Co-expression of CD69, CD49d, CD279 and CD20 in chronic lymphocytic leukemia cells is a new biomarker of active disease before or under therapy

Defined clinical and biological criteria are instrumental for the management of chronic lymphocytic leukemia (CLL), both at diagnosis and progression, and in terms of therapeutic choice and monitoring of response to therapy. Notwithstanding these advances, none of the currently used biomarkers proved effective in monitoring clinical response to BTK inhibitors (BTKi) such as ibrutinib, widely used as the gold standard treatment for CLL patients.

iwCLL consensus guidelines allow for precise diagnosis and staging of CLL and provide a decisional framework for indications of treatment. At diagnosis, Binet or RAI staging systems, defining sub-types of patients, are now completed by CLL-IPI prognostic index and ERIC score leading to either wait and watch strategy or initiation of therapy.[1] Recently, International Prognostic Score for early stage CLL (IPS-E) has been proposed to predict time to first treatment for asymptomatic patients (Binet stage A),[2] patients with unmutated immunoglobulin heavy chain variable region gene (UM-IGHV), high absolute lymphocyte count (ALC >15x10^9/L) and palpable lymph nodes being considered at high risk and therefore most prone to benefit from therapy.

In case of progression or symptomatic/active disease, additional criteria are defined to guide the therapeutic strategy.[1] For patients needing treatments, a wide range of therapies are available, ranging from cytostatic agents combined or not with monoclonal antibodies, to targeted therapies.[1] Assessment of clinical response mainly relies on multi-color flow cytometry, polymerase chain reaction (PCR), or next-generation sequencing (NGS) to detect measurable residual disease (MRD).[3,4] These methods are also useful for the assessment of response to BCL-2 inhibitors, such as venetoclax[5] but not to BTKi when used as monotherapy.[6] Indeed, prolonged high ALC was often observed in ibrutinib-treated patients[7] and the decrease of ALC was not correlated to a better outcome.[7,8] Under ibrutinib selective pressure-acquired resistance driven by *BTK* and/or *PLCγ2* mutations has been largely reported,[9] although these mutations were not detected in 30% of CLL relapse under ibrutinib.[10] Expression of either CD279, CD49d, or CD69 on B-leukemic cells has been reported, in separate studies, as associated to CLL progression under ibrutinib and linked to patient outcome.[11-14] Finally, cellular indexing of transcriptomes and epitopes by sequencing (CITESeq) single cell analyses revealed an increased expression of CD49d, CD69, CD279, CD20 at both genes (*ITGA4*, *CD69*, *PDCD1*, *MS4A1*) and proteins level in leukemic cells of patient experiencing progression under ibrutinib.[15] Nevertheless, no biological markers are available to monitor early disease progression in patients under targeted therapies, including ibrutinib.

In this study, we used multi-color flow cytometry to assess CD69, CD49d, CD279 and CD20 co-expression at the surface of $CD19^+/CD5^+$ B-leukemic cells with the aim of developing a monitoring strategy allowing early prediction of CLL progression under targeted therapies.

Surface expression of CD69, CD49d, CD279 and CD20 markers by $CD19^+/CD5^+$ B-leukemic cells was analyzed in peripheral blood samples, in a cohort of 90 patients (Table 1), treated or not with targeted therapies. Samples were obtained from the Hematology Department with written informed consent. Clinical and biological annotations of the samples have been reported to the Comité National Informatique et Liberté. For ibrutinib and venetoclax-treated patients, studies were approved by the competent authority (ANSM, number: 1551668A-11), the ethics committee (number: CPP16-004a) and registered as *clinicaltrials gov. Identifier: NCT02824159* and *NCT02005471*.

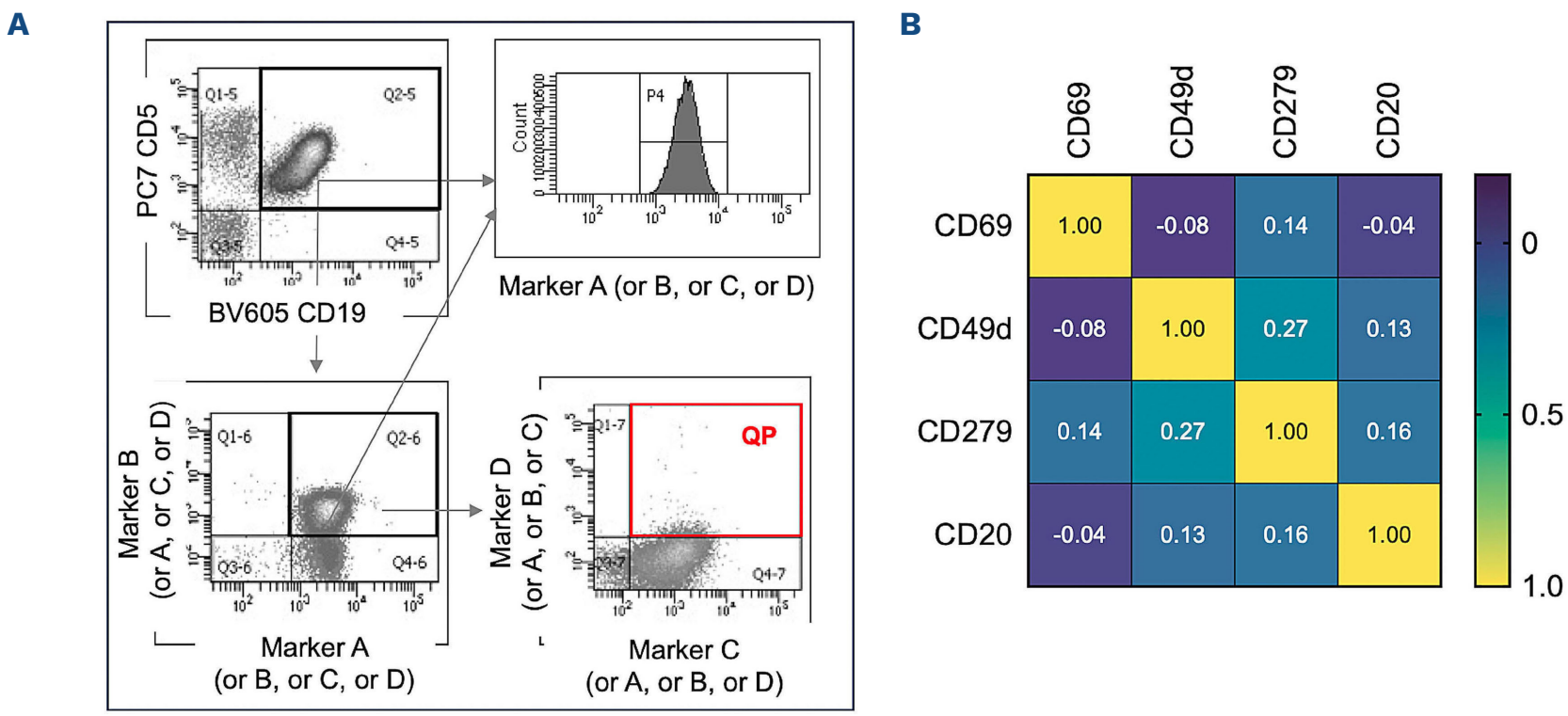
Cell surface staining was performed using fresh peripheral

Table 1. Clinical characteristics of the patients.

Patient characteristics	
Sex, N (%)	90
M	56 (62)
F	34 (38)
Median age in years (range)	69 (36-87)
Mutational status, N (%)	90
IGHV-M	29 (32)
IGHV UM	56 (62)
ND	5 (6)
Untreated patients, N (%)	36
Binet stage A	22 (61)
Binet stage B/C	14 (39)
Ibrutinib treatment, N (%)	54
Progressive disease/Richter transformation	28 (52)
BTK-M	13 (46.5)
BTK-UM	13 (46.5)
ND	2 (7)
No progressive disease	26 (48)
Venetoclax treatment (post-ibrutinib), N (%)	10
Progressive disease/Richter transformation	6 (60)
No progressive disease	4 (40)

M: male; F: female; IGHV-M: mutated immunoglobulin heavy chain variable region gene; IGHV-UM: unmutated immunoglobulin heavy chain variable region gene; BTK-M: mutated Bruton tyrosine kinase; BTK-UM: unmutated Bruton tyrosine kinase; ND: not determined.

blood mononuclear cells (PBMC) isolated from blood samples by density gradient sedimentation (Ficoll-Hypaque, GE Healthcare) or following red blood cell lysis (RBC Lysis buffer, BioLegend). Levels of expression of the markers were assessed in 11 samples and showed no statistical difference between Ficoll-Hypaque purified PBMC and lysed red blood cell samples (r=0.981), confirming that either method of sample preparation can be used for these experiments.



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	PD *vs* No PD	Stage B/C *vs* Stage A	PD *vs* Stage A	Stage B/C *vs* No PD	No PD *vs* Stage A	PD *vs* Stage B/C
CD69 >30%	0.38	-0.10	0.16	0.12	-0.22	0.26
CD49d >30%	0.30	0.68	0.77	0.21	0.47	0.09
CD279 >20%	0.48	0.23	0.25	0.46	-0.23	0.02
CD20 >20%	-0.01	0.07	0.04	0.03	0.05	-0.04
CD69 / CD49 >10%	0.45	0.43	0.69	0.19	0.24	0.26
CD69 / CD279 >10%	0.40	0.18	0.20	0.38	-0.20	0.02
CD69 / CD20 >10%	0.34	0.08	0.13	0.29	-0.21	0.05
CD49d / CD279>10%	0.56	0.38	0.60	0.34	0.04	0.23
CD49d / CD20 >10%	0.34	0.68	0.77	0.25	0.43	0.09
CD20 / CD279 >10%	0.52	0.43	0.40	0.55	-0.12	-0.03
CD69 / CD49d / CD279 >10%	0.28	0.00	0.28	0.00	0.00	0.28
CD69 / CD49d / CD20 >10%	0.53	0.00	0.68	-0.15	0.15	0.68
CD69 / CD279 / CD20 >10%	0.36	0.11	0.09	0.38	-0.27	-0.02
CD49d / CD279 / CD20 >10%	0.44	0.50	0.52	0.42	0.08	0.02
CD69 / CD49d / CD279 / CD20 >0.5%	0.96	1.00	1.00	0.96	0.04	0.00

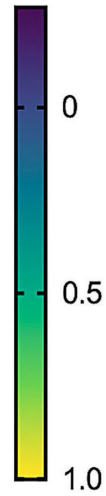


Figure 1. Analysis of CD69, CD49d, CD279 and CD20 expression in chronic lymphocytic leukemia patients. (A) Schematic representation of flow cytometry gating strategy for the analysis of markers alone or in combination in $CD19^+/CD5^+$ leukemic cells. $CD19^+/CD5^+$ population was defined compared to isotype control; for multiplex labeling, gates were defined on $CD19^+/CD5^+$ population compared to specific control (see methods). (B) Pearson r analysis of CD69, CD49d, CD279, CD20. (C) Yule coefficient of biomarkers (alone or in combination) in chronic lymphocytic leukemia (CLL) patients according to their clinical status (under or not ibrutinib therapy). (D) Youden test efficacy of bio-markers (alone or in combination) in CLL patients according to their clinical status as in (C). PD: progressive disease; no PD: no progressive disease; NS: not significant.

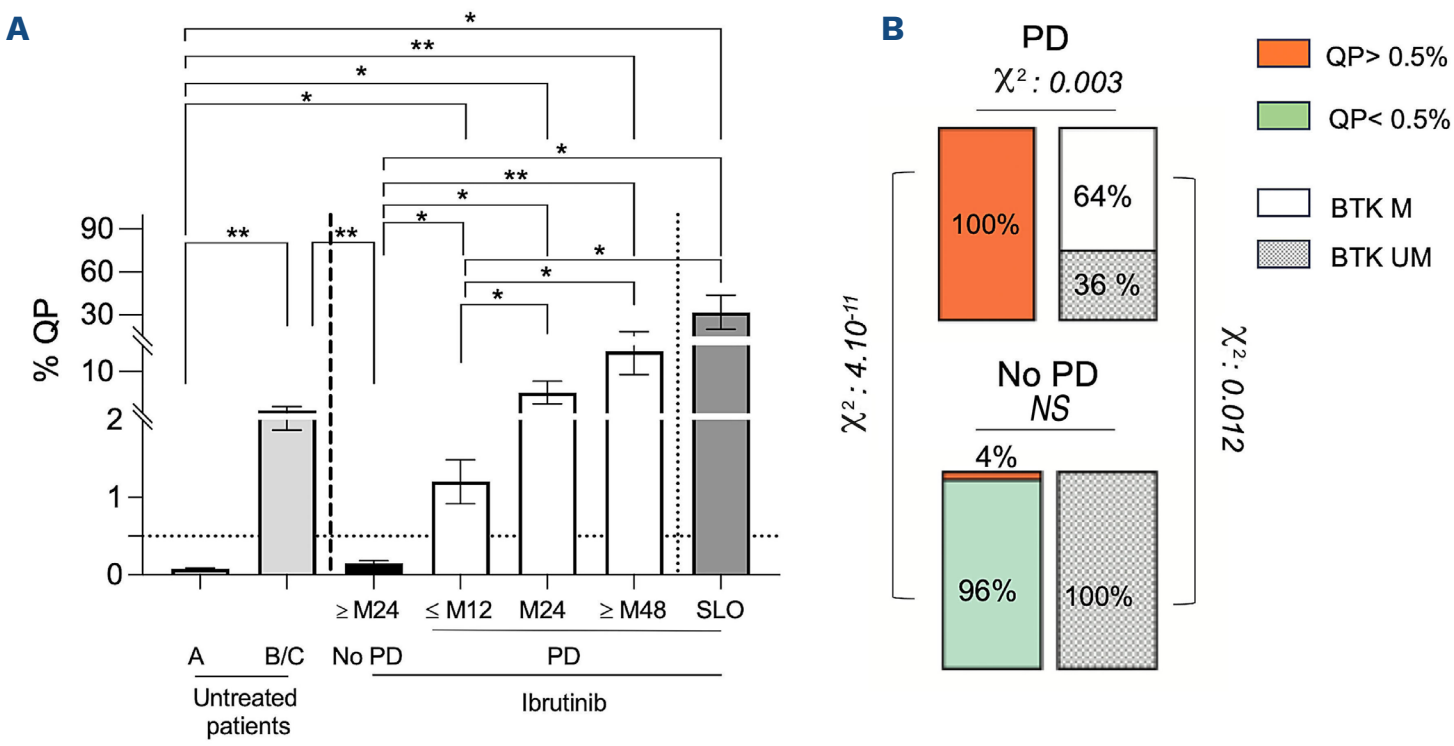
Cells were incubated with titrated antibodies (*Online Supplementary Table S1*): (i) isotype controls; (ii) anti-CD19/anti-CD5 antibodies + isotype controls for CD69, CD49d, CD279, CD20 (specific control); (iii) anti-CD19/anti-CD5 antibodies + anti-CD69, anti-CD49d, anti-CD279, anti-CD20 antibodies. Flow cytometry data were analyzed for each marker, alone or in combination, following the presented gating strategy (Figure 1A). Positivity for each marker was defined as previously described[11,13] or by frequency analysis in the whole cohort compared to specific control on $CD19^+/CD5^+$-gated B-leukemic cells for single or multiplex labeling. The weak correlations, measured by Pearson r analysis, between CD69, CD49d, CD279 and CD20 supported their independence (Figure 1B). In order to evaluate the best marker or combination of markers predicting CLL progression, we compared their expression among patients grouped according to disease stage or progression status using Yule coefficient, to measure association between variables, combined to χ^2 analysis. Although some markers, considered alone or in combination, could discriminate CLL stages or disease progression, co-expression of CD69/CD49d/CD279/CD20 (quadruple-positive population, QP) >0.5% of B-leukemic cells, stood out as the best combination in all comparisons (Figure 1C). This was further confirmed using Youden test efficacy (Figure 1D). Altogether, these data showed that QP >0.5% was the best marker linked to CLL progression in both untreated (stage B/C *vs*. stage A) and targeted therapy-exposed patients (relapsing *vs*. responding disease).

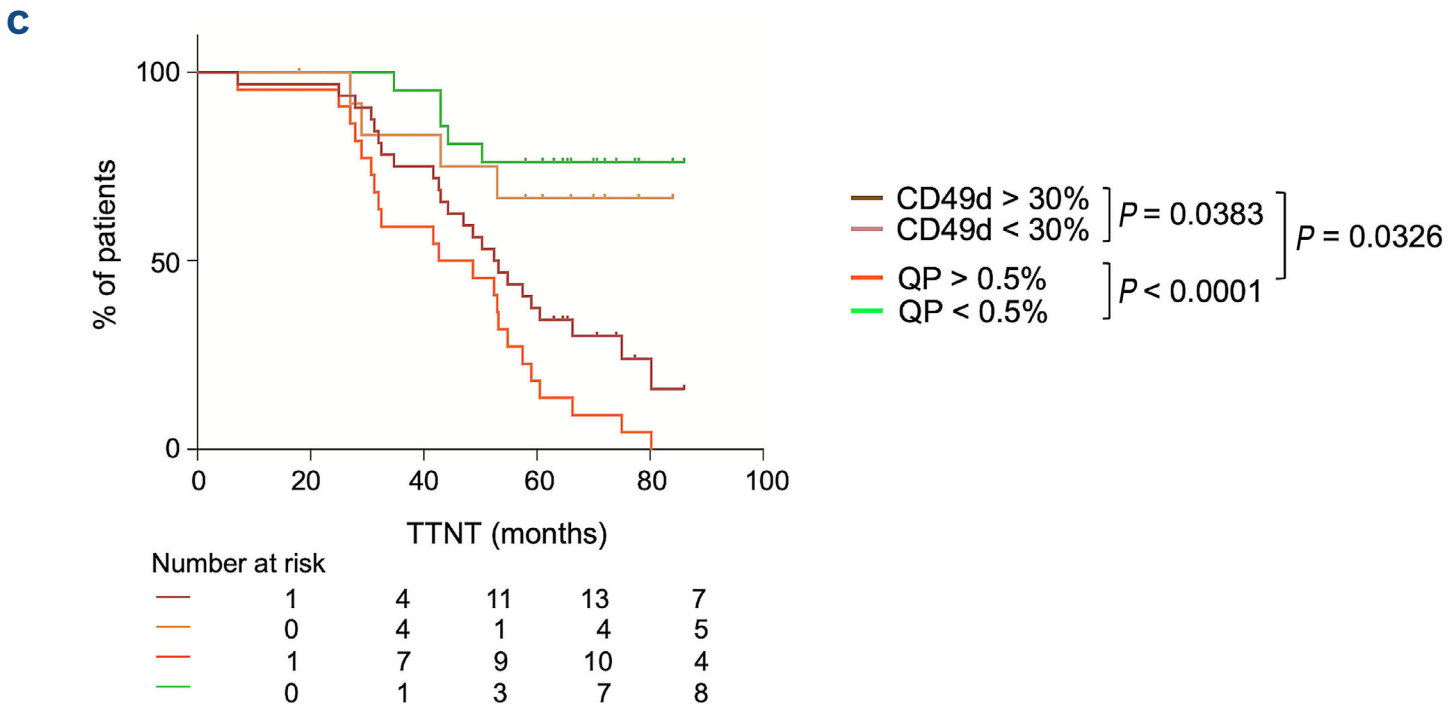
QP quantification was determined in all patients of the cohort. For untreated patients, QP was assessed either at diagnosis, or annual visit to the Hematology ward. Statistical analyses revealed an increase of this B-leukemic cell sub-population in stage B/C *versus* stage A (Figure 2A). After 24 months of ibrutinib exposure, 48% of patients under ibrutinib didn't progress and exhibited a QP <0.5%. (Figure 2A). In our cohort, 52% of ibrutinib-treated patients developed progressive disease (Table 1) and their QP systematically exceeded 0.5%, whatever the time of observation (Figure 2A). Among patients relapsing after ibrutinib, some were subsequently treated by venetoclax (Table 1) during 2 years and QP was quantified within 12 months following planned treatment stop. In this small cohort, resistance to venetoclax seemed also correlated to a QP >0.5% (mean QP=0.065±0.04% in venetoclax responding patients; mean QP=7.22 ± 2.1% in venetoclax refractory patients; *P*=0.022). For all sub-groups, no statistical correlation was found between QP and ALC (r=0.001; *P*=0.92) or IGHV mutational status (*P*=0.35). Interestingly, we observed a high percentage of QP in the secondary lymphoid organs of ibrutinib-treated patients (diagnosed as Richter transformation) (Figure 2A), suggesting that this sub-population putatively recirculates from niches where activation signals are delivered.

Progressive disease under ibrutinib was originally associ-

ated to a selection-induced resistance mechanism, driven by *BTK* and/or *PLCγ2* hotspot mutations.[9] However, 30% of ibrutinib-refractory patients do not display these mutations, leading to a delay in the switch of therapy.[10] We therefore compared *BTK* mutational status and QP proportions in ibrutinib-treated patients within our cohort. All patients experiencing progressive disease exhibited a QP >0.5%, whereas only 64% of them carried *BTK* mutations (Figure 2B). Among non-progressive patients, the only patient (1/25, 4%) displaying a QP >0.5% after 18 months under ibrutinib, showed decreased QP at a later time point (<0.5% after 2 years of therapy). These results strongly suggest that the QP population could represent a more reliable progression marker than *BTK* mutational status (preceding overt relapse by almost 1 year). Moreover, since none of the patients reached undetectable MRD under ibrutinib, due to permanent hyper-lymphocytosis (PR-L, partial response with lymphocytosis), we confirmed that QP >0.5% criterion was independent of the percentage (r=-0.17; *P*=0.42) or absolute number (r=0.012; *P*=0.44) of circulating B-leukemic cells and so doing, stands for a reliable dynamic proxy of long-lasting ibrutinib response.

CD49d/VLA-4 (integrin α4 chain) expression emerged as a microenvironmental factor that contributes to BTKi resistance in CLL.[13] We next compared the time to next treatment (TTNT) and overall survival (OS) according to CD49d expression and QP in the ibrutinib-exposed cohort. Although we also validated CD49d as predictor of treatment-free survival,[12,13] QP >0.5% was more significantly associated to a shorter TTNT in our cohort than CD49d >30% (Figure 2C) but not with OS (Figure 2D). Furthermore, swimmer plot analyses of TTNT were performed in a sub-group of patients from our cohort (N=24) in whom serial assessments were available at different time points along ibrutinib treatment. In these patients, detection of QP cells preceded overt relapse (Figure 2E). Interestingly,





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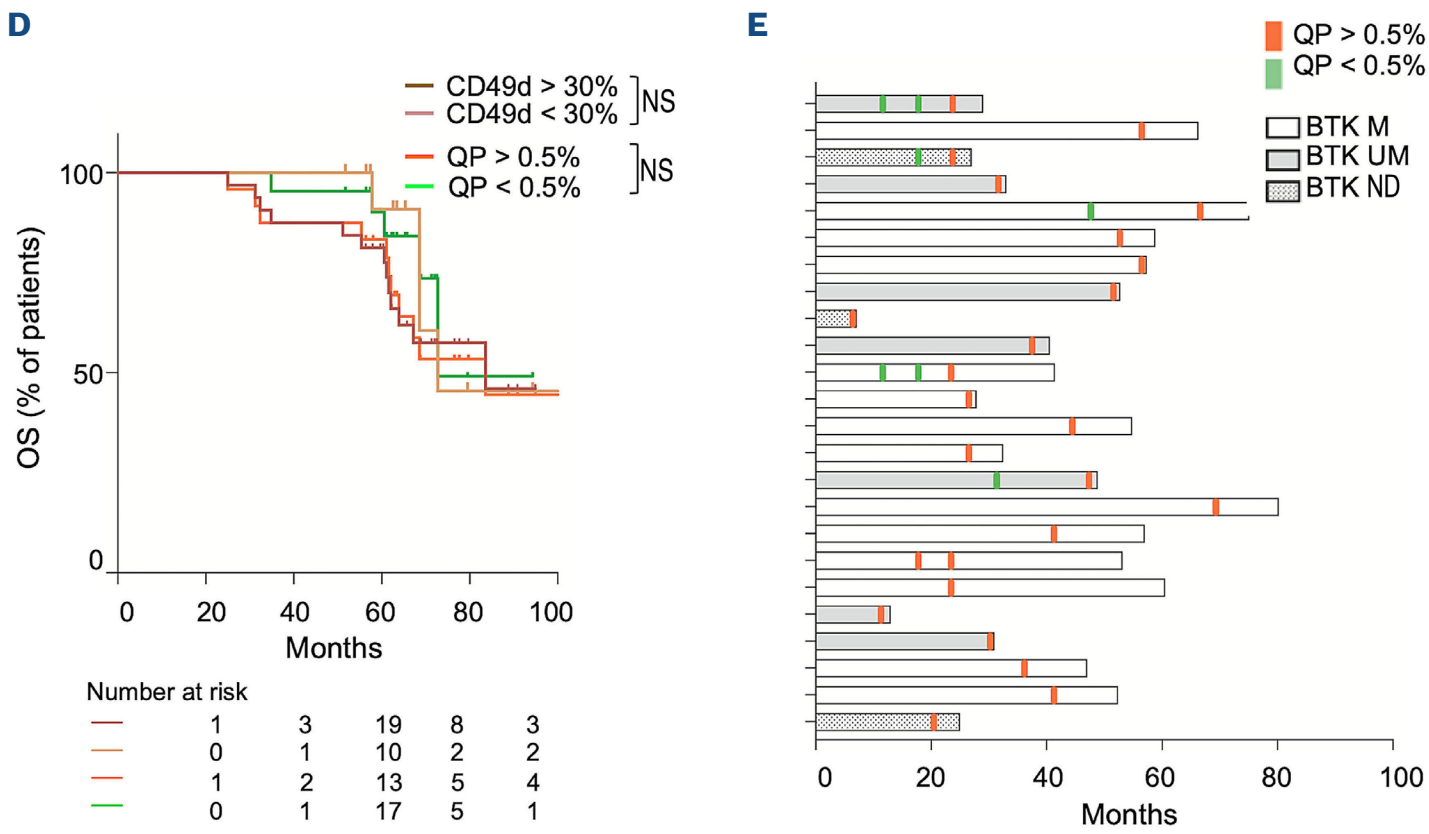

Figure 2. CD69/CD49d/CD20/CD279 co-expression (quadruple-positive population) as a predictive marker of chronic lymphocytic leukemia progression. (A) Quantification of quadruple-positive population (QP) according to clinical stages of chronic lymphocytic leukemia (CLL) patients under or not targeted therapies. Statistical analysis of biological data was done using one way Anova *P<0.05; **P<0.01. (B) BTK mutational status and QP frequency analysis in ibrutinib-treated patients according to progression and/or response. (C) Time-to-next treatment (TTNT) analysis according to CD49d and QP criteria in patients under ibrutinib treatment (N=50). (D) Overall survival analysis according to CD49d and QP criteria in patients under ibrutinib treatment (N=49). (E) Swimmer plot follow-up of QP in ibrutinib-progressive CLL patients. SLO: second lymphoid organs; M: months of treatment; No PD: no progressive disease; PD: progressive disease; BTK-M: mutated Bruton tyrosine kinase; BTK-UM: unmutated Bruton tyrosine kinase; NS: not significant; ND: not determined.

for all patients showing a QP >0.5%, decision to discontinue BTKi therapy was due to progressive disease or Richter transformation, and took place after a median of 47.2 months.

Although our study was based on a mono-centric cohort and should be validated in cohorts from other clinical centers, our data constitute an initial proof-of-concept supporting the use of QP >0.5% as predictor of CLL progression. QP stood out as predictor of clinical response to BTKi for which MRD is not applicable. Beyond this primary objective of our study, we showed that QP proportions were associated to progression prior to therapy. Determining the proportion of QP leukemic cells could be easily performed within routine patient care every 3 months. Combined or not with clinical markers of active disease suspicion cases, QP proportions could guide patient follow-up and therapeutic adjustments.

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Disclosures

No conflicts of interest to disclose.

Contributions

SC performed experiments, analyzed flow cytometry data, wrote the manuscript. LY provided CLL samples, advised on results, wrote the

manuscript. SL advised on statistical analyses. CL advised on results. AQM designed the research, performed experiments, analyzed flow cytometry data, wrote the manuscript and supervised the study. The authors read and approved the final manuscript.

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Data-sharing statement

All relevant data are within the manuscript and its *Online Supplementary Appendix.*

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