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A gene expression signature based on microenvironment signaling to predict progression in chronic lymphocytic leukemia

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Abstract:
Several gene expression profiles with a strong correlation with patient outcome have been previously described in chronic lymphocytic leukemia (CLL), although their applicability in clinical practice as biomarkers has been particularly limited. Here we describe the training and validation of a gene expression signature for predicting early progression of patients with CLL based on the analysis of 200 genes related to microenvironment signaling on the NanoString platform. In the training cohort (n=154), the CLL15 assay containing a 15-gene signature was associated with time to first treatment (TtFT) (HR: 2.83, 95%CI 2.17-3.68; p<0.001). The prognostic value of the CLL15 score (HR 1.71, [95%CI 1.15-2.52]; p=0.007) was further confirmed in an external independent validation cohort (n=112). Of note, the CLL15 score improved the prognostic capacity over the *IGHV* mutational status and the International Prognostic Score for asymptomatic early-stage (IPS-E) CLL. In multivariate analysis, the CLL15 score (HR: 1.83, 95%CI 1.32-2.56; p<0.001) and the IPS-E CLL (HR: 2.23, 95%CI 1.59-3.12; p<0.001) were independently associated with TtFT. The newly developed and validated CLL15 assay successfully translates previous gene signatures, such as the microenvironment signaling, into a new gene expression-based assay with prognostic implications in CLL.

Conflict of interest: COI declared - see note

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A gene expression assay based on chronic lymphocytic leukemia activation in the microenvironment to predict progression

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Running Title: CLL15 assay for time to progression in CLL

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Abstract:

Several gene expression profiles with a strong correlation with patient outcome have been previously described in chronic lymphocytic leukemia (CLL), although their applicability in clinical practice as biomarkers has been particularly limited. Here we describe the training and validation of a gene expression signature for predicting early progression of patients with CLL based on the analysis of 200 genes related to microenvironment signaling on the NanoString platform. In the training cohort (n=154), the CLL15 assay containing a 15-gene signature was associated with time to first treatment (TtFT) (HR: 2.83, 95%CI 2.17-3.68; p<0.001). The prognostic value of the CLL15 score (HR 1.71, [95%CI 1.15-2.52]; p=0.007) was further confirmed in an external independent validation cohort (n=112). Of note, the CLL15 score improved the prognostic capacity over the *IGHV* mutational status and the International Prognostic Score for asymptomatic early-stage (IPS-E) CLL. In multivariate analysis, the CLL15 score (HR: 1.83, 95%CI 1.32-2.56; p<0.001) and the IPS-E CLL (HR: 2.23, 95%CI 1.59-3.12; p<0.001) were independently associated with TtFT. The newly developed and validated CLL15 assay successfully translates previous gene signatures, such as the microenvironment signaling, into a new gene expression-based assay with prognostic implications in CLL.

Key Points

- The CLL15 signature, based on the expression of genes associated to microenvironment signaling, predict the risk of early progression in CLL
- The predictive power of the CLL15 signature is independent of the *IGHV* mutational status and of the IPS-E CLL score

Introduction

It is well accepted that patients with chronic lymphocytic leukemia (CLL) that are asymptomatic and in early clinical phase do not require therapy.(1) Nevertheless, cumulative data on the risk of clonal evolution (2-4) renewed the interest about an early therapeutic intervention on patients at diagnosis who are likely to progress rapidly.(5) Therefore, the identification at diagnosis of these patients has been an intense focus of clinical research in the field of CLL. Prognostication in this setting has classically relied upon a myriad of laboratory values, cytogenetic abnormalities, gene mutations or the mutational status of the immunoglobulin genes (*IGHV*).(6-10) More recently, the International Prognostic Score for Early-stage CLL [IPS-E] has been developed employing three covariates, unmutated *IGHV*, absolute lymphocyte count > 15 × 10^9/L, and presence of palpable lymph nodes.(11)

In spite of all this extensive investigation, the accuracy of these models may be improved.(11-16) In addition, the emergence of novel targeted agents has reignited interest in early treatment of patients with high risk of early progression.(5)

Gene expression profile and the clinical course of the patients with CLL have been correlated in different studies.(7, 8, 17-26) Regrettably, biomarkers based on gene expression profiles exhibit several caveats that preclude them from being widely applicable in the prognostication of CLL patients. These include the lack of reproducibility and standardization, and the complexity of bioinformatics analysis. Significantly, the prognostic value of clustering methods is limited by the fact that the

assignment of an individual can vary when different patients are included during the clustering process, thus impeding the use of these methods in real-time. In this regard, the development of new platforms that allow direct and reproducible quantification of gene expression such as NanoString nCounter should facilitate the attainment of gene expression biomarkers applicable in clinical settings.(27, 28) Among different gene signatures, and since CLL is a malignancy particularly dependent on the interaction with their microenvironment for survival and proliferation (25), *IGHV* mutational status signature (7, 8, 17, 18) and genes involved in the activation of malignant cells in the microenvironment, including the stimulation of the B cell receptor (BCR),(24-26, 29) are of particular interest. Indeed, this notion is reinforced by the standard use of different small molecules targeting CLL-microenvironment interactions, particularly BTK inhibitors.(30, 31)

Herein, we developed, evaluated and validated a multi-gene expression signature using genes associated with the activation of CLL cells in the microenvironment and the *IGHV* mutational status. This assay, based on a NanoString platfom, should facilitate its applicability in clinical settings.

Materials and Methods

Study design and patient population

The overall design of the process for developing and evaluating the new assay for the risk of progression in CLL patients is shown in Supplemental Fig 1. For the training cohort of the study, 156 untreated samples, 119 from University Hospital Vall d'Hebron and 37 from University of Salamanca were used. The assay was validated using 112 samples from an independent cohort of patients from the German Cancer Research Centre, Heidelberg. Details of the validation cohort have been reported elsewhere.(32) Samples were obtained at diagnosis whenever possible. For patients that did not have a sample at the moment of diagnosis, samples during the follow-up were collected but always before the patients received any treatment. Gene expression quantification was performed in blood samples from untreated patients diagnosed with CLL. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque Plus (GE Healthcare, Buckinghamshire, United Kingdom) density gradient and subsequently cryopreserved until analysis. Tumor cells were purified using immunomagnetic depletion by EasySep™ Human B Cell Enrichment Kit (StemCell Technologies), and the final tumor content was assessed by flow cytometry. The estimated median tumor content was 98.3% (range 80%-99.9%) in the training cohort, and 95.7% (range 86.8%-99.4%) in the validation cohort.

A written informed consent was obtained from all individuals in accordance with the declaration of Helsinki. The study was approved by the clinical research ethics committee of the Vall d'Hebron Barcelona Hospital Campus.

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149 ***Gene expression analysis***

150 Gene expression was quantified in 250 ng of RNA on the NanoString platform
151 (NanoString Technologies, WA) using the "high sensitivity" setting on the nCounter™
152 PrepStation and 555 fields of view (FOV) on the nCounter™ Digital Analyzer. One
153 hundred seventy-eight genes were selected from the literature, including genes related
154 to the activation of CLL cells in the microenvironment,(23-26) genes differentially
155 expressed according to the mutational status of *IGHV*,(7, 8, 17, 18) and other genes with
156 prognostic interest in CLL (Supplementary methods, Supplemental Table S1).
157 Normalization for RNA loading was performed using the geometric mean of 22
158 housekeeping genes (Supplemental Table S1). The normalized data were log10
159 transformed. Reference gene selection is further described in the Data Supplement.

160

161 ***Predictive gene expression score***

162 Detailed descriptions of model building and model performance assessment are
163 provided in the Data Supplement. In brief, we used the gene expression data from the
164 training cohort to produce a parsimonious predictive model for Time to First Treatment
165 (TtFT) using a penalized Cox model.(33) To evaluate the global performance of the
166 multivariate Cox model obtained from the selected genes, different diagnostic
167 parameters have been calculated and are summarized in the Data supplement (Table
168 S2), including R^2, the Brier score, iAUC (a summary measure of the area under the

receiver operating characteristic [ROC] curve calculated for the different times), and the Harrell's C-statistic, a generalization of the AUC.(34, 35) The graph obtained for the AUC values at the different time points is shown in supplemental Figure 2. For illustration purposes, we dichotomized the predictive gene expression score in three risk-groups using R *partykit* package.

Statistical Analysis

The statistical analysis plan was pre-specified prior to the evaluation of the gene expression from the training and validation cohort. The primary endpoint of the study was TtFT, defined as the time from the date of obtaining the sample to the date of treatment onset. To study the predictive capacity of the gene expression score, we relaxed the linearity assumption using restricted cubic splines by means of *rms* R package (Harrell, F. E. Jr. Package 'rms' [The Comprehensive R Archive Network, 2016]). The Harrell's C-statistic was calculated to compare the discrimination capacity of different models. The analysis of deviance (*anova* R function) was used to study if the inclusion of new factors had a significant improvement in the predictive capacity of the model. Survival curves were estimated using the Kaplan-Meier method to visualize gene expression risk-groups and compared by the log-rank test. Cox proportional-hazard models were used to obtain hazard ratios (HRs) with 95% CIs without dichotomizing continuous factors.(36) In order to select prognostic variables with the highest impact in TtFT, we performed a least absolute shrinkage and selection operator (LASSO) regression using package *glmnet* in R software to build the most parsimonious

multivariate model. Imputation of random missing values was carried out via the *mice* R package (Supplemental Table S3). The median follow-up was calculated using the reverse Kaplan-Meier method. All analyses were performed using R statistical software version 3.6.2.

Results

Generation of a prognostic model based on gene expression: the CLL15 assay

The training cohort was comprised of 156 patients with previously untreated CLL. Median age of the series was 66 years (range, 34 to 90 years) and 57% were men. A total of 37% of samples were obtained at diagnosis of CLL, while 63% of samples were obtained during the follow-up of the patients prior to any CLL treatment. Median time from CLL diagnosis to sample collection was 11.9 months (95%CI 7.1 – 22.6). The analysis of TTfT was calculated from the date of obtaining the sample to the date of treatment onset. The main clinical and biological characteristics of the series are shown in Table 1. Ninety-two cases (59%) were *IGHV* mutated, 54 cases (35%) were *IGHV* unmutated, whereas 9 cases (6%) were undetermined because of polyclonal, unproductive or biclonal rearrangement. In 1 case no *IGHV* mutational data was obtained.

Digital gene expression for 178 genes of interest and 22 housekeeping genes (Supplement Table S1) was determined in the 156 samples from the training cohort. Adequate gene expression was obtained in 154 (99%) samples. Two samples (1%) with not enough quality for expression testing were excluded from the analysis.

The expression of 76 genes was significantly associated with TtFT in univariate Cox regression analysis (adjusted p-value controlling for false discovery rate [FDR] <0.05), and 88 with a FDR <0.1. A total of 46 genes (FDR <0.1) met the pre-specified inclusion criteria and were selected for further analysis (see Methods). Among them, a total of 15 genes (*MYC, ITGA4, CERS6, ZNF471, ZNF667, SEPT10P1, ZAP70, LTK, CCL3,*

CNR1, EGR2, TNF, IL4R, FGL2, PPBP) were finally selected for a prognostic model of TtFT using a penalized Cox method. In addition, 15 housekeeping genes were also selected based on their low variance across the samples. A final model, named CLL15, to predict TtFT in the training cohort was developed using the expression of the 15 predictive genes normalized with the 15 housekeeping genes (Figure 1). Then, a linear equation comprising the log transformed normalized gene expression levels of the 15 genes multiplied by their respective regression coefficients was established, and calculated for each patient of the training cohort to obtain the CLL15 score. The C-statistic for the model was 0.77.

Figure 2A shows the shape of association between the CLL15 score and TtFT risk after relaxing the linearity assumption for continuous variables. As a continuous variable, the CLL15 assay score was associated with TtFT (HR: 2.83, 95%CI 2.17-3.68; p <0.001). To better stratify the risk of progression, optimal thresholds for defining 3 groups with differentiated outcomes (TtFT) were determined using the R *partykit* package. The low-risk group (score ≤2.718, comprising 55% of the cohort), had a 5-year estimated risk of treatment initiation of 30.5%. In the intermediate-risk group (score ≤3.535 and >2.718, comprising 20% of the cohort) the 5-year estimated risk of treatment initiation was 57.8% (HR 2.67, [95%CI 1.39-5.10]; p= 0.003). Finally, in the high-risk group (score >3.535, comprising 25% of the cohort) the 5-year estimated risk of treatment initiation was 93.4% (HR 10.9, [95% CI 6.12-19.3]; p <0.001) (Figure 2B). Notably, the CLL15 score exhibited similar prognostic capacity in the subgroup of patients with early clinical stage (n=116) with a 5-year estimated risk of treatment

initiation of 18.2%, 44.8%, and 79.54%, in the low, intermediate and high-risk groups, respectively (Figure 2C).

The prognostic value of the CLL15 score is independent of the IGHV mutational status, and of the International Prognostic Score for Asymptomatic Early-stage (IPS-E) CLL

We analyzed the association between the progression risk groups obtained by the CLL15 assay with known biological prognostic factors in CLL, including the most common chromosomal alterations determined by FISH (del17p, del11q and trisomy 12), the level of protein expression of ZAP-70 and CD38 determined by flow cytometry, the mutations of *TP53*, *NOTCH1*, *SF3B1 and MYD88 genes,* the mutational status of *IGHV,* the International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI) and the IPS-E CLL score.

In the univariate analysis, several factors such as the *SF3B1* mutations, *IGHV* status, the expression of ZAP-70 and CD38 by flow cytometry, clinical stage (RAI and Binet), the CLL-IPI and the IPS-E score were associated with TtFT (Figure 3). In the final multivariate analysis, the CLL15 score, the IPS-E CLL and Binet stage were the only factors that maintained their independent statistical significance (Figure 3).

We next explored the introduction of the mutational status of the *IGHV* (mutated/ unmutated) as a variable in the expression model, comparing its performance with the previous model of only gene expression. The C-statistic for the combined model was 0.79 and the analysis of deviance showed that the addition of *IGHV* status to

the gene expression score (and vice versa) provides significant predictive information (analysis of deviance p <0.001). According to these results, the model combining gene expression with the *IGHV* variable performed better in predicting TtFT than the models of gene expression and *IGHV* by themselves. In the pairwise multivariate Cox models, both variables, the *IGHV* mutational status and the categorized groups of progression risk according to the gene expression model did contribute prognostically (Figure 2D and Supplementary Table 4).

The inclusion of the CLL15 score also improved the capacity to predict TtFT of the IPS-E score. Figure 4A shows the increment in discrimination capacity in terms of C-statistic when CLL15 score was included in the model concurrently with the IPS-E score or *IGHV* status. Moreover, in pairwise multivariate Cox models the CLL-IPI and the CLL15 also independently contributed to TTfT in the training cohort, with a C-statistic for the CLL-IPI alone of 0.73, and of 0.81 for the combination. However, when the IPS-E score was included, the information of the CLL-IPI did not improve the model (Supplementary Table 4). Finally, i) CLL15 score, ii) *IGHV* status, and iii) IPS-E score are all independent factors that improve prediction of TtFT (all analysis of deviance pairwise comparison p <0.01) (Figure 4B).

Validation and reproducibility of the CLL15 assay

The CLL15 assay was then validated in cryopreserved samples from 112 patients from an independent cohort from Heidelberg (Supplemental Table S5). As a continuous

283 variable, the CLL15 score was significantly associated with TtFT (HR 1.71, [95%CI 1.15-
284 2.52]; p= 0.007). Figure 5A shows the association between CLL15 score and TtFT risk
285 after relaxing the linearity assumption in the validation cohort. Using the pre-
286 established cut-off in the training cohort, the assay assigned 22 (19.6%) patients to the
287 low-risk group, 42 (37.5%) to intermediate-risk and 48 (42.9%) to the high-risk group.
288 These three groups presented differentiated outcomes with 60-months estimated risk
289 of treatment initiation of 16.5%, 40% and 58.1% in the low, intermediate, and high-risk
290 groups, respectively (p= 0.03 overall log-rank test, Fig 5B). Moreover, as observed in the
291 training cohort, the gene expression information both as a continuous variable and as a
292 risk-group was an independent prognostic factor in the presence of *IGHV* mutational
293 status (Supplemental Table S6). The C-statistic for the IGHV mutational status and for
294 the gene expression model was 0.6 and 0.63, respectively, while the C-statistic for the
295 combined model was 0.67. As observed in the training cohort, three risk groups were
296 identified after combining the CLL15 score and the *IGHV* mutational status information
297 (Supplemental Figure S3).To determine the reproducibility of the CLL15 assay, we
298 selected 9 samples with scores distributed across the assay (low risk, intermediate, and
299 high risk). The RNA from each of these samples was run on the CLL15 assay in triplicate,
300 with each run performed on a different NanoString cartridge. The results showed 100%
301 concordance of risk group assignment across the triplicates (Supplemental Figure S4),
302 with a standard deviation of 0.073 points.
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Discussion

In this study we translated a gene expression prognostic signature composed by genes involved in the microenvironment activation and *IGHV* mutational status into a test applicable to categorize patients into differentiated risk of progression and requiring treatment for their CLL. The assay demonstrated the ability to identify patients with a high risk of requiring treatment in a short time or with an extremely stable disease.

On the basis of the enormous advances in the biology and treatment of CLL, classical staging systems have been complemented by a plethora of new prognostic parameters based on CLL genetics and biology, including gene expression profiles.(14, 15, 37, 38) In spite that gene expression profiles have been strongly correlated with the clinical course of the patients,(7, 8, 17-26) their translational value in clinical practice has been difficult to implement due to methodological reasons. The recent advent of new platforms such as the NanoString nCounter, capable of a digital, direct quantification on a real-time basis for individual patients, allows the attainment of gene expression analysis in a clinical setting.(27, 28) In this regard, we demonstrated the clinical strength and reproducibility of the CLL15 assay in an independent cohort of previously untreated patients with CLL, and its analytical reproducibility by showing a very low variability across repeated measurements.

Several *in vitro* and *in vivo* data indicate that CLL is a malignancy highly dependent on microenvironment signals for survival and proliferation, with BCR signaling being the

most prominent pathway activated in CLL cells isolated from lymph nodes.(25) The role of the microenvironment in CLL pathogenesis has been reinforced when molecules targeting CLL-microenvironment interactions have shown unprecedented therapeutic results.(30, 31) The CLL15 assay included genes coding for cytokines, chemokines and cytokines receptors, such as *CCL3*, *TNF*, *PPBP* and *IL4R*, integrins, such as *ITGA4*, and transcription regulatory factors, such as *MYC* and *EGR2*, which are involved in microenvironment activation in different studies in CLL.(23-26, 39-41) In addition, genes previously reported to be differentially expressed according to the *IGHV* mutational status, including *CERS6*, *CNR1*, *FGL2*, *LTK*, *SEPT10P1*, *ZAP70*, *ZNF471*, and *ZNF667,* were also selected in the CLL15 assay.(17, 18, 24, 26, 41-44) Of note, the levels of expression of the aforementioned genes could also be regulated in microenvironment activation processes.(18, 25, 45) Thus, *ZAP-70* expression has been associated with enhanced and prolonged BCR signaling,(46, 47) higher responsiveness to chemokines [56–58], and enhanced migration of CLL cells (48, 49), reinforcing the notion that increased *ZAP-70* expression is associated with a more aggressive clinical course of patients with CLL.(37) (50, 51)

It is worth mentioning that the CLL15 signature kept its predictive value independently of the *IGHV* mutational status, the CLL-IPI and the IPS-E CLL score. More importantly, the inclusion of the CLL15 score improved the discrimination capacity to predict TtFT when *IGHV* or *IPS-E* were included in the model, suggesting that the CLL15 signature could complement the prognostic value of these other variables. In addition, the combination of the CLL15 and the CLL-IPI had independent predictive information,

but with the inclusion of the IPS-E score, the information of the CLL-IPI did not contribute prognostically to the model. In this sense, the combination of the IPS-E and the CLL15 assay was highly discriminative for TtFT with a C-statistics of 0.85. It appears that combining a more clinical-based score as the IPS-E with a molecular score (CLL15) could increase the accuracy of both models. Unfortunately, the IPS-E score was not available for the validation cohort and this comparison could not be validated in this cohort.

On the other hand, the combination of IGHV and CLL15 also improved the predictive capacity of the model. Three clearly different risk groups were identified after combining CLL15 and IGHV status. However, a limited improvement of the C-statistic was observed and the lower statistical power in the validation cohort did not allow for validating all the findings.

Nowadays, one of the moving fields is the possibility of early treatment of patients at early stages that are likely to progress within a short period of time.(5) The selection of these patients is usually based on standard prognostic scores. The usage of more accurate methods for prognostication, such as the CLL15 score, should allow for better identification of patients with an increased risk of early progression and thus support future trials based on risk-adapted therapeutic intervention.

In conclusion, biological prognostication in CLL relies on the use of genetic aberrations together with the mutational status of *IGHV*. Unfortunately, the use of gene expression profiles has been difficult owing to its technical difficulties and reproducibility, precluding its penetration in clinical practice. The employment of newer

and more reproducible methods to assess gene expression could round off well-established prognostic parameters, appraising the entire biological profile in the prognostication of patients with CLL. The study presented herein successfully translates previously described gene expression signatures with strong prognostic value into a new gene expression-based assay, the CLL15, applicable in the routine diagnostic setting.

Authors' Disclosures

P.A. has received honoraria from Janssen, Roche, Celgene, Abbie and Astrazeneca. G.V has received research honoraria for speaker activities from MSD and advisory role from Astrazeneca. M.A has received honoraria for speaker activities from Astrazeneca, advisory role from Janssen and non-financial support from Janssen and Abbvie. AMN. has received honoraria from Janssen, Roche, Takeda, Gilead, Abbvie, Celgene for speaker activities and from Janssen, Takeda, Gilead, Kiowa Kirin, Astra-Zeneca and Beigene for participating in advisory boards. M.C. has received research funding from, Janssen, Roche and AstraZeneca. F.B has received honoraria and research grants from Roche, Celgene, Takeda, AstraZeneca, Novartis, AbbVie, Lilly, Beigene, and Janssen.

All remaining authors have declared no conflicts of interest.

Authors' contributions

Designed research and supervised the work: PA, TZ, MC and FB. Provided samples: PA, MC, JL, TZ, MA, MG, GI, SB, AMN. Performed experiments: DM, JC, JB, BTV. Analyzed and interpreted data: PA, GV, MC, FB. Wrote the manuscript: PA, GV, MC, FB. Revised the manuscript: all authors.

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	Total Cohort (n=154)	CLL15 categories			*P* value
		Low-Risk Group (n=85)	Intermediate-Risk Group (n=31)	High-Risk Group (n=38)	
Male	88 (57.1%)	47 (55.3%)	16 (51.6%)	25 (65.8%)	0.435
Female	66 (42.9%)	38 (44.7%)	15 (48.4%)	13 (34.2%)	
Age – median (range) years	70 (34 – 91)	72 (34 – 91)	69 (46 – 91)	64 (44 – 85)	0.05
Binet stage					
A	116 (76.3%)	79 (92.9%)	24 (80%)	13 (35.1%)	<0.01
B	27 (17.8%)	6 (7.1%)	4 (13.3%)	17 (45.9%)	
C	9 (5.9%)	0	2 (6.7%)	7 (18.9%)	
Missing	2	-	1	1	
Lymphocyte cell count,10^9/L – median (range)	16.8 (3.2 – 323)	16.8 (3.2 – 238)	15.6 (4.2 – 323)	22.1 (7.9 – 207.4)	0.18
Missing	38	3	7	28	
β2-microglobulin					<0.01
≤ 3.5 mg/dL	108 (74%)	68 (81.9%)	24 (85.7%)	16 (45.7%)	
> 3.5 mg/dL	38 (26%)	15 (18.1%)	4 (14.3%)	19 (54.3%)	
Missing	8	2	3	3	
CLL-IPI					<0.01
Low (0-1)	54 (43.9%)	38 (56.7%)	11 (42.3%)	5 (16.7%)	
Intermediate (2-3)	32 (20.8%)	16 (23.9%)	9 (34.6%)	7 (23.3%)	
High (4-6)	31 (20.1%)	12 (17.9%)	5 (19.2%)	14 (46.7%)	
Very high (7-10)	6 (3.9%)	1 (1.5%)	1 (3.8%)	4 (13.3%)	
Missing	31	18	5	8	
CLL IPS-E					0.785
Low (0)	24 (27.3%)	16 (25.4%)	7 (36.8%)	1 (16.7%)	
Intermediate (1)	44 (50%)	32 (50.8%)	9 (47.4%)	3 (50%)	
High (2-3)	20 (22.7%)	15 (23.8%)	3 (15.8%)	2 (33.3%)	
Missing	28	16	5	7	
IGHV mutational status					<0.01
Mutated	90 (62.5%)	58 (74.4%)	22 (71%)	10 (28.6%)	
Unmutated	54 (37.5%)	20 (25.6%)	9 (29%)	25 (71.4%)	
Undetermined	9	7	-	2	
Missing	1	-	-	1	
ZAP-70					0.121
<20%	88 (74.6%)	54 (78.3%)	20 (80%)	14 (58.3%)	
≥20%	30 (25.4%)	15 (21.7%)	5 (20%)	10 (41.7%)	
Missing	36	16	6	14	
CD38					0.011
<30%	117 (84.2%)	69 (90.8%)	24 (85.7%)	24 (68.6%)	
≥30%	22 (15.8%)	7 (9.2%)	4 (14.3%)	11(31.4%)	

Missing		15		9		3		3	
FISH analysis									
17 deletion		11 (7.9%)		6 (8.1%)		3 (10.3%)		2 (5.4%)	
11q deletion		14 (10%)		8 (10.8%)		3 (10.3%)		3 (8.1%)	
13q deletion		77 (55%)		38 (51.4%)		21 (72.4%)		18 (48.6%)	
Trisomy 12		26 (18.6%)		18 (24.3%)		5 (17.2%)		3 (8.1%)	
Missing		14		11		2		1	
Complex karyotype (≥3 abnormalities)									0.877
No		63 (90%)		42 (89.4%)		14 (93.3%)		7 (87.5%)	
Yes		7 (10%)		5 (10.6%)		1 (6.7%)		1 (12.5%)	
Missing		84		38		16		30	
TP53 mut									0.009
No		92 (92%)		61 (98.4%)		20 (83.3%)		11 (78.6%)	
Yes		8 (8%)		1 (1.6%)		4 (16.7%)		3 (21.4%)	
Missing		54		23		7		24	
NOTCH1 mut									0.351
No		86 (82.7%)		49 (79%)		23 (92%)		14 (82.4%)	
Yes		18 (17.3%)		13 (21%)		2 (8%)		3 (17.6%)	
Missing		50		23		6		21	
SF3B1 mut									0.066
No		92 (94.8%)		61 (98.4%)		21 (91.3%)		10 (83.3%)	
Yes		5 (5.2%)		1 (1.6%)		2 (8.7%)		2 (16.7%)	
Missing		57		23		8		26	
MYD88 mut									0.395
No		85 (94.6%)		60 (96.8%)		21 (91.3%)		7 (87.5%)	
Yes		5 (5.4%)		2 (3.2%)		2 (8.7%)		1 (12.5%)	
Missing		61		23		8		30	
Median follow up - months		43.8 m		43.6 m		43.8 m		45.9 m	0.61

Table 1: Patient and disease characteristics of the training cohort

Abbreviations: CLL-IPI: International Prognostic Index for Chronic Lymphocytic Leukemia; CLL IPS-E: International Prognostic Score for Asymptomatic Early-stage Chronic Lymphocytic Leukemia; *P* values are for comparisons across the 3 risk groups determined by the CLL15 score.

Figure Legends:

Figure 1. The gene expression-based model to predict Time to First Treatment in patients with chronic lymphocytic leukemia. Heatmap of the CLL15 assay with the 15 informative genes shown as rows and the 154 patient samples as columns. The three patient groups identified by the assay are shown below the heatmap together with the mutational status of the *IGHV* genes.

Figure 2. (A) Log relative hazard according to the CLL15 score. (B) Kaplan-Meier curves of the Time to First Treatment of the three patient groups identified by the CLL15 assay. (C) Kaplan-Meier curves of the Time to First Treatment of the three patient groups identified by the CLL15 assay in the subgroup of patients with early-stage disease (Binet A 0/I). (D) Kaplan-Meier curves of the Time to First Treatment according to CLL15 assay and *IGHV* mutational status.

Figure 3. Univariate and multivariate analysis for Time to First Treatment according to prognostic factors in CLL.

Figure 4. (A) Discrimination capacity in terms of C-statistic according to models including CLL15 score, *IGHV* mutational status, and IPS-E CLL score. (B) ANOVA pairwise comparisons.

Figure 5. (A) Log relative hazard according to the CLL15 score in the validation cohort. (B) Kaplan-Meier curves of the Time to First Treatment of the three patient groups in the validation cohort identified by the CLL15 assay.

Figure 1

Figure

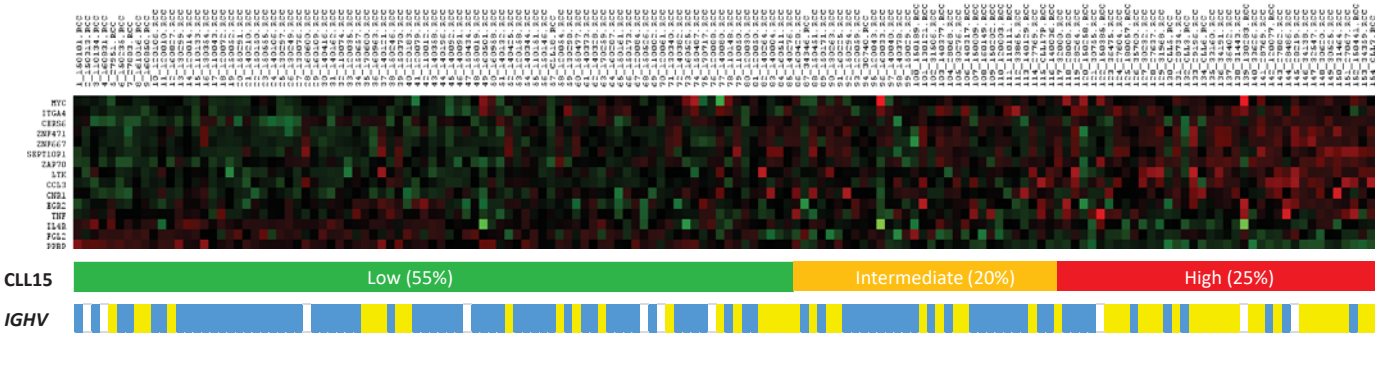
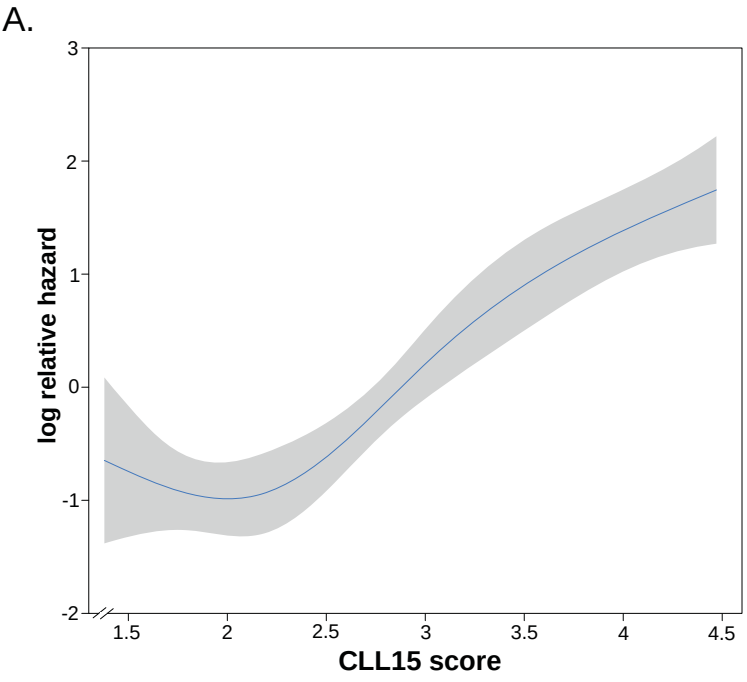
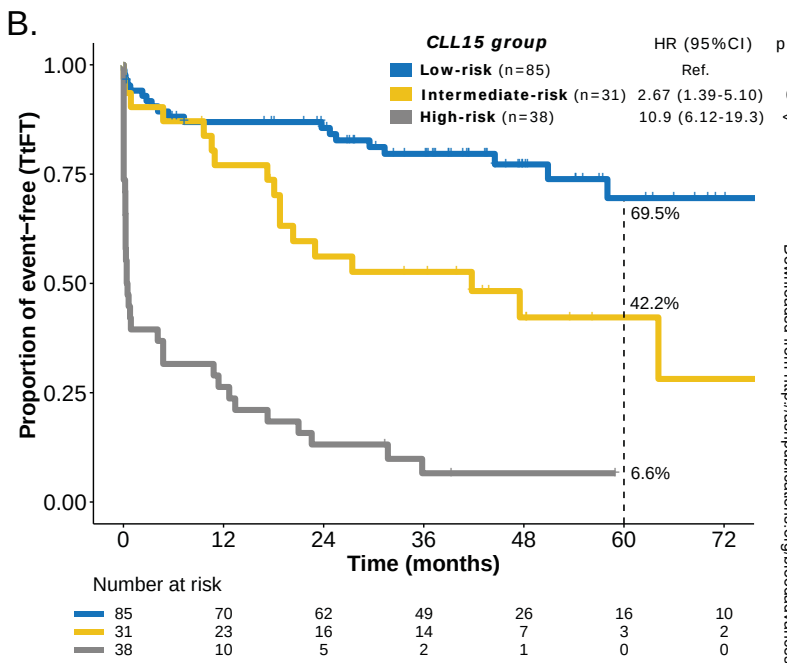
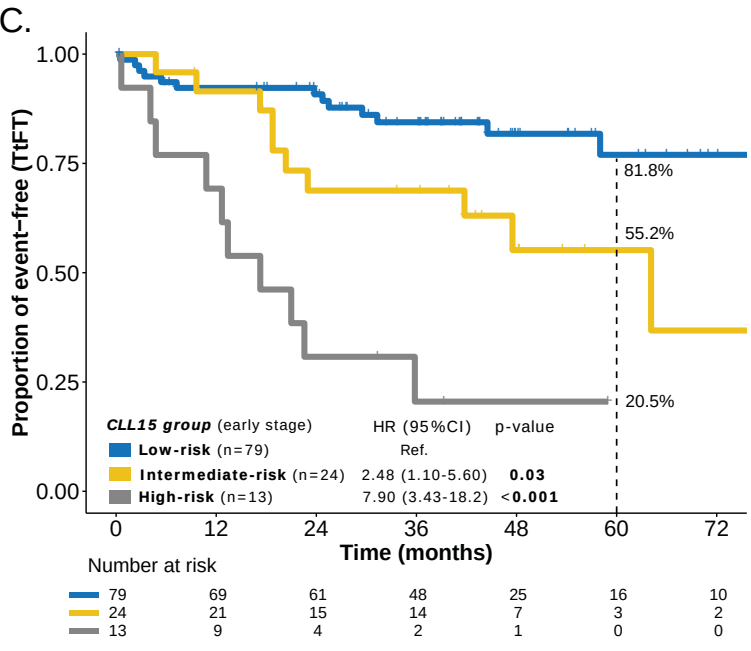


Figure 2







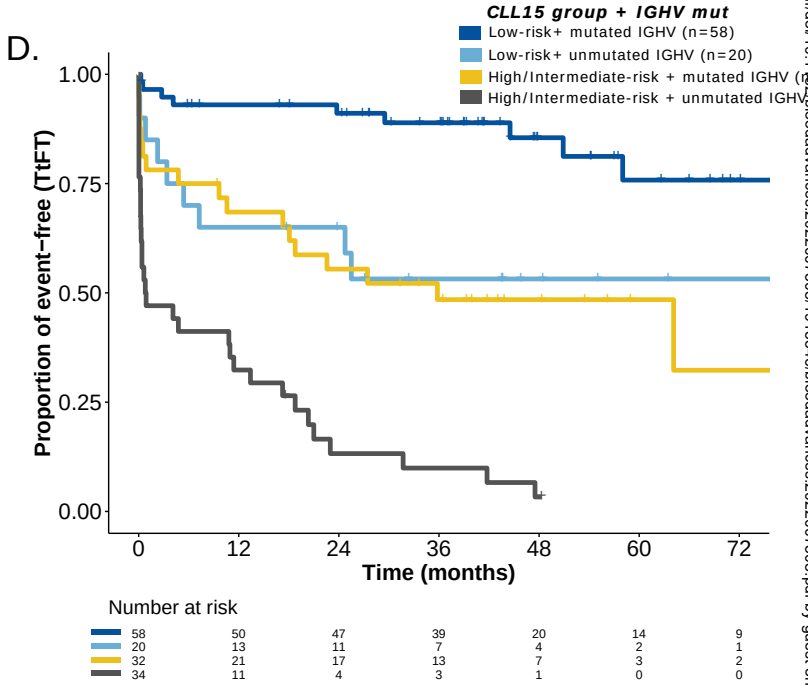


Figure 3

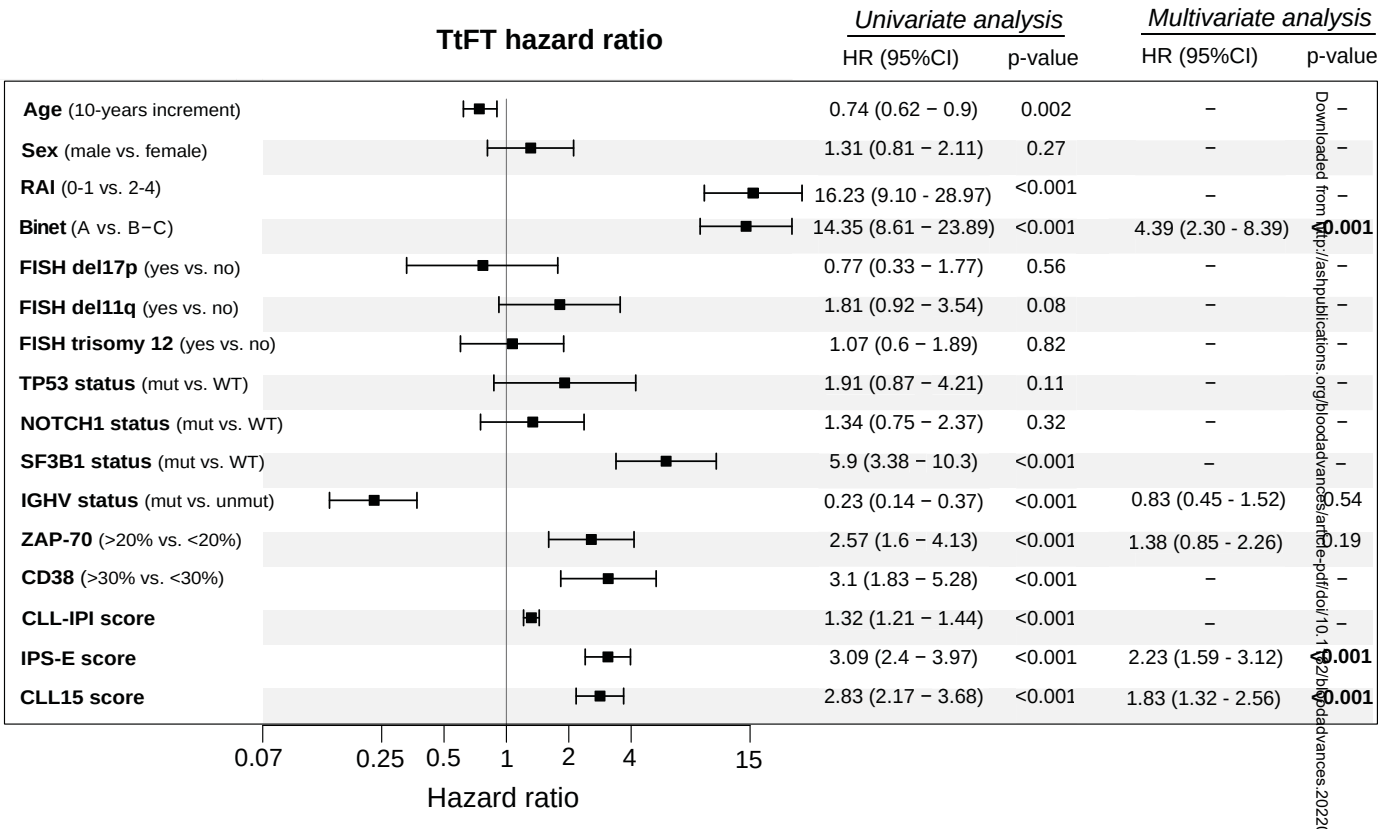
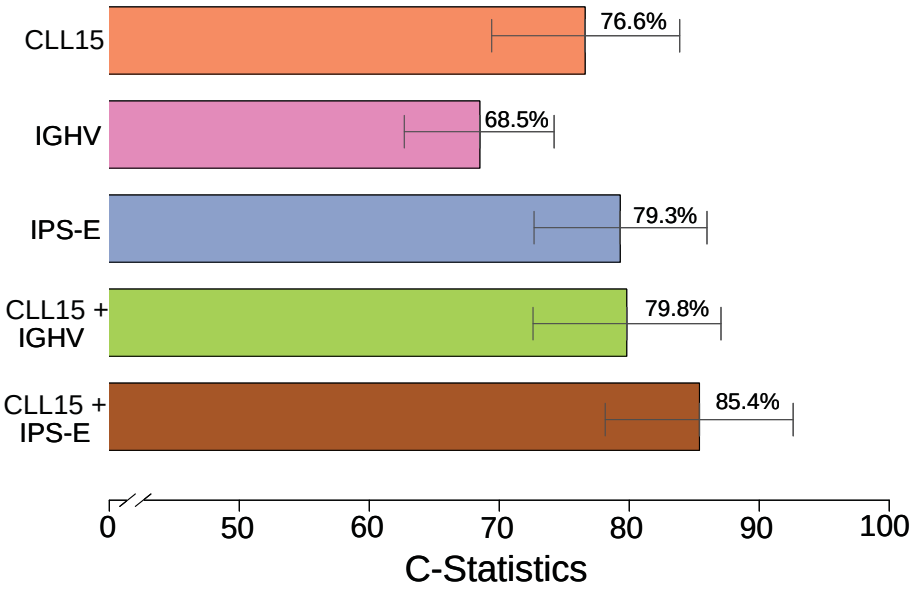


Figure 4

A.



B.

Analysis of deviance *(p-values)*			
-		*Complex model*	
		CLL15 score + IGHV status	CLL15 score + IPS-E score
Simple model	IGHV status	**<0.001**	X
	IPS-E score	X	**<0.001**
	CLL15 score	**0.003**	**<0.001**

Figure 5