SUPPLEMENTARY APPENDIX

CD49d is the strongest flow cytometry-based predictor of overall survival in chronic lymphocytic leukemia

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SUPPLEMENTARY APPENDIX CONTENT

Supplementary Figure Legends

Fig. S1. Scatter plot of CD49d expression in paired samples collected at two different time-points; grey squares, first sample at diagnosis, second samples before therapy (99 cases); black squares, first sample at diagnosis, second samples at relapse (5 cases).

Fig. S2. Funnel plot showing the relationship between the hazard ratio for overall survival of all studies (x-axis) and the precision of the study estimate (standard error, y-axis). Filled circles: published series; open triangles: unpublished series.

Fig. S3. OS (A) and TFS (B) Kaplan-Meier plot of CLL prognostic categories defined by expression of CD38, ZAP-70, *IGHV* mutational status, presence of high-risk chromosomal aberrations (Del17p or Del11q). IG M, mutated *IGHV*; IG UM, unmutated *IGHV*; FISH-, Del17p negative and Del11q negative; FISH+, Del17p positive or Del11q positive.

Fig. S4. OS Kaplan-Meier plot of patients with complete data (n=1,117) vs patients with at least one prognostic variable missing (n=1,855) (A); distribution of main clinico-biological prognostic variables between complete and missing data patient's subsets (B); p value<0.01 only for Del17p.

Fig. S5. Meta-analysis and funnel plot of ZAP-70 (A, B) and CD38 (C, D) as prognostic factors for overall survival. Solid boxes indicate the HR in each study with dimensions proportional to weights (inverse of variance), horizontal lines indicate 95% confidence interval, the diamond indicates the pooled HR. Filled circles: published series; open triangles: unpublished series.

Fig. S6. Tree model for flow-cytometry measured prognostic variables in early stage patients (A), patients below 65 years of age (B), patients belonging to the validation cohort only (C).

Supplementary Methods

Determination of the optimal CD49d prognostic cut-off and CD49d recoding

Data on CD49d were available both as a binary variable coded in each study according to the specific cut-off employed by the authors, and as a continuous variable, as percentage of CD49d positive CLL cells. To compare different CD49d cut-offs and to determine the optimal cut-point, we divided the IPD in two cohorts of published and unpublished data, to be used as test and validation set, respectively. In the training set, we first examined the functional form of the relation between CD49d and OS in martingale residual plot (1-3), which shows the excess mortality (y axis) against the whole range of CD49d percentage values (x axis), with a superimposed line of locally weighted scatterplot smoothing (lowess) fit. Second, to identify an optimal cut-off, we applied a data-driven method (i.e. median) and three outcome-driven methods in each study set individually and in the merged data set. The three outcome-driven methods used were (Maxstat) (4), recursive partitioning (Rpart) (5, 6) and maximal concordance index (C-index) (7) . The validation set was then used to test and compare the predictive accuracy of each candidate cut-off by evaluating the C-index (2) . Finally, data from published and unpublished cohorts were pooled after recoding all CD49d values with the validated cut-off. To assess the relative contribution of prognostic variables to the final multivariate Cox model we evaluated the change in the degree of correct classification by Net Reclassification Improvement (8) in models excluding each variable in turn.

Evaluation of heterogeneity and study quality in pooled analysis

Study heterogeneity was evaluated by Q test and I^2 statistic, considering a Q test p value <0.05 and an I^2 value >50% as indicative of substantial heterogeneity. Study quality was scored by the availability of the following information (9, 10): 1) inclusion criteria; 2) exclusion criteria; 3) prospective or retrospective study; 4) description of patients characteristics; 5) description of CD49d assay; 6) definition of end-point; 7) indication of follow-up time; 8) patients lost at follow-up. The highest score (8) identified the highest study quality. Risk of publication bias was assessed by inspection of funnel plots. Since the number of individual studies was relatively small, we did not test asymmetry on funnel plots (11, 12).

Recursive partitioning

Recursive partitioning (5, 6) was used to study the relative prognostic importance of CD38, ZAP-70 and CD49d. We initially allowed a full-grown tree by setting the complexity parameter at 0.00. Then, we pruned the tree using ten-fold cross-validation to determine the best tree size. The best number of splits was identified as that showing a cross-validation error lower than the smallest cross validation error plus the corresponding standard error (5). Tree instability was investigated with a bootstrapping approach as implemented in R in the package randomSurvivalForest (13). One thousand bootstrap samples were used to rank the importance of each variable by computing the prediction error increase associated to that variable and the minimal depth (how deep in the tree the split based on that variable occurs).

Supplementary Results

CD49d flow cytometry analysis

Three different anti-CD49d monoclonal antibody (mAb) clones from four different companies were used, either conjugated with phycoerythrin (PE) or fluorescein-isothiocyanate (FITC). Three-color and 2-color analyses were performed in five and three studies, respectively (Table S3). The use of different CD49d mAb clones and fluorochromes had no impact in the flow cytometry assay of CD49d. In particular, the fraction of CD49d positive cases was 37% for assays performed with the mAb clone 9F10 versus 36% with other mAb clones (χ^2: p=0.78), and 37% for assays performed with PE mAb vs. 35% with FITC (χ^2: p=0.57). Finally, the usage of specific mAb clones and fluorochromes had no effect in the prognostic power of CD49d (interaction p=0.81 and 0.15, respectively).

Prognostic impact of CD49d in validation cohort

In the validation cohort, we found nearly identical estimates to those of the pooled data. In detail, 5-year OS was 95% in $CD49d^-$ CLL versus 88% in $CD49d^+$ CLL,10-year OS was 85% in $CD49d^-$ CLL versus 70% in $CD49d^+$ CLL; 5-year TFS was 69% in $CD49d^-$ CLL versus 43% in $CD49d^+$ CLL, 10-year TFS was 53% in $CD49d^-$ CLL versus 25% in $CD49d^+$ CLL.

Supplementary References

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Table S1. PRISMA checklist; page 1 of 2

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	4
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	4
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	4, fig 1
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	4, fig 1
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	table 1
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5,6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5,6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	5,6

Table S1. PRISMA checklist; pag 2 of 2

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	5,6, fig S1
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	5,6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	4
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	fig 2, fig 3
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	8, fig 2, fig S5
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	8
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	9,10,11, fig4, fig S6
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	12,13
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	12,13
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	12,13
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	none

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

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Table S2. Individual study results

	CD49d predicts short OS	CD49d predicts short TFS	CD49d^{+} cases %	CD49d cut-off %	cut-off † method
published cohort					
Gattei et al. 2008	yes	yes	43	30	Maxstat
Shanafelt et al. 2008	yes	yes	23	45	Rpart
Rossi et al. 2008	nd	yes	39	30	ref #10
Nuckel et al. 2009	yes	yes	63	45	ROC
Kurtova et al. 2009	nd	nd	31	30	ref #10
Cro et al. 2010	nd	nd	29	30	ref #10
Shanafelt et al. 2011	nd	nd	33	45	ref #11
Majid et al. 2011	yes	yes	35	30	ref #10

tabulated values estimated on IPD included in metanalysis.
† CD49d cut off was determined by maximally selected rank statistics (Maxstat), recursive partitioning (Rpart), receiver operating characteristic (ROC) or chosen according to the indicated reference.
nd: not determined

Table S3. CD49d flow cytometry analysis and CD49d cut off determination according to different statistical procedures.

	Cytometry	Monoclonal Antibody			CD49d cut-off (%)			
					Statistical procedure[$]			
		Clone	Source	Format	Median	Maxstat	Rpart	C-index
Gattei et.al 2008	3-col	9F10	BD	PE	20	7	31	32
Shanafelt et al. 2008	2-col	9F10	BD	PE	6	43	45	48
Rossi et al. 2008	3-col	9F10	BD	PE	15	8	8	9
Nuckel et al. 2009	2-col	44H6	ACRIS	FITC	11	89	6	8
Kurtova et al. 2009	3-col	9F10	BD	PE	3	33	38	6
Cro et al. 2010	3-col	HP 2/1	BC	FITC	0	10	10	11
Shanafelt et al. 2011	2-col	9F10	BD	PE	12	85	85	86
Majid et al. 2011	3-col	44H6	SEROTEC	PE	9	22	23	23
merged data					10	31	31	23

[$] Maxstat, Rpart, C-index were applied on OS data in the cohorts related to each published study.

na: not available; PE, phycoerithrin; FITC, fluorescein; BD, Becton Dickinson; BC, Beckman Coulter; 3-col, 3 color; 2-col, 2 color

Table S4. Validation of CD49d cut-off

	Model-1: cut-off >=30%			Model-2: cut-off >=45%			Patients changing CD49d status[#]
	HR[$]	p	CD49d+ cases %	HR[$]	p	CD49d+ cases %	%
Shanafelt	2.38	0.00001	35	2.62	<0.00001	31	4
Pepper	2.87	0.01447	30	3.27	0.00921	21	9
Del Poeta	3.68	0.03075	43	2.80	0.07941	40	3
Rossi	3.84	0.00445	35	3.09	0.01569	34	1
merged data*	2.50	<0.00001	35	2.49	<0.00001	32	3

Analysis performed on the set of 1416 unpublished IPD.

[#] Patients changing CD49d status (from positive to negative) when using 30% versus 45% cut-off

[$] CD49d HR for shorter OS.

* Cox models stratified by study site. C-index (≥ 30% cut-off) = 0.61; C-index (≥45% cut-off) = 0.59 (p<0.0001)

Table S5. Multivariate Cox regression analysis of OS without CD49d

	Final reduced model			Initial full model			Univariate model		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Age >65years	2.97	2.01 - 4.39	5×10^{-8}	2.77	1.85 – 4.15	7×10^{-7}	2.75	1.87 - 4.02	2×10^{-7}
UM *IGHV*	1.95	1.28 - 2.99	0.0020	1.93	1.25 – 2.97	0.0029	2.64	1.81 - 3.85	5×10^{-7}
Del 17p	2.67	1.46 - 3.52	0.0002	2.24	1.42 – 3.54	0.0005	2.90	1.87 - 4.49	2×10^{-6}
Gender (M)	1.70	1.15 - 2.49	0.0071	1.67	1.13 – 2.45	0.0093	1.53	1.06 - 2.22	0.0241
ALC>15×10^9/L	1.55	1.04 - 2.31	0.0319	1.48	0.99 – 2.22	0.06	1.55	1.05 - 2.30	0.0290
ZAP-70	1.60	1.06 - 2.41	0.0240	1.55	1.03 – 2.36	0.0376	2.27	1.57 - 3.27	1×10^{-5}
β2M above ULN	-	-	-	1.34	0.85 – 2.10	0.21	2.23	1.47 - 3.40	0.0002
CD38	1.56	1.08 - 2.26	0.0176	1.53	1.06 – 2.22	0.0238	1.98	1.38 - 2.82	0.0002
Del 11q	-	-	-	1.10	0.64 – 1.88	0.73	1.35	0.82 - 2.23	0.24

Final model: multivariate analysis with backward stepwise elimination of non significant predictors. All models were stratified by study site and clinical stage. Total cases included: 1,117, events 137.
HR, hazard ratio; CI, confidence interval; UM, unmutated; M, male; ALC, absolute lymphocyte count; β2M, β2-microglobulin; ULN, upper limit of normal

Table S6. Matrix of pairwise correlations

		CD49d		CD38		ZAP-70		*IGHV*	
		neg	pos	neg	pos	neg	pos	M	UM
CD49d	neg			k=0.43 $p=3x10^{-10}$		k=0.43 p=0.018		k=0.22 p=0.12	
	pos								
CD38	neg	1316 (53)	398 (16)			k=0.32 $p=3x10^{-16}$		k=0.34 $p=3x10^{-10}$	
	pos	238 (10)	522 (21)						
ZAP-70	neg	1089 (44)	395 (16)	1209 (49)	275 (11)			k=0.42 p=0.32	
	pos	465 (19)	525 (21)	505 (20)	485 (20)				
IGHV	M	889 (43)	359 (17)	1011 (49)	237 (11)	948 (46)	300 (15)		
	U M	403 (19)	417 (20)	396 (19)	424 (21)	275 (13)	545 (26)		

Number (percentage) of cases are tabulated in the lower-left quadrants of the table; the relative statistics (k, Cohen kappa coefficient; p, McNemar test) are indicated in the symmetric correspondent upper-right quadrants.
M, mutated; UM, unmutated; pos, positive; neg, negative

Table S7. Prognostic importance of clinico-biological variables in the multivariate model.

Variable deleted	LL	LR	p	C-index	p	NRI (%)	p
none (final model)	450.7	-	-	0.76	-	-	-
Age >65years	469.0	36.6	$1x10^{-9}$	0.71	$2x10^{-8}$	-35	0.000
UM *IGHV*	461.5	21.6	$3x10^{-6}$	0.73	$2x10^{-5}$	-29	0.000
CD49d≥30%	460.5	19.7	$9x10^{-6}$	0.75	$7x10^{-5}$	-23	0.000
Del17p	455.8	10.1	0.0015	0.74	0.21	-15	0.21
Gender (M)	455.6	9.8	0.0017	0.75	$7x10^{-5}$	-10	0.09
ALC>$15x10^9$/L	453.8	6.2	0.013	0.75	$1x10^{-5}$	-12	0.02

All models were stratified by study site and clinical stage. Total cases included: 1,117, events 137.
Final model included: Age >65years, *IGHV,* CD49d≥30%, Del17p, Gender, ALC>$15x10^9$/L
LL, log-likelihood; LR, log-likelihood ratio, NRI: Net Reclassification Improvement; UM, unmutated; M, male; ALC, absolute lymphocyte count; β2M, β2-microglobulin; ULN, upper limit of normal

Table S8. Multivariate Cox regression analysis of OS. Cases selected after exclusion of ALC and β2M

	Final reduced model			Initial full model			Univariate		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
CD49d included									
Age >65years	2.62	1.98-3.47	$1x10^{-11}$	2.67	2.02-3.54	$8x10^{-12}$	2.61	1.98-3.44	$9x10^{-12}$
UM *IGHV*	1.66	1.24-2.23	0.0007	1.54	1.13-2.09	0.0064	2.25	1.70-2.98	$1x10^{-8}$
CD49d≥30%	2.12	1.57-2.86	$2x10^{-6}$	2.03	1.50-2.75	$4x10^{-6}$	2.58	1.97-3.39	$7x10^{-16}$
Del 17p	2.37	1.62-3.47	$9x10^{-6}$	2.32	1.58-3.40	$1x10^{-5}$	2.66	1.82-3.88	$4x10^{-7}$
Gender (M)	-	-	-	1.30	0.97-1.74	0.08	1.31	0.99-1.74	0.058
ZAP-70	-	-	-	1.29	0.94-1.76	0.11	2.13	1.62-2.81	$8x10^{-8}$
CD38	1.44	1.07-1.95	0.0169	1.40	1.03-1.89	0.0293	2.20	1.69-2.88	$6x10^{-9}$
Del 11q	1.74	1.18-2.57	0.0054	1.66	1.12-2.46	$2x10^{-5}$	1.80	1.24-2.60	0.0018
CD49d excluded									
Age >65years	2.63	1.99-3.48	$1x10^{-11}$	2.67	2.02-3.54	$8x10^{-12}$			
UM *IGHV*	1.59	1.17-2.15	0.0027	1.52	1.12-2.06	0.0079			
Del 17p	2.16	1.49-3.13	$5x10^{-5}$	2.27	1.55-3.33	$2x10^{-5}$			
Gender (M)	-	-	-	1.31	0.98-1.76	0.07			
ZAP-70	1.47	1.09-1.99	0.0126	1.56	1.04-1.92	0.0281			
CD38	1.87	1.41-2.47	$1x10^{-5}$	1.83	1.38-2.42	$2x10^{-5}$			
Del 11q	-	-	-	1.43	0.97-2.11	0.07			

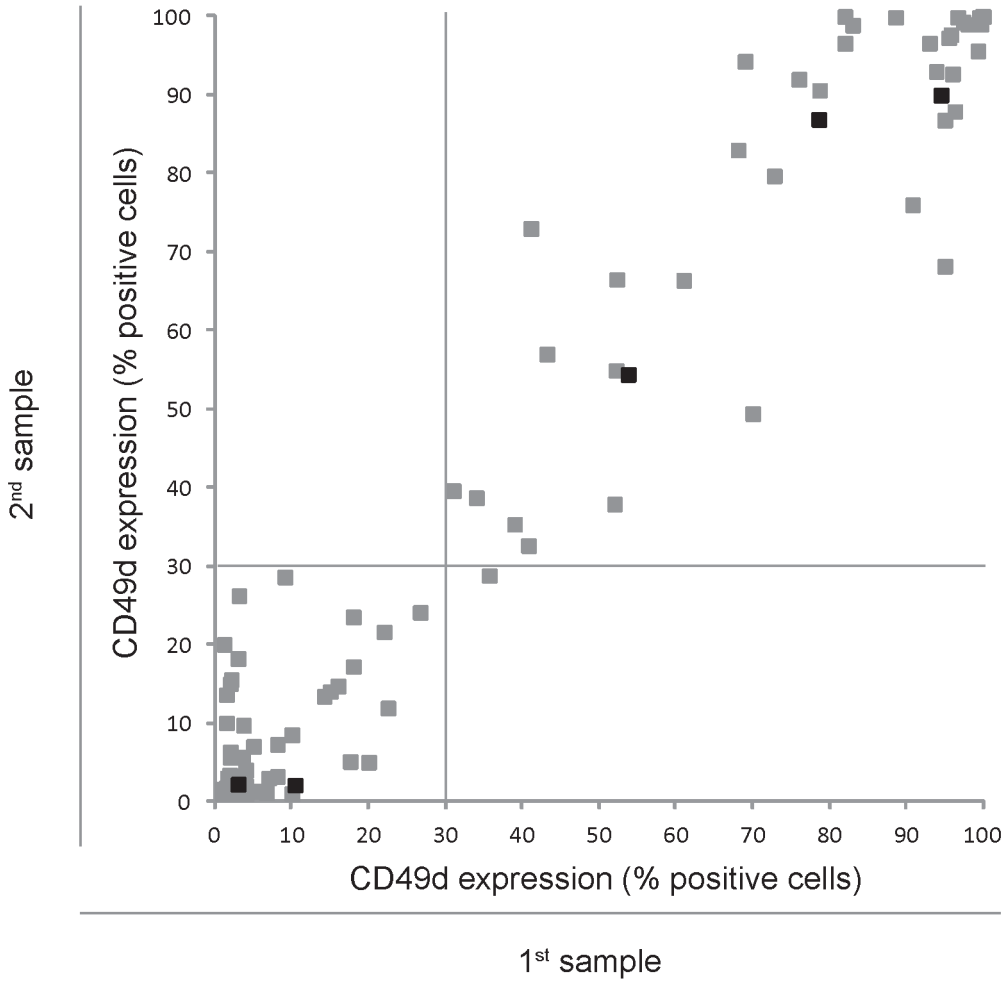
Final model: multivariate analysis with backward stepwise elimination of non significant predictors. All models were stratified by study site and clinical stage. Total cases included, 1,655; events, 246.
HR: hazard ratio; CI: confidence interval; UM, unmutated; M, male.
Final model with CD49d vs final model without CD49d: log-likelihood ratio chi-square test, $p<0.0001$; C-index: 0.731 vs 0.729, p=0.0004

Table S9. Multivariate Cox regression analysis of TFS

	final reduced model			initial full model			univariate model		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Age >65years	-	-	-	-	-	-	1.03	0.91 – 1.15	0.65
UM *IGHV*	1.73	1.40 - 2.14	$5x10^{-7}$	1.70	1.37 – 2.11	$1x10^{-6}$	2.65	2.33 - 3.02	$2x10^{-16}$
CD49d≥30%	1.68	1.38 - 2.04	$1x10^{-7}$	1.62	1.32 – 1.98	$3x10^{-6}$	1.89	1.68 - 2.12	$2x10^{-16}$
Del 17p	1.60	1.20 - 2.13	0.0012	1.62	1.22 – 2.16	0.0009	1.64	1.31 - 2.06	$1x10^{-5}$
Gender (M)	-	-	-	-	-	-	1.01	0.89 - 1.14	0.89
ALC>$15x10^9$/L	2.24	1.83 - 2.76	$1x10^{-14}$	2.24	1.82 – 2.75	$2x10^{-14}$	1.72	1.50 - 1.98	$1x10^{-14}$
ZAP-70	1.46	1.18 – 1.79	0.0004	1.43	1.16 – 1.76	0.0008	1.87	1.65 - 2.12	$2x10^{-16}$
β2M above ULN	1.65	1.32 – 2.08	$1x10^{-5}$	1.65	0.31 – 2.07	$2x10^{-5}$	2.37	1.99 - 2.82	$2x10^{-16}$
CD38	-	-	-	1.13	0.92 – 1.40	0.23	1.90	1.69 - 2.14	$2x10^{-16}$
Del 11q	1.48	1.14 – 1.94	0.0035	1.46	1.12 – 1.91	0.0055	2.02	1.68 - 2.41	$2x10^{-14}$

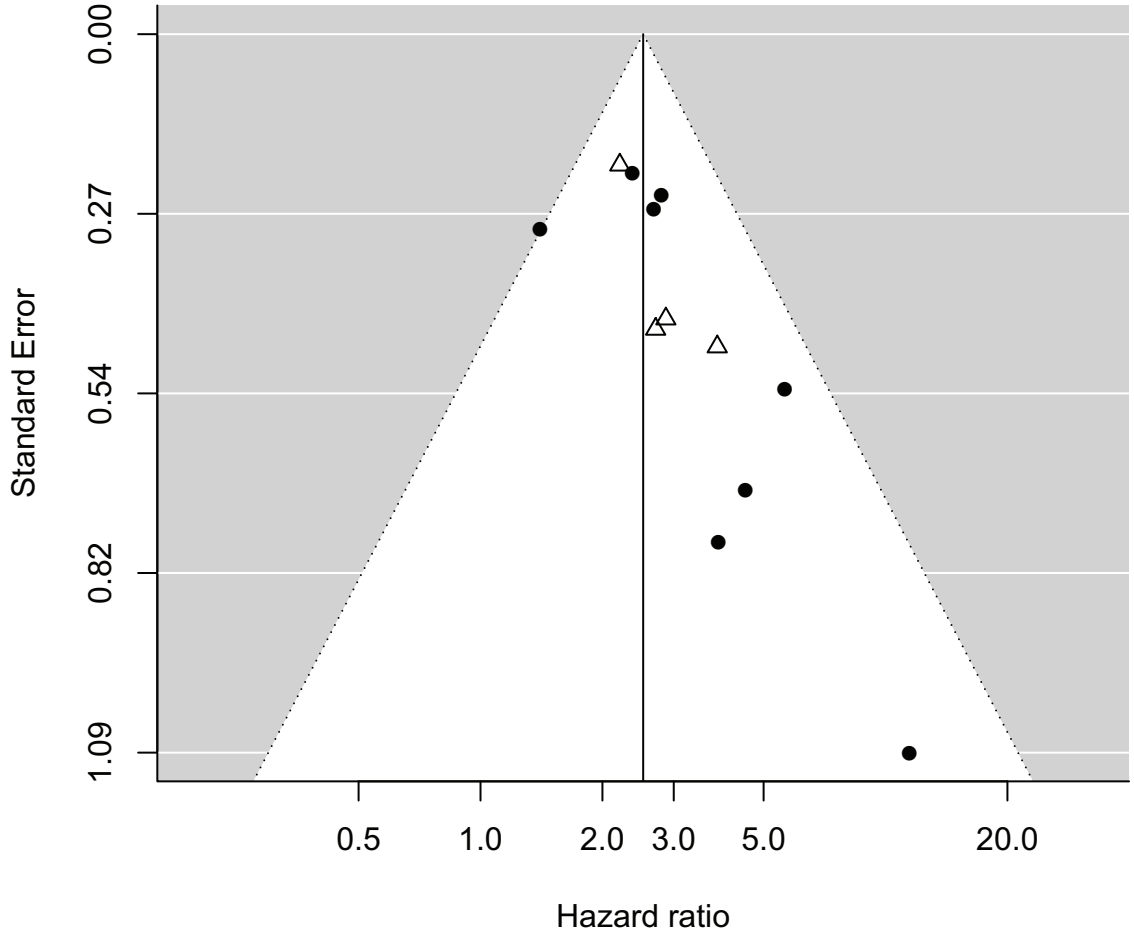
Final model: multivariate analysis with backward stepwise elimination of non significant predictors. All models were stratified by study site and clinical stage. Total cases included: 1,109, events 514.
HR, hazard ratio; CI, confidence interval; UM, unmutated; M, male; ALC, absolute lymphocyte count; β2M, β2-microglobulin; ULN, upper limit of normal

Figure S1



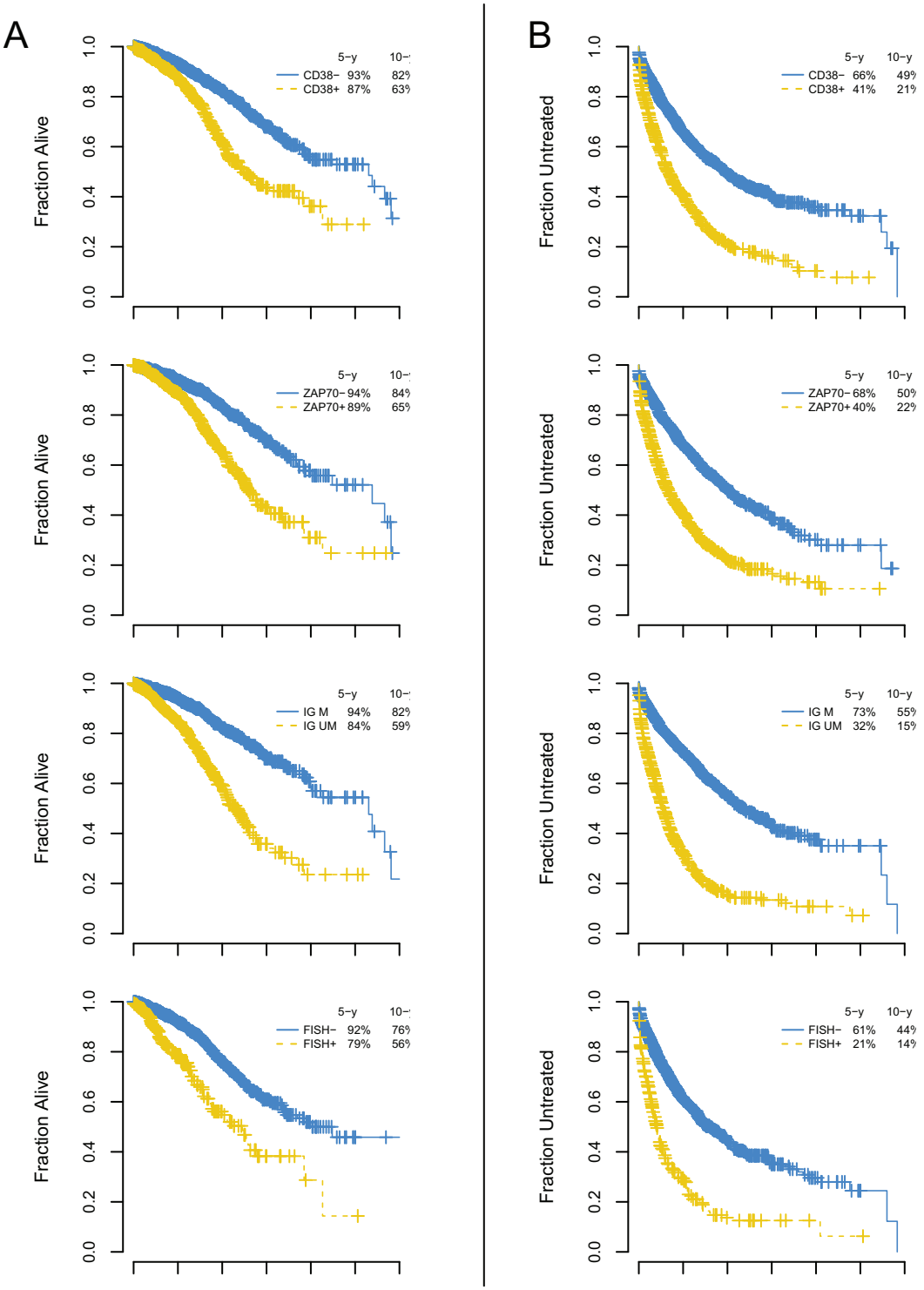
Scatter plot of CD49d expression in paired samples collected at two different time-points; grey squares, first sample at diagnosis, second samples before therapy (99 cases); black squares, first sample at diagnosis, second samples at relapse (5 cases).

Figure S2



Funnel plot showing the relationship between the hazard ratio for overall survival of all studies (x−axis) and the precision of the study estimate (standard error, y−axis). Filled circles: published series; open triangles: unpublished series.

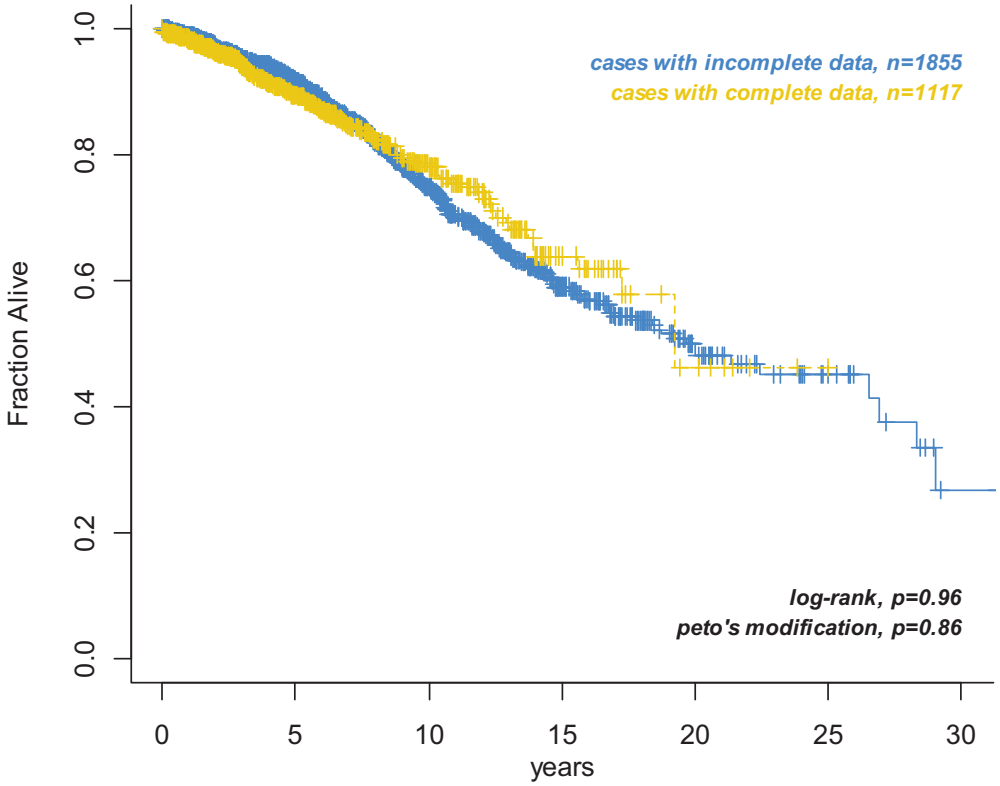
Figure S3



OS (A) and TFS (B) Kaplan−Meier plot of CLL prognostic categories defined by expression of CD38, ZAP−70, IGHV mutational status, presence of high−risk chromosomal aberrations (Del17 or Del11q). IG M, mutated IGHV; IG UM, unmutated IGHV; FISH−,Del17p negative and Del11q negative; FISH+, Del17p positive or Del11q positive.

Figure S4

A

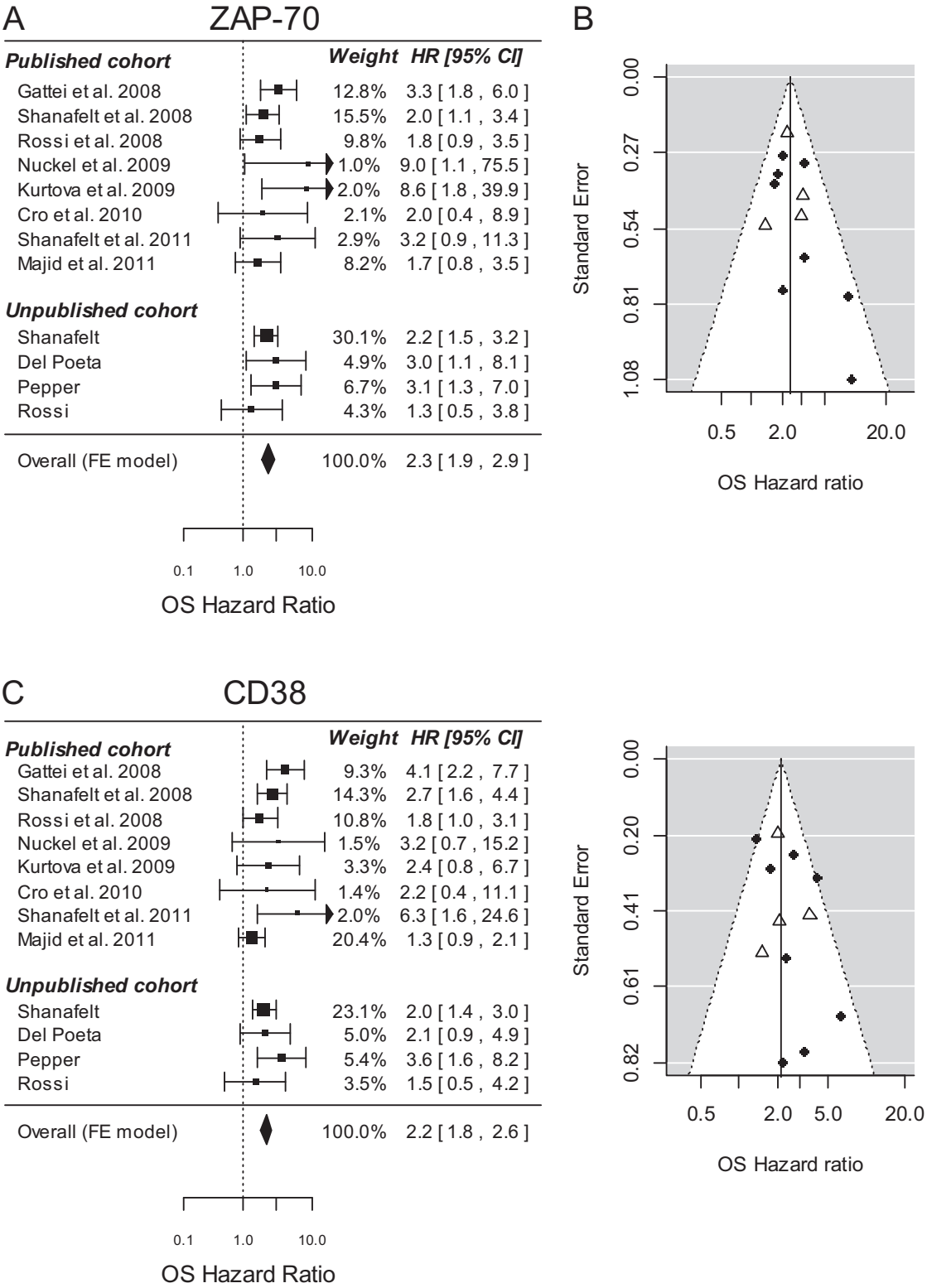


B

	complete series (n=1,117)	incomplete series (n=1,1855)	No. available
Age median, years	64	63	2965
Male gender, n (%)	678 (61)	1181 (64)	2972
Advanced stages, n (%)	81 (7)	128 (7)	2956
Del 11q, n (%)	115 (10)	108 (9)	2288
Del 17p, n (%)	99 (9)	51 (4)	2288
UM IGVH, n (%)	446 (40)	519 (39)	2444
ZAP70+, n (%)	433 (39)	571 (41)	2495
CD38+, n (%)	325 (29)	545 (31)	2866
CD49d+, n (%)	432 (39)	666 (36)	2972
5-year OS, %	89	92	2972
10-year OS, %	79	75	2972

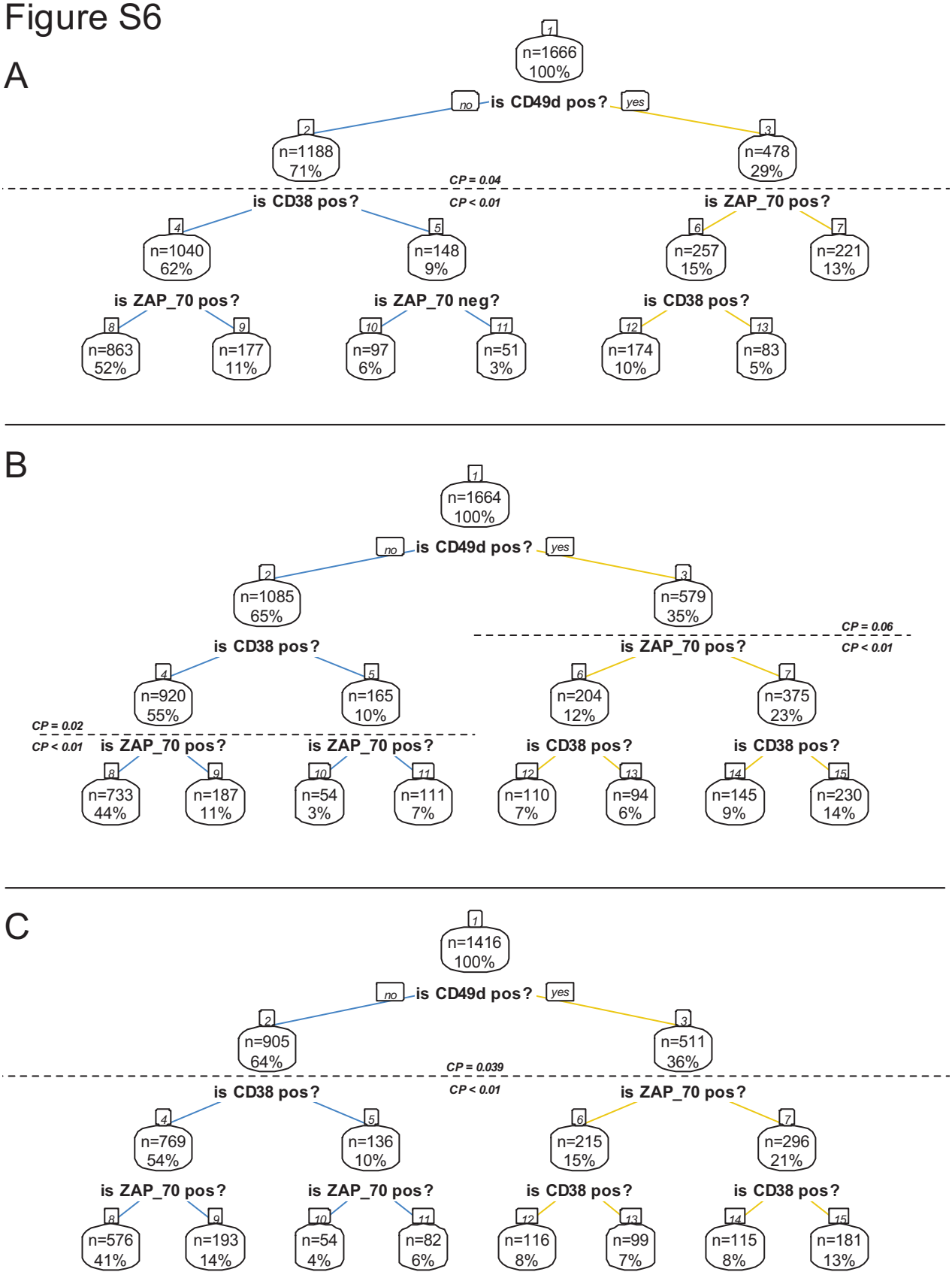
OS Kaplan-Meier plot of patients with complete data (n=1,117) vs patients with at least one prognostic variable missing (n=1,855) (A); distribution of main clinico-biological prognostic variables between complete and missing data patient's subsets (B); p value<0.01 only for Del17p.

Figure S5



Meta-analysis and funnel plot of ZAP-70 (A, B) and CD38 (C, D) as prognostic factors for overall survival. Solid boxes indicate the HR in each study with dimensions proportional to weights (inverse of variance), horizontal lines indicate 95% confidence interval, the diamond indicates the pooled HR. Filled circles: published series; open triangles: unpublished series

Figure S6



Tree model for flow-cytometry measured prognostic variables in early stage patients (A), patients below 65 years of age (B), patients belonging to the validation cohort only (C).