.

**CD31 density.** All patients were positive for CD31. The mean percentage expression (SD) was 95.9% (2.87). The mean (SD) of the ABC values was: 30218 (13243). There was no correlation between CD31 density and age (Spearman's rank: R=0.02284, P=0.8044) and CD31 was equally distributed between the male and female groups (Mann-Whitney U test, P=0.3024). There was a significantly higher level of CD31 expression within the stage A group of patients than those in stage B and C (Fig. 1a, P=0.0003, Mann-Whitney U test). The 75th percentile of CD31 density as ABC value (38294 ABC) was found to be the cutoff point that identified disease outcome (Fisher's exact). High density of CD31 (equal to or above the cutoff value of 38294 ABC) predicted for disease-specific survival (Fig. 2a; Kaplan-Meier, P=0.0087) and time to requiring treatment (TTT) (Fig. 2f; Kaplan-Meier, P=0.0064) and identified Binet stage A patients who had more aggressive disease (Figs. 2b and g; Kaplan-Meier: disease-specific survival, P=0.035; TTT, P=0.0716). All CLL-related deaths occurred in patients with low CD31 (Table I). No patient with Binet stage B/C and high CD31 density was identified (Table I). Low CD31 density predicted for a more aggressive clinical course irrespective of patient age (Fig. 2c, P=0.045 and 2d, P=0.021) or stage of disease progression (Fig. 2e, P<0.0001). Patients

Table I. Patient characteristics.

Patients	All patients	CD31 high	CD31 low	CD38 high	CD38 low
Number	120	30	90	27	93
Male(M):Female(F)	70:50	16:14	54:36	19:8	51:42
Median age at diagnosis (range)	70 (47-91)	73 (52-91)	69 (47-90)	68 (47-90)	71 (49-91)
Binet A (M:F)	103	30 (16:14)	73 (39:34)	14 (8:6)	89 (47:42)
Binet B (M:F)	8	-	8 (6:2)	7 (5:2)	1 (1:0)
Binet C (M:F)	9	-	9 (9:0)	6 (6:0)	3 (3:0)
CLL deaths	22	-	22	13	9

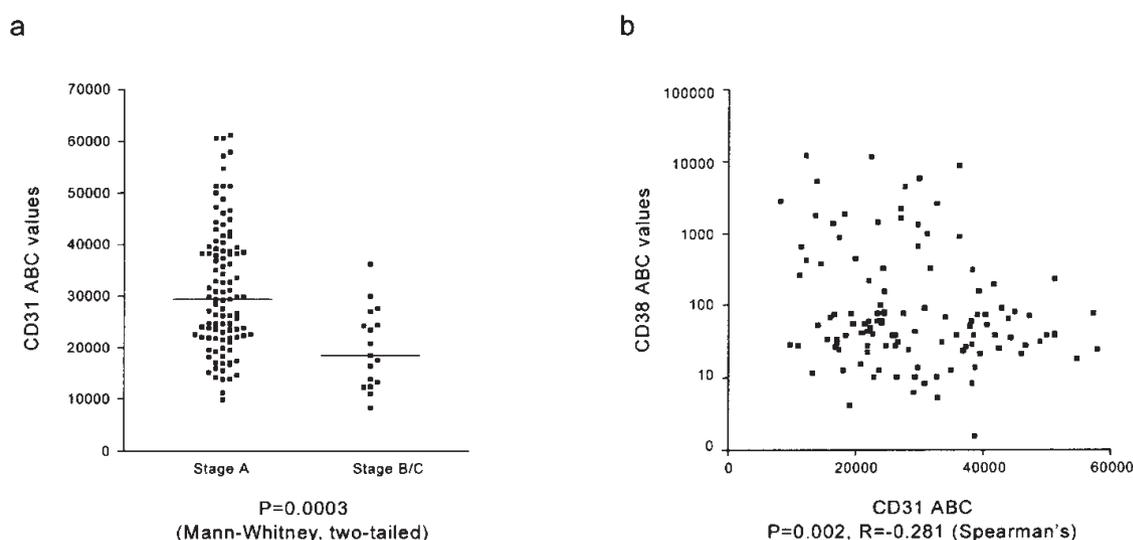


Figure 1. The density of CD31 expressed as ABC values was significantly higher in patients with Binet stage A disease (n=103) than in patients with progressive disease (Binet stage B/C; n=17) (a). The mean ABC values (SD) and confidence intervals (CI) were: stage A: 31141 ABC (12345); CI: 33541 and 28740; and stage B/C: 20241 ABC (7804); CI, 24400 and 16082. There was a weak but significant inverse correlation between the densities of CD31 and CD38 (b).

with low CD31 and in stage B/C or under 60 years of age had the worse prognosis (Figs. 2e, c and d, respectively).

**CD31 and CD38 densities.** When all patients were included in the analysis, there was a significant and inverse correlation between CD31 and CD38 densities (Fig. 1b).

To investigate the impact of CD31 density on the prognostic value of CD38 density, we used the CD38 ABC values of the same cohort of patients obtained in a previous study performed by our group (20). The cutoff point for CD38 density in this previous study was 250 ABC (20). Survival period of patients with low CD31 (below 38294 ABC) and high CD38 (above or equal to 250 ABC) was significantly shorter than all the other groups (Fig. 3a and b, Kaplan-Meier). When all patients were included in the analysis, low CD31 identified those with low CD38 who had more aggressive disease (Fig. 3a,  $P=0.0425$ ). No patient with high density of both CD31 and CD38 was identified in the cohort of 120. There was a suggesting trend that low CD31 density predicted for disease outcome for Binet stage A patients with low CD38 (Fig. 3b,  $P=0.0721$ ).

**Statistical analysis.** In Cox multivariate analysis when all patients were included and stage, age, CD31 and CD38 densities were analysed, stage was the most significant predictor of survival ( $P<0.0001$ ).

## Discussion

This is the first report to show that the level of expression (density) on the malignant cells of CD31, the physiological ligand for CD38, is a significant prognostic indicator for patients with B-CLL.

Low CD31 density predicted for poor disease outcome for all patients and for Binet stage A patients separately. Also, low CD31 identified the group of patients with good prognosis (i.e. low CD38) who were at high risk of disease progression. Interestingly, all disease-related deaths occurred in patients with low CD31 while, regarding CD38 expression, there was almost an equal distribution of deaths between the group with low and that with high CD38 density. In addition, the mean value of CD31 density of the group of patients with progressive disease was significantly lower than the mean

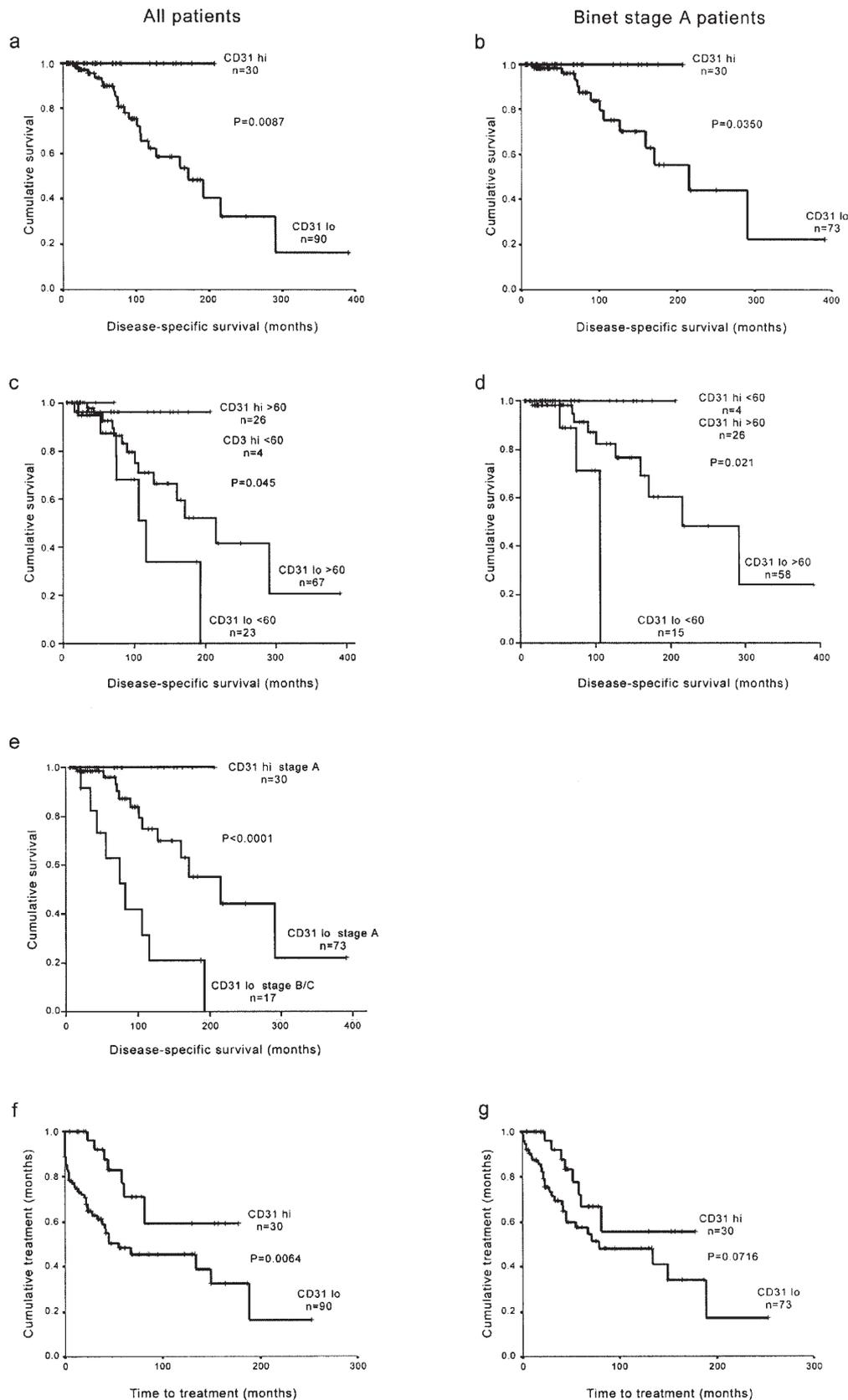


Figure 2. Disease-specific survival for all patients (a, c and e) and for Binet stage A patients (b and d) was plotted on the basis of high (hi) or low (lo) CD31 density taking the 75th percentile of expression (38294 ABC) as the cutoff point using Fisher's exact test. CD31 hi,  $\geq 38294$  ABC; CD31 lo,  $< 38294$  ABC. (a and b) Disease-specific survival curves for 'all' and Binet stage A patients, respectively, based on CD31 hi or lo density. (c and d) Disease-specific survival of 'all' and Binet stage A patients, respectively, based on CD31 density and patient age. CD31 hi and  $>60$  years vs CD31 lo and  $>60$  years: 'all patients',  $P=0.082$ ; Binet stage A,  $P=0.065$ . CD31 lo and  $>60$  years vs CD31 lo and  $<60$  years: 'all patients',  $P=0.086$ ; Binet stage A,  $P=0.050$ . (e) Survival curve of patients on the basis of CD31 hi or lo and Binet stage A or B/C. CD31 hi Binet stage A vs CD31 lo Binet stage A,  $P=0.035$ ; CD31 lo Binet stage A vs CD31 lo Binet stage B/C,  $P<0.0001$ . CD31 hi Binet stage A vs CD31 lo Binet stage B/C,  $P<0.0001$ . (f and g) Kaplan-Meier curves representing time to treatment for all patients and for Binet stage A patients, respectively, based on high ( $\geq 38294$  ABC) or low ( $< 38294$  ABC) CD31 density.

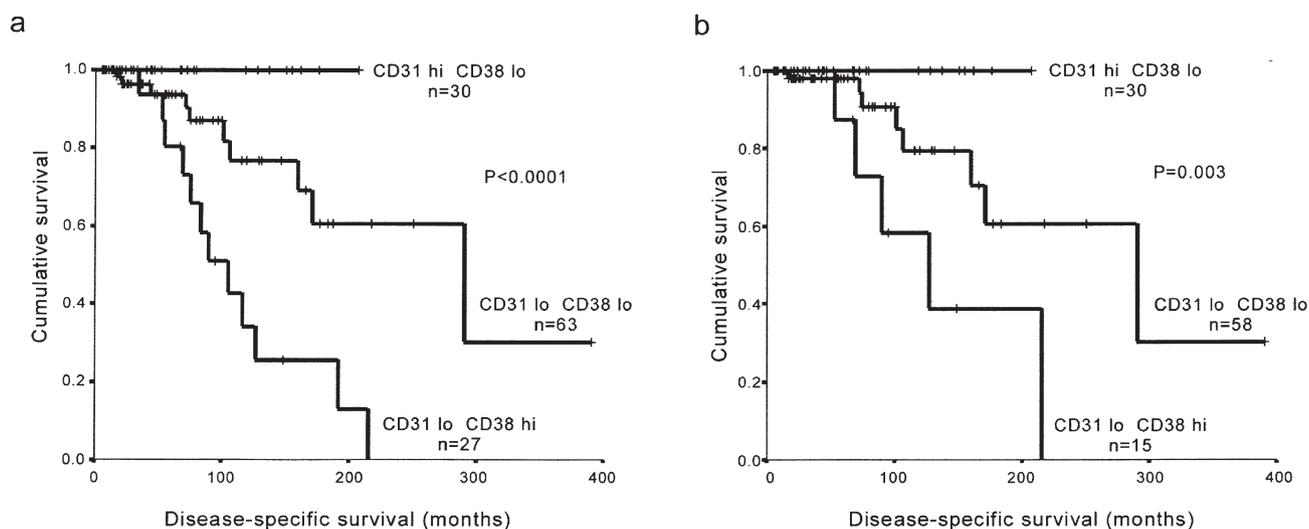


Figure 3. (a and b) Disease-specific survival of all patients and Binet stage A patients, respectively, on the basis of CD31 and CD38 densities. The cutoff points were: CD31 hi,  $\geq 38294$  ABC; CD31 lo,  $< 38294$  ABC and CD38 hi,  $\geq 250$  ABC; CD38 lo,  $< 250$  ABC, as assessed in a previous study (20). CD31 hi CD38 lo vs CD31 lo CD38 lo: 'all patients',  $P=0.0425$ ; Binet stage A,  $P=0.0721$ . CD31 lo CD38 lo vs CD31 lo CD38 hi: 'all patients',  $P=0.0010$ ; Binet stage A,  $P=0.0210$ . CD31 hi CD38 lo vs CD31 lo CD38 hi: 'all patients',  $P<0.0001$ ; Binet stage A,  $P=0.0030$ .

value of the group in stage A disease. No patient with high CD31 and in Binet stage B/C of disease progression was identified in this cohort of patients. These findings imply that low CD31 density may relate to disease overload and also that low CD31 is a better predictive indicator than high CD38 density. Furthermore, the presence of both adverse factors (i.e. high CD38 and low CD31) identified the group of patients with the highest risk of disease progression. Interestingly, no patient with high density of both factors was identified in this cohort. These findings prompt us to speculate that the level of expression of CD31 and its receptor CD38 by the malignant cells could partly govern disease aggression. Indeed, in patients with MM, CD38 and CD31 positivity (density) has been associated with the less aggressive forms of the disease while most patients with the more aggressive plasmablastic MM or plasma cell leukaemia express high levels of CD38 but CD31 is absent (16). Since CD31 is the counter-receptor for CD38 (9) and as both molecules are involved in the activation of cell survival (7,11,12) or apoptosis pathways (8,13), it is possible that these two molecules may interact with each other and the outcome of such interplay may affect disease progression by affecting the survival patterns of the malignant cells. Indeed, a study dating from 1996 reported that ligation of CD38 induced by its dimerization with a specific monoclonal antibody resulted in the activation of signal transduction pathways that inhibited proliferation and induced apoptosis of human immature B-cells (21). Since these activities were induced only upon CD38 ligation, these authors suggested the existence of a natural ligand for CD38 (21). In addition, a recent study showed that the ligation of CD31 with CD38 is involved in T-cell selection in the thymus and induces apoptosis of the double-positive thymocytes (22).

It is possible to hypothesise that in B-CLL, homophilic (with itself) or heterophilic (with CD38) interactions of CD31 (23) within the microenvironment of the lymph node

(LN) or BM may also lead to the activation of apoptotic pathways and the subsequent death of the leukaemic cells. Such interplay may act as a negative system to counteract the growth potential of the CD38<sup>+</sup> malignant cells within the biological setting of BM and/or LN microenvironment where cytokines and interactions of the malignant cells with endothelial (CD31<sup>+</sup>) and nurse-like cells enhance their proliferation and survival (24,25).

In the present study, it is likely that the affinity of such interaction and, indirectly, the density of receptor and its counter-receptor, may be an important factor that governs the activation of apoptosis pathways. Such interaction between CD31 and CD38, both being expressed on malignant cells, may also provide an explanation for the poor disease outcome observed in some patients with B-CLL who express different levels of CD38, as it has been suggested (18).

In contrast to the findings of the present study, Ibrahim *et al* reported that CD31 expression alone did not predict for disease outcome but low co-expression of both CD31 and CD38 identified patients with a good prognosis (17). However, these authors examined the predictive value of the percentage of the co-expressed factors, while the present study investigated the prognostic significance of the densities of CD31 and CD38, both factors expressed on malignant cells. Furthermore, although CD31 and CD38 were co-expressed in patients with B-CLL, the CD38/CD31 phenotype did not associate significantly with the stage of disease (18). Again, this study examined the role of the percentage rather than the level of expression of CD31 and CD38 (18).

In conclusion, the density of CD31 identifies a group of patients with high expression who have a major survival advantage over patients with low expression. Low levels of CD31 identify patients with low CD38 who have a worse prognosis. The mode of action by which CD31 and CD38 affect the clinical outcome in B-CLL merits further investigation.

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