Proteomic markers with prognostic impact on outcome of chronic lymphocytic leukemia patients under chemo-immunotherapy: results from the HOVON 109 study

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BRIEF COMMUNICATION

Proteomic markers with prognostic impact on outcome of chronic lymphocytic leukemia patients under chemo-immunotherapy: results from the HOVON 109 study

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Despite recent identification of several prognostic markers, there is still a need for new prognostic parameters able to predict clinical outcome in chronic lymphocytic leukemia (CLL) patients. Here, we aimed to validate the prognostic ability of known (proteomic) markers measured pretreatment and to search for new proteomic markers that might be related to treatment response in CLL. To this end, baseline serum samples of 51 CLL patients treated with chemo-immunotherapy were analyzed for 360 proteomic markers, using Olink technology. Median event-free survival (EFS) was 23 months (range: 1.25–60.9). Patients with high levels of sCD23 (>11.27, $p = 0.026$), sCD27 (>11.03, $p = 0.04$), SPINT1 (>1.6, $p = 0.001$), and LY9 (>8.22, $p = 0.0003$) had a shorter EFS than those with marker levels below the median. The effect of sCD23 on EFS differed between immunoglobulin heavy chain variable gene-mutated and unmutated patients, with the shortest EFS for unmutated CLL patients with sCD23 levels above the median. Taken together, our results validate the prognostic impact of sCD23 and

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highlight SPINT1 and LY9 as possible promising markers for treatment response in CLL patients.

The natural history of chronic lymphocytic leukemia (CLL) is highly heterogeneous. Some patients do not require treatment for decades, while others require direct treatment after diagnosis and experience diminished life expectancy because of CLL [1]. Since the introduction of multiple novel treatments, there is a growing need for informative prognostic markers with clinical significance that can influence the choice of standard of care [1].

To date, several prognostic markers have been identified, including immunoglobulin heavy chain variable (IGHV) gene mutation status, ZAP70, chromosomal alterations, CD38, CD40L, and biochemical parameters (e.g., lactate dehydrogenase and β2-microglobulin [β2M]) [1]. Yet, the identification of new prognostic parameters that are able to predict clinical outcome after treatment is important for patient management and may be useful in guiding therapeutic decisions.

Proteomic data are increasingly being used for biomarker discovery and for gaining mechanistic insight into lymphoid malignancies. Among the general population, an immune system environment that is characterized by elevated levels of B-cell stimulatory cytokines has been suggested to contribute to the development of B-cell lymphoma, including CLL [2–5]. In a study including 105 newly diagnosed and untreated CLL patients, high levels of soluble CD23 (sCD23) at the time of initial diagnosis were a strong predictor of progressive disease within the first year of disease presentation [6]. Other studies obtained similar results implicating sCD23 as a suitable marker with prognostic potential in CLL at diagnosis [7–9].

The aims of this pilot study were (1) to validate the prognostic ability of known proteomic markers (in particular B-cell activation markers such as sCD23 and sCD27) measured pretreatment for treatment response in CLL patients, and (2) to search for new proteomic markers and their biological pathways that might be related to treatment response.

Methods

Study subjects were selected from the HOVON 109 clinical study, which is a phase I/II trial designed for efficacy and safety of first-line therapy involving chlorambucil, rituximab, and lenalidomide in elderly patients and young frail patients with advanced CLL [10]. Total treatment duration was 12 months, and all patients were followed until 5 years after registration. Of 63 patients enrolled in the HOVON 109 study, 51 with an available sample at baseline were included in our current pilot study. Clinical data were collected from the HOVON database.

Four commercially available proteomic panels (Oncology, Inflammation, Immune response, and Development; Olink Bioscience, Uppsala, Sweden) including 360 low-abundance serum proteins were selected for this study, covering multiple proteomic markers previously associated with incidence or progression of CLL in published clinical and population-based studies [2–9]. Serum samples were analyzed using a multiplex proximity extension assay. Data were expressed in the arbitrary unit NPX (Normalized Protein eXpression) on a $\log_2$ scale and linearized using the formula 2^{NPX}, where a high NPX value corresponds to a high protein concentration (Supplementary Table E1, online only, available at www.exphem.org).

Statistical procedures are provided in detail in the Supplementary Data (online only, available at www.exphem.org). As the analyses of B-cell activation markers that were found to be predictive in the general population (sCD23 and sCD27) rely on a prior hypothesis, these were not corrected for multiple testing. All other p values were corrected for multiple testing.

Results

Clinical characteristics of the CLL patients (30 males, 21 females; median age: 71 years) are summarized in Supplementary Table E2 (online only, available at www.exphem.org). Until 5 years after registration, 26 events were recorded. We first evaluated measured levels of the proteomic markers in the context of other known prognostic factors. Mutated IGHV status was significantly associated with lower levels of sCD23 and higher levels of nuclear factor of activated T cells 3 (NFATC3), while a positive association between β2M levels and 58 markers was established (Supplementary Table E3, online only, available at www.exphem.org). No marker remained significantly associated with Rai and cytogenetic aberrations after multiple testing correction.

In log-rank testing of known prognostic factors, male patients had shorter EFS as compared with female patients, and IGHV-mutated CLL patients had longer EFS than unmutated CLL patients (Supplementary Figure E1, online only, available at www.exphem.org). No significant EFS effects were seen for β2M levels, Rai stage, or well-defined chromosome aberrations. The latter may be due to the small number of cases with these aberrations in our pilot study.

From the previously studied B-cell activation proteomic markers (Supplementary Table E4, online only, available at www.exphem.org), only the sCD23 level was significantly associated with EFS (HR = 1.56, 95% CI = 1.02–2.4, $p = 0.04$) in the univariate model, while none appeared significantly correlated to EFS in the models adjusted for gender and IGHV status. As

sCD23 level was related to IGHV status, the interaction term was further included in the adjusted model that resulted in a borderline significant association for sCD23 (hazard ratio [HR] = 0.23, 95% confidence interval [CI] = 0.04–1.14, $p = 0.07$).

When using median values as threshold, patients with sCD23 and sCD27 levels above the median did have a shorter EFS than those with marker levels below the median (Figure 1A,B). Notably, when combining these markers with significant CLL prognostic factors in this cohort (i.e., IGHV mutation status and gender), sCD23 or sCD27 levels above the median were associated with the lowest EFS in unmutated IGHV patients (Figure 1C,D). In contrast, survival distributions for male and female patients with sCD23 or sCD27 levels above the median did not differ significantly (Figure 1E,F).

Several newly studied proteomic markers were significantly associated with EFS in Cox regression models, albeit that upon multiple testing correction, even the top four proteomic markers from the models exhibited only a trend toward significance (Supplementary Table E5, online only, available at www.exphem.org). Nevertheless, the two markers with p values closest to significance, that is, serine peptidase inhibitor SPINT1 and surface antigen LY9, were associated with a significantly longer EFS in patients with marker levels equal or lower than the median (Figure 2). Moreover, CLL patients with higher levels of IFNLR1 had a shorter EFS than those patients with marker levels equal to or lower than the median.

Findings were independently validated via Lasso regression (details are in the Supplementary Data).

The stepwise Cox regression model including gender and IGHV mutation status plus sCD23 and the top four proteomic markers from the univariate and/or multivariable (model M2, Table 1) revealed a significant independent effect for sCD23 (HR = 0.27, 95% CI = 0.12–0.58, $p = 0.0008$), SPINT1 (HR = 3.6, 95% CI = 1.12–11.5, $p = 0.03$), LY9 (HR = 7.03, 95% CI = 1.6–30.8, $p = 0.009$), and CLEC7A (HR = 0.43, 95% CI = 0.27–0.7, $p = 0.0007$) over gender and IGHV status. The AUC of the model was higher (0.61) compared with that of the model including only gender and IGHV status (0.38). Because of the limited sample size of our study resulting in the wide confidence intervals, these findings should be interpreted with caution and require validation in larger studies.

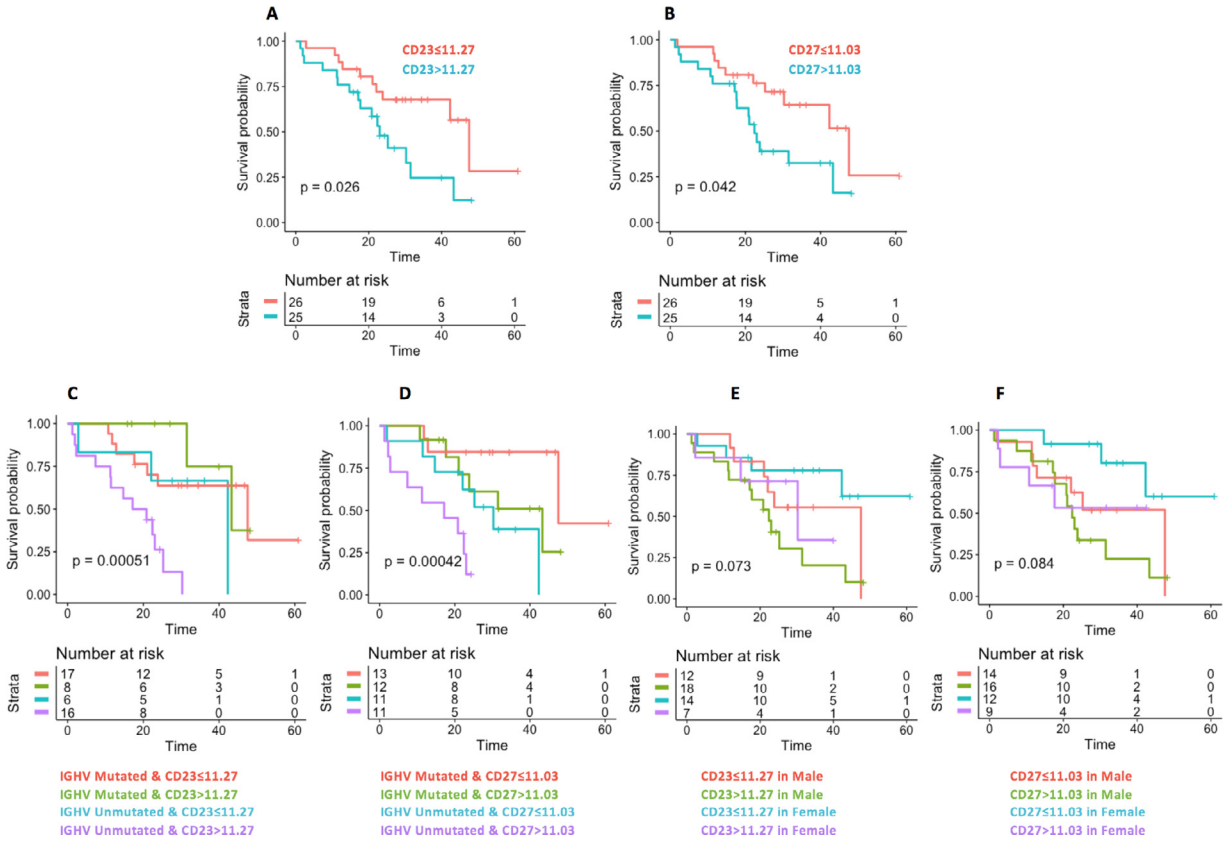

Figure 1. Kaplan–Meier curves for EFS related to the B-cell activation proteomic markers.

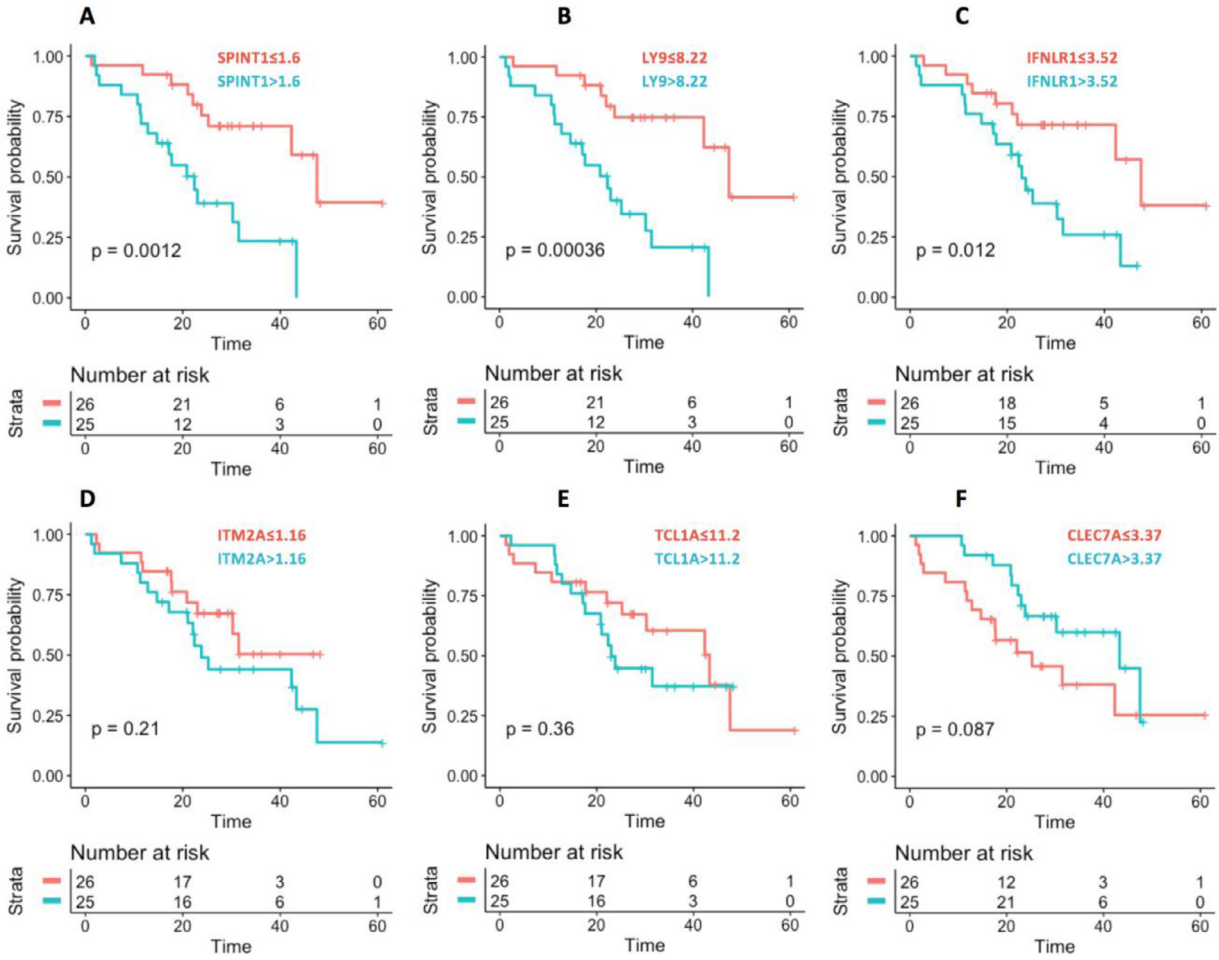

Figure 2. Kaplan–Meier curves for EFS related to the top four proteomic markers in univariate and/or multivariable Cox regression analysis.

Discussion

In this pilot study, we were able to validate sCD23 levels above the median as significantly associated with a shorter EFS, which is consistent with previous studies [7]. Soluble CD23 is released from activated B cells and can itself induce further B-cell stimulation as well

Table 1. Adjusted HRs for event-free survival for combination of the proteomic markers and known prognostic factors

	M1 (stepwise)		M2 (stepwise)	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Female	0.40 (0.16–0.98)	0.04	0.21 (0.06–0.66)	0.008
Unmutated IGHV	4.25 (1.68–10.8)	0.002	3.9 (1.28–11.9)	0.016
sCD23			0.27 (0.12–0.58)	0.0009
sCD23* IGHV			NS	
SPINT1			3.6 (1.12–11.5)	0.031
LY9			7.03 (1.6–30.8)	0.009
IFNLR1			NS	
ITM2A			NS	
CLEC7A			0.43 (0.27–0.7)	0.0007
TCL1A			NS	
R^2 (corrected[a])	0.28 (0.24)		0.60 (0.48)	
AUC (corrected[a])	0.40 (0.38)		0.68 (0.61)	

AUC=Area under the curve; *CI*=confidence interval; *NS*=nonsignificant.
[a]R^2 and AUC corrected for overfitting by 100 times bootstrapping.

as function as a potent mitogenic growth factor. Notably, sCD23 seems to be associated with IGHV status, since mutated CLL patients had significantly lower levels of sCD23, as compared with unmutated patients. When both markers are used, patients with unmutated IGHV genes with sCD23 levels above the median can be regarded as very poor prognostic group. Similarly, patients with unmutated IGHV genes and high levels of sCD27 were found to have an even poorer prognosis. High sCD27 levels were described to be associated with higher Rai stage, β2M, and LDH among CLL patients [11,12]. Here we found that high levels of sCD27 were associated with β2M levels and inferior prognosis.

Our study further indicated that higher levels of SPINT1 are associated with shorter EFS time and higher β2M levels. SPINT1, an enzyme that is encoded by the Kunitz-type protease inhibitor 1 gene, modulates matriptase proteolytic activity. Matriptase was identified in Burkitt lymphoma cells [13] and later in CLL [14]. In fact, it is highly upregulated in CLL and promotes cancer cell invasion either directly by degrading matrix proteins or indirectly by activating growth factors or through yet unknown mechanisms [14].

In our cohort, CLL patients with high LY9 levels had an inferior EFS as compared with patients with low LY9 levels. LY9 is known to interact with SLAM-associated protein that has been implicated in autoimmunity. It has been reported that LY9 is a naturally processed antigen in CLL and can serve as tumor-associated antigen in this disease [15]. It was reported that LY9-specific cytotoxic T cells from CLL patients efficiently recognized native and CD40L-activated autologous malignant CLL cells via MHC-I molecules. These findings provide strong evidence that LY9 can be employed for the design of T cell-based immunotherapeutic strategies of LY9-expressing malignancies including CLL [15] and, thus, underline the impact of the results from our current study.

A major strength of this pilot study is the large set of novel proteomic markers, which we measured and which were previously not extensively described in CLL patients. Despite the relatively small number of available cases, which had an impact on statistical power, our pilot study identified SPINT1 and LY9 as promising independent prognostic proteomic markers next to sCD23 and sCD27 in patients treated for CLL. Further studies with larger sample sizes are required to validate these results. Also, as proteomic markers were solely measured before treatment, changes in marker levels during treatment should be evaluated in new CLL patient cohorts.

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Conflict of interest disclosure

None of the authors declared a conflict of interest.

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SUPPLEMENTARY INFORMATION

Supplementary Methods

Written informed consent was obtained before enrollment in the trial. The study was approved by an accredited Ethical Committee and Institutional Review Board and was performed according to the Declaration of Helsinki, the International Conference on Harmonization Good Clinical Practice Guidelines and the European Union Clinical Trial Directive (2001/20/EG). The study was registered with EuraCT number 2010-022294-34 [1].

Protein measurements. Serum samples were analyzed using a multiplex proximity extension assay. In brief, 1 μL sample was incubated in the presence of proximity antibody pairs tagged with DNA-reporter molecules. Once the pair of antibodies is bound to their corresponding antigens, the respective DNA tails form an amplicon by proximity extension, which was quantified by high-throughput real-time PCR (BioMark^{TM} HD System, Fluidigm Corporation). Protein abundance directly correlates with the generated fluorescent signal, which is expressed in quantitation cycles produced by the BioMark's Real-Time PCR Software following the Proseek Multiplex protocol. To minimize variation within and between runs, data (Ct values) were normalized using both an internal control (extension control) and an interplate control, and then transformed using a pre-determined correction factor.

The limit of detection was determined for each biomarker based on the mean value of 4 controls analyzed in each run. Thirty-five markers were excluded from statistical analyses because of a high non-detection rate in the cohort (i.e. >30% of the cases) (Supplementary Table E1, footnote). For 35 samples, the non-detection rate ranged from 2% to 30% (median 7%) and those were set to the value of the lower limit of quantification divided by the square root of two. Around 83% of markers were detected in all samples. The list of biomarkers (n=322) included in the statistical analyses and their median value is shown in Supplementary Table E1.

Statistical analyses. Differences in marker distributions across different levels of prognostic factors were evaluated by Wilcoxon tests. Kaplan–Meier plot and Log-rank test were used to examine the survival distribution for protein markers (≤ median or >median) and other known prognostic factors. The median of the protein markers are shown in Supplementary Table E1.

Cox proportional hazard models were used for testing the effects of the markers for event-free survival (EFS; time from registration to induction failure, progression, or death from any cause, whichever comes first). Induction failure was defined as not having achieved at least a PR during/after a maximum of 12 cycles. Unadjusted analyses were carried out for each proteomic marker. Due to the limited sample size of the study, the Cox model of proteomic markers was further adjusted only for significant known prognostic factors (gender and IGHV status).

Of interest was the added prognostic value of proteomic markers that might help to assess prognosis in clinical practice. Two models, one containing only significant known prognostic markers and the other containing the first model plus significant proteomic markers were compared by estimating the area under a ROC (receiver operating characteristic) curve (AUC) for EFS. R square and AUC corrected for overfitting by 100 bootstrapping were reported.

Validation analysis. As standard regression models perform poorly in a situation with a data set containing a number of variables superior to the number of samples, we additionally applied the least absolute shrinkage and selection operator (Lasso) technique for variable selection [2]. It is a powerful method that perform two main tasks: regularization and feature selection. In order to do so the method applied a shrinking (regularization) process in which the coefficients of the regression variables were penalized, thus shrinking some of them to zero. During the feature selection process the variables that still have a non-zero coefficient after the shrinking process were selected to be part of the model. Optimal tuning parameter λ, which controls the strength of the penalty, was obtained by 5 folds cross-validation.

Supplementary Results

Validation analysis by means of Lasso regression. In total 8 proteomic markers were selected in association with EFS in Lasso analysis (Supplementary Table E6). Interestingly, five proteomic markers suggested in our Cox regression models (LY9, SPINT1, ITM2A, IFNLR1, and CLEC7A) were among the selected variables, thus supporting the validity of these markers as possible prognostic markers.

Fourteen proteomic markers [sCD23, TCL1A, ITM2A, CLEC7A, LY9, VEGFR-3, IFNLR1, SPINT1, TNF receptor-associated factor 2 (TRAF2), Semaphorin-7A (SEMA7A), signal-regulatory protein beta-1 (SIRPB1), C-type lectin domain family 4 member C (CLEC4C), tripeptidyl peptidase 1 (TPP1), monocyte chemoattractant protein 1 (MCP1)] found to be associated with EFS in the adjusted Cox regression models at $p<0.05$ and/or in Lasso analysis were subjected to computational functional analysis. Molecular functions, biological process, and cellular components related to the proteins are shown in Supplementary Figure E2. Due to the limited sample size and protein numbers, further pathway analyses and statistical enrichment detection were not possible.

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Supplementary Table E1. Proteomic markers with their median values included in the statistical analyses

Marker	Label	M*	Marker	Label	M*	Marker	Label	M*
DevOID01400	CTSF	2.32	DevOID01447	CD109	2.56	DevOID01490	TPP1	4.60
DevOID01401	MATN2	2.68	DevOID01448	VSIG4	5.13	DevOID01491	CD209	4.35
DevOID01402	HTRA2	0.68	DevOID01449	CRIM1	1.71	ImmOID00936	PPP1R9B	4.17
DevOID01403	MFGE8	3.72	DevOID01450	CGA	4.77	ImmOID00937	GLB1	0.72
DevOID01405	ACAN	2.97	DevOID01451	CLEC14A	3.82	ImmOID00938	PSIP1	5.78
DevOID01406	ITGB1	6.14	DevOID01452	CA6	0.37	ImmOID00939	ZBTB16	1.60
DevOID01407	DKK3	4.23	DevOID01453	MIF	7.56	ImmOID00940	IRAK4	2.02
DevOID01408	SPINT2	2.46	DevOID01454	CRELD2	2.50	ImmOID00941	TPSAB1	3.12
DevOID01409	APP	4.16	DevOID01455	ITGA5	1.79	ImmOID00942	HCLS1	5.49
DevOID01410	HAVCR2	3.21	DevOID01456	PEAR1	5.05	ImmOID00943	CNTNAP2	1.42
DevOID01411	DSC2	4.59	DevOID01457	MESDC2	2.14	ImmOID00944	CLEC4G	2.60
DevOID01412	TMSB10	6.03	DevOID01458	CD99L2	1.59	ImmOID00945	IRF9	3.80
DevOID01413	ANGPTL4	2.43	DevOID01459	SCARF1	6.74	ImmOID00946	EDAR	1.15
DevOID01414	INHBC	1.56	DevOID01460	LGMN	3.02	ImmOID00949	CLEC4C	4.28
DevOID01415	SPINK1	3.37	DevOID01461	SEMA7A	6.72	ImmOID00950	IRAK1	1.38
DevOID01416	CLEC11A	3.69	DevOID01462	COLEC12	3.57	ImmOID00951	CLEC4A	3.63
DevOID01417	PDGFRB	3.95	DevOID01463	GUSB	3.42	ImmOID00952	PRDX1	3.06
DevOID01418	COCH	5.47	DevOID01492	FUT3/FUT5	2.44	ImmOID00953	PRDX3	-0.18
DevOID01419	FCRL5	5.72	DevOID01465	STIP1	0.64	ImmOID00954	FGF2	1.95
DevOID01420	PILRA	2.84	DevOID01466	B4GAT1	4.18	ImmOID00955	PRDX5	6.64
DevOID01421	B4GALT1	3.07	DevOID01467	CD69	5.76	ImmOID00956	DPP10	0.54
DevOID01422	CD300LG	3.38	DevOID01468	CRHBP	2.07	ImmOID00957	TRIM5	2.85
DevOID01423	PARK7	4.27	DevOID01469	MSMB	2.64	ImmOID00958	DCTN1	5.06
DevOID01424	BLVRB	0.11	DevOID01470	SPINT1	1.59	ImmOID00960	CDSN	3.07
DevOID01426	CD74	2.69	DevOID01471	FSTL3	2.23	ImmOID00961	GALNT3	1.73
DevOID01427	CCL21	0.58	DevOID01472	IL13RA1	1.65	ImmOID00963	TRAF2	2.27
DevOID01428	PTPRF	2.72	DevOID01473	CD58	2.26	ImmOID00964	TRIM21	1.94
DevOID01429	BCAM	3.36	DevOID01474	SCGB3A1	2.34	ImmOID00965	LILRB4	3.26
DevOID01430	SIRPB1	3.17	DevOID01475	NOV	3.26	ImmOID00967	KRT19	3.10
DevOID01431	IGF2R	6.25	DevOID01476	CNTN4	3.52	ImmOID00968	ITM2A	1.16
DevOID01432	P4HB	0.28	DevOID01477	CA2	3.85	ImmOID00969	HNMT	0.64
DevOID01433	FUCA1	5.83	DevOID01478	XG	4.00	ImmOID00971	MILR1	2.44
DevOID01434	ESAM	3.21	DevOID01479	ARSA	2.93	ImmOID00972	EGLN1	4.24
DevOID01436	BST1	1.28	DevOID01480	PPIB	3.93	ImmOID00973	NFATC3	-0.07
DevOID01437	CST6	4.85	DevOID01481	SPINK5	0.04	ImmOID00974	LY75	3.11
DevOID01438	MYOC	3.19	DevOID01482	OMD	2.08	ImmOID00976	EIF4G1	5.16
DevOID01439	SNAP29	4.92	DevOID01483	PEBP1	5.74	ImmOID00977	CD28	1.13
DevOID01440	WFIKKN2	3.37	DevOID01484	PAMR1	3.78	ImmOID00978	PTH1R	1.47
DevOID01441	CDON	2.20	DevOID01485	ROBO1	2.08	ImmOID00979	BIRC2	0.53
DevOID01442	CD177	4.79	DevOID01486	CD23	11.3	ImmOID00980	HSD11B1	2.36
DevOID01443	NID2	1.27	DevOID01487	LAMA4	2.45	ImmOID00982	PLXNA4	3.31
DevOID01444	DAG1	2.75	DevOID01488	LAIR1	4.55	ImmOID00983	SH2B3	1.54
DevOID01445	CD97	5.71	DevOID01489	RELT	5.37	ImmOID00984	FCRL3	2.70
ImmOID00985	CKAP4	6.32	InfOID00483	IL-17C	0.99	InfOID00542	CD40	10.9
ImmOID00987	HEXIM1	4.31	InfOID00484	MCP-1	11.0	InfOID00545	FGF-19	8.29
ImmOID00988	CLEC4D	2.29	InfOID00486	CXCL11	8.67	InfOID00549	MCP-2	8.03
ImmOID00989	PRKCQ	0.27	InfOID00487	AXIN1	3.49	InfOID00550	CASP-8	4.73
ImmOID00990	MGMT	4.26	InfOID00490	CXCL9	9.31	InfOID00551	CCL25	6.09
ImmOID00991	TREM1	2.03	InfOID00491	CST5	5.89	InfOID00552	CX3CL1	5.63
ImmOID00992	CXADR	1.55	InfOID00494	OSM	5.38	InfOID00553	TNFRSF9	10.9
ImmOID00994	SRPK2	1.18	InfOID00496	CXCL1	8.40	InfOID00554	NT-3	1.14
ImmOID00995	KLRD1	5.69	InfOID00498	CCL4	8.93	InfOID00555	TWEAK	9.20
ImmOID00996	BACH1	1.84	InfOID00499	CD6	6.90	InfOID00556	CCL20	3.66
ImmOID00997	PIK3AP1	5.43	InfOID00501	IL18	8.73	InfOID00557	ST1A1	4.49
ImmOID00999	STC1	5.25	InfOID00502	SLAMF1	2.04	InfOID00558	STAMPB	4.77
ImmOID01001	FAM3B	4.04	InfOID00504	MCP-4	3.92	InfOID00560	ADA	3.74
ImmOID01002	SH2D1A	1.39	InfOID00505	CCL11	7.63	InfOID00561	TNFB	6.53
ImmOID01003	ICA1	1.44	InfOID00506	TNFSF14	7.08	InfOID00562	CSF-1	7.96
ImmOID01004	DFFA	5.64	InfOID00507	FGF-23	1.13	OncOID00655	TXLNA	6.14
ImmOID01005	DCBLD2	2.35	InfOID00508	IL-10RA	0.87	OncOID00657	CPE	3.82
ImmOID01006	FCRL6	2.07	InfOID00509	FGF-5	0.64	OncOID00658	KLK13	3.15

(*continued*)

Supplementary Table E1 (*Continued*)

Marker	Label	M*	Marker	Label	M*	Marker	Label	M*
ImmOID01007	NCR1	1.82	InfOID00510	MMP-1	13.6	OncOID00659	CEACAM1	6.66
ImmOID01010	IFNLR1	3.52	InfOID00511	LIF-R	2.60	OncOID00660	MSLN	4.01
ImmOID01011	DAPP1	1.30	InfOID00512	FGF-21	4.58	OncOID00661	TNFSF13	7.59
ImmOID01013	SIT1	3.79	InfOID00513	CCL19	8.59	OncOID00662	EGF	11.1
ImmOID01014	MASP1	1.08	InfOID00514	IL-15RA	0.56	OncOID00663	TNFRSF6B	6.60
ImmOID01015	LAMP3	3.72	InfOID00515	IL-10RB	7.30	OncOID00664	SYND1	6.92
ImmOID01016	CLEC7A	3.36	InfOID00517	IL-18R1	6.84	OncOID00665	TGFR-2	6.71
ImmOID01017	CLEC6A	2.38	InfOID00518	PD-L1	4.50	OncOID00666	IL6	3.83
ImmOID01018	DDX58	4.17	InfOID00519	Beta-NGF	1.53	OncOID00667	CD48	7.85
ImmOID01019	IL12RB1	2.32	InfOID00520	CXCL5	9.74	OncOID00668	SCAMP3	6.67
ImmOID01020	TANK	2.48	InfOID00521	TRANCE	3.97	OncOID00669	LY9	8.22
ImmOID01021	ITGA11	1.35	InfOID00522	HGF	8.63	OncOID00670	IFN-gamma-R1	5.34
ImmOID01023	LAG3	2.53	InfOID00523	IL-12B	4.65	OncOID00671	ITGAV	3.60
ImmOID01025	CD83	3.56	InfOID00527	MMP-10	5.44	OncOID00672	TRAIL	8.07
ImmOID01026	ITGB6	1.63	InfOID00528	IL10	3.27	OncOID00673	hK11	6.72
ImmOID01027	BTN3A2	1.98	InfOID00530	CCL23	9.95	OncOID00674	GPC1	5.05
InfOID00471	IL8	10.9	InfOID00531	CD5	8.18	OncOID00675	TFPI-2	8.10
InfOID00472	VEGFA	9.92	InfOID00532	CCL3	7.86	OncOID00676	hK8	7.29
InfOID00474	MCP-3	3.08	InfOID00533	Flt3L	9.08	OncOID00677	VEGFR-2	7.14
InfOID00475	GDNF	0.90	InfOID00534	CXCL6	8.46	OncOID00678	LYPD3	4.70
InfOID00476	CDCP1	3.33	InfOID00535	CXCL10	8.78	OncOID00679	PODXL	3.60
InfOID00477	CD244	5.69	InfOID00536	4E-BP1	7.53	OncOID00680	S100A4	3.26
InfOID00478	IL7	2.53	InfOID00538	SIRT2	3.35	OncOID00681	IGF1R	3.52
InfOID00479	OPG	10.6	InfOID00539	CCL28	0.94	OncOID00682	ERBB2	7.79
InfOID00480	LAP TGF-β-1	8.00	InfOID01213	DNER	7.36	OncOID00683	ERBB3	8.08
InfOID00481	uPA	10.2	InfOID00541	EN-RAGE	6.03	OncOID00684	SCF	9.28
OncOID00685	SPARC	6.06	OncOID00708	5′-NT	10.8	OncOID00730	CD207	2.81
OncOID00686	GZMH	5.97	OncOID00709	CDKN1A	2.46	OncOID00731	ICOSLG	6.45
OncOID00687	TGF-alpha	8.85	OncOID00710	DLL1	10.4	OncOID00732	WFDC2	8.68
OncOID00688	FURIN	9.21	OncOID00711	MK	5.92	OncOID00733	CXCL13	9.70
OncOID00689	CYR61	5.38	OncOID00712	ABL1	4.42	OncOID00734	MAD homolog 5	3.62
OncOID00690	hK14	6.94	OncOID00713	FGF-BP1	5.19	OncOID00735	ADAM-TS 15	1.56
OncOID00691	FADD	2.65	OncOID00714	TLR3	5.51	OncOID00736	CD70	6.96
OncOID00692	MetAP 2	5.38	OncOID00715	LYN	3.57	OncOID00737	RSPO3	3.45
OncOID00693	PVRL4	4.83	OncOID00716	RET	5.30	OncOID00738	FR-gamma	6.53
OncOID00694	FASLG	9.28	OncOID00717	VIM	6.17	OncOID00739	CEACAM5	1.24
OncOID00695	EPHA2	3.93	OncOID00718	TNFRSF19	4.59	OncOID00740	VEGFR-3	7.22
OncOID00696	ITGB5	7.09	OncOID00719	CRNN	4.47	OncOID00741	MUC-16	4.50
OncOID00697	Gal-1	7.07	OncOID00720	TCL1A	11.2	OncOID00742	WIF-1	4.94
OncOID00698	SEZ6L	3.48	OncOID00721	CD160	6.24	OncOID00743	GZMB	4.44
OncOID00749	GPNMB	6.85	OncOID00722	TNFRSF4	5.77	OncOID00744	FCRLB	1.42
OncOID00700	CAIX	4.02	OncOID00723	MIC-A/B	4.63	OncOID00745	ANXA1	4.90
OncOID00701	MIA	10.2	OncOID00724	WISP-1	6.27	OncOID00746	FR-alpha	6.72
OncOID00702	CTSV	3.44	OncOID00725	CXL17	3.91			
OncOID00703	CD27	11.0	OncOID00726	PPY	9.42			
OncOID00704	XPNPEP2	8.56	OncOID00727	S100A11	4.34			
OncOID00705	ERBB4	5.26	OncOID00728	AREG	3.40			
OncOID00707	ADAM 8	6.27	OncOID00729	ESM-1	8.58			

*Median (M) normalized protein expression level on log2 scale; The excluded markers due to a high non-detection rate were NFASC, KPNA1, BDNF, IL-1 alpha, IL-2, IL-4, IL-5, IL-13, IL-20, IL-24, IL-33, TSLP, EIF5A, IL-22 RA1, ARTN, PLA2G4A, IL-20RA, IFN-gamma, SPRY2, ARNT, IL-2RB, NTF4, PADI2, TNF, LIF, NRTN, JUN, DGKZ, FXYD5, NF2, IL-17A, PTPN6, ITGA6, NUDT5, CXCL12.

Supplementary Table E2. Characteristics of the study participants

	N=51
Age, years*	71 (60-83)
Gender	
Female, n	21
Male, n	30
Rai stage, n	
0	2
1	9
2	9
3	20
4	11
chromosome aberration	
Del17p13	7/49
Del13q14	24/51
Del11q22	7/51
Tri12	11/51
IGHV mutation status	
Mutated	25/47
Unmutated	22/47
Beta 2-Microglobulin (β2M), μg/mL*	3.8 (1.6 -10.4)
Survival status	
Alive	47
Dead	4
Survival duration*	31.7 (15.8-62.1)
Progression free survival (PFS) status	27
No progression	27
Progression	24
PFS duration*	24.4 (1.25-60.9)
Event free survival (EFS) status	
No event	25
Event	26
EFS duration*	23 (1.25-60.9)

*Median (range); Missing numbers: Del17p13 (n=2), Immunoglobulin heavy chain variable genes (IGHV) mutation status (n=4), β2M (n=10)

Supplementary Table E4. Cox models for EFS related to individual B-cell activation proteomic markers found to be predictive in population-based studies

Markers	Univariate		Multivariable*	
	HR (95% CI)	P	HR (95% CI)	P
CD23	**1.56 (1.02-2.4)**	**0.04**	1.21 (0.80-1.86)	0.37
CD27	2.9 (0.71-11.9)	0.14	2.45 (0.53-11.4)	0.25

*Each protein marker individually adjusted for gender and IGHV mutation status; There was a significant interaction effect between sCD23 and IGHV status (HR=2.75, 95% CI= 1.04-7.27, P=0.04) implying that the effect of sCD23 on EFS would be different between mutated and unmutated patients (HR of 0.23 for one unit increase in sCD23 in mutated male CLL patients vs. HR of 3.4 for unmutated male CLL patients).

Supplementary Table E5. Top 4 proteomic markers from the univariate and multivariable Cox regression models for EFS

Markers	Univariate	
	HR (95% CI)	P
SPINT1	3.16 (1.64-6.05)	0.0005
LY9	5.1 (1.94-13.41)	0.0009
IFNLR1	2.85 (1.51-5.40)	0.0013
ITM2A	3.40 (1.61-7.12)	0.0013
	Multivariable*	
TCL1A	0.70 (0.12-0.86)	0.0020
ITM2A	2.81 (1.29-6.13)	0.0095
CLEC7A	0.60 (0.40-0.90)	0.0145
LY9	3.10 (1.25-7.68)	0.0147

*Each protein marker individually adjusted for gender and IGHV mutation status; Bonferroni correction level: p=0.05/322=0.0002

Supplementary Table E3. Proteomic markers significantly associated with well-known CLL prognostic factors (corrected for multiple testing)

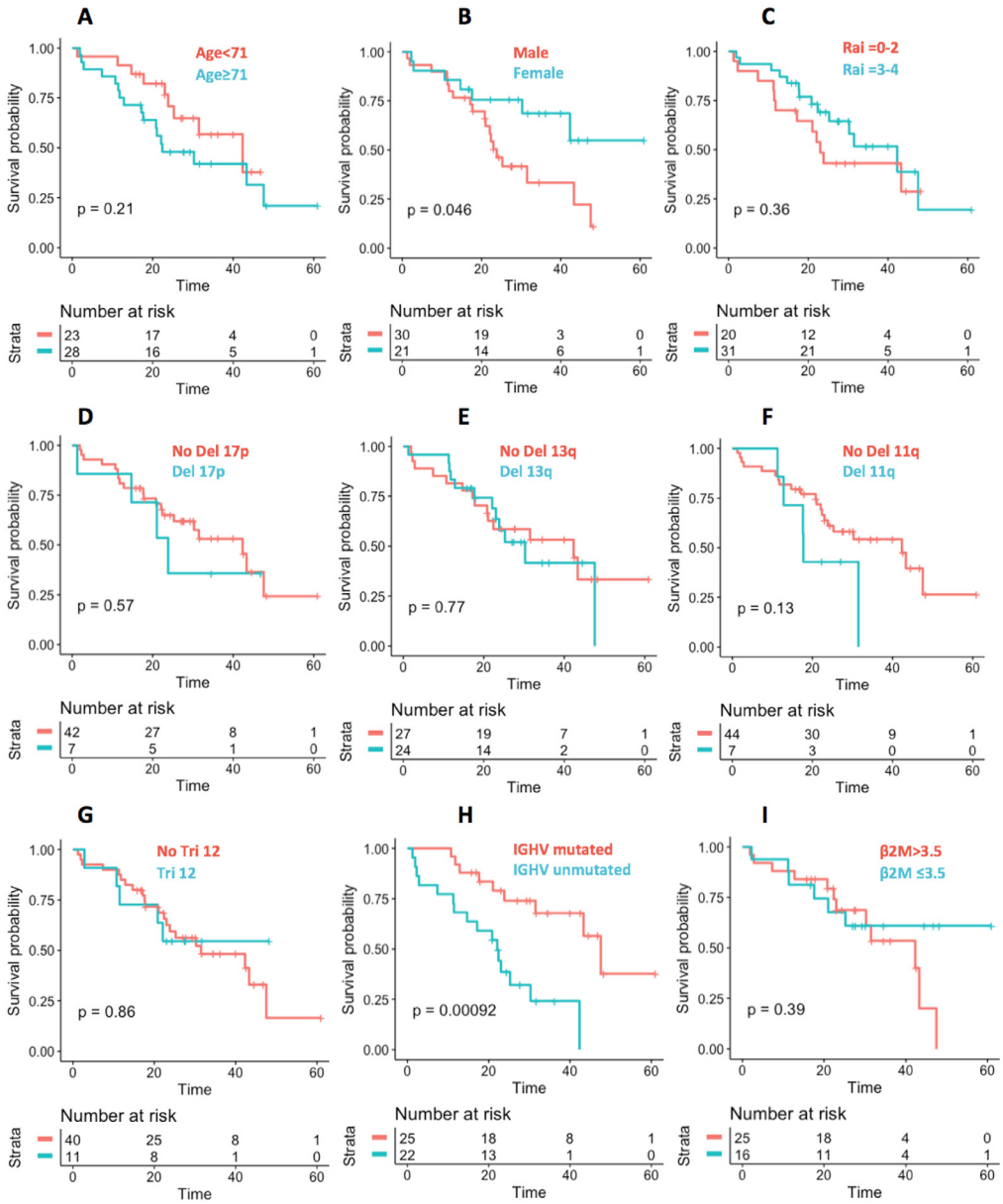
Sex	IGHV Mut.	β2M	β2M (cont.)	β2M (cont.)	β2M (cont.)	LDH	pblymph	Pblymph (cont.)
ANGPTL4	CD23	TNFRSF4	TNFRSF6B	FSTL3	LILRB4	BCAM	PSIP1	HEXIM1
CGA	NFATC3	ROBO1	SPINT1	CD74	CD28	ITGB1	DFFA	DDX58
		B4GALT1	TNFB	IFN-gamma-R1	BCAM	TFPI-2	ABL1	IFNLR1
		RELT	ANGPTL4	LAIR1	CD5	FSTL3	SIT1	MASP1
		EPHA2	TPP1	PD-L1	CSF-1	FGF-23	SRPK2	LY75
		DLL1	FCRLB	CD23	MIC-A/B	EPHA2	PPP1R9B	PEBP1
		DSC2	CD40	SEMA7A	FCRL6	ROBO1	CCL28	NT-3
		LAG3	IL12RB1	WISP-1	CD244	HTRA2	TMSB10	HCLS1
		CD97	CLEC4C	CD27	IL18	SCF*	IRF9	IRAK1
		ADAM 8	CXCL9	CST5	CD160	TNFRSF4	TXLNA	SCAMP3
		HAVCR2	MILR1	SIRPB1	CD209		CCL4	ICOSLG
		IL-15RA	ITGB1	CLEC11A	WFDC2		MGMT	
		TNFRSF9	SH2D1A	SLAMF1	PILRA			
		TNFRSF19	GALNT3	PDGFRB				
		IL-10RB	CD83	FGF-23				

*inverse association; Beta 2-Microglobulin (β2M); lactate dehydrogenase (LDH); peripheral B lymphocyte number (pblymph)

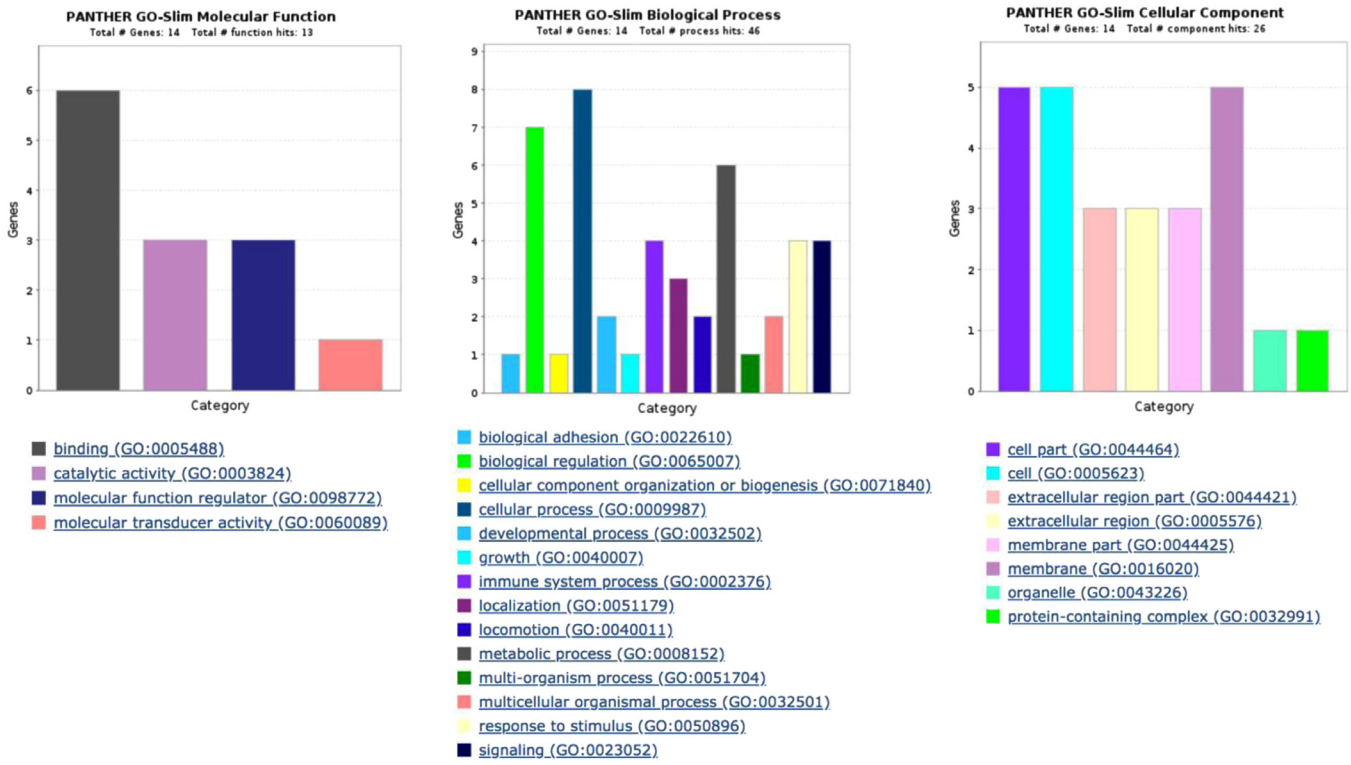
Supplementary Table E6. Proteomic markers selected by 5 folds cross-validated Lasso

Proteomic marker	Lasso
	Coefficient at lambda.min
LY9	0.1757
ITM2A	0.1749
IFNLR1	0.1025
TPP1	0.0968
SPINT1	0.0913
CLEC4C	0.0325
MCP-1	0.0056
CLEC7A	-0.2875
Lambda minimum	0.21
Alpha	1

The lambda.min refers to value of λ at the lowest cross-validation error.



Supplementary Figure E1. Kaplan-Meier curves for EFS related to well-known CLL prognostic factors



Supplementary Figure E2. Molecular functions, biological process, and cellular components related to 14 proteins (with P<0.05 in adjusted Cox model and/or Lasso model) using the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system