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by Sarah Cadot, Loic Ysebaert, Sebastien Lamy, Camille Laurent, and Anne Quillet-Mary

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

Sarah Cadot[1,2], Loïc Ysebaert[1,2,3], Sébastien Lamy[4,5], Camille Laurent[1,2,3], Anne Quillet-Mary[1,2,*]

[1]INSERM UMR1037, CNRS UMR5071, Université Toulouse III-Paul Sabatier, Centre de Recherches en Cancérologie de Toulouse, Toulouse, France

[2]Laboratoire d'Excellence 'TOUCAN-2', Toulouse, France

[3]Institut Universitaire du Cancer-Oncopole de Toulouse, Toulouse, France

[4]Tarn Cancer Registry, Claudius Regaud Institute, Institut Universitaire du Cancer-Oncopole de Toulouse, Toulouse, France

[5]CERPOP INSERM UMR1295, Université Toulouse III-Paul Sabatier, Toulouse, France

Running title: A new predictive biomarker for CLL progression

* Corresponding author: E-mail: anne.quillet-mary@inserm.fr

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Authorship 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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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 IGHV (UM-IGHV), high absolute lymphocyte count (ALC >15 x 10^9/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, PCR, or 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, 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 for the 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, n° 1551668A-11), the ethics committee (N° CPP16-004a) and registered by Clinical-Trials.gov (NCT02824159 and NCT02005471).

Cell surface staining was performed using fresh 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 red blood cells lysed samples ($r = 0.981$), confirming that either method of sample preparation can be used for these experiments. Cells were incubated with titrated antibodies (Supplemental Table 1): (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 (Fig. 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 (Fig. 1B). 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 Chi^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 (Fig. 1C). This was further confirmed using Youden test efficacy (Fig. 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 *vs* stage A (Fig. 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 (Fig. 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) (Fig. 2A), suggesting that this sub-population putatively re-circulates from niches where activation signals are delivered.

Progressive disease under ibrutinib was originally associated 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 (Fig. 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 timepoint (< 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 one 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% (Fig. 2C) but not with overall survival (Fig. 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 (Fig. 2E). Interestingly, 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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	Number of patients
Gender	**90**
M	56 (62%)
F	34 (38%)
Median age (yrs)	69 (range 36-87)
Mutational Status	**90**
IGHV-M	29 (32%)
IGHV UM	56 (62%)
nd	5 (6%)
Untreated patients	**36**
Binet Stage A	22 (61%)
Binet Stage B/C	14 (39%)
Ibrutinib treatment	**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)	10
Progressive Disease/Richter transformation	6 (60%)
No Progressive Disease	4 (40%)

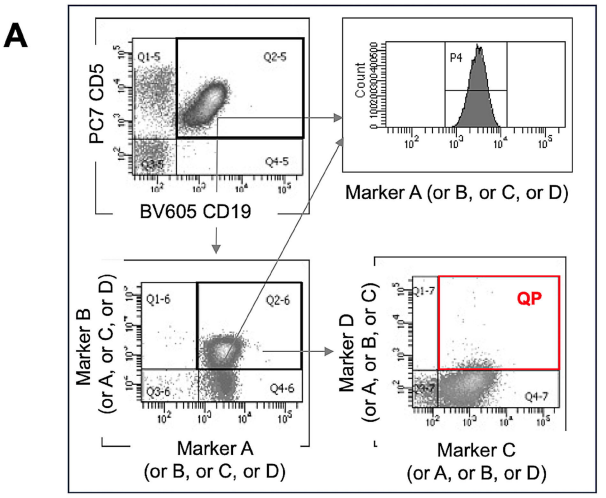
Table 1: Clinical characteristics of the patients.

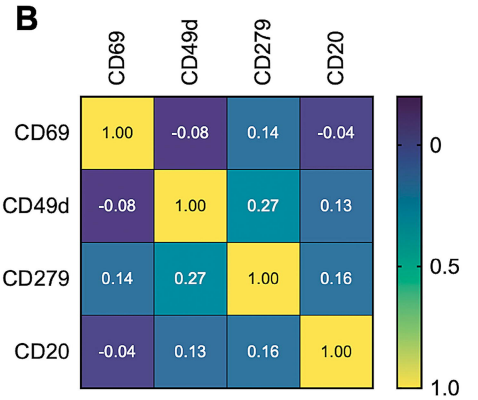
M: Male; F: Female; IGHV-M: mutated immunoglobulin heavy chain variable region gene; IGHV-UM: unmutated immunoglobulin heavy chain variable region gene; BTK-M: mutated Bruton Tyrosine Kinase; BTK-UM: unmutated Bruton Tyrosine Kinase; nd: not determined.

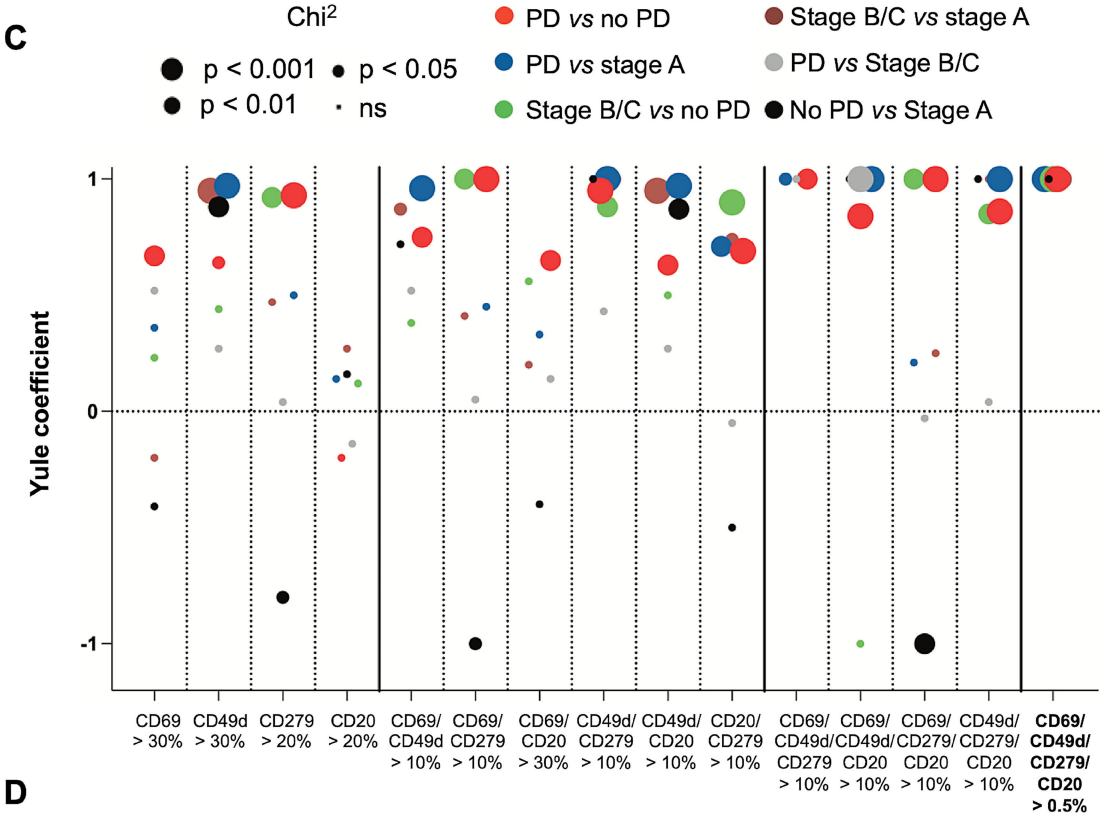
Figure Legends

Figure 1: Analysis of CD69, CD49d, CD279 and CD20 expression in CLL 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 bio-markers (alone or in combination) in CLL patients according to their clinical status (under or not ibrutinib therapy); PD: progressive disease, no PD: no progressive disease. *ns*: not significant. **(D)** Youden test efficacy of bio-markers (alone or in combination) in CLL patients according to their clinical status as in (C).

Figure 2: CD69/CD49d/CD20/CD279 co-expression (QP) as a predictive marker of CLL progression. (A) Quantification of QP according to clinical stages of CLL patients under or not targeted therapies. SLO: second lymphoid organs; M: months of treatment; No PD: no progressive disease; PD: progressive disease. 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. BTK-M: mutated Bruton Tyrosine Kinase; BTK-UM: unmutated Bruton Tyrosine Kinase **(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*). *ns*: not significant. **(E)** Swimmer plot follow-up of QP in ibrutinib-progressive CLL patients. TTNT: Time to next treatment.

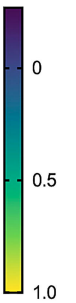


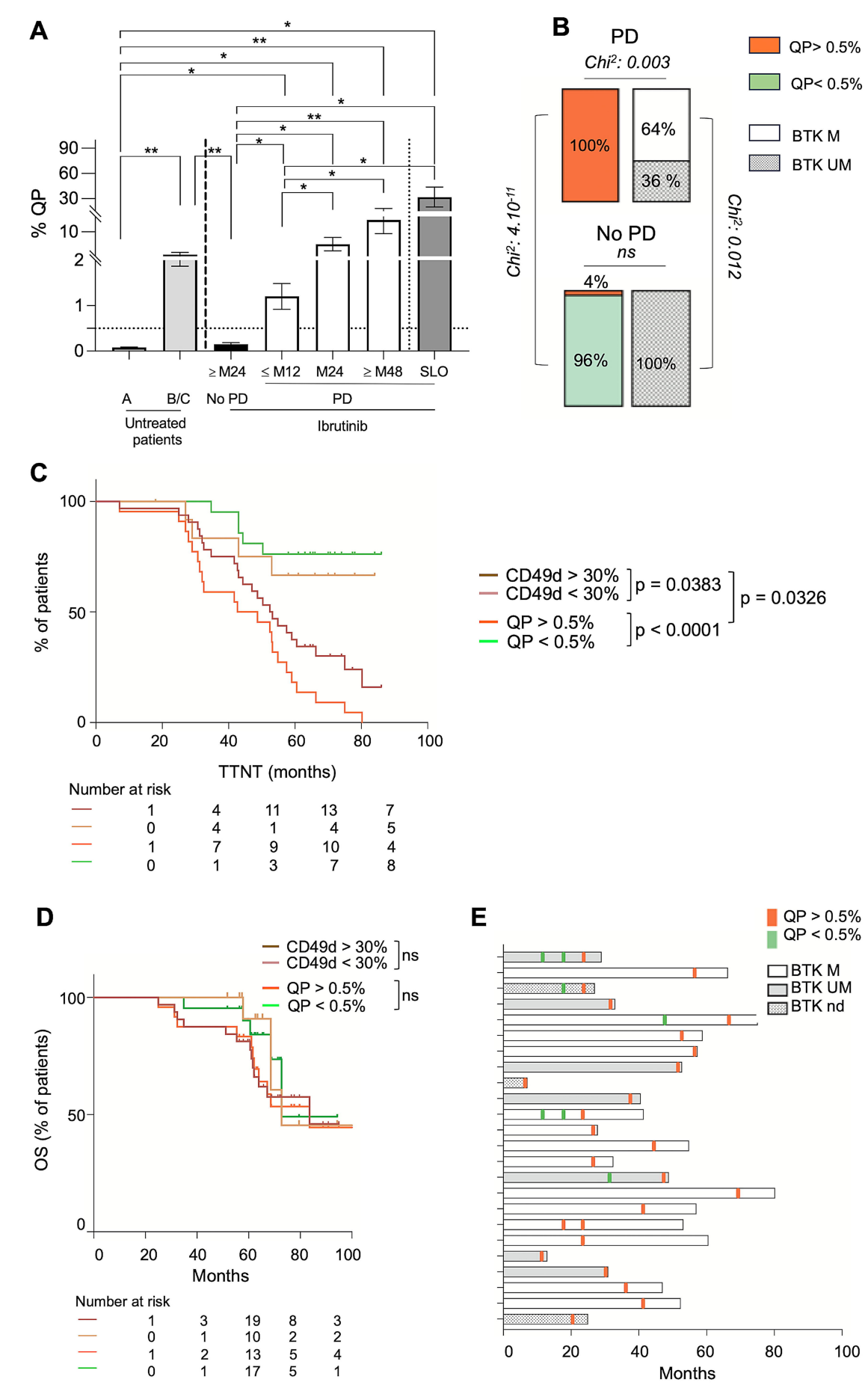




D

	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




A
% QP
90
60
30
10
2
1
0
≥ M24
≤ M12
M24
≥ M48
SLO
A
B/C
No PD
PD
Untreated patients
Ibrutinib
B
PD
Chi²: 0.003
100%
64%
36 %
No PD
ns
4%
96%
100%
Chi²: 4.10⁻¹¹
Chi²: 0.012
QP> 0.5%
QP< 0.5%
BTK M
BTK UM
C
% of patients
TTNT (months)
Number at risk
1 4 11 13 7
0 4 1 4 5
1 7 9 10 4
0 1 3 7 8
CD49d > 30%
CD49d < 30%
p = 0.0383
p = 0.0326
QP > 0.5%
QP < 0.5%
p < 0.0001
D
OS (% of patients)
Months
Number at risk
1 3 19 8 3
0 1 10 2 2
1 2 13 5 4
0 1 17 5 1
CD49d > 30%
CD49d < 30%
ns
QP > 0.5%
QP < 0.5%
ns
E
QP > 0.5%
QP < 0.5%
BTK M
BTK UM
BTK nd
Months

Company	Antibody	Clone	Catalogue number	Concentration used
BioLegend	BV 605 anti-human CD19	HIB19	302244	1 µg/mL
	PE-Cy7 anti-human CD5	UCHT2	300622	1 µg/mL
	BV 421 anti-human CD49d	9F10	304322	1 µg/mL
	APC anti-human CD20	2H7	302310	1 µg/mL
	BV 605 Mouse IgG1 isotype control	MOPC-21	400162	1 µg/mL
	PE-Cy7 Mouse IgG1 isotype control	MOPC-21	400126	1 µg/mL
	BV 421 Mouse IgG1 isotype control	MOPC-21	400158	1 µg/mL
	APC Mouse IgG2b isotype control	MPC-11	400322	1 µg/mL
BD Biosciences	FITC mouse anti-human CD279	MIH4	557860	10 µg/mL
	FITC Mouse IgG1 isotype control	MOPC-21	555748	10 µg/mL
Beckman Coulter	PE anti-human CD69	TP1.55.3	IM1943U	1/100
	PE Mouse IgG2isotype control	7T4-1F5	A09141	1/100

Supplemental Table 1: Antibodies used in the study