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LYMPHOID NEOPLASIA

CD49d promotes disease progression in chronic lymphocytic leukemia: new insights from CD49d bimodal expression

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KEY POINTS

- CLL with bimodal CD49d expression accounts for ~20% of CLL and displays distinct clinicobiological features.
- The prognostic impact of CD49d is increased by considering CD49d bimodal expression.

CD49d is a remarkable prognostic biomarker of chronic lymphocytic leukemia (CLL). The cutoff value for the extensively validated 30% of positive CLL cells is able to separate CLL patients into 2 subgroups with different prognoses, but it does not consider the pattern of CD49d expression. In the present study, we analyzed a cohort of 1630 CLL samples and identified the presence of ~20% of CLL cases (n = 313) characterized by a bimodal expression of CD49d, that is, concomitant presence of a CD49d^{+} subpopulation and a CD49d^{-} subpopulation. At variance with the highly stable CD49d expression observed in CLL patients with a homogeneous pattern of CD49d expression, CD49d bimodal CLL showed a higher level of variability in sequential samples, and an increase in the CD49d^{+} subpopulation over time after therapy. The CD49d^{+} subpopulation from CD49d bimodal CLL displayed higher levels of proliferation compared with the CD49d^{-} cells; and was more highly represented in the bone marrow compared with peripheral blood (PB), and in PB CLL subsets expressing the CXCR4^{dim}/CD5^{bright} phenotype, known to be enriched in proliferative cells. From a clinical standpoint, CLL patients with CD49d bimodal expression, regardless of whether the CD49d^{+} subpopulation exceeded the 30% cutoff or not, experienced clinical behavior similar to CD49d^{+} CLL, both in chemoimmunotherapy (n = 1522) and in ibrutinib (n = 158) settings. Altogether, these results suggest that CD49d can drive disease progression in CLL, and that the pattern of CD49d expression should also be considered to improve the prognostic impact of this biomarker in CLL. (*Blood*. 2020;135(15):1244-1254)

Introduction

CD49d, the rate-limiting α-chain of the CD49d/CD29 integrin heterodimer very late antigen-4, expressed in ~40% of chronic lymphocytic leukemia (CLL) cases, is a strong independent predictor of survival and treatment need in CLL.[1-6]

CD49d/CD29 mediates both cell-cell and cell-matrix interactions in CLL-involved tissues, delivering prosurvival signals and protecting CLL cells from drug-induced apoptosis.[7] Moreover, CD49d/CD29 has a key role in driving homing of CLL cells to lymphoid tissues.[8,9] This is confirmed by the association between high CD49d expression and the presence of lymphadenopathy at diagnosis, the development of lymphadenopathy during the course of the disease, and the almost universal CD49d expression in CLL subsets with prevalent nodal disease.[10-12] Lymph nodes (LNs) represent the predominant sites of CLL cell activation and proliferation.[13,14]

Concordantly, LN-derived CLL cells are characterized by a distinct gene-expression profile and phenotype, including higher CD49d expression than in paired peripheral blood (PB) samples.[8,15] A higher CD49d expression was also documented in PB CXCR4^{low}/CD5^{bright} CLL cells, a cell subset representing the proliferative LN-derived CLL cell fraction.[8,16]

In CLL, CD49d expression is variable, from completely negative cases to cases uniformly expressing the molecule at very high levels.[17-19] The 30% cutoff, which robustly separates CLL patients into 2 subgroups with different prognoses, has been extensively validated.[1-5] However, the simple use of the cutoff does not provide information on the expression pattern of CD49d. Indeed, we previously observed that CD49d may be expressed in a bimodal pattern in CLL, with the copresence of clearly distinct negative and positive groups of cells in the same sample.[20,21] In this context, CD49d$^+$ subpopulations, especially when represented in <30% of cells, raise the question of whether they may or may not impact disease progression.

Here, we analyzed CD49d expression in a large cohort of CLL cases with the following aims: (i) to determine the exact frequency of CLL cases with a bimodal pattern of CD49d expression; (ii) to highlight potential functional differences between the CD49d$^-$ and CD49d$^+$ cells in CLL expressing CD49d with a bimodal pattern; and (iii) to evaluate the clinical impact of CD49d bimodal expression.

Methods

CLL patients and primary CLL cell characterization

The study, performed under the institutional review board (IRB) approval of the Aviano Centro di Riferimento Oncologico (approval nos. IRB-05-2010 and IRB-05-2015), included 1630 CLL cases diagnosed and treated according to the current International Workshop on Chronic Lymphocytic Leukemia (iwCLL) guidelines,[22] from a consecutive series of 2045 multicenter patients all referred to the Clinical and Experimental Onco-Hematology Unit of the Centro di Riferimento Oncologico in Aviano for immunocytogenetic analyses between 2006 and 2017. For the purposes of clinical analyses, patients were split into 2 cohorts: cohort A (764 patients from a single center) and cohort B (866 patients from 5 different centers), whose clinical and biological features are summarized in supplemental Table 1 (available on the *Blood* Web site). This study also included 158 CLL patients (47 cases relapsed/refractory [RR] CLL from the cohort of 1630, and an additional 88 RR and 23 treatment-naive CLL) treated with ibrutinib in the context of a multicenter Italian named patient program; clinical and biological features of this cohort are summarized in supplemental Table 2. Informed consent was obtained in accordance with the Declaration of Helsinki. Patients' characterization included *IGHV* mutational status, cytogenetic abnormalities, *TP53* and *NOTCH1* mutations, all detected as previously reported,[2,23-27] and clinical parameters, such as age, Rai stage, and β-2-microglobulin (B2M) levels.

Immunophenotypic analyses, cell sorting, proliferation assay, and telomere-length measurements

Immunophenotypic analyses and cell sortings were performed on fresh PB, or paired PB and bone marrow (BM) samples at different time points using a FACSCanto I and a FACSAriaIII (BD Biosciences, La Jolla, CA) flow cytometer/cell sorter upon instrument calibration with CS&T beads (BD Biosciences) using the FACSDiva software (BD Biosciences). Detailed guidelines for the analysis and definition of CD49d bimodal expression[28] are reported in supplemental Materials and methods.

Proliferation and telomere-length assays were performed using previously published procedures.[29,30] Details on these assays, and on the statistics used for clinical correlations are included in supplemental Materials and methods.

Results

Frequency of CD49d bimodal expression in CLL

CD49d expression was analyzed in PB samples from 1630 CLL cases at diagnosis (supplemental Table 1). According to the 30% cutoff identified for clinical purposes,[1,2] 904 cases (55.5%) were CD49d$^-$ and 726 cases (44.5%) were CD49d$^+$. In 313 cases (19.2%), CD49d had a bimodal expression (bimCD49d), with 2 separate cell populations, 1 completely negative, with a fluorescence signal superimposable to that of the negative control, and the other positive, characterized by a fluorescence signal completely above the cutoff (Figure 1A; supplemental Figure 1). CLL cells from the remaining 1317 patients (80.8%) expressed CD49d in a homogeneous pattern (homCD49d) (Figure 1B). In terms of percentage of CD49d-expressing CLL cells, virtually all values (ranging from 1% to 99%) were represented both in the bimCD49d and homCD49d groups. However, although in bimCD49d cases all CD49d expression values were almost equally represented, 90% of CLL with homCD49d expression showed either very low (56% of cases with expression ≤20% CD49d) or very high (34% cases with expression ≥80% CD49d) expression levels, with as low as 2% of cases clustered around the 30% cutoff (Figure 1C-D), in keeping with previous observations.[1,2] Of note, the vast majority of cases expressing CD49d around the 30% cutoff (CD49d expression ranging between 20% and 40%) belonged to the bimCD49d group (67 of 94 cases; 71%).

The CD49d$^-$ and CD49d$^+$ cell subpopulations from bimCD49d CLL derive from the same clone

The *IGHV* mutational status was assessed by Sanger sequencing in 303 bimCD49d. In 283 of 303 cases (93.4%), a unique *IGHV* sequence was detected, whereas among the remaining 20 cases either multiple *IGHV* sequences were found (n = 14) or no *IGHV* sequence could be determined (n = 6). A detailed description of the *IGHV* gene usage and status is reported in supplemental Table 3. The *IGHV* sequence analysis was then repeated by next-generation sequencing in the sorted CD49d$^-$ and CD49d$^+$ fractions from 31 bimCD49d samples with a variable amount of CD49d$^+$ subpopulation, including 1 biclonal sample presenting 2 different immunoglobulin rearrangements by Sanger sequencing. Next-generation sequencing analysis confirmed a unique *IGHV* sequence in both CD49d$^-$ and CD49d$^+$ fractions in 30 cases, and the concomitant presence of 2 sequences in the biclonal sample, without differences between the CD49d$^-$ and CD49d$^+$ fractions (supplemental Table 3).

The proportion of the CD49d$^+$ subpopulation in bimCD49d CLL tends to increase over time

Sequential samples were available in 94 bimCD49d and in 322 homCD49d patients (median sample interval: 34 months [range, 3-116 months] for bimCD49d and 35 months [range,

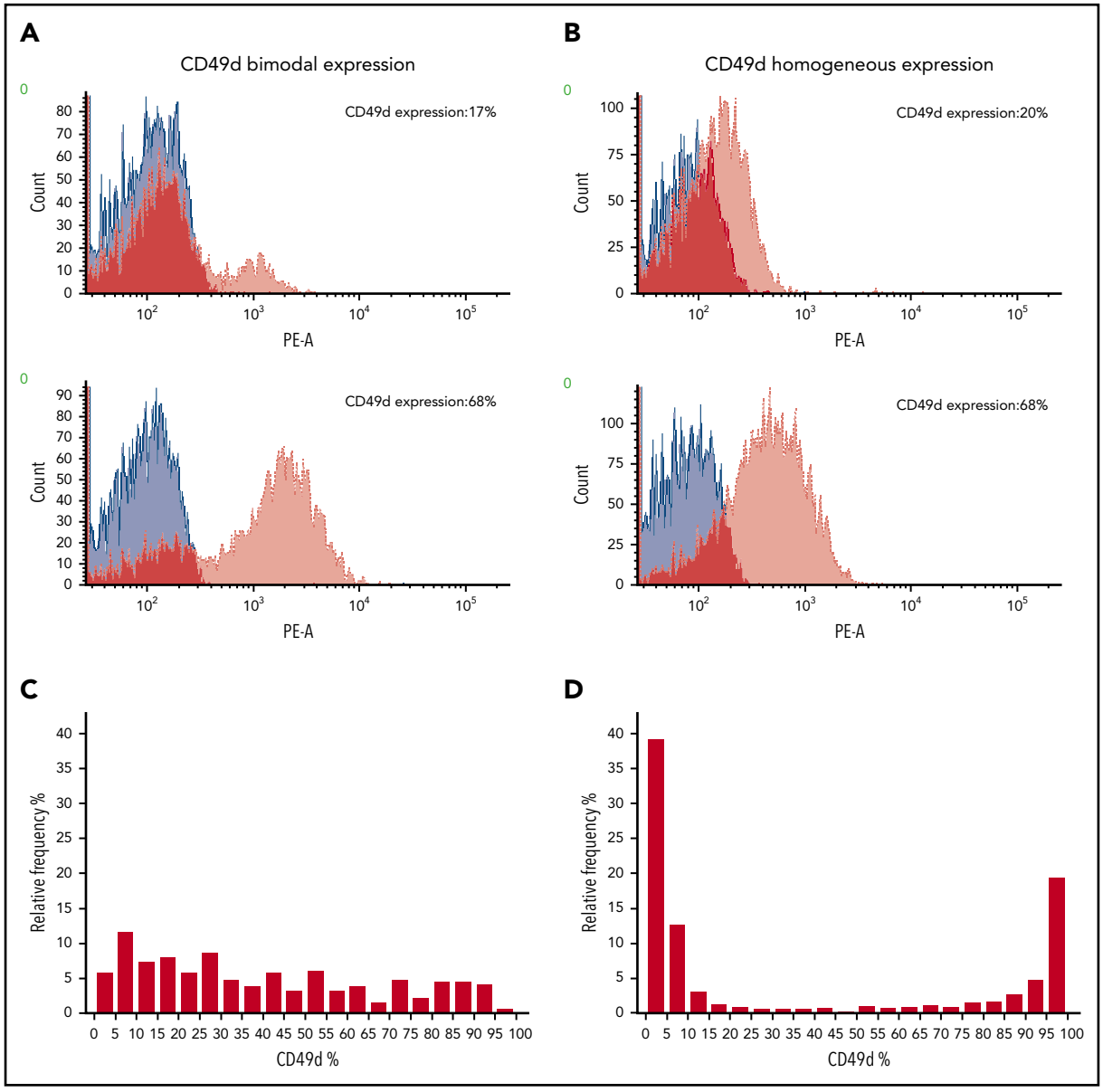

Figure 1. CD49d bimodal and homogeneous expression. (A-B) Histogram plots of CD49d expression (red) in 2 representative CLL cases with CD49d bimodal expression (A) and in 2 representative CLL cases with CD49d homogeneous expression (B). Blue histograms refer to unstained cells. The dark red color corresponds to the overlap of CD49d and unstained histogram plots. (C-D) Relative percentage of frequency of CD49d expression in CLL with CD49d bimodal (C) and CD49d homogeneous (D) expression.

6-161 months] for homCD49d). Forty-two patients (44.7%) and 131 patients (40.7%) from the bimCD49d and homCD49d groups, respectively, had received 1 line of therapy after the first sampling, whereas the remaining patients received no treatment before the 2 samplings.

Compared with homCD49d CLL, where CD49d expression was highly stable over time (supplemental Figure 2A-B), bimCD49d cases were characterized by a higher variability of CD49d expression between the first and the second sample (supplemental Figure 2C-D). Despite this, the proportion of CD49d$^+$ cells, almost stable between the first and the second sample in untreated bimCD49d cases (Figure 2A), increased after the first line of treatment (P = .03) (Figure 2B). Moreover, a detailed analysis of CD49d expression in sequential samples from additional bimCD49d CLL patients receiving 2 to 4 lines of therapy (n = 13; median sample intervals = 33 months) highlighted an increasing proportion of CD49d$^+$ cells with a tendency toward plateau as the number of treatment lines increased (Figure 2C).

The proportion of CD49d$^-$ and CD49d$^+$ cell subpopulations varies among different tissue compartments

The proportion of CD49d$^-$ and CD49d$^+$ subpopulations was analyzed in paired PB and BM samples from 13 bimCD49d cases. A higher amount of CD49d$^+$ cells was observed in BM compared with PB samples (P = .0006). Notably, in 3 cases, the CD49d$^+$ subset increased from <30% in the PB to >30% in the BM (Figure 3A).

We next analyzed the amount of CD49d$^+$ cells in the context of subpopulations with variable reciprocal densities of CXCR4/CD5

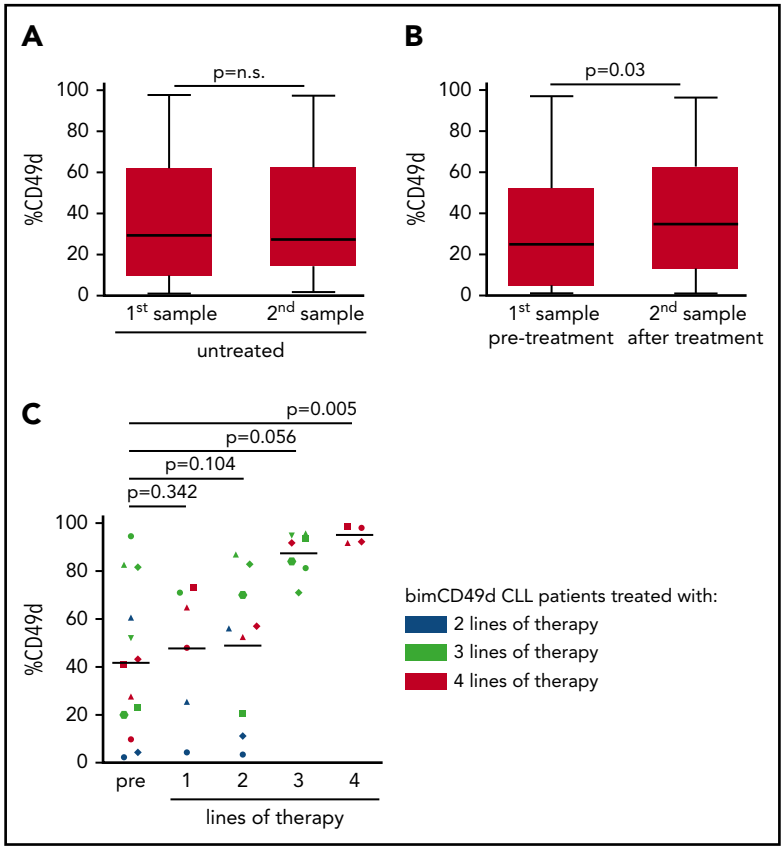

Figure 2. CD49d expression over time. CD49d expression was evaluated in 2 sequential samples from 94 CLL cases with CD49d bimodal expression either untreated (n = 52; A) or treated once between the samples (n = 42; B). (C) Variation of the amount of CD49d$^+$ cells in sequential samples from 13 CD49d bimodal CLL treated with 2 lines of therapy (n = 3; blue symbols), 3 lines of therapy (n = 6; green symbols), or 4 lines of therapy (n = 4; red symbols). Each symbol corresponds to a different case; *P* values refer to the paired Wilcoxon test (A-B) and to the Bonferroni-corrected Student *t* test (α = 0.0125).

(supplemental Figure 3), representing cell subsets defined as "recent emigrants from tissue sites" (CXCR4^{dim}/CD5^{bright}) or "attempting to reenter the tissue sites" (CXCR4^{bright}/CD5^{dim})[16] in 147 bimCD49d CLL. A median of 8% (range, 3% to 35%) and 7% (range, 3% to 14%) of total CLL populations was identified as CXCR4^{dim}/CD5^{bright} and CXCR4^{bright}/CD5^{dim}, respectively. Overall, the CXCR4^{dim}/CD5^{bright} fractions were characterized by significantly higher proportions of CD49d$^+$ cells compared with their paired CXCR4^{bright}/CD5^{dim} fractions ($P < .0001$) (Figure 3B).

CD49d$^+$ cells display a higher propensity to proliferate than CD49d$^-$ cells

The CXCR4^{dim}/CD5^{bright} cell fraction is known to mark CLL subsets with increased proliferation compared with CXCR4^{bright}/CD5^{dim} cells.[16] Consistently, proliferation experiments using cells from bimCD49d CLL (n = 10), evidenced a higher proportion of proliferating cells in the CD49d$^+$ compared with the CD49d$^-$ fractions ($P = .004$) (supplemental Figure 4A-B), and no acquisition or loss of CD49d expression by the CD49d$^-$ and CD49d$^+$ fractions, respectively (supplemental Figure 5).

We next examined the telomere length in paired CD49d$^-$ and CD49d$^+$ subpopulations from 15 CLL samples (supplemental Figure 6). Despite an intraclonal variation in the length of individual telomeres, all subpopulations tested showed overall short telomeres, indicating that they had undergone a large number of cell divisions,[31] without substantial differences between the paired CD49d$^+$ and CD49d$^-$ cells (mean telomere length, 3.1 kb and 3.2 kb, respectively). Of note, in 2 cases (CLL#7 and CLL#8; supplemental Figure 6 arrows) the CD49d$^+$ subpopulations displayed shorter telomeres (>1 kb difference)

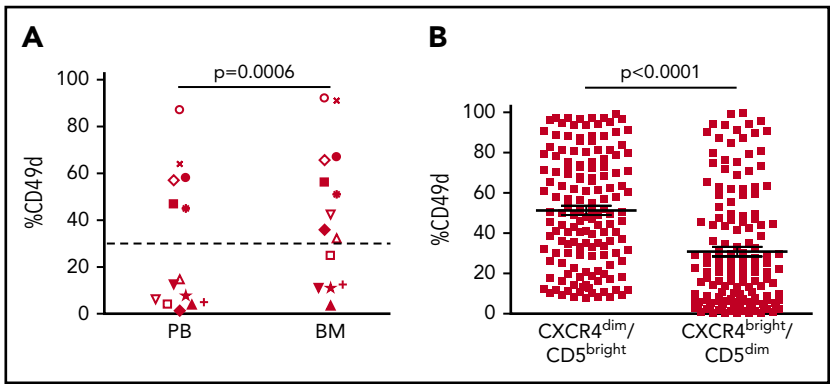

Figure 3. Amount of CD49d$^+$ cells in different tissue compartments and in intraclonal populations from bimCD49d CLL. (A) Percentage of CD49d$^+$ cells in paired PB and BM samples from 13 CLL cases with CD49d bimodal expression. Each symbol corresponds to a different case. (B) Amount of CD49d$^+$ cells in intraclonal subpopulations with variable reciprocal densities of CXCR4/CD5 expression (CXCR4^{dim}/CD5^{bright} and CXCR4^{bright}/CD5^{dim} fractions) from 147 CLL cases with CD49d bimodal expression; *P* values refer to the paired Wilcoxon test.

compared with the CD49d$^-$ cells, suggesting a greater proliferative history for the CD49d$^+$ cell fractions.

Finally, we performed a comprehensive cytogenetic and mutation analysis in the sorted CD49d$^-$ and CD49d$^+$ cell fractions from 56 bimCD49d CLL cases characterized by 1 or more genetic abnormalities (75 total tests).[4,32] In this context, significant associations between CD49d$^+$ cells with trisomy 12 and *NOTCH1* mutations, as we previously described,[20,21] and between CD49d$^-$ cells and 17p deletion, were detected (supplemental Tables 4 and 5).

CLL patients with CD49d bimodal expression have clinical behavior similar to CD49d$^+$ CLL in the context of standard chemoimmunotherapy

To study the clinical behavior of bimCD49d CLL in terms of overall survival (OS), CLL cases were split in 2 homogeneous cohorts (cohort A and cohort B, see "Methods" and supplemental Table 1). To exclude a potential bias due to improved survival for patients treated with novel agents including ibrutinib, only patients treated with standard chemoimmunotherapy were included in these analyses (n = 1522).

Notably, when comparing the clinical outcomes of bimCD49d$^-$ (ie, with a CD49d$^+$ component <30% cutoff) and bimCD49d$^+$ (ie, with a CD49d$^+$ component ≥30% cutoff) CLL, no OS differences were found in the separate and combined cohorts (supplemental Figure 7A-B; Figure 4A). Moreover, no other cutoff values able to split bimCD49d cases into 2 groups with different OS could be selected by receiver operating characteristic curve analyses (supplemental Figure 8A-B). Conversely, the main clinical and biological parameters showed the expected trends of OS in both cohorts (supplemental Figure 9). Given the superimposable clinical behavior of bimCD49d$^-$ and bimCD49d$^+$ CLL, we merged them in a single group and compared their OS probabilities with those of homCD49d CLL split according to the 30% cutoff. bimCD49d cases displayed significantly shorter OS compared with homCD49d$^-$ CLL ($P < .0001$ for cohort A, $P = .0013$ for cohort B, and $P < .0001$ for the combined cohort), and no difference with homCD49d$^+$ CLL in both the separate and combined cohorts (supplemental Figure 7C-D; Figure 4B).

The same analyses were performed using the treatment-free survival readout. The results obtained, overall similar to those obtained using the OS readout, are summarized in supplemental Figures 10-12.

CD49d bimodal expression has independent prognostic impact in patients treated with standard chemoimmunotherapy

To test the independent prognostic impact of bimCD49d expression, patients were split into 3 groups: homCD49d$^-$, homCD49d$^+$, and bimCD49d. As summarized in supplemental Table 6, the frequency of various negative clinical and biological features in bimCD49d CLL was usually intermediate between that observed in homCD49d$^-$ and homCD49d$^+$ CLL, with the exclusion of del17p/*TP53* disruption, which showed the lowest frequency in bimCD49d CLL. By univariable analysis, both homCD49d$^+$ and bimCD49d had a significantly increased hazard of death (hazard ratio [HR] = 3.18 [95% confidence interval (CI), 2.41-4.19] and HR = 2.37 [95% CI, 1.71-3.3], respectively) compared with homCD49d$^-$ patients (HR = 1.0, reference; Table 1). By Cox analysis for OS, both homCD49d$^+$ and bimCD49d remained independent predictors after adjusting for Rai stage, age, *IGHV* mutation status, del17p, del11q, *TP53* and *NOTCH1* mutation status in a model that did not include B2M (Table 1, model I, n = 1045) or did include B2M (Table 1, model II, n = 753). The same was observed in multivariable models considering *TP53* disruption instead of del17p and *TP53* mutations (supplemental Table 7).

Comparable results were obtained in a Cox analysis for treatment-free survival. Again, homCD49d$^+$ and bimCD49d had an increased risk of being treated in models that either included B2M (n = 831) or not included B2M (n = 1140) (supplemental Table 8).

Finally, we compared the prognostic power of CD49d considered either as dichotomous according to the canonical 30% cutoff, or merging homCD49d$^+$ plus bimCD49d CLL, both in univariable analysis and in the context of multivariable analyses that included Rai stage, age, *IGHV*, and *TP53* mutation status (supplemental Table 9). In both analyses, merging homCD49d$^+$ and bimCD49d CLL outperformed the 30% cutoff (C index = 0.63 [95% CI, 0.59-0.67] vs 0.64 [95% CI, 0.60-0.67]; $P < .001$) and improved the prognostic power of the Cox model (C index = 0.799 [95% CI, 0.748-0.809] vs 0.803 [95% CI, 0.750-0.814]; $P < .001$). Of note, bimCD49d cases that would have been misassigned to a low-risk group accounted for 9% of cases, corresponding to 147 cases in our cohort.

CD49d bimodal expression correlated with shorter PFS in ibrutinib-treated patients

The impact of CD49d bimodal expression on progression-free survival (PFS) under ibrutinib was evaluated in a cohort of 158 cases treated with ibrutinib in the context of an Italian multicenter named patient program, with a median follow-up of 16 months (supplemental Table 2). Among 158 patients, 29 were from a previous study of ours,[33] with follow-up information here updated. In all cases, CD49d expression was evaluated before starting ibrutinib.

The ibrutinib cohort was characterized by 39 bimCD49d cases (24.7%) and 119 homCD49d cases (75.3%). Also in this context, the CD49d 30% cutoff split homCD49d but not bimCD49d CLL into 2 groups with different prognoses (Figure 4C; supplemental Figure 13A). Moreover, bimCD49d cases displayed shorter PFS than homCD49d$^-$ CLL ($P = .004$), and no different PFS when compared with homCD49d$^+$ CLL (Figure 4C). Consistently, homCD49d$^+$ and bimCD49d CLL combined in a single group showed reduced PFS compared with homCD49d$^-$ CLL ($P = .0052$; supplemental Figure 13B).

The prognostic impact of homCD49d$^+$/ bimCD49d CLL was then tested in multivariable analyses. homCD49d$^+$/ bimCD49d CLL retained independent negative prognostic impact together with *TP53* disruption and >1 previous therapies, both in a multivariable model that considered the whole cohort of ibrutinib-treated patients (n = 158; Table 2), and in a subgroup of RR CLL (n = 124; Table 3) in which information on other prognostic factors[34] was available (Tables 2 and 3).

We then verified whether the proportion of CD49d$^+$ cells varied over time during ibrutinib treatment in patients with

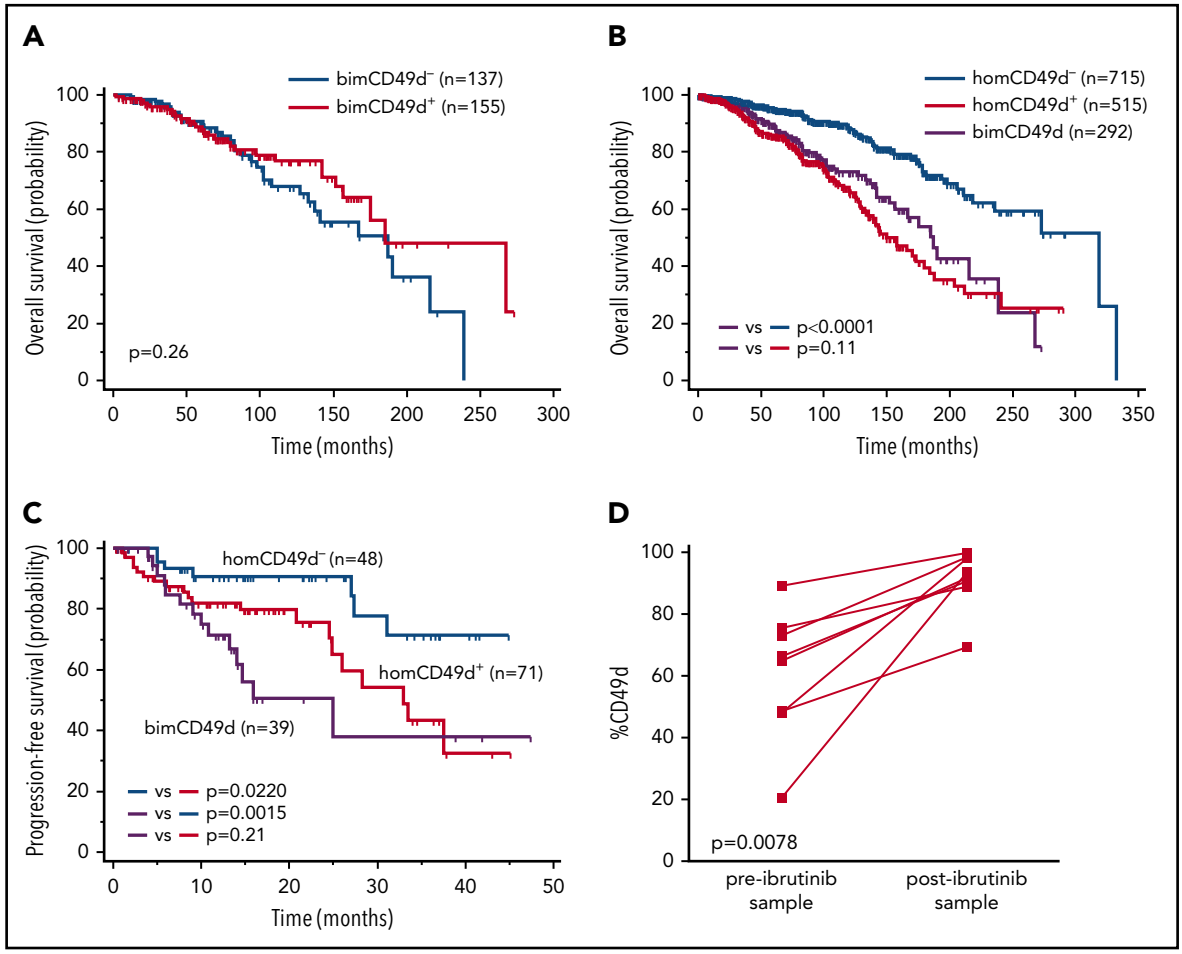

Figure 4. Clinical impact of CD49d bimodal expression in the context of chemoimmunotherapy and ibrutinib treatment. (A) OS Kaplan-Meier curves of all bimCD49d CLL cases split in bimCD49d$^-$ (blue curves) and bimCD49d$^+$ (red curves) groups according to the 30% cutoff. (B) OS Kaplan-Meier curves of CLL cases with bimodal CD49d expression (bimCD49d, purple curves), homogeneous-negative (homCD49d$^-$, blue curves), and homogeneous-positive (homCD49d$^+$, red curves) CD49d expression. (C) PFS Kaplan-Meier curves of ibrutinib-treated CLL cases with bimodal CD49d expression (bimCD49d, purple curve), negative (neg; blue curve), and positive (pos; red curve) homogeneous CD49d expression (homCD49d). (D) CD49d expression in 2 sequential samples from 8 CLL cases with CD49d bimodal expression treated with ibrutinib between samplings; *P* values refer to the log-rank test (A-C) and to the paired Wilcoxon test (D).

bimCD49d expression. Two consecutive samples were available in 8 bimCD49d cases, with a median time between the preibrutinib and postibrutinib sample collection of 28 months (range, 8-50 months). In all cases, an increased proportion of CD49d$^+$ cells emerged after treatment ($P = .0078$; Figure 4D; supplemental Figure 14).

CD49d expression and CLL-IPI risk categories

We finally tested whether CD49d expression had an impact on OS prediction in the context of the risk categories identified by the International Prognostic Index (CLL-IPI).[35] Complete data to score CLL cases according to the CLL-IPI were available in 878 patients from our cohort. The CLL-IPI was able to efficiently segregate CLL patients in 4 risk categories characterized by significantly different OS (Figure 5A). In the context of each risk category, we further split patients into 2 groups according to CD49d expression, considering homCD49d$^+$ and bimCD49d CLL as a single group (CD49d$^+$/bimCD49d). CD49d$^+$/bimCD49d CLL showed reduced OS in all CLL-IPI risk categories with the exception of the very-high-risk group, in which the survival probability of a very few CD49d$^-$ patients (n = 16) was similar to that of CD49d$^+$/bimCD49d patients (Figure 5B-E).

Discussion

This study was designed to investigate the functional and clinical implications of the bimodal expression of CD49d in CLL. In previous studies, we indicated the presence of CLL characterized by bimodal CD49d expression,[20,21] but the frequency and the clinical impact of CD49d bimodal expression has never been addressed. Here, using a large cohort of patients, we showed that the frequency of CD49d bimodal cases accounted for ~20% of total cases. The CD49d$^-$ and CD49d$^+$ subpopulations from the large majority of these bimodal cases shared the same *IGHV* rearrangement, confirming a common clonal origin.

CD49d in CLL is expressed at either very low or very high levels in most cases, with few cases displaying expression levels around the 30% cutoff.[1,2] This characteristic, associated with a low probability of misclassifying patients, is further emphasized by the results from the present study. Indeed, after sorting out CLL cases with CD49d bimodal expression, the frequency of CLL cases with 20% to 40% CD49d expression in the homCD49d group turned out to be even lower (from 6% to 2%, ie, 27 of 1630 cases).

Table 1. Cox regression analysis of OS

Factor	Univariable analysis				Multivariable analysis: model I (n = 1045)			Multivariable analysis: model II (n = 753)		
	Cases	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
homCD49d⁺*	1522	3.18	2.41-4.19	<.0001	2.13	1.52-2.99	<.0001	2.65	1.76-3.98	<.0001
bimCD49d†	1522	2.37	1.71-3.30	<.0001	2.12	1.44-3.12	.0001	2.70	1.74-4.17	<.0001
Age > 65 y	1522	3.16	2.46-4.06	<.0001	4.05	2.96-5.54	<.0001	3.72	2.55-5.44	<.0001
Rai stage II-IV	1515	2.68	2.10-3.43	<.0001	1.82	1.32-2.44	.0001	n.i.	n.i.	n.i.
UM *IGHV*	1385	3.88	3.02-5.00	<.0001	2.41	1.79-3.24	<.0001	2.78	1.99-3.91	<.0001
del17p	1407	4.81	3.29-7.03	<.0001	n.i.	n.i.	n.i.	2.21	1.44-3.38	.0003
del11q	1407	2.56	177-3.69	<.0001	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
tri12	1407	1.82	1.29-2.56	.0006	2.32	1.64-3.29	<.0001	0.65	0.43-0.98	.0423
TP53 mutated	1253	3.04	2.23-4.16	<.0001	2.41	1.79-3.24	<.0001	n.i.	n.i.	n.i.
NOTCH1 mutated	1444	2.02	1.54-2.65	<.0001	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
β2M > ULN	1017	4.12	3.01-5.63	<.0001	—	—	—	1.73	1.18-2.52	.0048

All factors used in univariable analyses were entered in the multivariable analysis.

—, variable not included in the model; β2M, β-2 microglobulin; CI, confidence interval; del, deletion; HR, hazard ratio; n.i., not included in the model after stepwise selection; tri, trisomy; ULN, upper limit of normal; UM, unmutated.

*homCD49d⁺ refers to CLL cases with ≥30% homogeneous expression of CD49d.

†bimCD49d refers to CLL cases with bimodal expression of CD49d.

The temporal stability, another important feature of CD49d expression,[1,2,36] has been here confirmed, particularly in the context of CLL completely lacking CD49d, in line with the methylation-dependent regulation of CD49d expression.[21] On the other hand, a fine-tune regulation of CD49d expression, observed in CLL cases with moderate to high CD49d expression, has been already reported in previous studies showing that microenvironmental stimuli are able to increase CD49d expression.[20,37] Because our flow cytometry analyses, based on the acquisition of at least 10 000 CLL events, could reach the 1% sensitivity, we cannot exclude the presence of lower percentages of CD49d⁺ cells in CLL grouped as homCD49d⁻. However, in none of these cases with sequential samples available did a measurable CD49d⁺ clone emerge. At variance with what was reported in homCD49d CLL cases, bimCD49d cases showed an overall increase of the CD49d⁺ subpopulation after treatment, with a tendency toward a plateau subsequent to multiple lines of therapy. This observation may be explained by the selection of CD49d⁺ CLL cells that find protection from therapy and/or gain proliferative advantage in the context of microenvironmental niches.[38] Consistently, the CD49d⁺ subpopulation of bimodal cases was increased in the BM, which is a privileged site of relapse in CLL,[39,40] compared with PB. Even though we did not have the chance to analyze paired LN-derived samples from CD49d bimodal cases, the analysis of CD49d expression in the context of the proliferative fraction CXCR4^{low}/CD5^{bright}, which was indicated as the group of cells recently egressed from lymphoid tissues,[16] highlighted a higher proportion of CD49d⁺ cells compared with their paired fractions. Consistently, bimCD49d cases presented with LN involvement at diagnosis more frequently compared with homCD49d⁻ CLL, but less frequently than CD49d⁺ CLL, in which baseline lymphadenopathy accounted for ~62% of cases, in line with previously reported data.[10] In this context, it remains to be determined whether bimCD49d CLL may be more prone than homCD49d⁻ CLL to develop lymphadenopathy during the course of the disease.[10]

Table 2. Cox regression analysis for PFS in ibrutinib-treated CLL cohort (whole cohort, n = 158)

Factor	Univariable analysis			Multivariable analysis*		
	HR	95% CI	*P*	HR	95% CI	*P*
CD49d⁺†	3.03	1.34-6.87	.0079	2.63	1.13-6.10	.0242
UM *IGHV*	1.71	0.75-3.88	.199	—	—	—
TP53 disruption	2.63	1.37-5.06	.0038	2.10	1.06-4.14	.0327
Rai stage III-IV	2.43	1.31-4.49	.0046	n.i.	n.i.	n.i.
Lines of therapy >1‡	4.49	2.39-8.45	<.0001	4.80	2.54-9.08	<.0001

*All factors with a significant *P* value in univariable analyses were entered in the multivariable analysis.

†CD49d⁺ cases were obtained by combining cases with ≥30% homogeneous CD49d expression and cases with CD49d bimodal expression.

‡Comparison between 0 and 1 vs >1 lines of therapy.

Table 3. Cox regression analysis for PFS in ibrutinib-treated CLL (R/R cohort, n = 124)

Factor	Univariable analysis			Multivariable analysis*		
	HR	95% CI	*P*	HR	95% CI	*P*
CD49d⁺†	3.42	1.39-8.43	.0075	3.41	1.32-8.79	.0112
UM *IGHV*	1.94	0.67-5.58	.2191	—	—	—
TP53 disruption	4.01	1.82-8.82	.0006	3.06	1.34-6.97	.0079
Hemoglobin, g/L <110 for women <120 for men	3.73	1.79-7.78	.0004	—	—	—
Rai stage III-IV	2.87	1.40-5.91	.0041	—	—	—
β2M ≥5 mg/L	1.09	0.44-2.68	.8513	—	—	—
LDH > ULN	2.14	1.05-4.40	.0373	—	—	—
Time from last therapy <24 mo	1.98	0.90-4.38	.0907	—	—	—
Lines of therapy >1‡	5.08	2.34-11.03	<.0001	6.68	2.91-15.35	<.0001

LDH, lactose dehydrogenase.

*Significant features for multivariate analysis were selected through a Cox LASSO (least absolute shrinkage and selection operator) regression, selecting variables with nonzero coefficient under the best λ selected by the model.[48]

†CD49d⁺ cases were obtained by combining cases with ≥30% homogeneous CD49d expression and cases with CD49d bimodal expression.

‡Comparison between 1 and >1 lines of therapy.

In keeping with the association with a CXCR4^{low}/CD5^{bright} phenotype,[16] CD49d⁺ subpopulations also showed a higher proliferation potential than the CD49d⁻ counterpart. The reasons behind this phenomenon cannot be explained by the mere expression of CD49d, but rather can be sought in the preferential expression by the CD49d⁺ cells of other costimulatory molecules. Even though the present study has not addressed this issue, previous reports by our and other groups showed high correlation and close relationship between expression of CD49d and CD38,[19,41-43] a molecule that was reported to label a subset enriched in proliferating cells within CLL clones, and associated with the risk of disease progression even when expressed in a subfraction of CLL cells.[44,45] Moreover, here we corroborated our previous findings of a significant association between CD49d⁺ cells with trisomy 12 and *NOTCH1* mutations,[26,27] pointing to a possible role of *NOTCH1* mutations in driving cell survival and proliferation in CD49d⁺ cells through NF-κB pathway activation.[20,37]

Despite the higher proliferative capacity of the CD49d⁺ subpopulations observed in vitro, CD49d⁻ and CD49d⁺ cells did not show different replicative histories in the majority of the investigated cases, pointing to a balanced proliferation rate in vivo. However, different telomerase activity between CD49d⁻ and CD49d⁺ cells can be hypothesized,[46] and that different telomere shortening may occur in follow-up samples cannot be ruled out, as it was reported in CLL cases with clonal evolution.[47]

In light of all previous considerations, it was not completely unexpected that bimCD49d CLL patients, even in the presence of a small CD49d⁺ subpopulation, followed a clinical outcome similar to that of homCD49d⁺ patients in CLL cases treated with conventional chemoimmunotherapy. Of note, both homCD49d⁺ and bimCD49d expression retained independent prognostic impact in multivariate models, which included the main clinical and biological prognosticators.[22] Consistently, the combination of homCD49d⁺ with bimCD49d CLL in 1 single group outperformed the 30% cutoff as OS predictor both in univariable and multivariable analyses.

The negative prognostic impact of CD49d in the ibrutinib setting was previously reported by us in CLL patients from 2 independent ibrutinib-treated cohorts.[33] In the present study, we could confirm CD49d expression as a negative prognostic marker in patients treated with ibrutinib, and showed that both homCD49d⁺ CLL, and bimCD49d CLL experienced significantly shorter PFS than CD49d⁻ CLL. Moreover, the homCD49d⁺ and bimCD49d categories turned out both independent prognosticators in the context of multivariable models that included the main clinical and biological covariates with a clinical impact in the ibrutinib setting.[34] Further validation studies with longer follow-up and larger cohorts of patients are needed to definitely validate CD49d as OS predictor in the context of target therapies.

Our previous study showed that, after 1 year of ibrutinib treatment, the LN mass reduction was lower in CD49d⁺ compared with CD49d⁻ CLL.[33] Consistently, the increasing proportion of the CD49d⁺ subpopulation observed here in 8 CLL cases with CD49d bimodal expression after ibrutinib treatment strongly supports the hypothesis of a higher resistance to treatment of CD49d-expressing cells. In light of these data, it would be interesting to evaluate whether higher percentages of the CD49d⁺ subpopulation could be found in tissue compartments other than PB.

Overall, our data point to a potential relevant role of CD49d to clinical practice, also in light of its ability to separate patients with different OS probability in the context of the CLL-IPI risk categories.[35] In this regard, further validation studies are needed to test whether including CD49d expression, either homogeneous or bimodal, in a comprehensive prognostic risk score may help to better stratify patients treated with chemoimmunotherapy or targeted therapy.

In summary, in the present study, we have reported for the first time the presence of CD49d bimodal expression in ~20% of CLL cases. The CD49d⁺ cells from bimodal CD49d CLL displayed higher levels of proliferation compared with the CD49d⁻ cells, and were more highly represented in the BM compared with PB, and in CLL subsets enriched in proliferative cells. The higher proliferative capacity of CD49d⁺ cells was in keeping with the tendency of the CD49d⁺ subpopulation to increase over time especially after therapy. In this regard, it is tempting to speculate that the CD49d⁺ subpopulation, even if it represents a minority of the tumor, may support the progression of the entire clone. Indeed, from a clinical point of view, the presence of small cell CD49d⁺ populations was consistently associated with bad prognosis, suggesting that bimCD49d and homCD49d⁺ CLL be

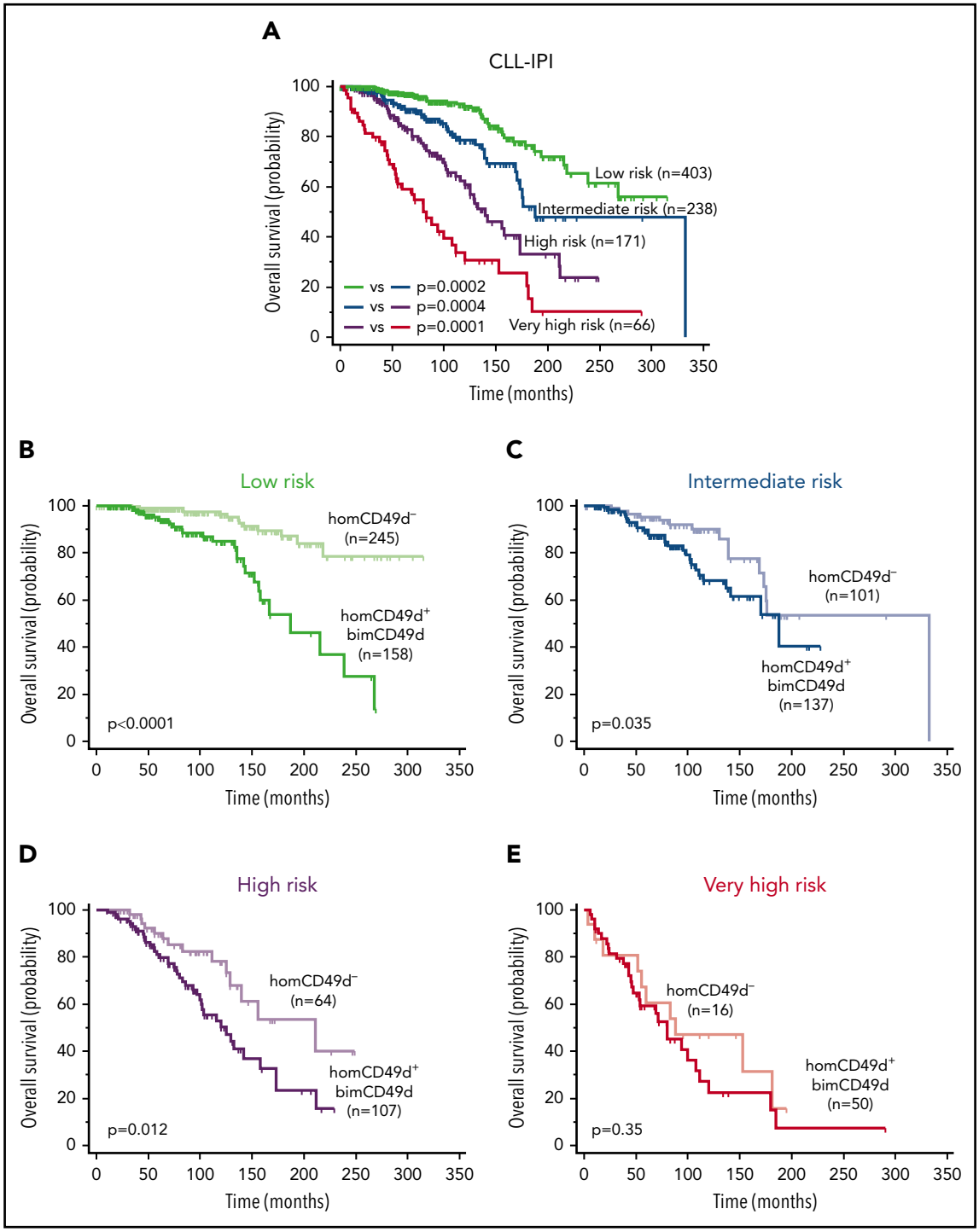

Figure 5. Clinical impact of CD49d expression in the context of CLL-IPI categories. (A) OS Kaplan-Meier curves of CLL cases split in the 4 risk categories of the CLL IPI. (B-E) OS curves of CLL cases with homogeneous-negative CD49d expression (homCD49d$^-$, light curve) and the merging of homogeneous-positive (homCD49d$^+$) and bimodal CD49d (bimCD49d) expression (dark curve) in the context of the low-risk (B), intermediate-risk (C), high-risk (D) and very-high-risk (E) categories of the CLL-IPI. *P* values refer to the log-rank test.

merged together to improve the prognostic power of CD49d in the clinical setting.

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Authorship

Contribution: A.Z. and V.G. designed the study, coordinated the experiments, and wrote the manuscript; E.T., F.P., and D.B performed immunophenotypic analyses, analyzed the data, and contributed to the writing of the manuscript; C.C., T.B., F.M.R., and R.B. performed cell sorting, performed proliferation experiments, and contributed to molecular characterization of the samples; P.N., H.C., I.C., and E.Z. performed immunophenotypic and molecular analyses; K.N. performed telomere-length analyses; J.P. and G.T. performed statistical analyses; M.G., R.M., E.S., I.I., J.O., G.D., L.L., F.Z., G.P., A.C., F.D.R., D.R., G.G., and G.D.P. provided well-characterized biological samples and contributed to the revision of the manuscript; and C.P. and T.N.H. contributed to scientific discussion, data interpretation, and the revision of the manuscript.

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Footnotes

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