Supplemental Materials for

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

Supplemental information:

- **Supplemental materials and methods**

- **Supplemental Tables:**

- **Supplemental Figures:**

Supplemental materials and methods

Immunophenotypic analysis

CD49d expression was analysed by 3-color immunofluorescence, by combining anti-CD49d-PE (clone 9F10), anti-CD5-FITC, and anti-CD19-PerCP-Cy5.5 or anti-CD19-APC (all from BD Biosciences). CD49d expression data were reported as percent of $CD5^{+}CD19^{+}$ CLL cells displaying specific fluorescence intensity greater than the 98% to 99% of the same unstained population. The detection of CLL subpopulations with different CXCR4/CD5 intensity of expression was determined by a four-color immunofluorescence that included an anti-CXCR4-APC mAb (BD Bioscience) to the above reported antibody combination. The $CXCR4^{dim}/CD5^{bright}$ and the $CXCR4^{bright}/CD5^{dim}$ populations were defined as described.[1] For each specimen, at least 10 000 $CD5^{+}CD19^{+}$ events were collected.

Definition of CD49d bimodal expression

The gating strategy used for the analysis of CD49d expression, depicted in Supplemental Figure 1A, was the following: 1) the lymphocyte population was selected on a forward- versus side-scatter (FSC/SSC) plot (R1); 2) CLL cells (CD19+CD5+) were selected on a CD19 versus CD5 dot plot gated in R1. The CLL region was drawn close to the CD19+CD5+ population in order to exclude the few CD19+CD5- cells corresponding to residual normal B cells that express CD49d at high levels;[2] 3) CD49d expression was displayed both in an histogram plot and in a CD19 versus CD49d dot plot, gated in the CLL region.

Bimodality for CD49d was defined as the presence of either two well-defined peaks (left panels on Supplemental Figure 1B) or two adjacent but distinct peaks (right panels on Supplemental Figure 1B) within the malignant clone always separated by a "nadir" in histogram plots viewed in both linear scale and in logarithmic (log) scale. Due to the possible appearance in log scale of a second mode despite the actual unimodal distribution in linear scale, all the cases of the present study were labeled as "bimodal" when a clear bimodal pattern (i.e. two peaks separated by a "nadir") was confirmed in both log and linear scale, according to reported recommendations.[3] In text and figures, either the linear or the log scales were used, for the sake of clarity. The lower limit of detection of a distinct $CD49d^{pos}$ population was 1%, corresponding to at least 100 $CD49d^{pos}$ cells over 10,000

total cells (example in Supplemental Figure 1C). The concomitant analysis of CD49d expression on a dot plot and on a histogram plot allowed to better detect the presence of small sub-populations (dot plot), and to better discriminate the separation of two distinct peaks (histogram plot) (Supplemental Figure 1B). All cases were analyzed independently by two operators (AZ and FP), and any discrepancy was resolved by review of the histogram plots by both parties.

Cell sorting

Cell sorting of CLL CD49d^{neg} and CD49d^{pos} subpopulations was performed using a FACSAriaIII (BD Biosciences) using a CD5-FITC, CD19-PerCP-Cy5.5, CD49d-APC or CD5-FITC, CD19-APC, CD49d-PE antibody combinations (all from BD Biosciences). A purity >95% was obtained for all samples.

Proliferation assays

Either total or CD49d^{neg}, and CD49d^{pos} sorted CLL cells were cultured on a layer of M210B4 cells (ATCC, Manassas, VA, USA) in complete RPMI with 7.5 mg/ml phosphorothioate CpG/ODN oligonucleotide 2006 (5-TCGTCGTTTTGTCGTTTTGTCGTT-3; Microsynth, Balgach, Switzerland) plus interleukin-2 (IL-2; R&D Systems, Minneapolis, MN, USA) in the presence of 10 μM 5-ethynyl-2'-deoxyuridine (EdU) for 48 hours. After harvesting, cells were washed, stained with anti-CD5-FITC, anti-CD19-PerCP-Cy5.5 and anti-CD49d-APC, fixed, permeabilized and stained with an anti-EdU-Pacific blue reagent following the manufacturer's instructions (Click-iT® EdU, ThermoFisher Scientific, Waltham, MA, USA).

Telomere length analysis

The high throughput STELA assay used in this study,[4] adapted from the previously published STELA protocol[5,6] used the telomere-adjacent primers specific for the XpYp telomere (XpYpC: 5′-CAGGGACCGGGACAAATAGAC-3′) and the 7q telomere (7qK1: 5′-GGGCACTGCCTCGCTTTGA-3′), in triplicate 30μl PCR reactions each containing 30ng of genomic DNA. Amplified fragments were resolved using capillary gel 100 electrophoresis and mean telomere length determined using PROSize software (AATI, Ankeny, Iowa, USA).

Statistics

Statistical analyses were performed using the MedCalc software (MedCalc Software, Mariakerke, Belgium) or the R statistical package (http://www.r-project.org/). Normally distributed data were compared using t-tests (paired or unpaired), non-parametric data using Mann Whitney test (independent) or Wilcoxon high rank test (paired analysis). Counts and frequency distributions were performed for discrete parameters. Significance of association between considered factors was assessed using χ^2 test. The overall concordance correlation coefficient (CCC) was used to evaluate the stability of CD49d expression overtime. For clinical analyses, the primary end points were overall survival (OS) and treatment-free survival (TFS), defined as described.[7] CLL patients treated with ibrutinib were not included in the analyses of OS and were analyzed separately using the progression-free survival (PFS) end point, defined as the time from the start of ibrutinib until progression or death. Treatment response was evaluated according to 2008 revised International Workshop on CLL (IWCLL) incorporating the 2012 clarifications for patients treated with kinase inhibitors as previously reported.[8-10] TFS, OS and PFS probabilities were estimated by the Kaplan-Meier method, and patients alive and treatment/progression-free were censored at the last follow-up. Log-rank test was used to compare TFS, OS and PFS probabilities between subgroups. Median follow-up was computed using the OS database and applying the inverted censoring method. Cox models were used to verify independent prognostic power of each parameter. Model minimization was performed by stepwise backward elimination. A P-value of 0.05 was considered to be statistically significant. Departure from proportionality in hazard was tested in all Cox models. The predictive power of the Cox models was evaluated according to Harrell's c-index, with corresponding 95% confidence intervals. The difference in the predictive power of the two CD49d categorizations was tested comparing 500 replicates using a bootstrap resampling procedure through *t*-test.

References

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Supplemental Table 1. Baseline characteristics of the whole cohort and of cohort A and cohort B CLL patients.

Factor*	whole cohort		cohort A		cohort B		p values†
		cases (n)		cases (n)		cases (n)	
age, median (range), y	64 (31-88)	1,630	64 (33-88)	764	64 (31-88)	866	0.39
>65	773 (47.4)		366 (47.9)		407 (47)		0.75
male sex	987 (60.5)	1,630	447 (58.5)	764	540 (62.3)	866	0.12
median follow-up, months	85		95		76		0.23
OS events	279 (18.3)	1,522	128 (18.5)	692	151 (18.2)	830	
median TFS, months	77		75		83		0.73
treated	804 (49.3)	1,630	395 (51.7)	764	409 (47.5)	861	
Rai stage II-IV	378 (23.3)	1,623	187 (24.5)	763	191 (22.2)	860	0.30
β2M> ULN	545 (49.5)	1,100	343 (50.1)	677	202 (47.8)	423	0.32
CD49d expression‡		1,630		764		866	
bimCD49d^{neg}	147 (9.0)		66 (8.6)		81 (9.3)		
bimCD49d^{pos}	166 (10.2)		70 (9.2)		96 (11.1)		0.13
homCD49d^{neg}	757 (46.4)		344 (45)		413 (47.7)		
homCD49d^{pos}	560 (34.4)		284 (37.2)		276 (31.9)		
UM *IGHV*	593 (39.8)	1,490	269 (37.6)	715	324 (41.8)	775	0.11
FISH category§		1,514		658		856	
del17p	126 (8.3)		58 (8.8)		68 (7.9)		0.61
del11q	156 (10.3)		66 (10)		90 (10.6)		0.82
tri12	234 (15.4)		99 (15.0)		135 (15.8)		0.70
del13q	582 (38.4)		262 (39.8)		320 (37.4)		0.33
normal	416 (27.5)		173 (26.3)		243 (28.4)		0.36
TP53 mutated	132 (9.8)	1,352	73 (10.5)	693	59 (9.0)	659	0.33
TP53 disrupted	190 (14.7)	1,290	94 (15.4)	610	96 (14.1)	680	0.51
NOTCH1 mutated	288 (18.6)	1,547	133 (17.5)	759	155 (19.7)	788	0.31

Cohort A includes CLL patients from a single Institution (S. Eugenio Hospital and University of Tor Vergata, Rome); cohort B includes patients from 5 Institutions (Ferrarotto Hospital, Catania; Amedeo Avogadro University of Eastern Piedmont, Novara; Azienda Ospedaliera

Universitaria S. Maria Misericordia, Udine; Maggiore General Hospital, University of Trieste, Trieste; Centro di Riferimento Oncologico della Basilicata, I.R.C.C.S., Rionero in Vulture).

*For all reported factors, the values refer to number of cases (%) unless otherwise specified; †all p values (for cohort A and B comparisons) refer to the χ^2 test, with the exception of those associated to age (Mann-Whitney test), and to OS and TFS (log-rank test); ‡CLL cases were split in four groups according to the level and pattern of CD49d expression: bimCD49d^{neg}, <30% bimodal; bimCD49d^{pos}, ≥30% bimodal; homCD49d^{neg}, <30% homogeneous; homCD49d^{pos}, ≥30% homogeneous; §FISH categories were as reported by Dohner et al (Dohner H, N Engl J Med. 2000;343:1910-6); §

β2M indicates beta-2 microglobulin; del, deletion; TFS, treatment-free survival; tri, trisomy; ULN, upper limit of normal; UM, unmutated.

Supplemental Table 2. Baseline characteristics of CLL patients treated with ibrutinib.

Factor*		cases available
age, median (range), y	69 (36-90)	158
>65, n (%)	102 (64.5)	158
male sex, n (%)	102 (64.5)	158
median follow-up, months	16	158
progression events, n (%)	41 (25.9)	
Rai stage III-IV, n (%)	48 (30.4)	158
Treatment status, RR	135 (85.4)	158
Lines of therapy >1	86 (54.8)	157
β2M ≥5 mg/L, n (%)	60 (38.2)	157
Hemoglobin <110 g/L for women/<120 g/L for men	41 (33.1)	124
LDH >ULN	43 (34.7)	124
Time from last therapy <24 months	72 (58.1)	124
CD49d expression†		158
bimCD49d^{neg}	12 (7.6)	
bimCD49d^{pos}	27 (17.1)	
homCD49d^{neg}	48 (30.4)	
homCD49d^{pos}	71 (44.9)	
UM *IGHV*	111 (73.5)	151
FISH category‡		138
del17p	47 (34.0)	
del11q	21 (15.2)	
tri12	19 (13.8)	
del13	23 (16.7)	
normal	28 (20.3)	
TP53 mutated	57 (38.3)	149
TP53 disrupted	76 (54.3)	140
NOTCH1 mutated	50 (33.6)	149

*For all reported factors, the values refer to number of cases (%) unless otherwise specified;†CD49d categories are as reported in footnote "‡" in Supplemental Table 1; ‡ FISH categories are as reported in footnote "§" in Supplemental Table 1.
RR, relapsed/refractory; all other abbreviations are explained in Supplemental Table 1.

Supplemental Table 3. *IGHV* gene usage and status in CLL with bimodal CD49d expression.

sample	*IGHV* status	V gene*allele	D gene*allele	J gene*allele
CT100†	UM	1-2*02	3-3*01	5*02
CT104	UM	7-4-1*02	5-24*01	4*02
CT114	UM	3-11*01	3-3*01	6*02
CT115†	UM	1-69*01	3-3*01	6*02
CT118	M	3-21*01	na	6*02
CT121†	UM	1-2*02	2-2*01	6*02
CT124	M	3-30*18	3-10*01	6*02
CT125	M	3-23*01	1-1*01	3*02
CT126	UM	3-9*01	3-16*01	na
CT129†	M	3-74*01	3-16*01	4*02
CT138†	M	3-11*01	5-5*01	6*02
CT142	UM	3-30*03	3-3*01	6*02
CT147	M	3-15*01	2-15*01	4*02
CT149	UM	1-69*12	3-3*01	6*03
CT15	UM	3-9*01	3-3*01	6*02
CT151	UM	1-69*01	4-23*01	6*02
CT155	M	3-7*01	3-10*01	2*01
CT157	M	3-33*05	1-26*01	3*01
CT168	UM	5-a*01	6-25*01	4*02
CT190†	M	3-72*01	2-2*02	4*02
CT193	M	3-7*01	5-24*01	1*01
CT194	M	3-11*01	3-9*01	6*02
CT208	M	1-3*01	5-18*01	4*02
CT213	UM	1-2*04	2-15*01	6*02
CT219	UM	3-23*04	3-9*01	4*02
CT227	M	2-26*01	2-21*01	3*02
CT23	M	4-39*01	2-2*01	5*02
CT237	M	4-59*08	3-10*01	6*02
CT240	UM	1-2*02	3-9*01	4*02
CT252	M	4-39*01	3-9*01	5*02

CT255	UM	3-33*01	3-3*01	4*02
CT268	UM	1-69*01	3-22*01	2*01
CT276†	UM	3-11*01	3-3*01	6*02
CT283†	M	4-34*01	2-15*01	5*01
CT285	M	3-30*02	6-25*01	4*02
CT287†	M	1-46*01	3-10*01	3*01
CT292	M	4-34*01	6-19*01	3*02
CT295	UM	3-30*03	3-3*01	6*02
CT302	UM	1-69*06	3-16*02	3*02
CT306	UM	3-30-3*01	3-10*01	6*02
CT314	M	3-7*01	6-19*01	3*01
CT315	UM	3-30*08	3-3*01	4*02
CT316	M	4-34*01	3-9*01	4*02
CT33	M	4-59*01	6-19*01	4*02
CT335	UM	1-69*01	3-3*01	6*02
CT338	M	4-31*03	4-17*01	3*02
CT339	UM	1-69*01	3-16*02	3*02
CT353	M	1-8*01	2-2*01	5*02
CT357	M	3-15*07	3-16*01	4*02
CT373	UM	1-69*01	3-3*01	6*02
CT378	UM	4-59*01	3-22*01	6*03
CT380	M	3-23*04	3-22*01	4*02
CT381	UM	5-51*01	3-22*01	4*02
CT388	UM	3-30*03	6-13*01	3*02
CT410	UM	3-33*01	6-13*01	4*02
CT412	UM	3-30-3*01	2-15*01	4*02
CT418	UM	1-2*02	1-26*01	6*02
CT42	UM	1-69*06	3-16*02	3*02
CT447	UM	1-69*06	3-16*02	3*02
CT48	M	4-34*04	1-26*01	3*02
CT50	M	3-7*01	6-19*01	4*02
CT71	UM	1-69*01	3-3*01	6*02

CT72	M	3-33*01	2-15*01	4*02
CT94	UM	3-15*01	2-2*02	6*03
RIO14	M	3-21*01	3-10*01	3*02
RIO17	UM	3-21*02	5-24*01	6*02
RIO2	UM	1-69*01	2-2*01	6*02
RIO22	UM	3-23*01	3-10*01	4*02
RIO27†	M	3-64*01	5-12*01	6*03
RIO29	M	3-15*01	2-2*01	3*01
RIO4	M	4-34*01	2-15*01	4*02
RIO45	M	3-74*03	3-22*01	4*02
RIO5	M	3-11*04	2-21*01	6*02
RIO53	UM	1-69*01	2-8*02	6*02
RIO55	UM	3-48*03	2-15*01	6*02
RIO66	UM	1-46*01	3-10*01	5*02
RM108	M	3-30*03	3-16*01	4*02
RM120	UM	1-69*01	3-3*01	6*03
RM129	UM	03-Nov	5-24	6
RM133	M	1-46*01	2-15*01	5*02
RM138†	M	4-39*01	3-22*01	4*02
RM139	M	3-53*01	2-15*01	5*02
RM150	M	3-74*01	4-23*01	5*02
RM164	UM	1-69*01	3-3*01	6*02
RM17	M	3-23*01	2-15*01	1*01
RM184	M	4-34*01	1-26*01	4*02
RM185†	UM	1-69*01	2-15*01	6*02
RM204†	M	3-7*01	2-2*01	4*02
RM209	M	3-30*03	3-10*01	4*02
RM212†	M	1-2*06	2-15*01	5*02
RM213	M	3-7*01	2-2*02	4*02
RM214	M	3-23*04	3-16*01	4*02
RM235	M	4-34*01	3-9*01	4*02
RM236	UM	3-23*01	3-10*01	4*02

RM238	M	3-23*01	1-1*01	4*02
RM24	UM	1-3*01	5-12*01	6*02
RM247	UM	4-31*03	3-3*01	5*02
RM25	UM	3-48*01	5-24*01	6*03
RM258	M	3-30-3*01	1-26*01	6*02
RM290†	M	3-21*01	5-24*01	6*02
RM292	UM	1-2*02	3-22*01	6*02
RM305	M	4-4*02	4-17*01	4*02
RM307	M	3-30*19	5-12*01	3*01
RM33	M	3-64*05	2-15*01	4*02
RM342	UM	2-5*01	3-22*01	6*02
RM349†	M	3-30*03	6-19*01	4*02
RM37	M	3-7*01	na	4*02
RM376	M	3-7*02	2-15*01	4*02
RM377	M	2-5*04	6-13*01	4*02
RM382	M	3-7*01	3-22*01	4*02
RM394	M	3-30*01	3-3*01	4*02
RM402†	M	3-13*01	1-7*01	4*02
RM409	UM	1-69*01	3-9*01	6*03
RM429	M	1-69*06	3-22*01	4*02
RM436	M	3-9*01	1-26*01	4*02
RM445†	M	1-8*01	3-10*01	6*03
RM470	UM	3-7*01	3-10*01	4*02
RM474	UM	3-33*01	3-3*01	5*02
RM491	M	3-49*03	3-22*01	6*02
RM503	M	3-30*18	3-16*02	6*02
RM518	M	4-39*01	2-2*01	4*02
RM528	M	4-30-4*01	6-19*01	1*01
RM529	M	3-7*01	5-24*01	2*01
RM53	M	4-34*01	6-13*01	4*02
RM537	UM	3-33*01	3-3*01	4*02
RM543	M	3-23*01	6-19*01	6*02

RM547	M	4-34*01	3-22*01	4*02
RM555	UM	4-39*01	1-20*01	5*02
RM560	UM	3-53*01	3-22*01	6*02
RM566	M	3-30*03	1-26*01	4*02
RM576	UM	4-39*01	3-22*01	4*02
RM583†	M	3-53*01	6-19*01	4*02
RM591	UM	1-69*01	3-10*01	6*02
RM594	M	1-3*01	5-12*01	4*02
RM596	M	3-33*01	6-13*01	6*02
RM598	M	3-33*01	2-15*01	4*02
RM623	M	3-72*01	1-26*01	3*02
RM624	M	3-33*01	1-7*01	3*01
RM626	M	3-74*02	1-20*01	4*02
RM631	UM	5-51*01	3-22*01	4*02
RM640†	UM	1-3*01	6-19*01	4*02
RM648	M	4-34*01	2-2*02	4*02
RM652	M	1-2*02	7-27*01	3*02
RM653	UM	5-51*01	3-22*01	4*02
RM654	M	3-30-3*01	4-17*01	6*02
RM675	M	4-34*01	5-12*01	2*01
RM681	UM	4-39*01	6-13*01	5*02
RM685	M	4-34*01	5-24*01	4*02
RM692	UM	1-69*01	3-3*01	6*02
RM693	M	1-3*01	2-21*01	5*02
RM697	M	2-5*10	4-17*01	4*02
RM698	M	1-8*01	4-23*01	5*01
RM705	M	2-5*01	1-7*01	4*02
RM706	M	4-34*01	5-18*01	3*01
RM711	M	4-34*01	2-8*01	4*02
RM714	M	3-72*01	1-1*01	5*02
RM722	M	3-15*01	1-26*01	4*02
RM726†	M	3-23*01	5-18*01	4*02

RM735	M	4-34*08	1-26*01	5*01
RM749	M	3-30*18	6-19*01	6*02
RM75	M	3-23*01	6-19*01	4*02
RM771	UM	3-23*01	2-2*01	4*02
RM773	M	4-30-4*03	6-19*01	4*02
RM79	UM	1-2*02	1-26*02	6*02
RM86	UM	3-49*05	3-9*01	4*02
RMPTV10	UM	4-39*01	3-3*01	4*02
RMPTV100†	M	4-30-4*01	3-9*01	5*02
RMPTV122†	UM	4-34*01	2-15*01	6*04
RMPTV133	UM	3-33*01	2-2*01	6*02
RMPTV137	UM	3-15*01	3-16*01	5*02
RMPTV138	M	6-1*01	6-19*01	5*01
RMPTV140	UM	1-69*01	3-10*01	6*02
RMPTV143	M	4-34*01	2-2*01	4*02
RMPTV154	UM	1-69*06	6-19*01	1*01
RMPTV156	UM	1-69*01	3-3*01	6*02
RMPTV165	M	3-7*03	3-22*01	3*01
RMPTV168	UM	3-74*01	4-23*01	6*02
RMPTV17	M	3-30-3*01	3-16*01	3*02
RMPTV176	M	4-34*01	5-18*01	3*02
RMPTV18	UM	3-15*01	3-3*01	6*02
RMPTV182	M	6-1*02	2-15*01	5*01
RMPTV191	M	3-23*04	6-13*01	4*02
RMPTV195	M	2-2*02	2-21*01	4*02
RMPTV2	UM	1-69*01	2-15*01	4*02
RMPTV209	M	3-21*01	1-14*01	6*02
RMPTV211	UM	1-3*01	5-12*01	4*02
RMPTV22	UM	1-69*01	2-2*01	6*03
RMPTV39	UM	3-33*01	3-10*01	6*02
RMPTV42	UM	1-69*01	3-16*01	3*02
RMPTV43	M	3-74*01	5-12*01	4*02

RMPTV53	M	3-33*01	3-10*01	3*01
RMPTV56†	UM	3-48*02	4-17*01	4*02
RMPTV58	M	3-23*04	1-26*01	4*02
RMPTV67	UM	1-3*01	6-19*01	4*02
RMPTV84	M	3-15*01	4-17*01	4*02
RMPTV94	M	3-74*01	1-14*01	4*01
RMPTV95	UM	1-69*01	2-2*01	6*02
RMPTV97	UM	4-39*01	1-26*01	6*02
TS110	M	3-48*03	3-3*01	4*02
TS122	M	4-34*01	2-15*01	6*03
TS131	UM	1-69*01	2-2*02	5*02
TS142†	M	3-23*01	2-15*01	4*02
TS145	M	4-4*02	1-17*01	5*02
TS164	M	4-34*01	3-16*02	4*02
TS166†	UM	3-30*03	3-3*01	4*02
TS177†	UM	3-7*01	4-17*01	6*02
TS178	M	3-7*01	2-21*01	3*02
TS181	M	3-23*01	3-16*01	4*02
TS182	M	4-34*01	6-19*01	6*02
TS189	UM	4-34*01	3-22*01	2*01
TS19†	UM	3-48*03	2-2*02	6*02
TS28	UM	4-34*01	2-8*02	6*02
TS63	UM	3-23*01	3-10*01	6*02
TS69	M	4-34*01	6-13*01	6*02
TS84	M	4-34*01	6-13*01	6*02
TS85	M	3-23*04	3-9*01	4*02
UD31	UM	1-2*02	6-19*01	4*02
UD35	M	3-74*01	6-13*01	4*02
UD4†	M	3-21*01	na	6*02
UD57	M	3-7*01	6-13*01	6*02
UD75†	M	4-31*02	3-10*01	6*02
UD82	M	3-23*01	6-19*01	4*02

NO39	M	3-33*01	na	na
NO66	M	3-23*01	na	na
NO69	UM	4-30-4*01	na	na
NO72	M	1-46*01	na	na
NO73	M	4-34*01	na	na
NO76	M	4-34*01	na	na
NO82	M	3-23*01	na	na
NO83	M	4-30-4*01	na	na
NO88	M	2-5*10	na	na
NO91	M	4-4*02	na	na
NO93	M	2-5*10	na	na
NO94	M	3-53*01	na	na
NO97	M	3-33*01	na	na
NO104	M	3-9*01	na	na
NO105	M	3-48*01	na	na
NO106	UM	1-69*01	na	na
NO109	M	5-51*01	na	na
NO113	M	3-74*01	na	na
NO114	M	3-30*02	na	na
NO116	M	3-11*01	na	na
NO118	M	1-69*01	na	na
NO120	UM	6-1*01	na	na
NO121	UM	3-48*01	na	na
NO123	M	4-34*01	na	na
NO125	M	1-8*02	na	na
NO129	M	3-48*01	na	na
NO130	UM	3-23*01	na	na
NO132	M	4-34*01	na	na
NO134	UM	2-5*01	na	na
NO136	UM	1-2*02	na	na
NO137	M	1-69*06	na	na
NO138	M	3-21*01	na	na

NO139	M	5-51*01	na	na
NO140	M	4-39*01	na	na
NO141	UM	1-69*06	na	na
NO142	UM	1-3*01	na	na
NO143	UM	6-1*01	na	na
NO144	M	1-8*01	na	na
NO145	M	4-34*01	na	na
NO147	UM	1-3*02	na	na
NO148	M	3-7*01	na	na
NO149	M	4-34*01	na	na
NO150	M	4-34*01	na	na
NO151	UM	4-39*01	na	na
NO152	UM	3-30-3*01	na	na
NO157	UM	1-3*01	na	na
NO158	M	3-33*01	na	na
NO159	UM	4-34*01	na	na
NO160	M	4-34*03	na	na
NO162	M	4-34*01	na	na
NO163	M	4-34*01	na	na
NO165	UM	1-69*01	na	na
NO167	UM	1-69*01	na	na
NO169	M	1-2*02	na	na
NO171	UM	3-21*01	na	na
NO172	M	4-61*02	na	na
NO173	M	1-3*01	na	na
NO174	M	3-21*01	na	na
NO177	UM	3-48*03	na	na
NO189	M	3-48*03	na	na
NO170	UM	1-2*02	na	na
UD47†‡	UM/M	3-43*01/4-34*03	3-22*01/5-18*01	6*02/4*02
RIO52‡	UM/UM	1-18*04/3-33*01	6-19*01/6-19*01	6*02/6*02
RM207‡	UM/M	4-4*07/3-7*02	3-10*01/2-8*01	6*02/3*02

RM273‡	M/M	3-74*01/4-39*01	5-5*01/2-21*01	4*02/4*02
RM230‡	M/M	3-23*01/1-46*01	2-21*01/5-24*01	6*02/4*02
RM556‡	M/M	4-61*02/1-3*01	5-12*01/1-26*01	4*02/4*02
RM542‡	UM/M	4-34*01/3-74*01	2-15*01/3-16*02	6*02/4*02
RM390‡	M/M	1-2*02/3-7*01	1-1*01/3-22*01	6*02/5*01
RM667‡	UM/UM	1-69*01/5-a*03	2-2*01/3-3*01	6*02/6*02
RM625‡	M/M	2-5*10/6-1*01	1-1*01/4-17*01	4*02/3*02
RM578‡	M/M	3-30*04/3-30*04	1-1*01/1-7*01	4*02/4*03
CT337‡	M/M	3-30*02/3-48*04	1-1*01/2-21*02	5*01/6*02
RMPTV147‡	M/M	4-34*01/3-30*03	1-26*01/6-13*02	3*01/4*02
CT423‡	UM/UM	1-69*01/1-69*01	3-3*01/2-2*01	6*02/6*02

IGHV genes were sequenced by Sanger sequencing and analysed with the IMGT/V-QUEST tool (http://www.imgt.org);
†cases used for *IGHV* sequence analysis by NGS in the sorted $CD49d^{neg}$ and $CD49d^{pos}$ fractions; in all cases, NGS analysis confirmed the reported *IGHV* sequences in both $CD49d^{neg}$ and $CD49d^{pos}$ fractions;
‡cases displaying two concomitant *IGHV* sequences (bi-clonal samples);
M, mutated *IGHV*, na, not available, UM, unmutated *IGHV*.

Supplemental Table 4. FISH analysis in sorted CD49d^{neg} and CD49d^{pos} CLL cells.

sample	cytogenetic aberration	% aberration on CD49d^{neg} cells	% aberration on CD49d^{pos} cells	p values*
CT72	tri12	3	50	
CT388	tri12	73	71	
CT378	tri12	4	75	
RMPTV251	tri12	4	82	
CT329	tri12	3	69	
RM805	tri12	30	70	
RMPTV22	tri12	4	75	
RM185	tri12	67	69	0.0026
RM474	tri12	74	71	
TOS50	tri12	57	72	
RM207	tri12	6	31	
CT276	tri12	77	71	
RMPTV122	tri12	71	82	
RM591	tri12	55	72	
CT126	tri12	73	76	
CT412	del13	11	17	
CT353	del13	35	83	
RM654	del13	59	27	
RM735	del13	22	17	
RM33	del13	64	16	
CT447	del13	21	85	
TOS83	del13	9	49	0.33
RMPTV56	del13	14	94	
RM139	del13	59	50	
TOS33	del13	100	98	
RM17	del13	26	8	
RM207	del13	52	69	
RM290	del13	85	98	

RM583	del13	95	90	
RIO27	del13	15	30	
CRO173	del13/ biallelic	20/78	21/74	
RM445	del13/ biallelic	51/45	42/52	
RM212	del13/ biallelic	85/7	23/29	
UD75	del13/ biallelic	21/79	7/93	0.62
TS142	del13/ biallelic	90/10	69/31	
CT129	del13/ biallelic	0/81	0/40	
TOS118	del13/ biallelic	0/46	0/12	
CT219	del11	76	15	
RMPTV56	del11	5	91	
TOS105	del11	25	39	0.81
CT219	del11	87	52	
RMPTV137	del11	88	7	
CT72	del17	8	6	
CT219	del17	4	3	
RMPTV53	del17	50	35	
CT447	del17	57	10	0.02
RMPTV56	del17	7	4	
CT115	del17	15	12	
CT129	del17	46	42	

*p values refer to the paired Wilcoxon test.

Supplemental Table 5. Mutation analysis in sorted CD49d^{neg} and CD49d^{pos} CLL cells.

sample	mutated gene	mutation description	% mutation on CD49d^{neg} cells	% mutation on CD49d^{pos} cells	p values*
RIO66	NOTCH1	c.*7668+371A>G	0	2.2	0.0122
TS63	NOTCH1	c.7541_7542del2, p.P2514Rfs*4	2.9	3.1	
TOS69	NOTCH1	c.7544_7545delCT, p.P2515fs*4	49.9	44.2	
RM185	NOTCH1	c.7544_7545delCT, p.P2515fs*4	5.2	48.5	
NO141	NOTCH1	c.7544_7545delCT, p.P2515fs*4	0.3	3.1	
NO159	NOTCH1	c.7544_7545delCT, p.P2515fs*4	0	3.7	
RM474	NOTCH1	c.7544_7545delCT, p.P2515fs*4	54.5	56.7	
RM207	NOTCH1	c.7544_7545delCT, p.P2515fs*4	5.7	31.3	
RMPTV122	NOTCH1	c.7544_7545delCT, p.P2515fs*4	14.2	46.1	
RIO27	NOTCH1	c.7544_7545delCT, p.P2515fs*4	3.0	3.9	
TS142	NOTCH1	c.7544_7545delCT, p.P2515fs*4	3.6	9.8	
CT115	NOTCH1	c.7544_7545delCT, p.P2515fs*4	0.4	1.2	
RM238	SF3B1	c.2098A>G,p.K700E	1.6	2.8	0.19
		c.2110A>T,p.I704F	4.0	9.2	
		c.2219G>A,p.G740E	5.0	1.2	
		c.2223G>C,p.G741N	1.7	3.0	
		c.2225G>A,p.G742D	1.7	0	
RMPTV147	SF3B1	c.2110A>T,p.I704F	0	3	
		c.2111T>C,p.I704T	0	9.9	
		c.2324G>T,p.R775L	5.8	1.9	
NO141	SF3B1	c.1996A>G p.K666E	4.1	11.2	
RM290	SF3B1	c.2098 A>G p.K700E	35.8	46.8	
RMPTV53	TP53	c.560-1G>A,p.?	65.6	72.7	0.27
RM528	TP53	c.712T>A,p.C238S	19.7	35.3	
RM108	TP53	c.646G>A,p.V216M	3.3	0	
RM692	TP53	c.596G>T,p.G199V	5.8	0	
CT338	TP53	c.472_477del,p.R158_A159del	8.5	0	
		c.479_487del,p.M160_Y163delinsN	8.5	0	
		c.763A>T,p.I255F	4.2	0	
RIO22	TP53	c.406C>T,p.Q136X	0	5.3	
NO121	TP53	c.797G>A p.G266E	16.0	0.3	

NO177	TP53	c.817C>T p.R273C	48.0	0.1	
RM185	BIRC3	c.1639_1640delCA p.Q547fs*11	7.8	0	na
RIO1	BIRC3	c.1654delC p.Q552fs*16	5.9	4.0	

*p values refer to the paired Wilcoxon test; na, not applicable.

Supplemental Table 6. Baseline characteristics of CLL with homogeneous or bimodal CD49d expression.

	homCD49d^{neg}*		homCD49d^{pos}†		bimCD49d‡		p values§	
		cases available		cases available		cases available	bimCD49d‡ vs homCD49d^{neg}*	bimCD49d‡ vs homCD49d^{pos}†
age, median (range), y	63 (32-88)	757	65 (31-88)	560	64 (33-88)	313	0.04	0.76
>65, n (%)	340 (44.9)		282 (50.4)		151 (48.2)		0.29	0.60
male sex, n (%)	438 (57.8)	757	355 (63.4)	560	194 (62.0)	313	0.24	0.73
Rai stage II-IV, n (%)	111 (14.7)	755	191 (34.4)	555	71 (22.7)	313	0.002	0.0004
β2M> ULN, n (%)	206 (38.1)	540	202 (47.8)	340	116 (52.7)	220	0.0003	0.14
Lymphadenopathy, n (%)‖	333 (44.0)	757	343 (61.6)	557	151 (48.2)	313	0.20	0.0001
UM *IGHV*, n (%)	194 (28.1)	691	324 (64.2)	505	115 (39.1)	294	0.0008	<0.0001
FISH category¶		705		510		299		
del17p, n (%)	45 (6.4)		63 (12.4)		18 (6.0)		0.94	0.005
del11q, n (%)	83 (11.8)		49 (9.6)		24 (8.0)		0.09	0.53
tri12, n (%)	14 (2.0)		158 (31.0)		62 (20.7)		<0.0001	0.002
del13, n (%)	385 (54.6)		99 (19.4)		98 (32.8)		<0.0001	<0.0001
normal, n (%)	178 (25.2)		141 (27.6)		97 (32.4)		0.02	0.17
TP53 mutated, n (%)	44 (8.7)	503	71 (17.9)	397	23 (10.1)	227	0.64	0.01
TP53 disrupted, n (%)	54 (9.9)	547	73 (18.2)	401	21 (7.7)	273	0.31	0.0001
NOTCH1 mutated, n (%)	62 (8.6)	723	159 (30.1)	528	67 (22.6)	296	<0.0001	0.09

*homCD49d^{neg} indicates cases with <30% homogeneous CD49d expression; †homCD49d^{pos} indicates cases with ≥30% homogeneous CD49d expression; ‡bimCD49d indicates cases with bimodal CD49d expression; §all p values refer to the χ^2 test, with the exception of p values associated to age that refer to the Mann-Whitney test; ‖lymphadenopathy was evaluated at diagnosis; ¶FISH categories were as reported in footnote "‡" in Supplemental Table 1.
Abbreviations are explained in Supplemental Table 1.

Supplemental Table 7. Cox regression analysis of overall survival including *TP53* disruption.

Factor	Multivariable analysis (n=1,058) model I			Multivariable analysis (n=761) model II		
	HR	95% CI	*p*	HR	95% CI	*p*
homCD49d^{pos}*	2.09	1.50-2.92	<0.0001	2.07	1.40-3.06	0.0002
bimCD49d†	2.10	1.43-3.09	0.0001	2.33	1.52-3.56	0.0001
age >65	3.74	2.75-5.08	<0.0001	3.41	2.36-4.93	<0.0001
Rai stage II-IV	1.85	1.38-2.49	<0.0001	n.i.	n.i.	n.i.
UM *IGHV*	2.54	1.90-3.40	<0.0001	2.72	1.95-3.79	<0.0001
TP53 disruption	2.10	1.52-2.90	<0.0001	2.01	1.38-2.92	<0.0001
del11q	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
tri12	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
NOTCH1 mutated	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
β2M >ULN	-	-	-	1.98	1.38-2.82	0.0002

All listed factor, with a significant p value by univariable analysis (see Table 1), were entered in the multivariable analysis;

*homCD49d^{pos} refers to CLL cases with ≥30% homogeneous expression of CD49d;† bimCD49d refers to CLL cases with bimodal expression of CD49d.

CI indicates confidence interval; HR, hazard ratio; n.i., not included in the model after stepwise selection; all other abbreviations are explained in Supplemental Table 1.

Supplemental Table 8. Cox regression analysis of treatment-free survival.

Factor		Univariable analysis			Multivariable analysis (n=1,140) model I			Multivariable analysis (n=831) model II		
	cases	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
homCD49d^{pos}*	1,625	2.35	2.01-2.75	<0.0001	1.72	1.42-2.10	<0.0001	1.37	1.06-1.76	0.015
bimCD49d†	1,625	1.74	1.41-2.11	<0.0001	1.63	1.30-2.03	<0.0001	1.48	1.13-1.93	0.0039
UM *IGHV*	1,485	3.7	3.19-4.30	<0.0001	2.67	2.21-3.23	<0.0001	2.62	2.08-3.30	<0.0001
del17p	1,509	2.84	2.26-3.58	<0.0001	1.61	1.17-2.21	0.0034	1.97	1.38-2.83	0.0002
del11q	1,509	3.33	2.72-4.09	<0.0001	1.81	1.44-2.27	<0.0001	1.75	1.32-2.34	0.0001
tri12	1,509	2.2	1.81-2.65	<0.0001	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
TP53 mutated	1,349	2.25	1.83-2.78	<0.0001	1.37	1.03-1.82	0.0290	1.50	1.06-2.12	0.0203
NOTCH1 mutated	1,543	2.24	1.91-2.63	<0.0001	1.32	1.08-1.60	0.0049	1.32	1.02-1.69	0.0311
β2M >ULN	1,098	3.36	2.79-4.06	<0.0001	-	-	-	2.45	1.96-3.05	<0.0001

All factors used in univariable analyses were entered in the multivariable analysis;
*homCD49d^{pos} refers to CLL cases with ≥30% homogeneous expression of CD49d; †bimCD49d refers to CLL cases with bimodal expression of CD49d.
Abbreviations are explained in Supplemental Tables 1 and 6.

Supplemental Table 9. Comparison of multivariable Cox regression analyses of overall survival (n=1,045).

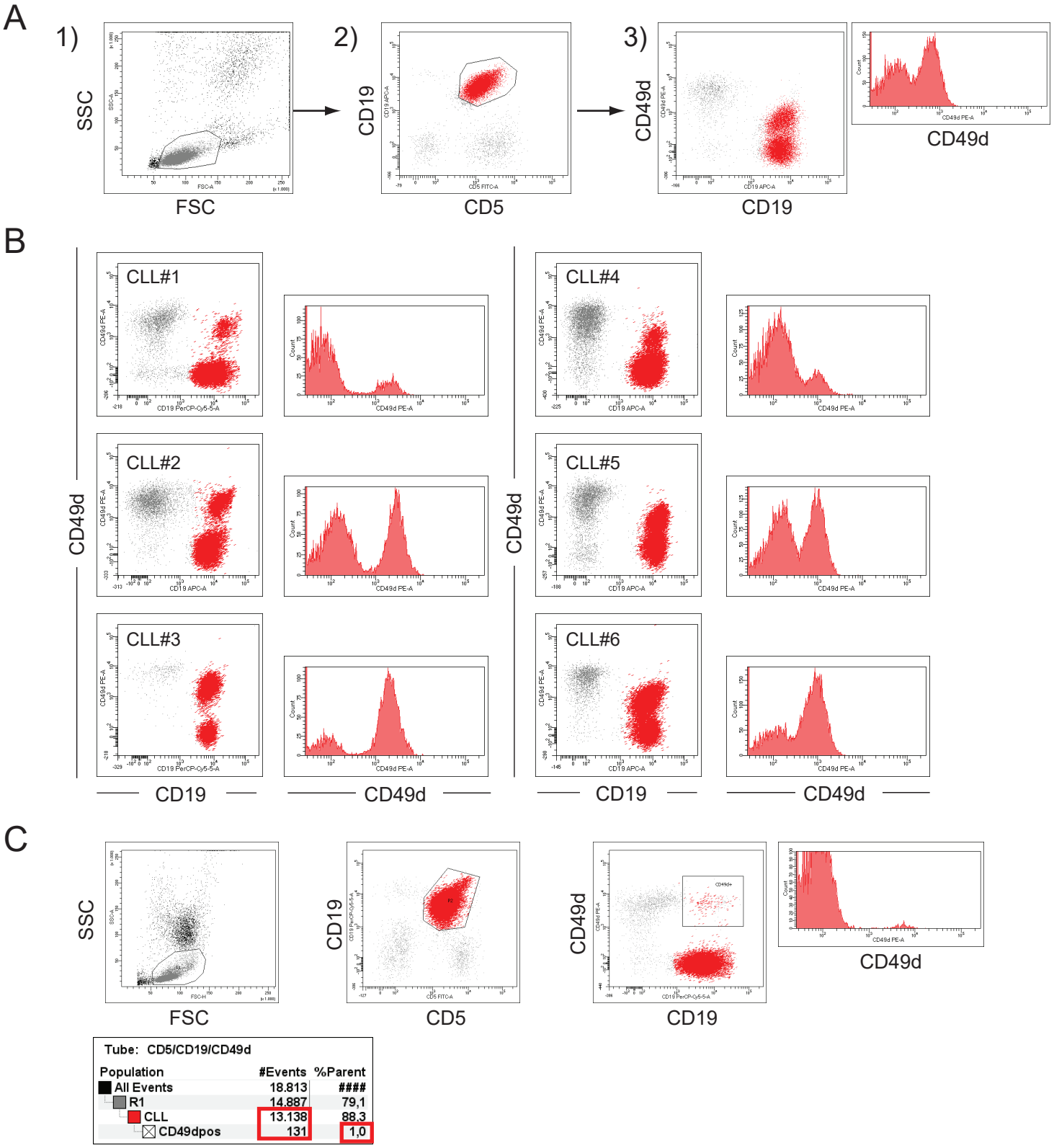
Factor	CD49d^{pos}= CD49d≥30%*			CD49d^{pos}=homCD49d^{pos}+bimCD49d†		
	HR	95% CI	*p*	HR	95% CI	*p*
CD49d^{pos}	1.68	1.26-2.25	0.0005	2.13	1.55-2.91	<0.0001
age >65	4.04	2.95-5.52	<0.0001	4.06	2.97-5.55	<0.0001
Rai stage II-IV	1.87	1.39-2.53	<0.0001	1.82	1.35-2.45	<0.0001
UM *IGHV*	2.45	1.82-3.28	<0.0001	2.42	1.80-3.23	<0.0001
TP53 mutated	2.32	1.64-3.28	<0.0001	2.33	1.65-3.29	<0.0001

The factors entered in the multivariable analyses were those resulting from the multivariable model I reported in Table 1.

*CD49d^{pos} cases were defined as cases with CD49d expression ≥30% cut-off; †CD49d^{pos} cases were obtained by combining cases with ≥30% homogeneous CD49d expression and cases with CD49d bimodal expression.

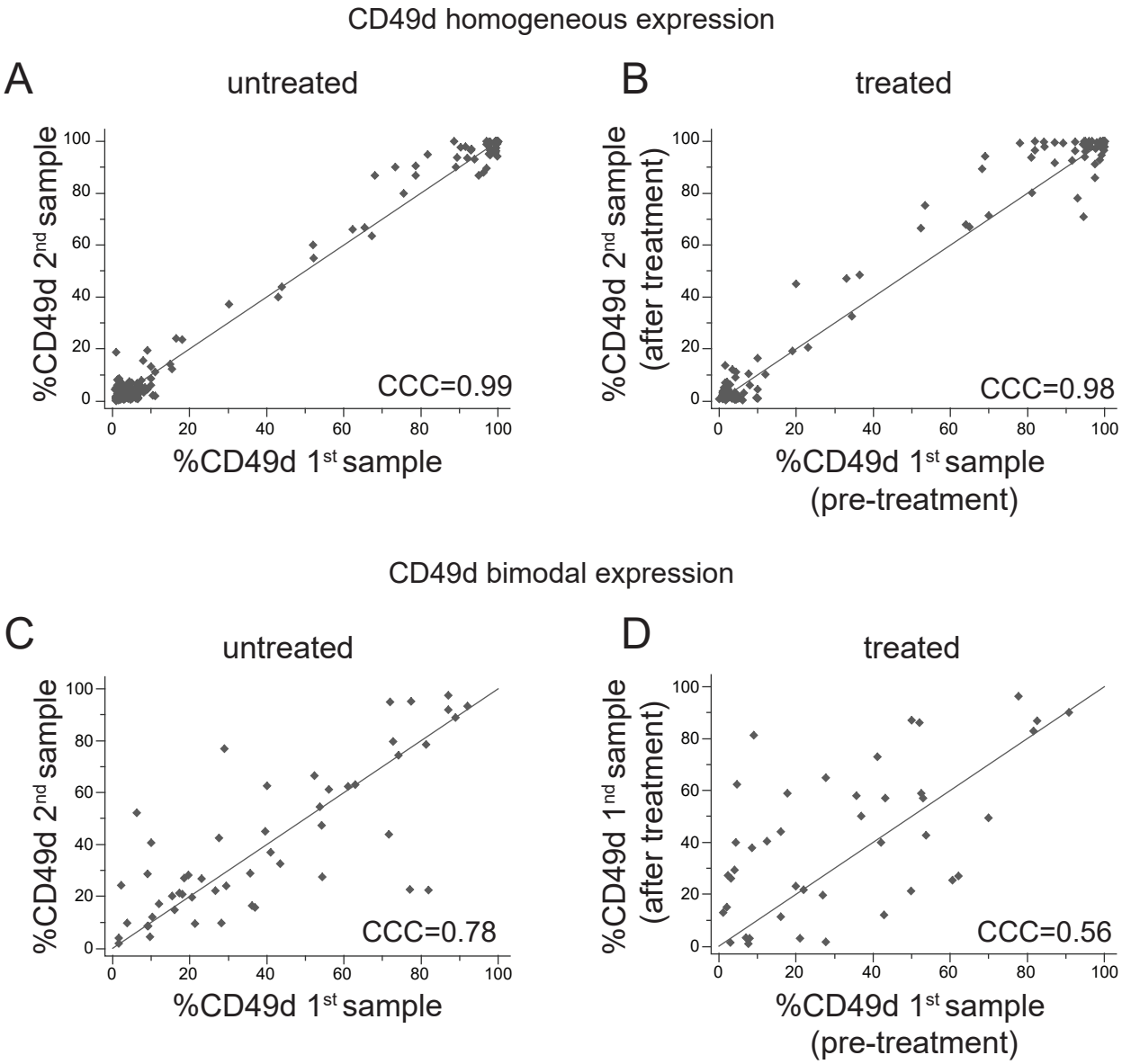
Abbreviations are explained in Supplemental Tables 1 and 6.

Supplemental Figure 1



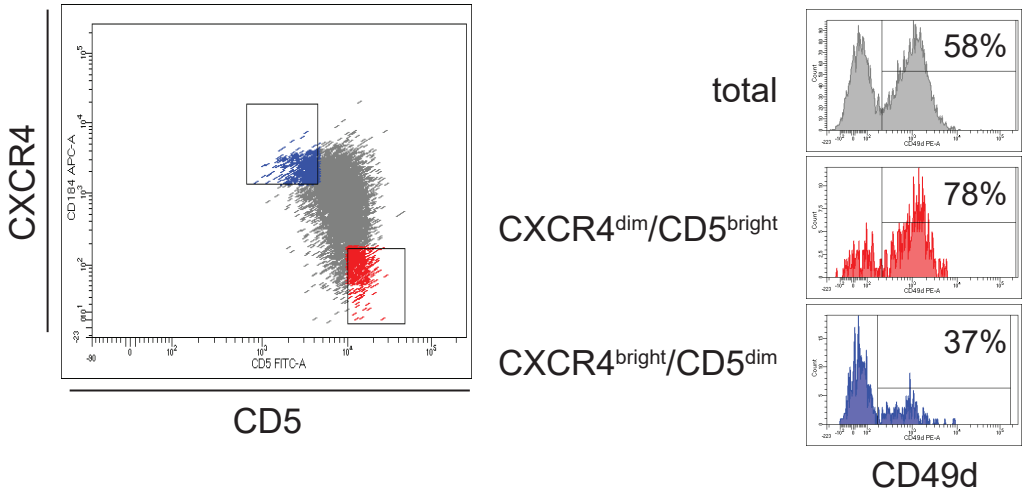
Supplemental Figure 1. Guidelines for the analysis of CD49d bimodal expression. (A) Gating strategy. 1) The lymphocyte population is selected on a forward- versus side-scatter (FSC/SSC) plot (R1); 2) CLL cells (CD19+CD5+) are selected on a CD5 versus CD19 dot plot gated in R1; 3) CD49d expression is displayed both in a CD19 versus CD49d dot plot and in a histogram plot in linear scale, gated in the CLL region; (B) Bimodality for CD49d is defined as the presence of either two well-defined and separate peaks (see representative CLL cases #1-3, left panels) or two adjacent but distinct peaks within the malignant clone (see representative CLL cases #4-6, right panels); (C) Flow cytometry analysis following the gating strategy reported in (A) of a representative CLL sample with CD49d bimodal expression characterized by the presence of 1% CD49d^{pos} cells. The 1% value corresponds to the lower limit of detection of a distinct CD49d^{pos} population, i.e. at least 100 CD49d^{pos} cells over 10,000 total cells (lower panel, red boxes).

Supplemental Figure 2



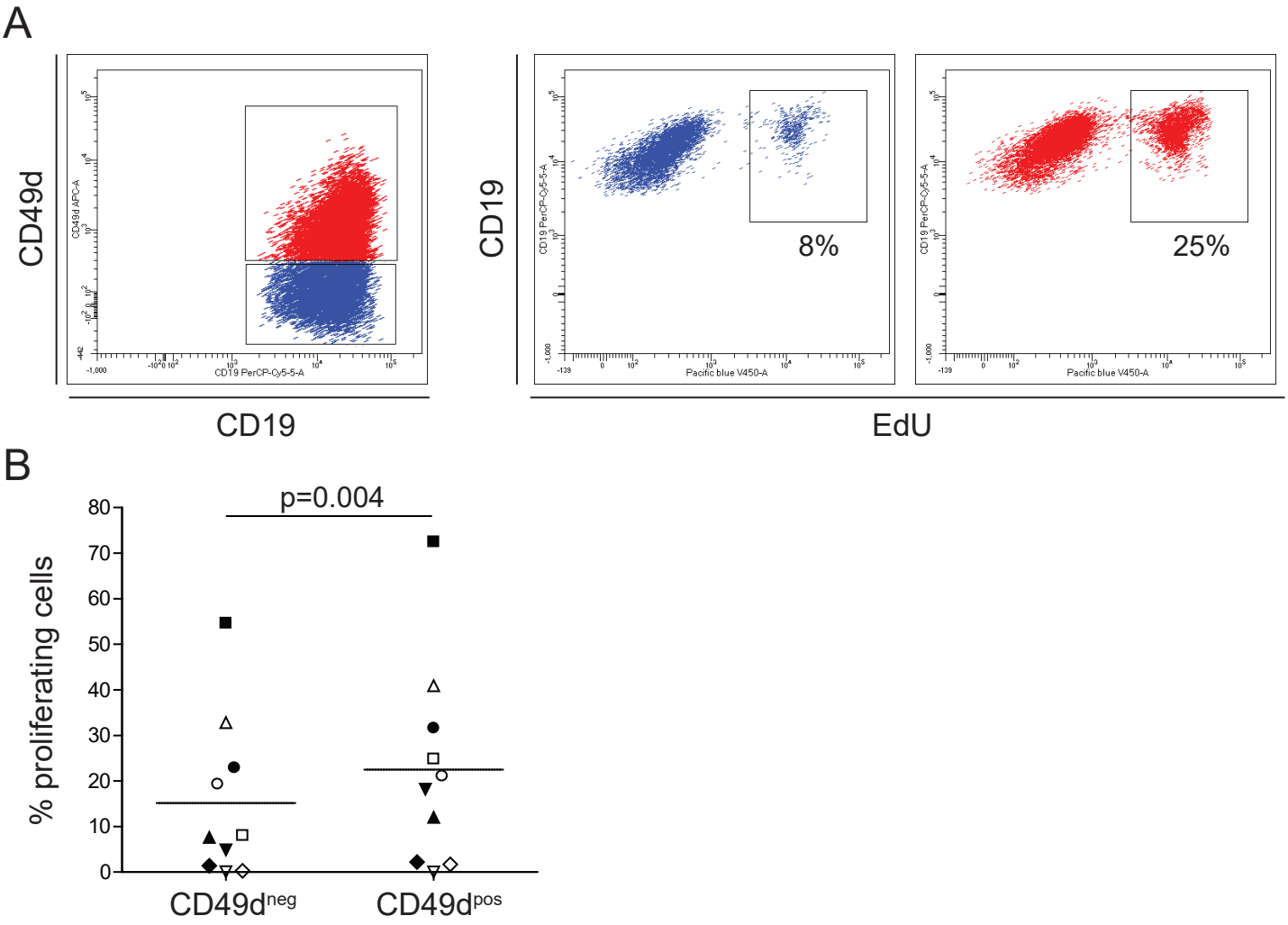
S**upplemental Figure 2. CD49d expression over time.** CD49d expression was evaluated in two sequential samples from 322 CLL cases with homogeneous CD49d expression (A-B) and 94 CLL cases with CD49d bimodal expression (C-D), either untreated (A, C) or treated between the samples (B, D). CD49d % expression values of the first and second samples are reported in x-axis and y-axis respectively. The concordance correlation coefficient (CCC) values are reported in each graph.

Supplemental Figure 3



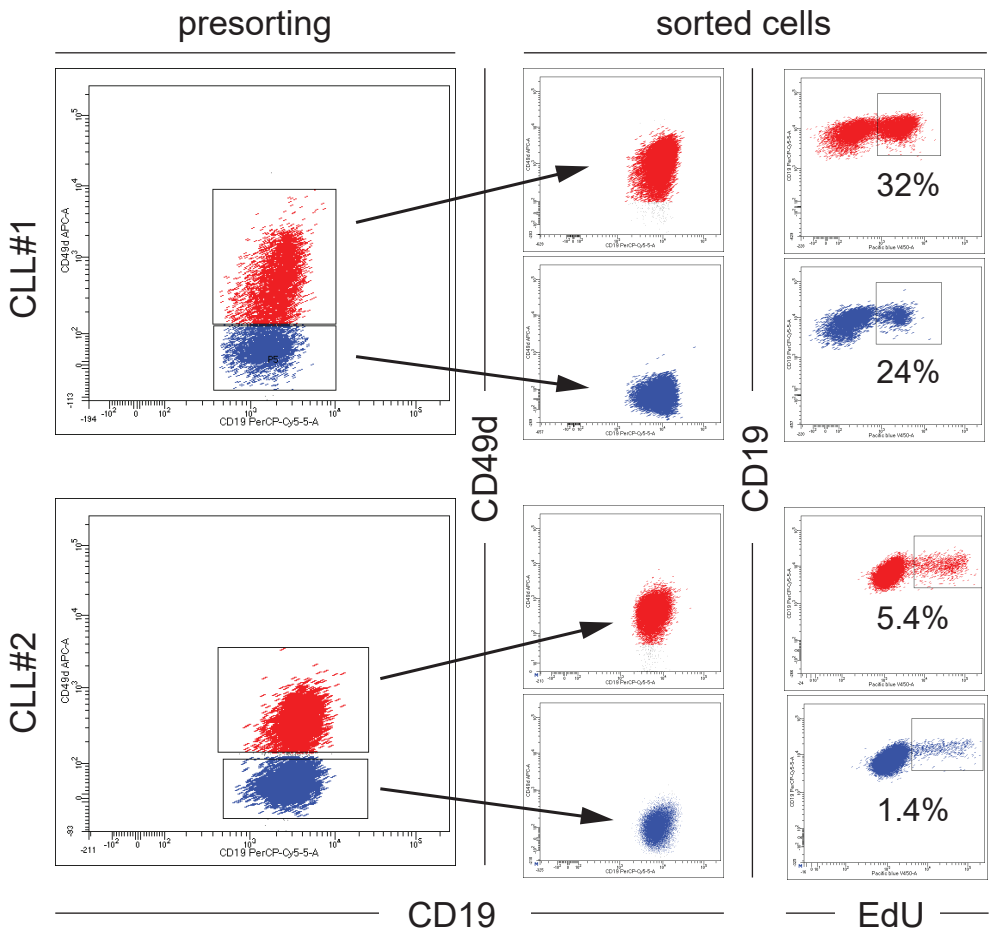
Supplemental Figure 3. Flow cytometry analysis to determine the amount of CD49d^{pos} cells in intra-clonal populations from bimCD49d CLL. Flow cytometry analysis of intra-clonal sub-populations with variable reciprocal densities of CXCR4/CD5 in one representative CLL case with CD49d bimodal expression. CLL subpopulations were gated on the basis of a CXCR4^{dim}/CD5^{bright} (red) and a CXCR4^{bright}/CD5^{dim} (blue) expression. The histogram plots on the right show CD49d expression in the context of the total CLL population (grey), and in the CXCR4^{dim}/CD5^{bright} (red) and CXCR4^{bright}/CD5^{dim} (blue) CLL fractions;

Supplemental Figure 4



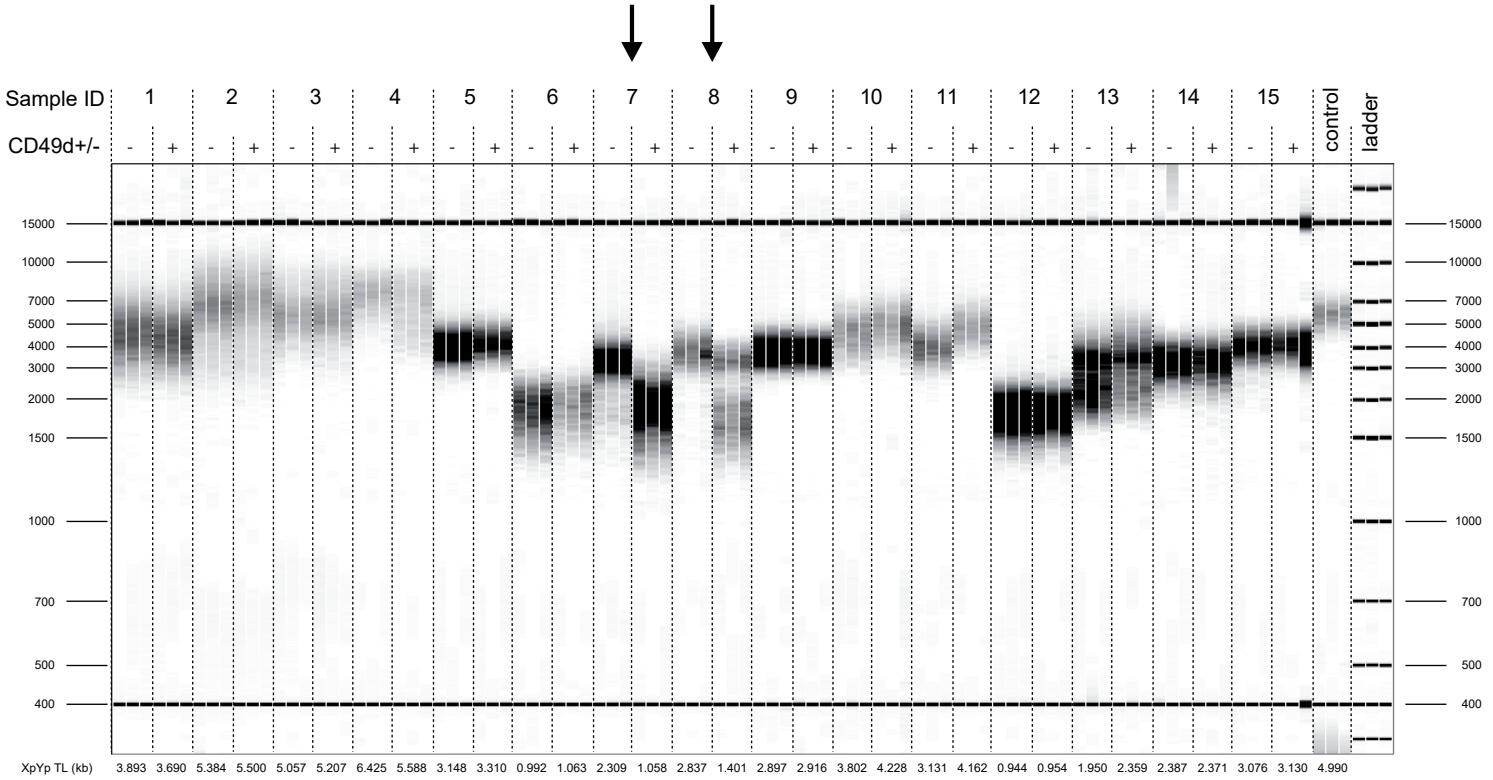
Supplemental Figure 4. Proliferation rates in CD49d^{neg} and CD49d^{pos} fractions from bimCD49d CLL. Percent of proliferating cells in CD49d^{neg} and CD49d^{pos} CLL fractions, as identified by the incorporation of 5-ethynyl-2'-deoxyuridine (EdU) following 48 hours co-culture with stromal cells and concomitant stimulation with the TLR9 agonist CpG. (A) Flow cytometry analysis in one representative CLL case with CD49d bimodal expression. CD49d^{neg} (blue) and CD49d^{pos} (red) CLL population were gated on a CD19 versus CD49d dot plot. Proliferating cells were then identified by positive staining with an anti-EdU-Pacific blue reagent on an EdU versus CD19 dot plot. (B) Percent of proliferating cells in the CD49d^{neg} and CD49d^{pos} fractions from 10 CLL cases; p value refers to the Mann Whitney test.

Supplemental Figure 5



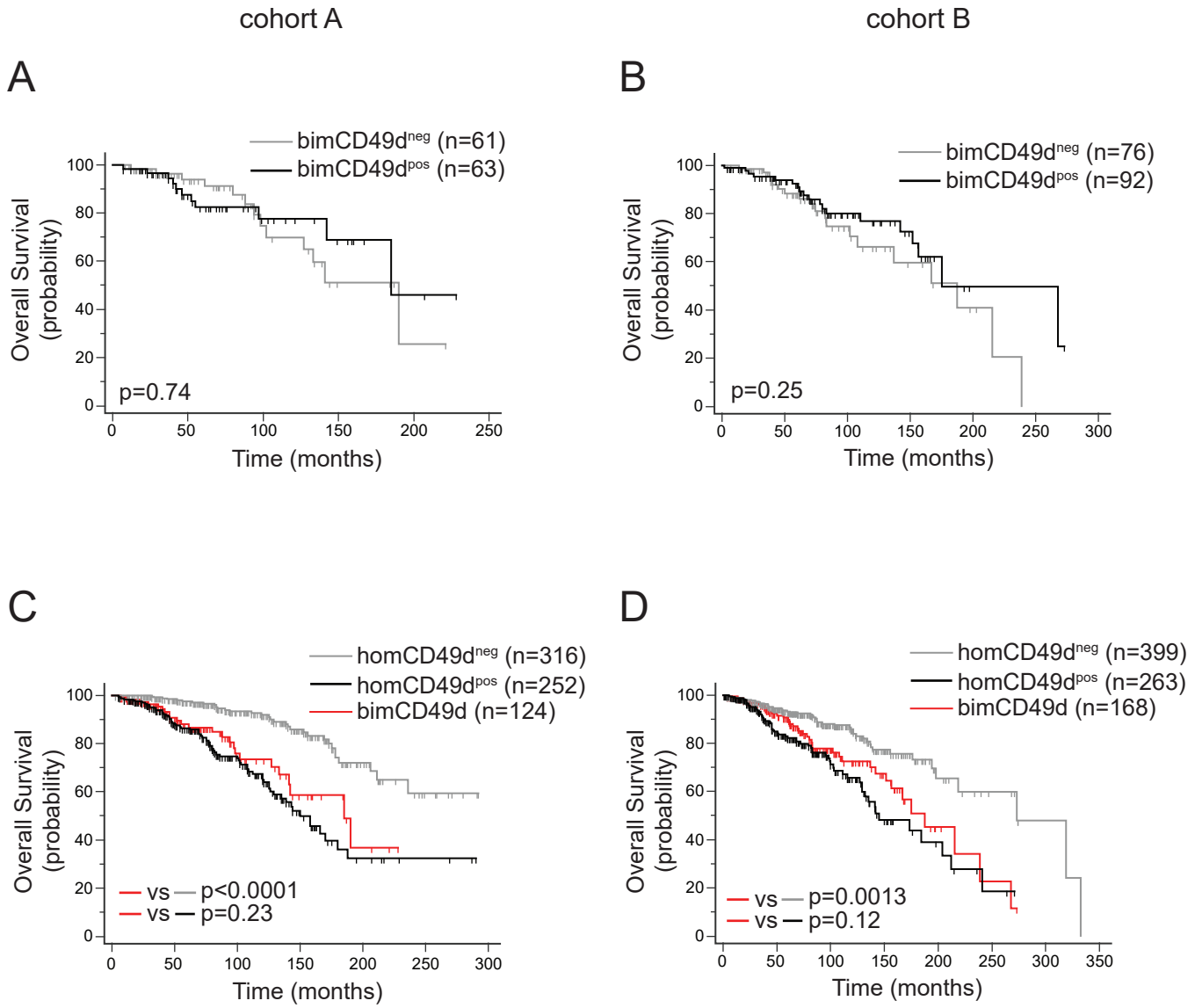
Supplemental Figure 5. Flow cytometry analysis of proliferation rates in CD49d^{neg} and CD49d^{pos} sorted fractions from bimodal CD49d CLL. Sorted CD49d^{neg} and CD49d^{pos} fractions from two CLL cases with bimodal CD49d expression were cultured for 48 hours with stromal cells and concomitant stimulation with the TLR9 agonist CpG. For cell sorting, CD49d^{neg} (blue) and CD49d^{pos} (red) CLL population were gated on a CD19 versus CD49d dot plot (dot plots on the left). Dot plots in the middle show CD19 versus CD49d staining in the sorted fractions after 48 hours co-culture. Dot plots on the right show the proliferating cells in the sorted CD49d^{neg} and CD49d^{pos} fractions identified by positive staining with an anti-5-ethynyl-2'-deoxyuridine (EdU)-Pacific blue reagent on a EdU versus CD19 dot plot.

Supplemental Figure 6



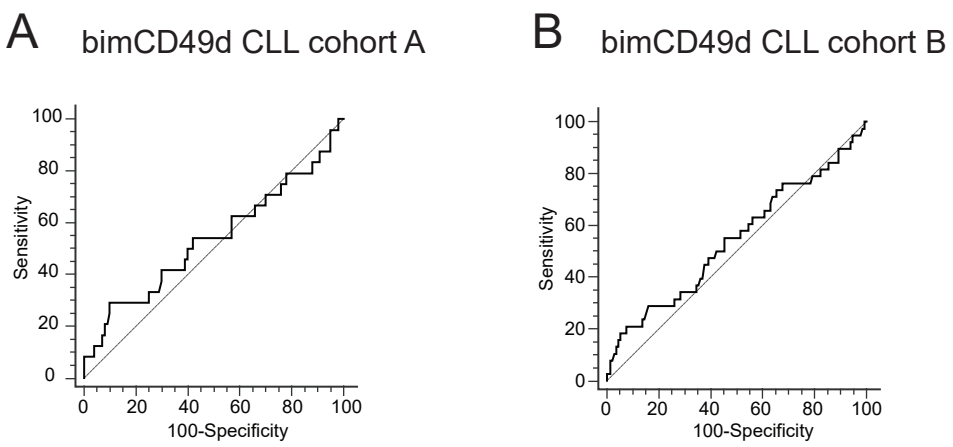
Supplemental Figure 6. Telomere length analysis in paired CD49d^{neg} and CD49d^{pos} CLL sub-populations. Paired analysis of telomere lengths (XpYp) of CD49d^{neg} and CD49d^{pos} sub-populations derived from 15 CLL patients were assessed by single telomere length analysis assay. The mean telomere length for each paired samples, analysed in triplicates, is reported below the gel image. Arrows indicate the two CLL samples (CLL#7 and CLL#8) displaying lower telomere length (>1kb difference) in CD49d^{pos} vs CD49d^{neg} sub-populations.

Supplemental Figure 7



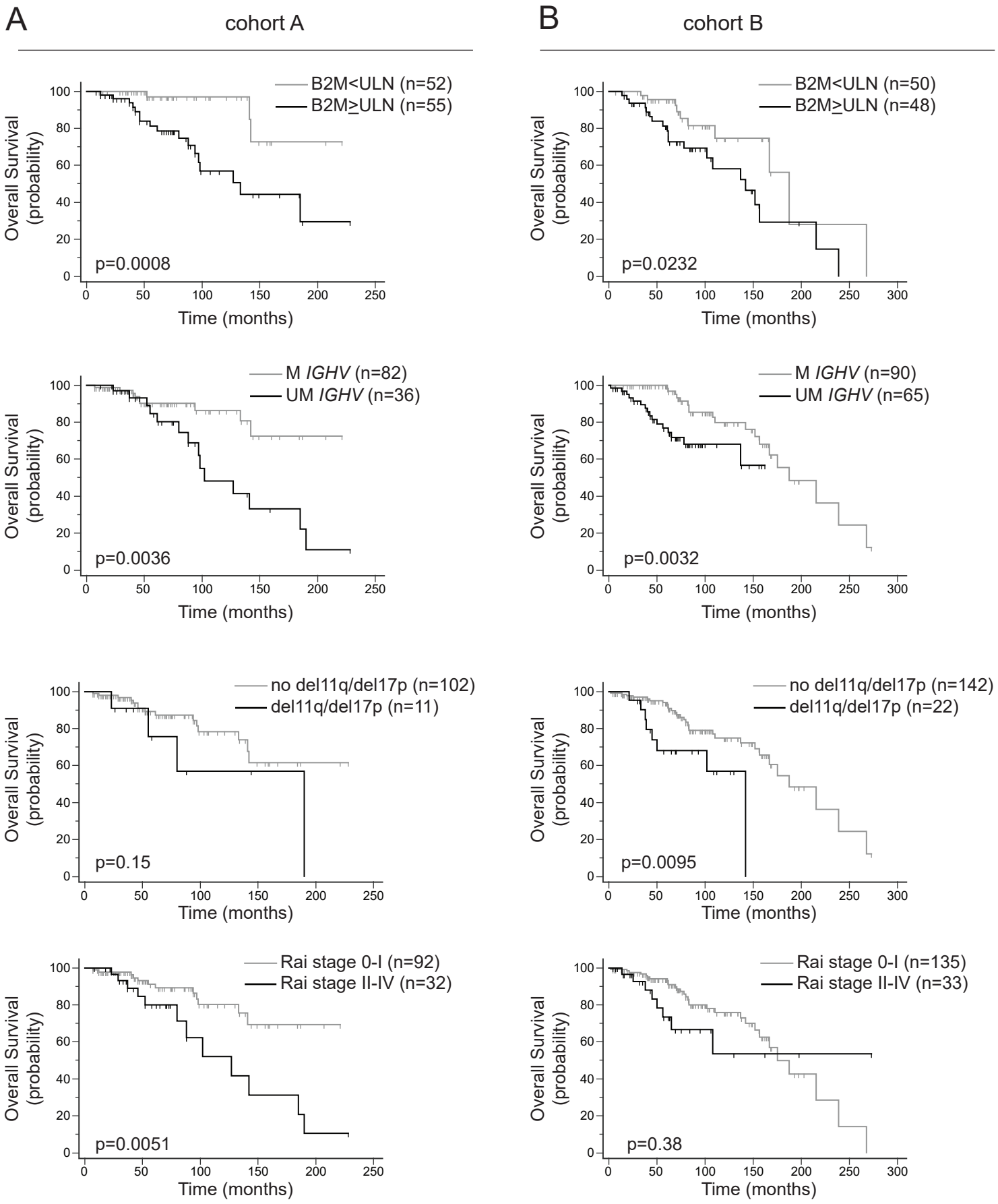
Supplemental Figure 7. Clinical impact of CD49d bimodal expression. (A-B) Overall survival Kaplan-Meier curves of bimCD49d CLL cases from cohort A (A) and cohort B (B) split in bimCD49d^{neg} (grey curves) and bimCD49d^{pos} (black curves) groups according to the 30% cut-off; (C-D) Overall survival Kaplan-Meier curves of CLL cases with bimodal CD49d expression (bimCD49d, red curves), homogeneous negative (homCD49d^{neg}, grey curves) and homogeneous positive (homCD49d^{pos}, black curves) CD49d expression in cohort A (C) and cohort B (D); p values refer to the log-rank test.

Supplemental Figure 8



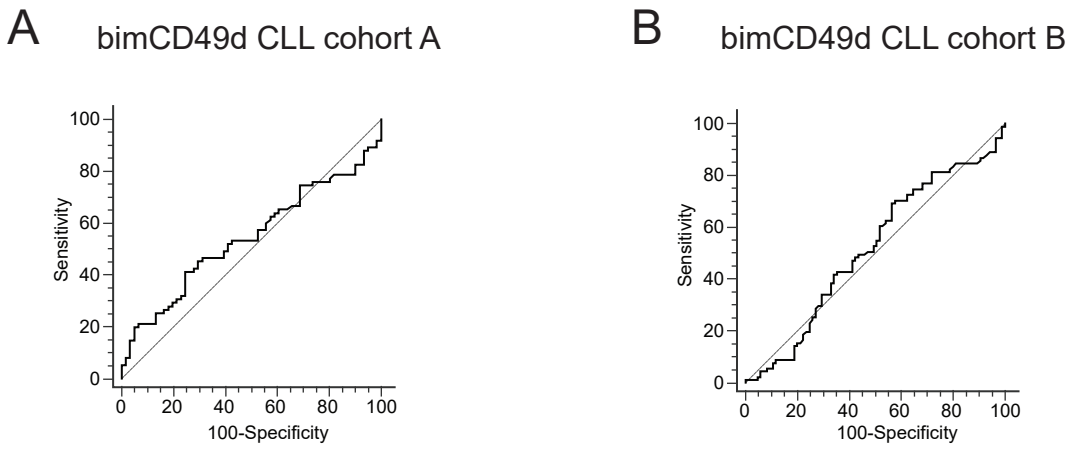
Supplemental Figure 8. Receiver Operating Characteristics' (ROC) curve analysis in CLL with CD49d bimodal expression using the overall survival readout. ROC curve analysis was performed with bimCD49d CLL from cohort A (A) and cohort B (B) to find possible cut-off values able to separate CLL in two groups with different overall survival probabilities.

Supplemental Figure 9



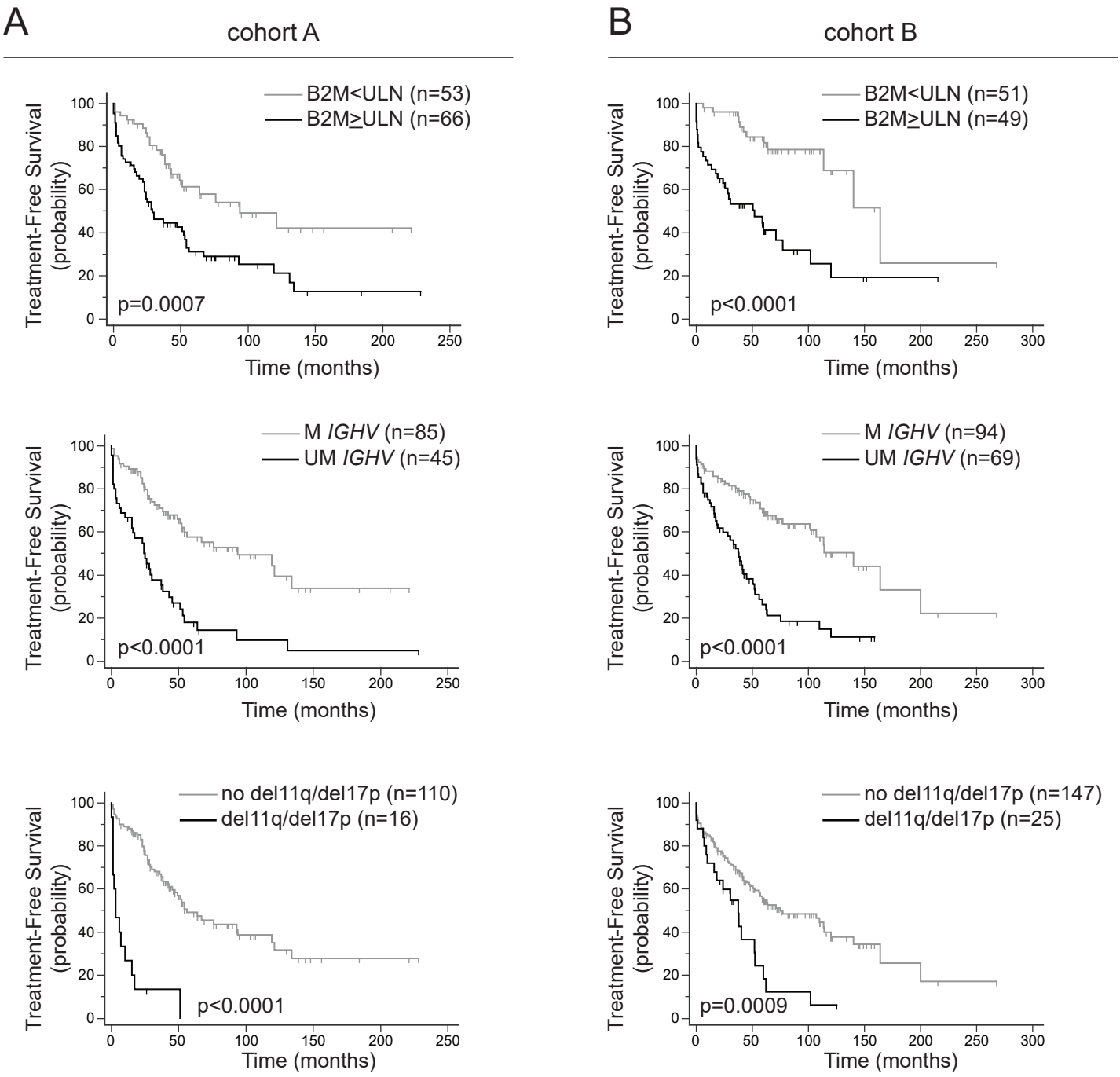
Supplemental Figure 9. Clinical impact as OS predictors of the main clinical and biological parameters in CLL from cohort A and cohort B. Overall survival Kaplan-Meier curves of bimCD49d CLL from cohort A (A) and cohort B (B) split in two groups according to: beta-2 microglobulin (B2M) levels (ULN=upper limit of normal), *IGHV* mutational status, presence of either del11q or del17p by FISH, Rai stage; p values refer to the log-rank test.

Supplemental Figure 10



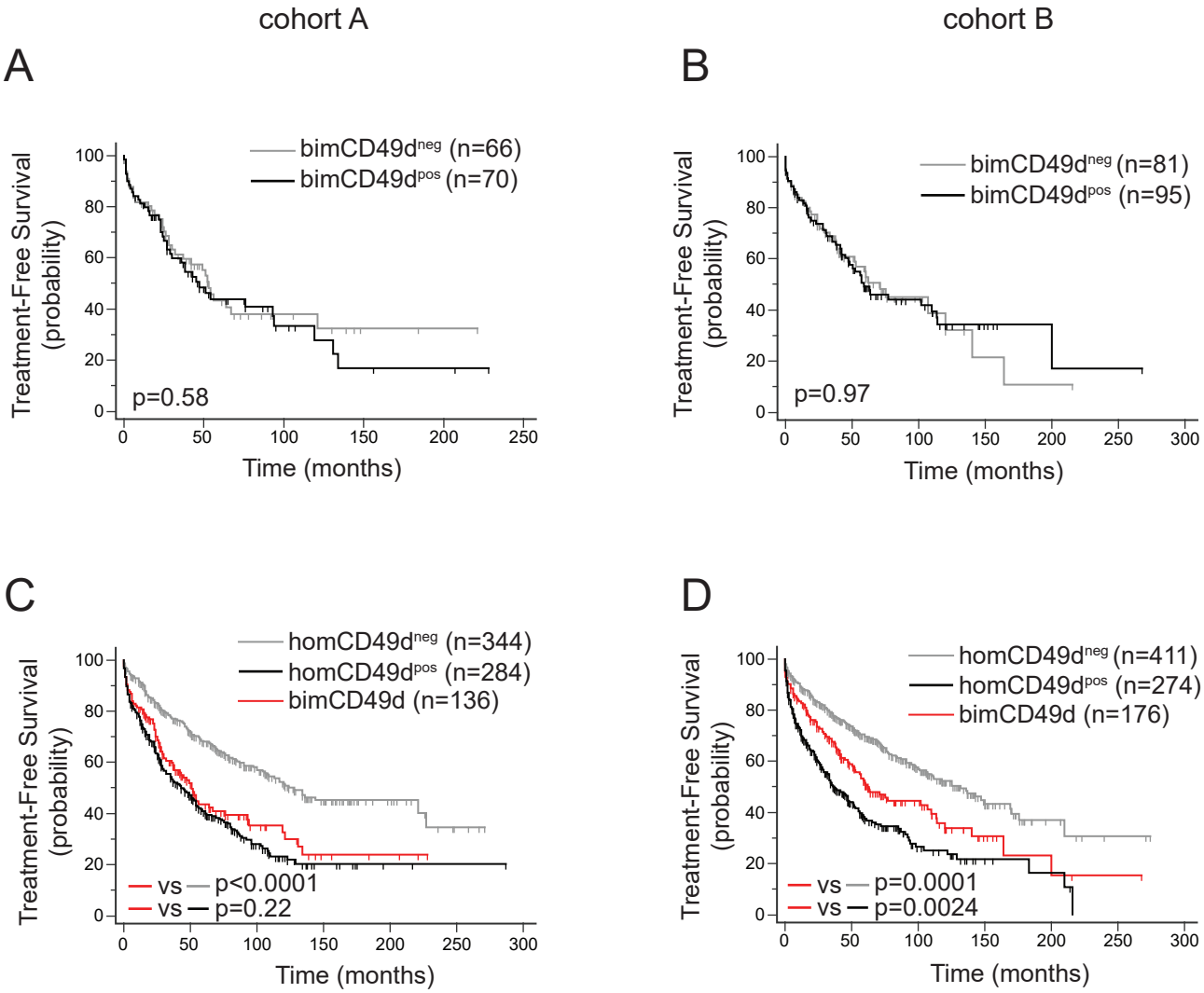
Supplemental Figure 10. Receiver Operating Characteristics' (ROC) curve analysis in CLL with CD49d bimodal expression using the treatment-free survival readout. ROC curve analysis was performed with bimCD49d CLL from cohort A (A) and cohort B (B) to find possible cut-off values able to separate CLL in two groups with different treatment-free survival probabilities.

Supplemental Figure 11



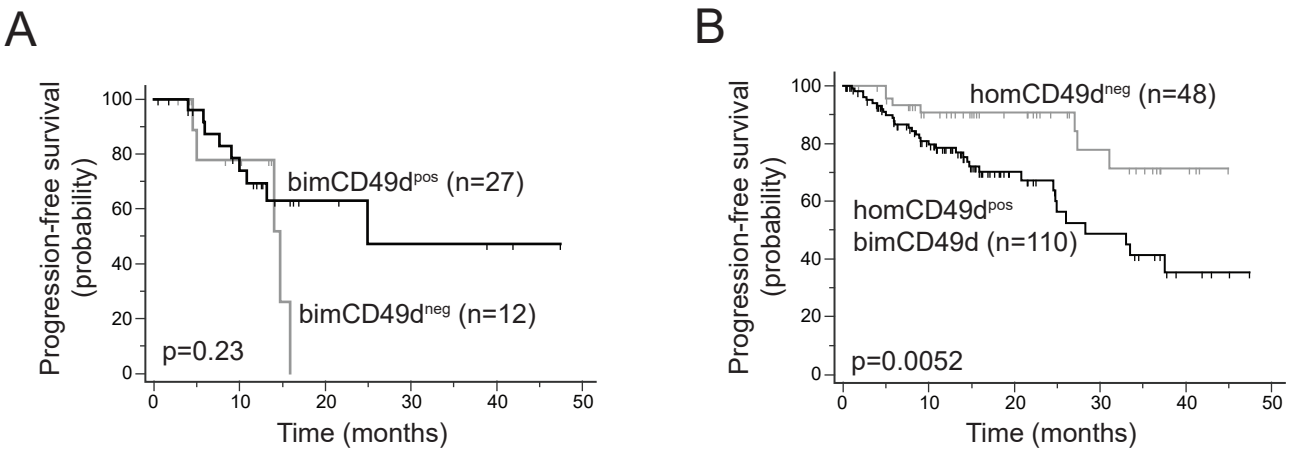
Supplemental Figure 11. Clinical impact as TFS predictors of the main clinical and biological parameters in CLL from cohort A and cohort B. Treatment-free survival Kaplan-Meier curves of bimCD49d CLL from cohort A (A) and cohort B (B) split in two groups according to: beta-2 microglobulin (B2M) levels (ULN=upper limit of normal), *IGHV* mutational status, presence of either del11q or del17p by FISH; p values refer to the log-rank test.

Supplemental Figure 12



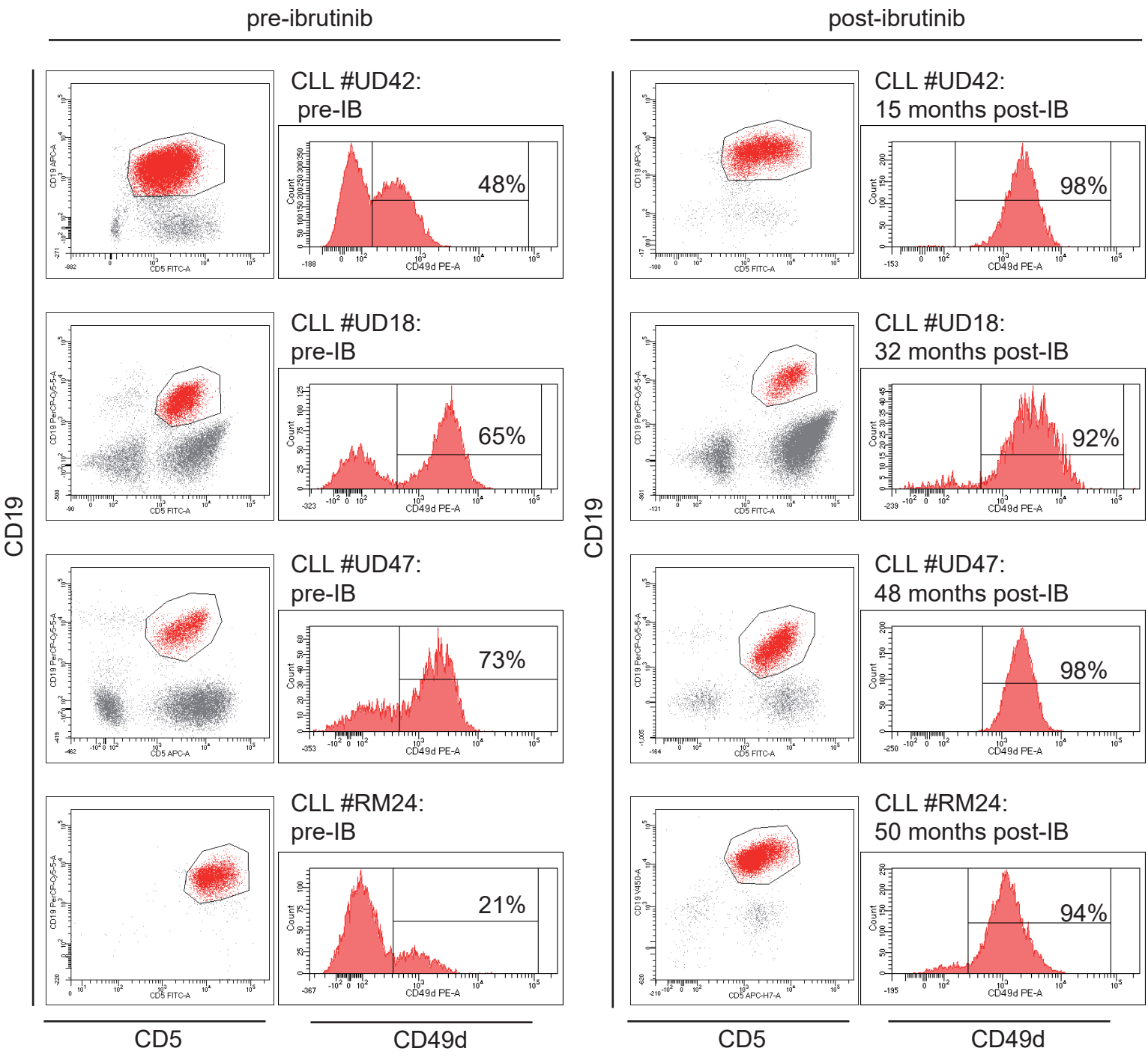
Supplemental Figure 12. Clinical impact of CD49d expression in bimCD49d in terms of treatment-free survival. (A-B) Treatment-free survival Kaplan-Meier curves of bimCD49d CLL cases from cohort A (A) and cohort B (B) split in bimCD49d^{neg} (grey curves) and bimCD49d^{pos} (black curves) groups according to the 30% cutoff; (C-D) Treatment-free survival Kaplan-Meier curves of CLL cases with bimodal CD49d expression (bimCD49d, red curves), homogeneous negative (homCD49d^{neg}, grey curves) and homogeneous positive (homCD49d^{pos}, black curves) CD49d expression in cohort A (C) and cohort B (D); p values refer to the log-rank test.

Supplemental Figure 13



S**upplemental Figure 13. Clinical impact of CD49d expression in the context of ibrutinib treatment.** (A) Progression-free survival Kaplan-Meier curves of ibrutinib-treated CLL cases with CD49d bimodal expression split in CD49d^{neg} (grey curve) and CD49d^{pos} (black curve) groups according to the 30% cut-off; (B) Progression-free survival Kaplan-Meier curves of ibrutinib-treated CLL cases with homogeneous negative CD49d expression (homCD49d^{neg}, grey curve) and the merging of homogeneous positive (homCD49d^{pos}) and bimodal CD49d (bimCD49d) expression (black curve).

Supplemental Figure 14



Supplemental Figure 14. CD49d expression at pre-treatment and after ibrutinib therapy. CD49d expression was analysed in the context of the CLL population defined by CD5+CD19+ expression. All samples were analysed at pre-treatment (pre-IB) and after the reported months of ibrutinib treatement (post-IB).