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Implementation of International Prognostic Index with flow cytometry immunophenotyping for better risk stratification of chronic lymphocytic leukemia

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Abstract

Background: Current chronic lymphocytic leukemia (CLL) International Prognostic Index (IPI) stratifies patients based on clinical, molecular, and biochemical features; however, B-cell markers also influence CLL outcomes. Here, prognostic roles of CD11c, CD38, and CD49d were first evaluated, and then an immunophenotypic score was combined with CLL-IPI for risk stratification of CLL patients.

Methods: A total of 171 CLL subjects were included, and surface marker expression was assessed by flow cytometry. Levels ≥30% were chosen as cut-off of positivity to a marker; then values of 1 (for CD11c and CD38) or 3 (for CD49d) were assigned and scores determined for each patient's clone immunophenotype.

Results: CD49d positivity was significantly associated with simultaneous expression of CD11c and/or CD38, unmutated *IGHV* status, and higher β2-microglobulin levels compared to those with CD49d negativity. Moreover, CD49d^{+} patients experienced a shorter progression-free survival and time to treatment. When the immunophenotypic score was combined with CLL-IPI, patients with high-risk immunophenotype had a significantly lower time-to-treatment regardless CLL-IPI.

Conclusions: Our results suggested clinical utility of an integrated prognostic score for better risk stratification of CLL patients. These results require further validation in prospective larger studies.

KEYWORDS
chronic lymphocytic leukemia, flow cytometry, prognosis, risk stratification

1 | INTRODUCTION

Chronic lymphocytic leukemia (CLL), a heterogeneous group of clonal B-cell lymphoproliferative diseases with various clinical course and molecular and biological features, accounts for almost 20% of all leukemia cases in Western Countries with a median age at diagnosis of 72 years old.[1,2] Neoplastic B cells frequently have a mature B lymphocyte phenotype showing positivity for CD5, CD19, and CD23, a weak monoclonal light chain immunoglobulin (SmIg) expression, and low levels of CD20.[3–5] CLL cells can also carry chromosomal and

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genetic abnormalities, including del(13q14),[6] immunoglobulin heavy-chain variable region gene (*IGHV*) mutational status and somatic mutations in *TP53*.[7] B cells with mutated *IGHV* likely derive from a B lymphocyte that has underwent to IGV somatic hypermutation and IGH class-switch recombination, two essential processes for B cell maturation and Ig production after antigen exposure and B-cell receptor (BCR) activation.[7,8] Indeed, mutated CLL cells frequently show a $CD5^{+}CD27^{+}$ memory B cell phenotype, while unmutated *IGHV* cells a $CD5^{+}CD27^{-}$ B cell phenotype related to a T-cell independent B lymphocyte activation.[8] Other somatic mutations occurring at the hematopoietic stem cell (HSC) level are also related to an increased risk of CLL development, such as mutations in *SF3B1*, *NOTCH1* *NFKB1E*, and other genes related to the NF-κB signaling pathway.[8–10]

Tumor microenvironment also plays an important role in CLL development as promotes CLL cell survival, proliferation, homing to lymphoid tissues and bone marrow (BM), and disease progression.[11–13] Several cell types and molecules are involved in the deep cross-talk between leukemic cells and tumor environment, such as T helper (Th) 17 lymphocytes or nurse-like cells, by directly interacting with tumor cells through ligand-receptor interactions (e.g., a4β1 integrin binding to vascular cell adhesion molecule-1 [VCAM1], ICAM, and others), or by indirectly releasing cytokines and chemokines (e.g., CXCL12 or CXCL13), facilitating tumor cell migration, homing and survival.[14,15] On the other hand, CLL cells can express several adhesion molecules influencing leukemic cell behavior and ultimately disease outcome.[11,15,16] For example, CD11c, an αxβ2 integrin that is a fibrinogen receptor mediating cell–cell and cell-matrix interactions, is variously expressed on CLL cells (4%–89% of cases) and is associated with less aggressive disease and better outcome.[16–21] Conversely. CD49d, an alpha4 integrin forming the very late antigen-4 (VLA-4), is frequently found on resting CLL cells and is a negative independent prognostic factor because high expression is associated with short overall survival and time to first treatment in CLL.[22–26] VLA-4 is involved in BM homing and retention of HSCs through VCAM-1 interaction on BM stromal cells.[23,27,28] CD49d also interacts with CD38 forming the macromolecular complex involved in trans-endothelial rolling, invasion, arrest, and survival of CLL cells in BM and lymphoid tissues, as CD49d mediates rolling and arrest on the endothelium, and CD38 favors survival and proliferation of leukemic cells.[23,29–33]

Risk stratification and prognostication are performed using the CLL-International Prognostic Index (CLL-IPI) that combines clinical, chromosomal, and molecular alterations, and laboratory findings: age; disease stage; β2-microglobulin levels; presence of del(17p); and *IGHV* mutational status.[34] Rai and Binet staging systems are based on clinical features and tumor load and estimate prognosis while not the rapidity of disease evolution.[1] Unmutated CLL had shorter survival and remission duration compared to M-CLL,[35] as well as patients with del(17p)/*TP53* mutations, even though clinical management of these subjects has markedly improved after the introduction of targeted therapies, such as ibrutinib, idelalisib, and venetoclax.[35] Moreover, the use of an integrated prognostic index has uniformed disease classification across clinical trials and has combined modern prognostic factors to historical clinical staging systems.[34] In this single-center retrospective study, we confirmed the independent prognostic role of CD49d as a single marker in CLL patients and in combination with other well-established CLL markers, and we evaluated the prognostic role of an integrated scoring system based on immunophenotypic and CLL-IPI features.

2 | PATIENTS AND METHODS

2.1 | Patients

A total of 171 patients were included in this retrospective study after informed consent was obtained in accordance with the Declaration of Helsinki. Subjects received a diagnosis of CLL according to the 2008 and 2016 WHO criteria[1,36] at the Hematology and Transplant Center, University Hospital "San Giovanni di Dio e Ruggi d'Aragona" of Salerno, Italy, from September 2013 to November 2021. Patients were staged according to the Rai and Binet systems, and risk stratification was calculated according to the CLL-IPI.[34] Inclusion criteria were age ≥18 years old and diagnosis of B-cell CLL. The median age at diagnosis was 68 years old (range, 39–91) and males were 59% ($N = 101$). Clinical characteristics are summarized in Table 1.

Patients received chemotherapy according to current guidelines.[37,38] In particular, 52.6% of subjects ($N = 90$) were on "wait and watch," while of the remaining 47.4% of patients ($N = 81$), 30 (37%) received standard chemotherapy (chlorambucil, $N = 26$; fludarabine, $N = 3$; and cyclophosphamide + vincristine + prednisone, CVP, $N = 1$) as first-line therapy, 12 small molecules (ibrutinib, $N = 10$; and venetoclax, $N = 2$), 36 rituximab-based regimens (rituximab alone, $N = 1$; rituximab + fludarabine + cyclophosphamide, R-FC, $N = 4$; rituximab + bendamustine, $N = 20$; rituximab + cyclophosphamide + doxorubicin + vincristine + prednisone, R-CHOP, $N = 2$; rituximab + fludarabine, $N = 6$; rituximab + chlorambucil, $N = 1$; and rituximab + venetoclax, $N = 2$), two subjects received afutuzumab, and one patient alemtuzumab + fludarabine. Therapeutic strategies are summarized in Table 1. Of these 81 patients, 33 of them (40.7%) received a second-line treatment with small molecules (ibrutinib, $N = 10$; and idelalisib, $N = 1$), rituximab-based regimens (rituximab alone, $N = 1$; rituximab + bendamustine, $N = 9$; rituximab + venetoclax, $N = 1$; rituximab + CVP, $N = 1$; and R-FC, $N = 1$), or standard chemotherapy (chlorambucil, $N = 4$; fludarabine, $N = 3$; cyclophosphamide, $N = 2$). Of these 33 patients, 15 of them (45.5%) required a third-line treatment, and three of them (20%) a fourth-line therapy (Table 1).

2.2 | Flow cytometry

Heparinized or ethylenediaminetetraacetic acid peripheral blood (PB) specimens were stained within 12 h from collection using a whole blood lysis technique and a panel of directly conjugated antibodies. Briefly, antibodies were directly added to 200 μl of whole blood and incubated for 20 min at 4°C. After incubation, 3 ml of red blood cell lysis buffer was added and samples were incubated for

TABLE 1 Clinical characteristics

Characteristics	$N = 171$	Range, %
Mean age, years	68	39–91
M/F	101/70	59%/49%
Disease stage—Rai system		
0	63	36.8%
1	24	14%
2	32	18.7%
3	14	8.2%
4	23	13.5%
Not evaluable	15	8.8%
Mean Hb, g/dl	13.3	8.6–17.2
Mean platelets/μl	180 104	19 100–325 000
Mean WBC, cells/μl	20 056	2482–96 090
Mean LDH, mU/ml	307.2	60–1079
Mean β2-microglobulin	2.99	1.1–6.04
IGHV mutational status		
Unmutated	20	11.7%
Mutated	19	11.1%
Not performed	132	77.2%
Chromosomal abnormalities		
del(13q14)	23	13.4%
del(17p)	8	4.7%
del(11q)	3	1.8%
+12	12	7%
≥2 abnormalities	16	9.4%
Not performed/evaluable	59	34.5%
Normal	50	29.2%
No treatment	90	52.6%
First-line therapy	81	47.4%
Standard chemotherapy	30	37%
Rituximab-based	36	44.4%
Small molecules	12	14.8%
Others	3	3.7%
Second-line therapy	33/81	40.7%
Standard chemotherapy	9	22.3%
Rituximab-based	13	39.4%
Small molecules	11	33.3%
Third-line therapy	15/33	45.5%
Standard chemotherapy	3	20%
Rituximab-based	1	6.7%
Small molecules	9	60%
Others	2	13.3%
Fourth-line therapy	3/15	20%
Small molecules	3	100%

Abbreviations: del, deletion; Hb, hemoglobin; IGHV, immunoglobulin heavy-chain variable region gene; LDH, lactate dehydrogenase; WBC, white blood cells.

15 min at room temperature, centrifuged, and cell pellets resuspended in 500 μl of PBS for acquisition. Antibodies were used according to manufacturers' instructions and were directed against: CD45; CD4; CD8; CD3; CD56; CD19; CD5; CD23; CD10; CD11c; CD20; CD103; CD38; CD49d; SmIg-Kappa; and SmIg (all from Beckman Coulter). Sample acquisition was performed on a five-color FC500 cell analyzer cytometer (Beckman Coulter) or a 10-color three-laser Beckman Coulter Navios Flow Cytometer (Beckman Coulter). Post-acquisition analysis was performed using CPX, Navios tetra software, or Kaluza Analysis Flow Cytometry software v2.1.1 (Beckman Coulter). Instrument daily quality control was carried out using Calibrite Beads or Flow-Check Pro Fluorospheres (Beckman Coulter), and external quality control by UK NEQAS for Leucocyte Immunophenotyping. Compensation was monthly checked using specific compensation kits (Beckman Coulter). Samples were run using the same PMT voltages, and at least 1 million events were recorded.

2.3 | Statistical analysis

Data were collected from a computerized database and chart review and were analyzed using Prism (v.9.0; GraphPad software). Differences in distributions of dichotomous variables were analyzed by chi-square or Fisher's exact test. Flow cytometry data were reported as percent of positive cells, and 30% of positive cells were used as a cut-off. Pearson analysis was employed for studying correlations, unpaired two-tailed t-tests for two group comparison. Log-rank (Mantel-Cox) test was used for survival analysis between groups. A p value <.05 was considered statistically significant.

3 | RESULTS

3.1 | Clinical feature distribution based on CD49d positivity

To investigate associations between CD49d expression levels on CLL cells and other clinical, phenotypical, and molecular features, immunophenotyping was performed at diagnosis and expression levels of CD49d were correlated with complete blood counts (CBCs), flow cytometry immunophenotyping, cytogenetics abnormalities, and clinical features (e.g., disease stage or age) (Table 2). In our CLL cohort, CD49d negativity was the most frequent condition ($N = 130$, 76%). However, no differences in age ($p = .1602$), sex ($p = .1015$), chromosomal abnormalities ($p = .1018$), disease stage ($p = .1016$), and risk stratification ($p = .2280$) distribution were described between $CD49d^+$ and $CD49d^-$ CLL. No differences were also observed for hemoglobin (Hb) levels ($p = .2173$), white blood cell (WBC; $p = .1087$), and platelet counts ($p = .1087$) between groups. Moreover, no correlations were found between CD49d expression levels and age at diagnosis ($p = .4700$), Hb levels ($p = .3247$), WBC ($p = .1707$) or platelet ($p = .9976$) counts, and lactate dehydrogenase levels (LDH; $p = .2413$).

TABLE 2 Feature distribution based on CD49d expression

Feature	CD49d+ (*N* = 41)	CD49d− (*N* = 130)	*p*
Mean age, years (range)	70 (39–90)	67 (39–91)	.1602
M/F	29/12	72/58	.1015
CD38+	20 (48.8%)	19	<.0001
CD11c+	20 (48.8%)	36	.0211
$CD38^{+}CD49d^{-}$ or $CD38^{-}CD49d^{+}$	21 (51.2%)	19	
$CD38^{+}CD11c^{+}$	14 (34.1%)	7	<.0001
$CD20^{+}$	39 (95.1%)	122	>.9999
$CD5^{+}$	39 (95.1%)	124	>.9999
Chromosomal abnormalities			.1018
Normal	12 (29.3%)	38 (29.2%)	
del(13q14)	3 (7.3%)	20 (15.4%)	
+12	6 (14.6%)	6 (4.6%)	
del(17p)	1 (2.4%)	7 (5.4%)	
del(11q)	0 (0%)	3 (2.3%)	
≥2 abnormalities	6 (14.6%)	9 (6.9%)	
Not performed/evaluable	12 (29.3%)	47 (36.2%)	
CLL-IPI			.2280
Low	23 (56.1%)	76 (58.5%)	
Intermediate	8 (19.5%)	36 (27.7%)	
High/very high	10 (24.4%)	18 (13.8%)	
IGHV mutational status			.0094
Unmutated	6 (100%)	14 (42.4%)	
Mutated	0 (0%)	19 (57.6%)	
Del(17p) (yes/no)	2/39	11/118	.4437
RAI stage			.1016
0	12 (29.3%)	51 (39.2%)	
1	3 (7.3%)	21 (16.2%)	
2	9 (21.9%)	23 (17.7%)	
3	4 (9.8%)	10 (7.7%)	
4	10 (24.4%)	13 (10%)	
Not evaluable	3 (7.3%)	12 (9.2%)	
Hb, g/dl (range)	12.9 (10.1–17.2)	13.3 (8.6–16.7)	.2173
WBC, cells/μl (range)	16 208 (2110–54 820)	23 681 (2600–105 230)	.1087
Platelets/μl (range)	189 647 (45000–321 000)	179 331 (19100–414 000)	.1087
β2-microglobulin, mg/L (range)	3.87 (2–5.6)	2.60 (1.1–6.04)	.0095
β2-microglobulin increased/normal	7/3	3/17	.0048
Treatments			.0454
Wait and Watch	16 (39%)	74 (56.9%)	
Therapy	25 (61%)	56 (43.1%)	

Abbreviations: CLL-IPI, chronic lymphocytic leukemia-International Prognostic Index; del, deletion; Hb, hemoglobin; WBC, white blood cells.

3.2 | Flow cytometry feature distribution based on CD49d positivity

Conversely, $CD49d^{+}$ CLL cells more frequently displayed a simultaneous positivity for CD38 (48.8% vs. 14.6%, percent of patients with $CD49d^{+}CD38^{+}$ vs. $CD49d^{-}CD38^{+}$ CLL cells; $p < .0001$), CD11c (48.8% vs. 27.7%, percent of patients with $CD49d^{+}CD11c^{+}$ vs. $CD49d^{-}CD11c^{+}$ CLL cells; $p < .0211$), or positive for CD38 and CD11c together ($CD49d^{+}CD38^{+}CD11c^{+}$ vs. $CD49d^{-}CD38^{+}CD11c^{+}$, 34.1% vs. 5.4%; $p < .0001$), while no associations were described with CD20 and CD5 (all $p > .05$). A positive correlation was described between CD49d and CD38 ($r = .4190$; $p < .0001$) or CD11c ($r = .2095$; $p = .0095$) expression levels, while a slight negative correlation was found between CD49d and CD5 levels ($r = -.1601$; $p = .0473$) (Figure 1A).

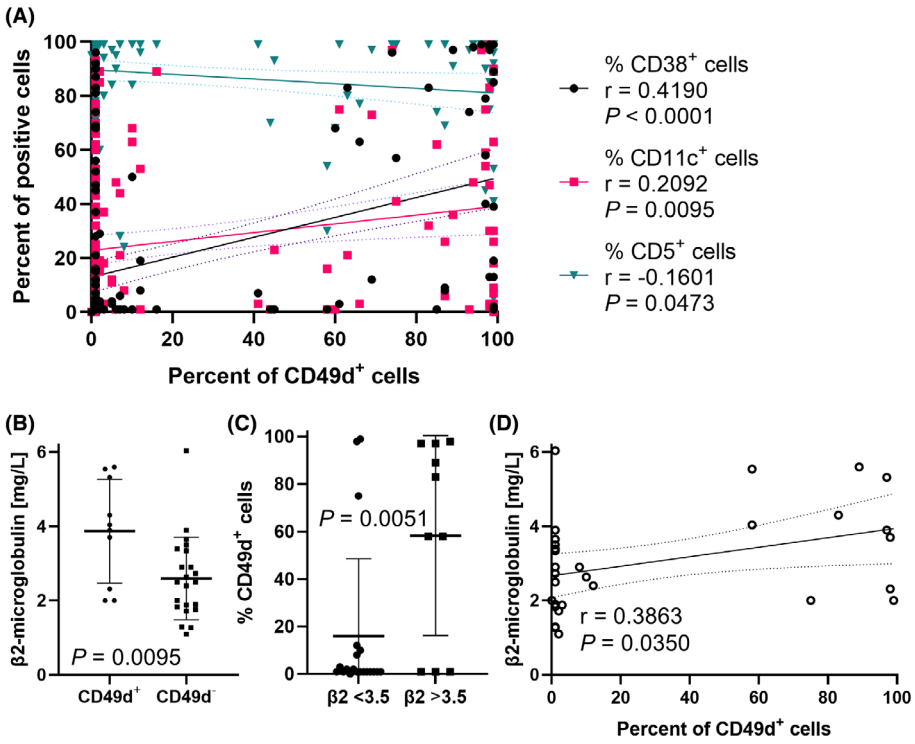


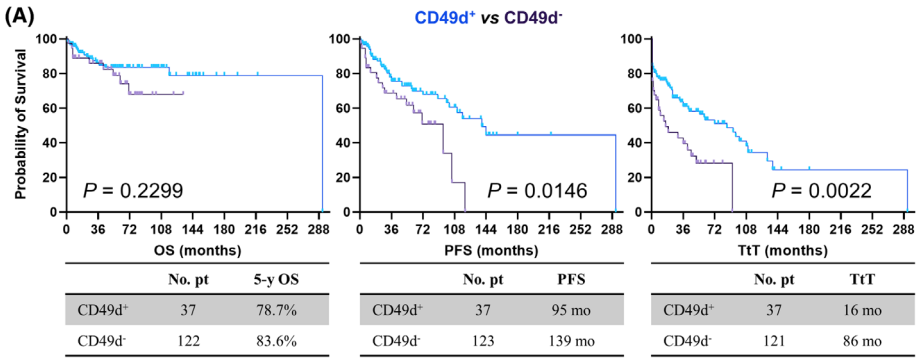
FIGURE 1 Correlations of CD49d expression levels. (A) Pearson correlation analysis was performed between CD49d expression and CD38 or CD11c. (B) Serum β2-microglobulin levels were compared between patients with or without CD49d positivity by unpaired *t* test (data are presented as mean ± SD). (C) CD49d expression was compared between patients with increased and normal β2-microglobulin levels (cut-off, 3.5 mg/L) (data are shown as mean ± SD). (D) Serum β2-microglobulin levels were correlated with CD49d expression levels by Pearson analysis. A $p < .05$ was considered statistically significant.

3.3 | Prognostic CLL-IPI biomarker distribution based on CD49d positivity

Patients with CD49d positivity also showed significantly higher concentrations of circulating β2-microglobulin compared to those subjects with CD49d negativity (mean ± SD, 3.87 ± 1.4 mg/L vs. 2.6 ± 1.1 mg/L, $CD49d^+$ vs. $CD49d^-$; $p = .0095$) (Figure 1B). Moreover, patients with β2-microglobulin levels ≥3.5 mg/L (cut-off used for CLL-IPI stratification) showed a higher expression (% of $CD49d^+$ cells, mean ± SD, 58.3 ± 42.13% vs. 15.9 ± 32.67%, increased vs. normal β2-microglobulin levels; $p = .0051$) (Figure 1C) and distribution of CD49d positivity compared to those subjects with normal β2-microglobulin levels ($p = .0048$; chi-square test performed) (Table 2). In addition, serum levels of β2-microglobulin were positively correlated with CD49d expression levels ($r = .3863$; $p = .0350$) (Figure 1D). In the subgroup of patients with available *IGHV* mutational status, unmutated *IGHV* condition was more frequently found in patients with CD49d positivity (100% vs. 42.4%, $CD49d^+$ vs. $CD49d^-$; $p = .0094$); however, the number of patients was too limited for drawing univocal conclusions. Moreover, patients with CD49d positivity were more likely to receive chemotherapy compared to those with $CD49d^-$ (61% vs. 43%, respectively; $p = .0454$).

3.4 | Clinical outcomes based on CD49d positivity

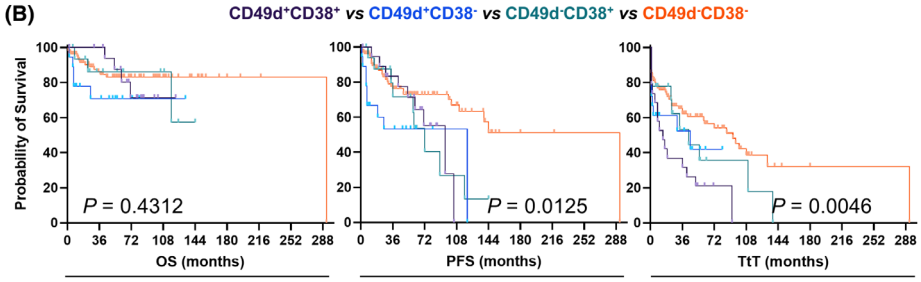
To investigate and confirm the prognostic role of CD49d in CLL clinical outcome, patients were divided based on CD49d positivity, and overall survival (OS), progression-free survival (PFS), and time-to-treatment (TtT) were compared between groups (Figure 2A). No differences were observed in OS between $CD49d^+$ and $CD49d^-$ patients (5-year OS, 78.7% vs. 83.6%, respectively; $p = .2299$; hazard ratio [HR], 1.62; 95% confidential interval [CI], 0.6654–3.931). Conversely, patients with CD49d positivity showed a shorter PFS compared to those with CD49d negativity (95 months vs. 139 months, respectively; $p = .0146$; HR, 1.96; 95%CI, 1.012–3.804), and a shorter TtT (16 months vs. 86 months, $CD49d^+$ vs. $CD49d^-$; $p = .0022$; HR, 2.54; 95%CI, 1.401–4.619). Next, because of the well-known negative prognostic role of CD38 in CLL outcome and the significant association between CD49d and CD38 levels described above, patients were divided into four groups based on concomitant expression of CD49d and CD38 ($CD49d^+CD38^+$, $CD49d^+CD38^-$, $CD49d^-CD38^+$, and $CD49d^-CD38^-$), and clinical outcomes compared (Figure 2B). No variations were described between groups for OS (5-year OS, 87.5% vs. 70.7% vs. 86.2% vs. 83.1%, $CD49d^+CD38^+$ vs. $CD49d^+CD38^-$ vs. $CD49d^-CD38^+$ vs. $CD49d^-CD38^-$; $p = .4312$); while patients with CD38 positivity with or without CD49d expression displayed the shortest PFS compared to those with CD38 negativity (95 months vs. 120 months vs. 72 months vs. 292 months, $CD49d^+CD38^+$ vs. $CD49d^+CD38^-$ vs. $CD49d^-CD38^+$ vs. $CD49d^-CD38^-$; $p = .0125$). Interestingly, patients with concomitant expression of CD49d and CD38 experienced the shortest TtT compared to those with discordant expression ($CD49d^+CD38^-$ or $CD49d^-CD38^+$) or negative for both markers (14 months vs. 45 months vs. 43 months vs. 93 months, $CD49d^+CD38^+$ vs. $CD49d^+CD38^-$ vs. $CD49d^-CD38^+$ vs. $CD49d^-CD38^-$; $p = .0046$). Similarly, CD49d expression was combined with CD11c, patients were divided in four groups ($CD49d^+CD11c^+$, $CD49d^+CD11c^-$,



	No. pt	5-y OS
CD49d+	37	78.7%
CD49d-	122	83.6%

	No. pt	PFS
CD49d+	37	95 mo
CD49d-	123	139 mo

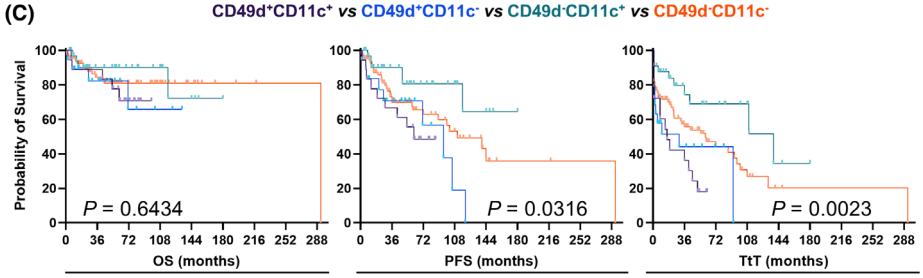
	No. pt	TtT
CD49d+	37	16 mo
CD49d-	121	86 mo



	No. pt	5-y OS
CD49d+CD38+	19	87.5%
CD49d+CD38-	18	70.7%
CD49d-CD38+	18	86.2%
CD49d-CD38-	104	83.1%

	No. pt	PFS
CD49d+CD38+	19	95 mo
CD49d+CD38-	18	120 mo
CD49d-CD38+	18	72 mo
CD49d-CD38-	105	292 mo

	No. pt	TtT
CD49d+CD38+	19	14 mo
CD49d+CD38-	18	45 mo
CD49d-CD38+	18	43 mo
CD49d-CD38-	103	93 mo



	No. pt	5-y OS
CD49d+CD11c+	18	77.8%
CD49d+CD11c-	19	82.3%
CD49d-CD11c+	34	90.2%
CD49d-CD11c-	88	81.1%

	No. pt	PFS
CD49d+CD11c+	18	55.6 mo
CD49d+CD11c-	19	70.7 mo
CD49d-CD11c+	34	80.7 mo
CD49d-CD11c-	89	65.6 mo

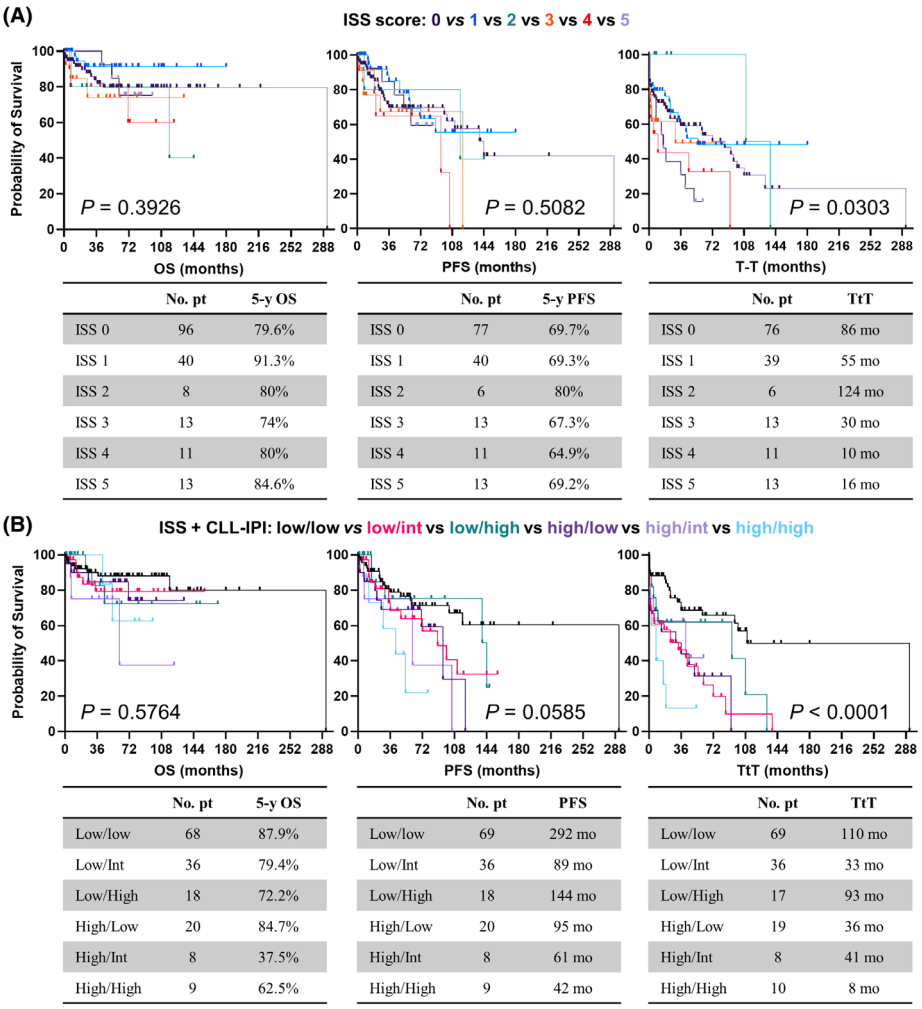
	No. pt	TtT
CD49d+CD11c+	18	16 mo
CD49d+CD11c-	19	30 mo
CD49d-CD11c+	33	138 mo
CD49d-CD11c-	88	60 mo

FIGURE 2 Clinical outcomes of CLL patients based on CD49d positivity. Five-year overall survival (5-y OS), progression-free survival (PFS), and time-to-treatment (TtT) of CLL patients were compared between groups of patients based on (A) CD49d positivity, (B) CD49d and CD38, or (C) CD49d and CD11c expression. The number of censored subjects (No. pt) for each group is also reported. A $p < .05$ was considered statistically significant. CLL, chronic lymphocytic leukemia; mo, months

CD49d$^-$CD11c$^+$, and CD49d$^-$CD11c$^-$), and clinical outcomes compared (Figure 2C). Also for CD11c, no variations were described between groups for OS (5-year OS, 77.8% vs. 82.3% vs. 90.2% vs. 81.1%, CD49d$^+$CD11c$^+$ vs. CD49d$^+$CD11c$^-$ vs. CD49d$^-$CD11c$^+$ vs. CD49d$^-$CD11c$^-$; $p = .6434$); while patients with concomitant expression of CD11c and CD49d displayed the shortest PFS compared to those with CD11c and/or CD49d negativity (55.6 months vs. 70.7 months vs. 80.7 months vs. 65.6 months, CD49d$^+$CD11c$^+$ vs. CD49d$^+$CD11c$^-$ vs. CD49d$^-$CD11c$^+$ vs. CD49d$^-$CD11c$^-$; $p = .0316$). Moreover, patients with concomitant expression of CD49d and CD38 experienced the shortest TtT compared to those with discordant expression or negative for both markers (16 months vs. 30 months vs. 138 months vs. 60 months, CD49d$^+$CD11c$^+$ vs. CD49d$^+$CD11c$^-$ vs. CD49d$^-$CD11c$^+$ vs. CD49d$^-$CD11c$^-$; $p = .0023$).

3.5 | Risk stratification using an immunophenotype score

Next, we sought to investigate the potential role of an immunophenotype scoring system (ISS) for risk stratification of CLL patients. Negativity for CD49d, CD38, or CD11c received a value of 0, while positivity for CD38 or CD11c had a value of 1, and positivity for CD49d had a value of 3. Therefore, the immunophenotype score ranged from 0 to 5, and each patient received a score based on CLL cell immunophenotyping. Subsequently, subjects were divided into six groups (from 0 to 5), and clinical outcomes were compared between groups (Figure 3A). No differences were observed in 5-year OS (79.6% vs. 91.3% vs. 80% vs. 74% vs. 80% vs. 84.6%, score 0 vs. 1 vs. 2 vs. 3 vs. 4 vs. 5; $p = .3926$) or 5-year PFS (69.7% vs. 69.3% vs. 80%



	No. pt	5-y OS
ISS 0	96	79.6%
ISS 1	40	91.3%
ISS 2	8	80%
ISS 3	13	74%
ISS 4	11	80%
ISS 5	13	84.6%

	No. pt	5-y PFS
ISS 0	77	69.7%
ISS 1	40	69.3%
ISS 2	6	80%
ISS 3	13	67.3%
ISS 4	11	64.9%
ISS 5	13	69.2%

	No. pt	TtT
ISS 0	76	86 mo
ISS 1	39	55 mo
ISS 2	6	124 mo
ISS 3	13	30 mo
ISS 4	11	10 mo
ISS 5	13	16 mo

	No. pt	5-y OS
Low/low	68	87.9%
Low/Int	36	79.4%
Low/High	18	72.2%
High/Low	20	84.7%
High/Int	8	37.5%
High/High	9	62.5%

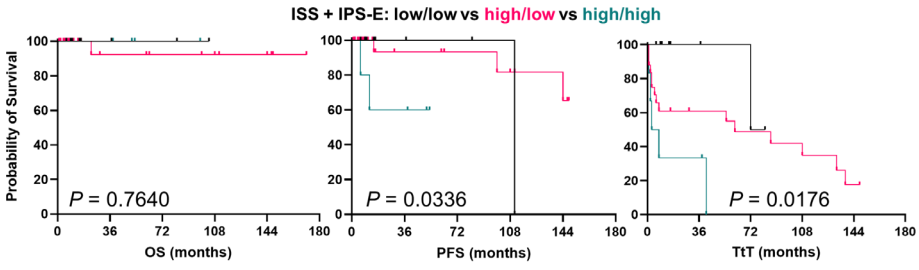
	No. pt	PFS
Low/low	69	292 mo
Low/Int	36	89 mo
Low/High	18	144 mo
High/Low	20	95 mo
High/Int	8	61 mo
High/High	9	42 mo

	No. pt	TtT
Low/low	69	110 mo
Low/Int	36	33 mo
Low/High	17	93 mo
High/Low	19	36 mo
High/Int	8	41 mo
High/High	10	8 mo

FIGURE 3 Clinical outcomes of CLL patients based on immunophenotypic scoring system (ISS). Five-year overall survival (5-y OS), progression-free survival (PFS), and time-to-treatment (TtT) of CLL patients were compared between groups based on (A) ISS score and (B) combination of ISS and chronic lymphocytic leukemia-International Prognostic Index (CLL-IPI) score. The number of censored subjects (no. pt) for each group is also reported. A $p < .05$ was considered statistically significant. CLL, chronic lymphocytic leukemia; int, intermediate; mo, months

vs. 67.3% vs. 64.9% vs. 69.2%, score 0 vs. 1 vs. 2 vs. 3 vs. 4 vs. 5; $p = .5082$); while patients with the highest scores (3–5) experienced the shortest TtT compared to those subjects with lower scores (86 months vs. 55 months vs. 124 vs. 30 vs. 10 vs. 16, score 0 vs. 1 vs. 2 vs. 3 vs. 4 vs. 5; $p = .0303$). Scores ranging from 0 to 2 identified patients with low risk of disease progression (low ISS), while scores from 3 to 5 with worse outcomes (high ISS). Therefore, we combined this immunophenotype score to CLL-IPI for each patient, and six groups were identified: low ISS/low CLL-IPI; low ISS/intermediate CLL-IPI; low ISS/high or very-high CLL-IPI; high ISS/low CLL-IPI; high ISS/intermediate CLL-IPI; and high ISS/high or very-high CLL-IPI. Clinical outcomes were then compared between groups (Figure 3B). No differences were observed in 5-year OS (87.9% vs. 79.4% vs. 72.2% vs. 84.7% vs. 37.5% vs. 62.5%, low ISS/low CLL-IPI vs. low ISS/intermediate CLL-IPI vs. low ISS/high or very-high CLL-IPI vs. high ISS/low CLL-IPI vs. high ISS/intermediate CLL-IPI vs. high ISS/high or very-high CLL-IPI; $p = .5764$) or PFS (292 months vs. 89 months vs. 144 vs. 95 vs. 61 vs. 41, low ISS/low CLL-IPI vs. low ISS/intermediate CLL-IPI vs. low ISS/high or very-high CLL-IPI vs. high ISS/low CLL-IPI vs. high ISS/intermediate CLL-IPI vs. high ISS/high or very-high CLL-IPI; $p = .0585$), even though patients with high ISS/high or very-high CLL-IPI displayed the shortest PFS. Interestingly, patients with high ISS/high or very-high CLL-IPI had the shortest TtT as well as subjects with high ISS and intermediate or low CLL-IPI compared to those subjects with lower scores (110 months vs. 33 months vs. 93 vs. 36 vs. 41 vs. 8, low ISS/low CLL-IPI vs. low ISS/intermediate CLL-IPI vs. low ISS/high or very-high CLL-IPI vs. high ISS/low CLL-IPI vs. high ISS/intermediate CLL-IPI vs. high ISS/high or very-high CLL-IPI; $p < .0001$).

Finally, our ISS score was combined with the International Prognostic Score for Early-stage CLL (IPS-E) score that includes *IGHV* mutational status, lymphocytosis, and presence of lymphadenopathies.[39] Three groups were identified: low ISS/low IPS-E; high ISS/low IPS-E; and high ISS/high IPS-E. Interestingly, no low ISS/high IPS-E patients were observed. Clinical outcomes were then compared between groups (Figure 4). No differences were observed in 5-year OS (100% vs. 92.3% vs. 100%, low ISS/low IPS-E vs. high ISS/low IPS-E vs. high ISS/high IPS-E; $p = .7640$). Conversely, patients with high ISS and high IPS-E had the shortest 5-year PFS (100% vs. 93.3% vs. 60%, low ISS/low IPS-E vs. high ISS/low IPS-E vs. high ISS/high IPS-E; $p = .0336$), and the shortest TtT (77 months vs. 61 months vs. 5.5 months, low ISS/low IPS-E vs. high ISS/low IPS-E vs. high ISS/high IPS-E; $p = .0176$).



	No. pt	5-y OS
Low/low	8	100%
High/Low	25	92.3%
High/High	6	100%

	No. pt	5-y PFS
Low/low	8	100%
High/Low	25	93.3%
High/High	6	60%

	No. pt	TtT
Low/low	8	77 mo
High/Low	25	61 mo
High/High	6	5.5 mo

FIGURE 4 Clinical outcomes of CLL patients based on immunophenotypic scoring system (ISS) and International Prognostic Score for Early-stage CLL (IPS-E). Five-year overall survival (5-y OS), progression-free survival (PFS), and time-to-treatment (TtT) of CLL patients were compared between groups based on (A) ISS score and (B) combination of ISS and IPS-E score. The number of censored subjects (no. pt) for each group is also reported. A $p < .05$ was considered statistically significant.

4 | DISCUSSION

B-cell CLL is a heterogeneous group of hematological malignancies with various biological features and clinical courses.[1] Actual Rai and Binet staging systems are based on clinical features and tumor load and estimate prognosis while not the rapidity of disease evolution; conversely, current prognostic scoring systems (CLL-IPI) better stratify patients[34]; however, these systems are continuously evolving because of novel discovery shading lights on disease biology, improvements in diagnostic procedures (e.g., introduction of sequencing methods), and incorporation of molecular and biochemical prognostic markers.[40,41] In this study, neoplastic cell immunophenotypic features were combined with current international prognostic systems to improve risk stratification of CLL patients showing that inclusion of three surface markers (CD49d, CD38, and CD11c) in CLL-IPI score could better identify patients with worse outcomes, especially those who experienced shorter PFS and TtT.

CLL is the most common lymphoproliferative disorder in elderly and in Western Countries and is characterized by clonal expansion of mature B lymphocytes that accumulate in the BM, PB, lymph nodes, spleen, liver, and, occasionally, also in extra-lymphoid tissues.[1,2] Patients display a wide range of clinical presentations and outcomes, with 5-year survivals ranging from 93% to 23%.[1] Cytogenetic abnormalities also contribute to this heterogeneity, as patients carrying del (17p) or del(11q) have a more aggressive disease compared to those a del(13q).[42] Moreover, unmutated *IGHV* status, the presence of neoplastic clones harboring somatic mutations in *TP53*, *ATM*, or *NOTCH1* genes, as well as increased expression of certain serum markers (e.g., β2-microglobulin), microRNAs, and/or surface proteins have been related to worse outcomes in CLL.[43] For example, high zeta-chain-associated protein kinase 70 and CD38 expression are associated with poor outcomes in CLL patients, especially in those with unmutated IGHV status.[22] Moreover, CD38 positivity is related to an increased frequency of anemia, thrombocytopenia, and leukocytosis worsening prognosis of these subjects[44]; however, discordant findings on CD38 clinical utility have been reported.[44,45] Similarly, CD49d is another independent prognostic markers, as increased expression on the neoplastic clone is related to poor OS.[25,46,47] In our study, we investigated surface expression of CD11c, CD38, and CD49d and their prognostic impact on 171 consecutive CLL patients. Our results showed that CD49d negativity was the most recurrent condition in CLL frequently associated with CD38 and/or CD11c negativity and lower serum levels of β2-microglobulin, a well-established CLL prognostic marker, compared to those subjects with CD49d positivity who more commonly expressed higher levels of CD38 and CD11c -also concomitantly-, and displayed unmutated *IGHV* status. Moreover, patients with CD49d positivity were more likely to receive chemotherapy with a shorter TtT compared to those with CD49d negativity, as well as a reduced PFS. In addition, we also confirmed the negative prognostic role of CD38 in CLL as patients who simultaneously expressed CD49d and CD38 displayed the shortest TtT and had reduced PFS compared to those subjects without the expression of these two markers. Our data added evidence to the independent negative prognostic role of both CD38 and CD49d and, underlined the importance to extend current prognostic systems by also including immunophenotypic characteristics.[48]

The evolution of prognostic systems is a central theme in hematology. Indeed, a fraction of patients stratified at low risk of evolution have a more aggressive clinical presentation resembling more a high-risk disease thus suggesting that current prognostic scores are still not specific and sensitive for a correct risk stratification likely because our knowledge is still limited.[41] For example, risk stratification in myelodysplastic syndromes has gone through a revolution starting in 1976 with the French-American-British (FAB) classification, passing through the 2016 World Health Organization classification system and the revised international prognostic scoring system likely coming up in the next few years with the inclusion of next-generation sequencing data.[40,49] Because of CLL's great heterogeneity in clinical course and outcomes, also CLL prognostic scoring systems require continuous improvements for prompt identification of patients with more aggressive diseases requiring an early initiation of high-dose chemotherapy or targeted treatments.[41] For example, the IPS-E stratifies patients just based on three parameters (unmutated IGHV, absolute lymphocyte count $>15 \times 10^9$ cells/L, and palpable lymph nodes), and can identify subjects at high risk of early treatment start (5-year cumulative risk, 8.4%, 28.4%, and 61.2%, low-, intermediate-, and high-risk patients).[39] The first staging systems, Rai and Binet, stratify patients based on clinical parameters, such as the presence of anemia,

thrombocytopenia, or the number of involved lymph nodes.[1] In 2016, the introduction of the new CLL-IPI prognostic score in clinical practice has markedly improved risk stratification of CLL patients by combining clinical features, such as age and stage according to Rai and/or Binet systems, serum values of β-2-microglobulin, and molecular markers, such as IGHV mutational status, presence of del(17p) or somatic mutations in *TP53*.[34] Using the CLL-IPI, patients can be grouped at the time of diagnosis in four prognostic groups with estimated 5-year survival ranging from 93.2% (low) to 23.3% (very high).[34] Based on the well-known prognostic impact of certain surface markers that can be easily measured by flow cytometry during routinely diagnostic evaluation, we implemented current CLL-IPI with an immunophenotypic score (ISS) including CD11c, CD38, and CD49d expression on neoplastic cells. For each selected parameter, positivity was defined when percentage of marker-expressing cells was ≥30%, and a value of 1 (for CD38 and CD11c) or 3 (for CD49d) was assigned. The 30% cut-off was selected according to literature.[17,18] Based on neoplastic cell phenotype, each patient received a score ranging from 0 (all three markers were negative) to 5 (all positive), and clinical outcomes were compared between groups showing that subjects with CD49d positivity with one or two additional markers had a shorter TtT compared to CD49d negative patients thus identifying a high-risk ISS. Similar results were also observed when ISS score was combined with the IPS-E demonstrating that our integrated prognostic scoring system was significantly more effective in identifying patients who faster experienced disease progression and/or required an earlier treatment and supporting the need to further integrate current prognostic scoring systems.

In conclusion, our results confirmed the independent negative prognostic role of CD38 and CD49d in CLL, and their clinical utility for a better risk stratification when incorporated in currently used international prognostic scoring systems.[39,40,47] Moreover, our study added evidence to the need to create integrated prognostic systems based on clinical, molecular, and phenotypic aspects. Indeed, looking from a single point of view is limited for understanding disease biology and outcomes, especially nowadays when biological data are high-dimensional and high-content, and high-throughput technologies are available making "historic" risk stratification systems unable to stratify and accurately define prognosis of hematological patients.[1,2,34] However, prospective studies on larger patient cohorts are needed to validate our data especially in the view of the increasing use of targeted therapies, and to identify additional marker combinations that can be included in routinely clinical practice.

AUTHOR CONTRIBUTIONS

Conceptualization: Valentina Giudice and Carmine Selleri. Clinical data: Matteo D'Addona, Luca Pezzullo, Laura Mettivier, Valentina Giudice, Francesca D'Alto, and Bianca Serio. Flow cytometry data: Angela Bertolini, Valentina Giudice, and Marisa Gorrese. Cytogenetics data: Rosalba Fumo and Pio Zeppa. Data curation: Valentina Giudice and Francesca D'Alto. Writing—original draft preparation: Valentina Giudice and Matteo D'Addona. Writing—review and editing: Carmine Selleri. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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