

# *IGHV*-associated methylation signatures more accurately predict clinical outcomes of chronic lymphocytic leukemia patients than *IGHV* mutation load



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## ABSTRACT

Currently, no molecular biomarker indices are used in standard care to make treatment decisions at diagnosis of chronic lymphocytic leukemia (CLL). We used Infinium MethylationEPIC array data from diagnostic blood samples of 114 CLL patients and developed a procedure to stratify patients based on methylation signatures associated with mutation load of the *IGHV* gene. This procedure allowed us to predict the time to treatment with a hazard ratio (HR) of 8.34 (95% confidence interval [CI]: 4.54-15.30), as opposed to a HR of 4.35 (95% CI: 2.60-7.28) using *IGHV* mutation status. Detailed evaluation of 17 cases for which the two classification procedures gave discrepant results showed that these cases were incorrectly classified using *IGHV* status. Moreover, methylation-based classification stratified patients with different overall survival (HR=1.82; 95% CI: 1.07-3.09), which was not possible using *IGHV* status. Furthermore, we assessed the performance of the developed classification procedure using published HumanMethylation450 array data for 159 patients for whom information on time to treatment, overall survival and relapse was available. Despite 450K array methylation data not containing all the biomarkers used in our classification procedure, methylation signatures again stratified patients with significantly better accuracy than did *IGHV* mutation load regarding all available clinical outcomes. Thus, stratification using *IGHV*-associated methylation signatures may provide better prognostic power than *IGHV* mutation status.

## Introduction

Most patients diagnosed with chronic lymphocytic leukemia (CLL) have asymptomatic, early-stage disease at the time of diagnosis but the subsequent disease course is highly variable, with some patients experiencing early progression and others living for many years with indolent disease.<sup>1</sup> Immediate treatment after diagnosis does not seem to improve patients' survival.<sup>2-5</sup> Consequently, to reduce

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unnecessary harmful complications following therapy, the majority of CLL patients are managed with a “watch and wait” strategy,<sup>6</sup> and treatment is only initiated at disease progression. This is assessed according to clinical symptoms defined by the Rai and Binet staging systems.<sup>7-9</sup> However, with the advent of new therapies it is well-recognized that some patients can potentially benefit from earlier intervention.<sup>9</sup>

Molecular biomarker-based indices, as opposed to clinical staging, are likely to reflect the complex biology of CLL and, therefore, predict patients’ outcomes more accurately.<sup>10</sup> However, the development of biomarker-based indices in CLL is still ongoing. Recent large multicenter studies, investigating the prognostic power of various known molecular and clinical biomarkers, have proposed two new biomarker indices: the International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI) and the International Prognostic Score for Early-stage CLL (IPS-E).<sup>11,12</sup> The CLL-IPI index is based on *TP53* aberrations, *IGHV* mutation status,  $\beta$ 2-microglobulin concentration, clinical Rai/Binet stage, and age, with *TP53* aberrations predicting overall survival (OS) most accurately in multivariable modeling.<sup>11</sup> However, lesions affecting the *TP53* locus are rather rare and other studies have shown that *IGHV*-mutated patients with *TP53* locus aberrations experience a rather indolent disease course.<sup>13-15</sup>

The IPS-E index was developed for early-stage patients with asymptomatic disease and time to first treatment (TTFT) as a primary outcome.<sup>12</sup> This index includes *IGHV* status, absolute lymphocyte count and palpable lymph nodes. *TP53* status did not show independent prognostic power in this index, which indicates that this biomarker may provide no clinical relevance for predicting TTFT for early-stage patients.

In both of the above indices, stratification of patients into mutated (M-CLL) or unmutated (U-CLL), according to *IGHV* mutation load, plays a central role.<sup>16</sup>

It is well-established that the CLL methylome reflects, to a large extent, the natural history of the B cell.<sup>17-20</sup> Recent studies have also shown that the CLL methylome can guide the stratification of patients experiencing different clinical outcomes both at diagnosis<sup>18,19,21</sup> and in clinical trials.<sup>20</sup> Specifically, Kulis *et al.* and, subsequently, Queirós *et al.* have shown that methylation signatures can stratify CLL patients into three groups experiencing different clinical outcomes: the n-CLL (naïve B-cell-like CLL), i-CLL (intermediate CLL), and m-CLL (memory B-cell-like CLL) subgroups.<sup>18,21</sup> The identified methylation signatures were closely related to *IGHV* mutation status, with the n-CLL and m-CLL subgroups consisting mainly of U-CLL patients and M-CLL patients, respectively. The new i-CLL subgroup included borderline M-CLL and U-CLL patients, as they were found to display both an intermediate load of mutations in the *IGHV* gene and intermediate clinical outcomes.<sup>18,21</sup> Further studies of i-CLL patients have shown that certain molecular features are enriched in this group of patients, such as poor-prognostic subset #2 characteristics.<sup>21,22</sup> The subset #2 i-CLL cases seem to constitute an aggressive subgroup of i-CLL with clinical prognosis resembling the prognosis of n-CLL patients.<sup>22</sup> Thus, the diagnostic utility of this classification needs to be studied further.

The above findings clearly indicate that methylation signatures of CLL cells are largely associated with the mutation load of the *IGHV* gene and that they have prog-

nostic significance. In this study, we developed a procedure for classifying patients based on methylation changes associated with *IGHV* mutation load, comparing the prognostic power of this classification procedure to predict clinical outcomes with that of patients’ stratification based on *IGHV* mutation load alone.

## Methods

### Clinical material

Our cohort of patients has already been described;<sup>23,24</sup> the patients’ clinicobiological characteristics are summarized in Table 1 (see *Online Supplementary File, Patient Cohort Section*). The Ethics Committee of the Region of Southern Denmark approved the study (approval number: S-20100128).

### Genome-wide DNA methylation analysis

To assess genome-wide DNA methylation, we analyzed 400 ng of DNA with the Illumina Infinium MethylationEPIC Beadchip (EPIC) array. Raw data were processed in R using the RnBeads package<sup>25</sup> with default filtering settings including the removal of probes, which were: (i) outside CpG context; (ii) overlapping single-nucleotide polymorphisms; (iii) targeting sex chromosomes; (iv) missing  $\beta$ -values; (v) showing a standard deviation of  $\beta$ -values  $<0.005$ ; and (vi) cross-reactive probes.<sup>26</sup>  $\beta$ -values were normalized using the BMIQ method<sup>27</sup> followed by noob background correction.<sup>28</sup> We assessed the sample purity using the methylomic data,<sup>19</sup> and included only patients’ samples with at least 85% B cells ( $n=114$ ) to limit the impact of cell-type composition.

### Bioinformatic and statistical analyses

Bioinformatic and statistical analyses were performed in R version 3.6.1, Stata/SE 15.0 (StataCorp, TX, USA), and QluCore Omics Explorer 3.4 (QluCore, Lund, Sweden). We used linear regression to test the association between methylation levels at individual CpG loci ( $\beta$ -values) and mutation load of *IGHV* (as percentage identity to germline sequence to avoid specific cut-off<sup>29</sup>) for a total of 671,684 CpG, using  $P<10^{-8}$  as recommended for methylomic studies.<sup>30</sup> Only CpG with qualitative methylation changes defined as an interquartile range of minimum 0.80 were included in subsequent analysis (*Online Supplementary File, Section 1*).

The primary clinical endpoint used to develop the classification procedure was time to treatment (TTT). CpG with methylation levels associated with TTT were selected using Cox regression with the significance threshold of  $P<10^{-7}$ ; this was chosen to identify the most associated CpG and to control for false-positive results. CpG independently associated with TTT were identified in a multivariable Cox regression model using a backward elimination procedure with  $P<0.05$ . Classification of *IGHV* mutation load (*IGHV* status) into mutated (M-CLL) and unmutated (U-CLL) was based on 98% identity cutoff to the germline sequence.<sup>16</sup> The strength of association between two classification methods was quantified by the odds ratio (OR) using Woolf approximation to calculate 95% confidence intervals (CI).

Secondary clinical endpoints were OS and relapse.<sup>31</sup> The prognostic accuracy of a classification method in predicting the clinical outcomes was evaluated using hazard ratios (HR) from univariate and multivariable Cox regression models, and by Kaplan-Meier plots combined with log-rank tests and estimation of median time to event. The Cox regression model assumptions were tested using Schoenfeld residuals, and  $P$  values  $<0.05$  were considered as statistically significant results.

### Validation of EPIC microarray data with methylation-sensitive high resolution melting

The microarray data were validated using methylation-sensitive high-resolution melting.<sup>32</sup> The details of the assay design can be found in *Online Supplementary Methods, Section 2*.

### Stratification of patients using IGHV-associated methylation signatures from 450K data

We used data from an independent CLL cohort (n=159)<sup>33</sup> previously published by Kulis *et al.*<sup>18</sup> and Queirós *et al.*<sup>21</sup> to test whether HumanMethylation450 BeadChip (450K) data are sufficient to stratify patients using our procedure.

## Results

### Identification of methylation signatures that independently predict short time to treatment

To investigate whether IGHV-associated methylation signatures can more accurately classify patients with aggressive disease at diagnosis than IGHV mutation status, we first used linear regression and identified 4,518 sites (CpG) in the EPIC array dataset at which the methylation levels ( $\beta$ -values) were associated with the IGHV mutation load (Figure 1A). Due to both technical and biological limitations of quantitative methylation measurements in clinical material (for a detailed description, see *Online Supplementary File, Section 1*), we focused our analysis on 147 sites of the 4518 CpG at which we also observed qualitative methylation changes (defined as an interquartile range of  $\beta$ -values >0.8) (Figure 1B). As TTT was the primary clinical indicator of aggressive disease in our study, we then used Cox regression to identify 44 CpG among these 147 sites at which the level of methylation ( $\beta$ -values) were associated with an increased hazard of short TTT (Figure 1C). Moreover, as biomarkers that independently predict clinical outcomes are most useful in clinical practice, we applied multivariable Cox regression analysis, performed as a backward elimination model, to select CpG sites at which the methylation levels independently predicted TTT (Figure 1D). This analysis resulted in a final set of nine CpG sites with six CpG located in gene bodies of *REPS1* (cg21740960), *RRM12B* (cg00395579), *SMYD3* (cg07395110), *IL1B* (cg07250315), *UBE2R2* (cg02198280), and *ATP9B* (cg21394039); two CpG did not annotate to any known gene (cg03282117 and cg00185137) and one CpG was located in the S-shelf of a CpG island in the *LMBR1* promoter (cg12032915).

### Development of a methylation-based classification procedure

Next, we assessed whether the methylation status of one of the nine selected CpG sites is sufficient to stratify the patients accurately into two groups with different TTT, or whether combining the information from all CpG sites stratifies patients more accurately. A detailed description of these analyses is provided in the *Online Supplementary File, Section 3*. Briefly, we used TTT as the primary outcome and estimated the power of the methylation changes at each CpG site to predict TTT using the HR from the Cox regression analysis. These analyses showed that hypomethylation predicted short TTT for one CpG site (cg07395110), while hypermethylation was associated with short TTT for the remaining CpG (*Online Supplementary Figure S1*). We then compared the HR of the individual CpG sites.

This analysis also showed that methylation status of the individual CpG sites predicted the clinical outcomes of patients with very similar accuracy (*Online Supplementary File, Figures S2 and S3*), and that none of the CpG sites was uniformly informative to predict short TTT (*Online Supplementary Figure S1*). Then, to combine the information from all nine CpG sites, we counted the number of CpG that predicted a short TTT for each patient and compared the HR between groups of patients with a different number of the CpG sites predicting short TTT. We performed this analysis for a series of different  $\beta$ -value cutoffs for individual CpG sites to allow us to establish a  $\beta$ -value cutoff at which the final stratification of patients was most accurate (*Online Supplementary Figures S4 and S5*).

**Table 1. Clinicobiological characteristics of the patients with chronic lymphocytic leukemia.**

Variable	N (%)
Age	114
Median [range], years	71 [49-92]
Age $\leq$ 65 years	37 (32 %)
Age >65 years	77 (68 %)
Sex	114
Male	72 (63 %)
Female	42 (37 %)
Binet stage	114
A	77 (68 %)
B+C	37 (32 %)
ZAP70 expression*	114
Low	67 (59 %)
High	47 (41 %)
CD38 expression <sup>†</sup>	114
Low	84 (74 %)
High	30 (26 %)
Trisomy 12	112
Absent	101 (90 %)
Present	11 (10 %)
Del(11q)	114
Absent	101 (89 %)
Present	13 (11 %)
Del(13q)	113
Absent	55 (49 %)
Present	58 (51 %)
NOTCH1 mutation	114
Absent	110 (96 %)
Present	4 (4 %)
TP53 aberration <sup>‡</sup>	114
Absent	103 (90 %)
Present	11 (10 %)
IGHV status**	114
M-CLL	72 (63 %)
U-CLL	42 (37 %)
Time to treatment	114
Median [range], months	51.3 [0.1-126]
Number of treated patients	64 (56 %)
Overall survival	114
Median [range], months	98.2 [0.4-144]
Median follow-up time [range], months	98.9 (0.4-144)
Number of deceased patients	57 (50 %)

Del: deletion; M-CLL: chronic lymphocytic leukemia with mutated IGHV; U-CLL: chronic lymphocytic leukemia with unmutated IGHV; \* ZAP70 expression is considered to be high when >20% cells are positive; <sup>†</sup>CD38 expression is considered to be high when >30% cells are positive; <sup>‡</sup>including TP53 mutation and del(17p); \*\*germline homology >98 % is considered U-CLL.

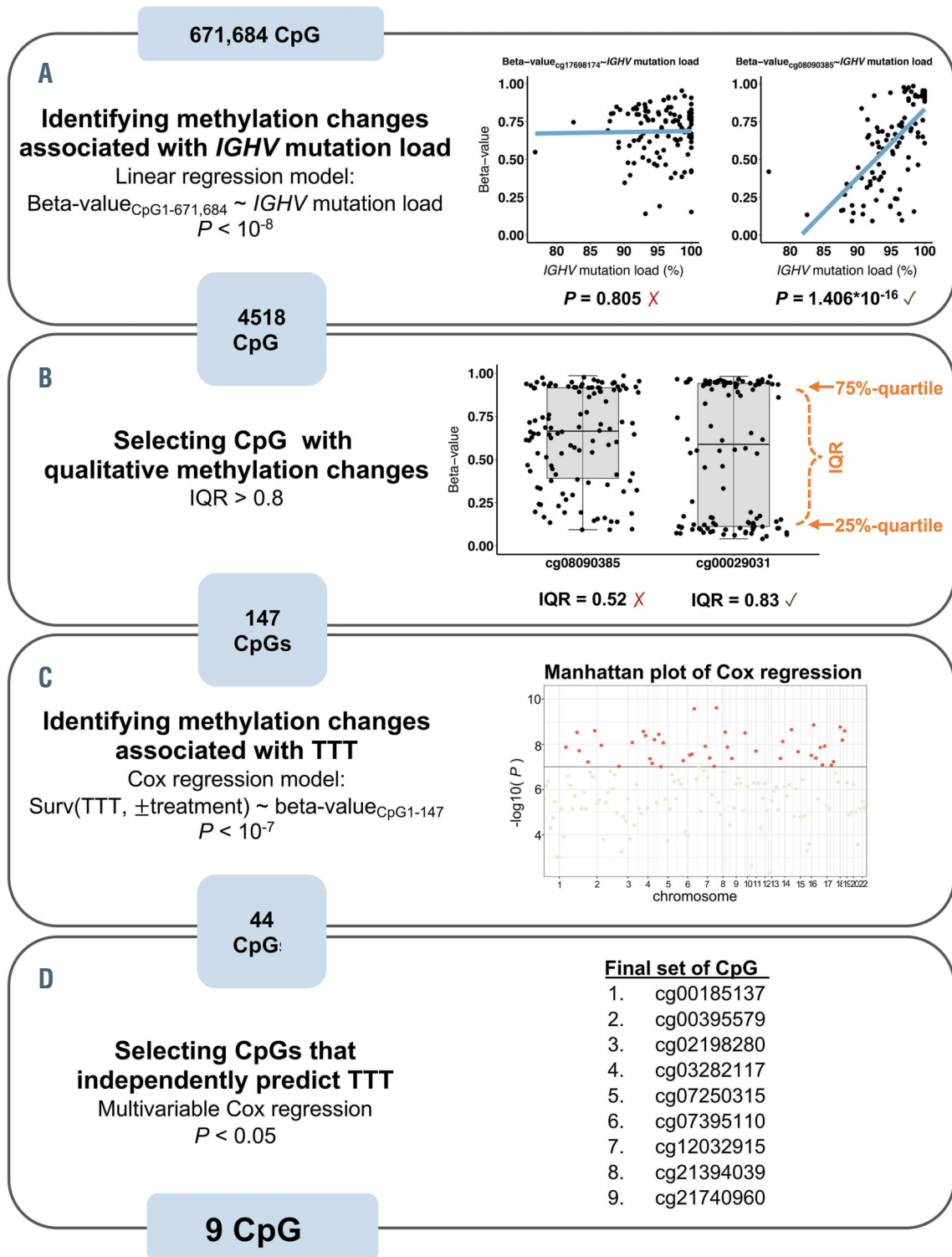


Figure 1. Identification of methylation changes that independently predict short time to treatment. (A) Test of the association between the methylation level (as  $\beta$ -value) with the *IGHV* mutation load (as percentage identity to germline sequence) using a linear regression model for 114 patients and 671 684 CpG sites. Two scatterplots display a non-significant association for cg17698174 (left) and a significant association for cg08090385 (right) between the  $\beta$ -values on the y-axis and *IGHV* mutation load on the x-axis. A total of 4 518 CpG showed significant association between  $\beta$ -values and *IGHV* mutation load using a significance threshold of  $P < 10^{-8}$ . (B) Selection of CpG with qualitative methylation changes. The boxplots display the distribution of methylation at cg08090385 (left) and cg00029031 (right), where each black dot represents a patient ( $n=114$ ) with the  $\beta$ -value for the specific CpG indicated on the y-axis. The box displays the 25-, 50- and 75-percentiles. An interquartile range (IQR)  $> 0.80$  was defined as a qualitative methylation change (*Online Supplementary Methods, Section 1*), and CpG with an IQR  $> 0.80$  were selected for further analyses ( $n=147$ ). (C) Univariate Cox regression analysis of the association between methylation level (as a continuous  $\beta$ -value) and time to treatment (TTT) in 114 patients for 147 CpG. The Manhattan plot displays the significance to predict TTT for each of the 147 CpG (dots), with the  $-\log_{10}(P)$  value from Cox regression analysis on the y-axis against the chromosomal location of the CpG on the x-axis. A total of 44 CpG (red dots) showed a significance level below the threshold of  $P < 10^{-7}$ . (D) Results from the multivariable Cox regression of 44 CpG to identify CpG sites that independently predict TTT performed using backward elimination. A final set of nine CpG sites was identified with a statistical significance of  $P < 0.05$ .



Overall, this data modeling showed that the combination of the information from all nine CpG had a considerably stronger prognostic power to predict TTT than had information from individual CpG sites. Specifically, the stratification for patients displaying two or more CpG sites with methylation status indicating short TTT (poor prognosis) versus patients with none or one CpG site (favorable prognosis) identified patients experiencing short TTT with a HR of 8.34 (95% CI: 4.54-15.30;  $P < 0.001$ ) (Online Supplementary Figure S5b-f). This HR was a clear improvement, as the power to identify patients with short TTT for the individual CpG sites stratified patients with HR ranging from 4.10 (95% CI: 2.46-6.85;  $P < 0.001$ ) to 6.60 (95% CI: 3.76-11.58;  $P < 0.001$ ) (Online Supplementary Figure S3A-I). The overview of the developed classification procedure is shown in Figure 2.

**Methylation-based classification predicts time to treatment with significantly higher accuracy than does IGHV mutation status**

Next, we compared the power to predict TTT of the methylation-based classification with stratification using IGHV mutation status (using the most frequent cutoff at 98% germline identity<sup>16</sup>). In our cohort, the methylation-based classification identified 53 patients with a poor prognosis and median TTT of 13.1 months (95% CI: 4.1-20.1), and 61 patients with a favorable prognosis for whom the median TTT was not reached. At the same time, stratification based on IGHV status identified 42 U-CLL patients with a median TTT of 10.1 months (95% CI: 3.4-21.1) and 72 M-CLL patients for whom the median TTT was not reached. Cox regression analyses showed that the methylation-based classification was significantly more accurate in predicting the need for treatment as described by a HR of 8.34 (95% CI: 4.54-15.30;  $P < 0.001$ ), compared to a HR of 4.35 (95% CI: 2.60-7.28;  $P < 0.001$ ) for IGHV status. This was further corroborated by the Kaplan-Meier analyses shown in Figure 3A, B.

The two stratification methods provided discrepant classifications for 17 patients (Online Supplementary Figure S6). The methylation-based classification predicted a

poor prognosis for 14 M-CLL cases. Those patients, however, experienced a significantly shorter median TTT of 16.2 months (95% CI: 3.9-37.9), than the median TTT of the remaining M-CLL patients (n=58) who did not reach the median TTT ( $P < 0.0001$ ) (Figure 3C, dotted curves). Similarly, the median TTT for the three U-CLL patients predicted to have a favorable prognosis according to the methylation-based classification was 70.0 months (95% CI: 64.7-not reached), and significantly longer than the median TTT of the remaining U-CLL patients (n=39), which was 8.0 months (95% CI: 1.9-20.1;  $P = 0.0188$ ) (Figure 3C, dashed curves). These Kaplan-Meier curves clearly indicate that the methylation-based classification predicted TTT more accurately for the discrepantly classified patients. We further analyzed the IGHV mutation load of the discrepant cases, and found that they displayed an intermediate level of IGHV mutations; this was significantly different and closer to the 98% cutoff than that of the remaining patients with similar IGHV status (Online Supplementary Figure S7). This may indicate a limitation of the IGHV mutation-based stratification of these cases.

**Accuracy of methylation-based classification to predict overall survival**

We then compared the accuracy of the two classifications to predict OS. Overall, 57 out of 114 patients in our study cohort experienced events and the median follow-up time was 98.9 months (95% CI: 94.4-117.6). Cox regression and Kaplan-Meier analyses showed that patients stratified using the methylation-based classification had significantly different OS (Cox regression: HR=1.82; 95% CI: 1.07-3.09;  $P = 0.027$ ; Kaplan-Meier:  $P = 0.0246$ ) (Figure 3D). At the same time, IGHV status-based stratification was not able to identify patients with different OS in our cohort (Cox regression: HR=1.35; 95% CI: 0.80-2.28;  $P = 0.263$ ; Kaplan-Meier:  $P = 0.2608$ ) (Figure 3E). We did not find significant differences in OS for the 17 patients with discrepant classification between the IGHV status- and methylation-based classifications (Figure 3F and Online Supplementary Figure S6). However,

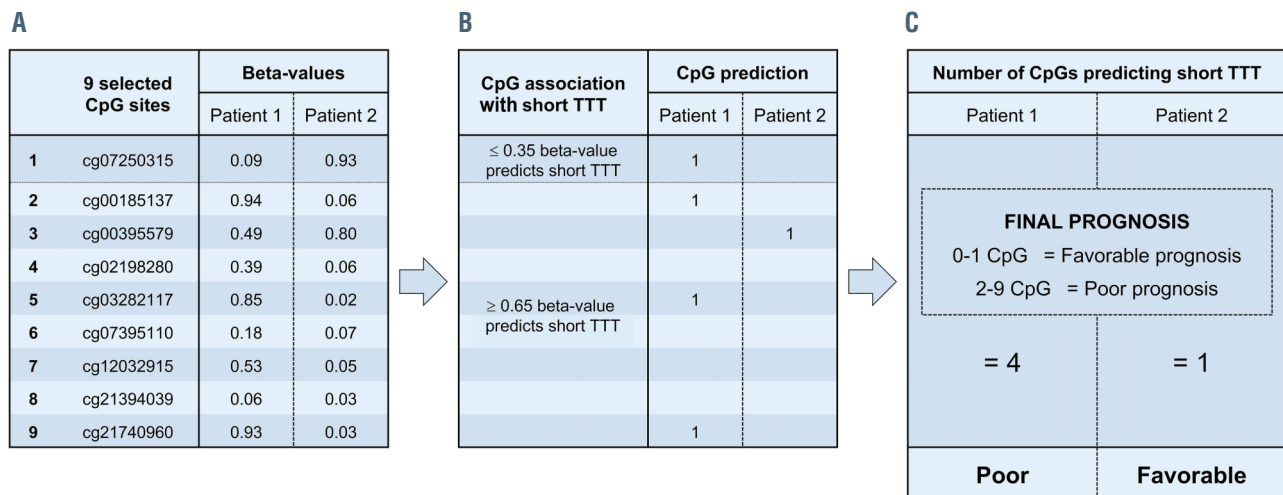


Figure 2. The methylation classification procedure based on the nine selected CpG sites. The final procedure for methylation-based classification of patients into having a favorable or poor prognosis. (A) Methylation levels (β-values) at nine selected CpG sites obtained from the EPIC array for two random patients. (B) Classification of the β-value according to the cutoff as 1 if the β-values predict short time to treatment (TTT). (C) The number of CpG predicting short TTT is counted for each patient, and the patient is classified as having a favorable prognosis if 0-1 CpG predicts a short TTT, or as having a poor prognosis if 2-9 CpG predict a short TTT.

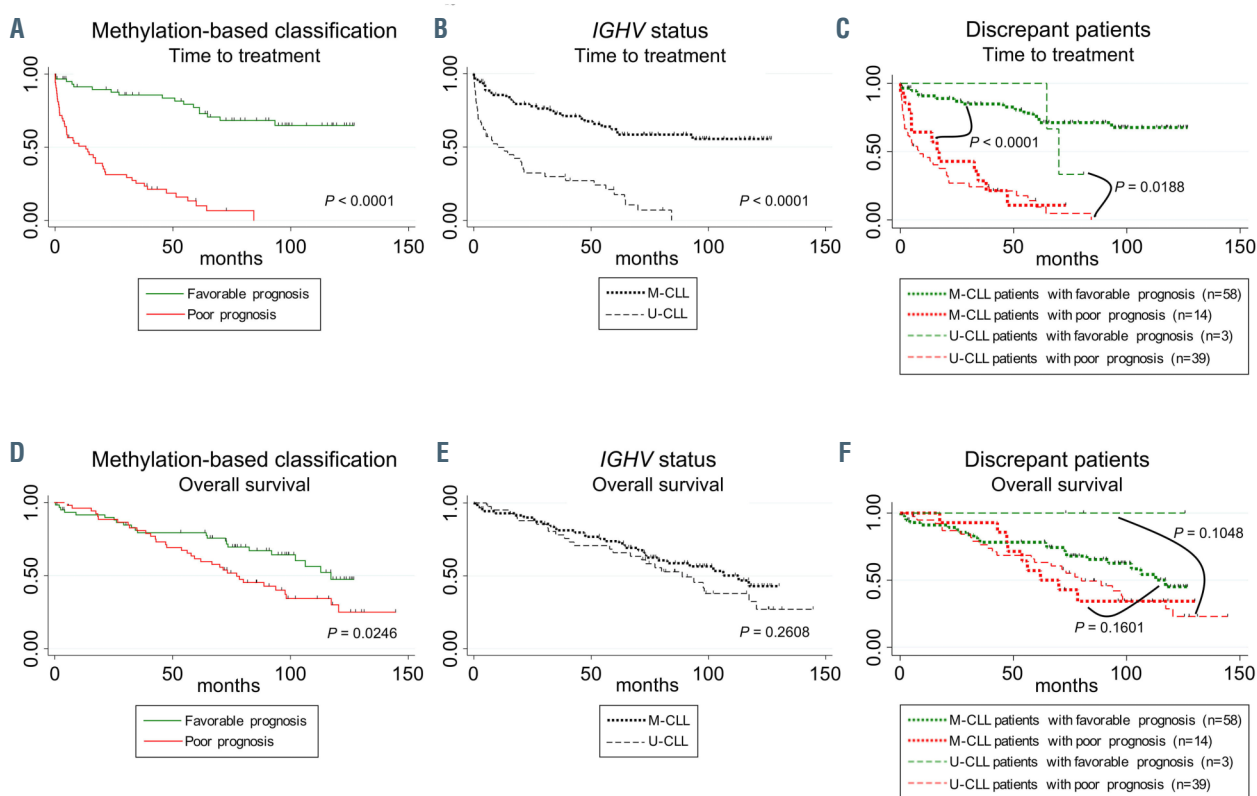
the follow-up time in our cohort was relatively short and an increased number of events is likely needed to increase the power of this analysis.

### Methylation-based stratification of patients from 450K array data

The cohort size available in this study did not allow us to divide patients into discovery and validation cohorts, which would be the most accurate way of assessing the prognostic power of a proposed procedure for stratifying CLL patients. Furthermore, we were not able to identify a publicly-available EPIC array dataset from a similar CLL cohort that could be used to validate our findings. The majority of genome-wide methylation profiling studies in CLL have, so far, been performed using the 450K array; a previous generation of the methylomic microarray. We assessed whether limited data obtained using the 450K BeadChip, which contained only three of the nine CpG sites we used to classify patients (cg00395579, cg12032915, and cg21394039), allow for the accurate stratification of patients according to the classification procedure we developed. The data we used here have been previously published and came from 159 CLL patients with TTT data available for 138 patients (34 events), and OS data for 139 patients (33 events)<sup>18,21</sup>. Relapse data were available for a subset of the patients in this cohort (74 patients/74 events), allowing us to make a

preliminary assessment of the power of methylation-based classification to predict relapse. Even with the limited data available for this cohort, our methylation-based classification procedure was able to stratify patients into two groups with different TTT (*Online Supplementary Figure S8A*) with a similar strength to that observed in our cohort: HR=8.41 (95% CI: 3.74-18.89;  $P<0.001$ ). The methylation classification also stratified patients with different OS with HR=6.03 (95% CI: 2.65-13.73;  $P<0.001$ ), and a different likelihood of relapse with HR=2.38 (95% CI: 1.33-4.25;  $P=0.003$ ) (*Online Supplementary Figure S8B, C*).

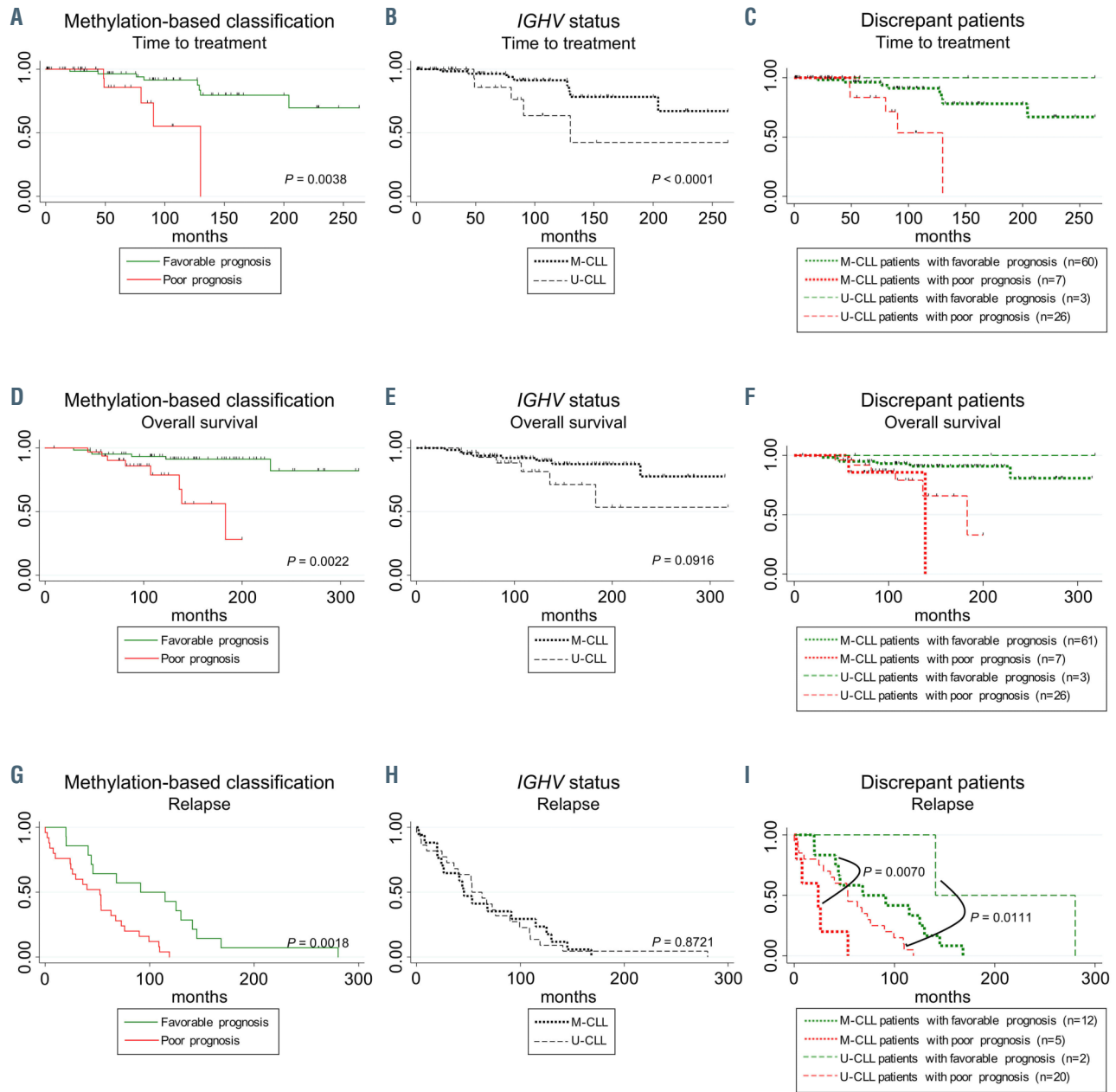
In this cohort, we also compared the performance of the methylation-based classification with that of stratification using *IGHV* status. The methylation-based classification stratified patients (96 patients/13 events) with different TTT with HR=5.20 (95% CI: 1.53-17.71;  $P=0.008$ ) (Figure 4A;  $P=0.0038$ ), as opposed to *IGHV* status which only stratified patients with borderline statistical significance: HR=2.97 (95% CI: 0.96-9.17;  $P=0.059$ ) (Figure 4B;  $P<0.0001$ ). The analysis of OS for patients in this cohort (97 patients/14 events) showed similar results to those observed in our CLL cohort, among whom only the methylation-based classification was able to stratify patients with different OS (HR=5.18; 95% CI: 1.62-16.53;  $P=0.006$ ) (Figure 4D;  $P=0.0022$ ), and *IGHV* status was not informative (HR) 2.46; 95% CI: 0.84-7.24;  $P=0.102$ )



**Figure 3.** Kaplan-Meier analyses of time to treatment and overall survival for the methylation-based classification and *IGHV* status stratifications. (A-C) Kaplan Meier curves describing time to treatment, and (D-F) Kaplan-Meier curves describing overall survival for stratification of patients using methylation-based classification (A and D), *IGHV* status (B and E), or both stratification methods (C and F) in our cohort of patients. In (C and F), the curves represent patients classified according to both stratification procedures: patients with mutated *IGHV* (dotted line) and unmutated *IGHV* (dashed lines) are represented by different colors according to the prediction by the methylation classification into favorable (green) or poor (red) prognosis. The log-rank test for equality was performed between all groups in (C and F), and all P values are listed below: green/dotted curve versus red/dotted curve (C:  $P<0.0001$ ; F:  $P=0.1601$ ); green/dotted curve versus green/dashed curve (C:  $P=0.3698$ ; F:  $P=0.2236$ ); green/dotted curve versus red/dashed curve (C:  $P<0.0001$ ; F:  $P=0.0675$ ); red/dotted curve versus green/dashed curve (C:  $P=0.0719$ ; F:  $P=0.0945$ ); red/dotted curve versus red/dashed curve (C:  $P=0.5284$ ; F:  $P=0.9315$ ); green/dashed curve versus red/dashed curve (C:  $P=0.0188$ ; F:  $P=0.1048$ ). M-CLL: patients with chronic lymphocytic leukemia with mutated *IGHV*; U-CLL: patients with chronic lymphocytic leukemia with unmutated *IGHV*.

(Figure 4E;  $P=0.0916$ ). Moreover, despite a limited number of patients with available *IGHV* status and relapse data (39 patients/39 events), the methylation-based classification still stratified patients experiencing different times to relapse with a HR=3.55 (95% CI: 1.54-8.18;  $P=0.003$ ) (Figure 4G;  $P=0.0018$ ), whereas *IGHV* status was not informative (HR=1.05; 95% CI: 0.55-2.01;  $P=0.872$ ) (Figure 4B;  $P=0.8721$ ).

Ten patients were classified discrepantly by the two classification procedures. The statistical analyses of data for those patients were of very limited power. However, three U-CLL patients classified as likely to have a favorable prognosis according to methylation signature did not experience an event but participated long enough in the study to speculate that they did indeed have both a favorable TTT and OS, as indicated by the dashed green



**Figure 4.** Kaplan-Meier analyses of time to treatment and overall survival for methylation-based classification and *IGHV* status stratifications in the cohort for which 450K data were available. (A-C) Kaplan-Meier curves describing time to treatment, (D-F) Kaplan-Meier curves describing overall survival, and (G-I) Kaplan-Meier curves describing relapse for patients stratified using the methylation-based classification (A, D, and G), *IGHV* status (B, E, and H), or both stratification methods (C, F, and I) in an independent cohort of patients (450K data). In (C, F, and I) the curves represent patients classified according to both stratification procedures: chronic lymphocytic leukemia patients with mutated *IGHV* (dotted) and unmuted *IGHV* (dashed) were colored according to the prediction by the methylation classification into favorable (green) or poor (red) prognosis. In (C), the case with mutated *IGHV* with a favorable prognosis (dotted/red curve) is visually difficult to spot behind the other curves in the top left of the Kaplan-Meier plot. The log-rank test for equality was performed between all groups in (C, F, and I), and all  $P$  are listed below: green/dotted curve versus red/dotted curve (C:  $P=0.7598$ ; F:  $P=0.0323$ ; I:  $P=0.0070$ ); green/dotted curve versus green/dashed curve (C:  $P=0.4285$ ; F:  $P=0.5530$ ; I:  $P=0.0880$ ); green/dotted curve versus red/dashed curve (C:  $P=0.0033$ ; F:  $P=0.0134$ ; I:  $P=0.0306$ ); red/dotted curve versus green/dashed curve (C:  $P$ =not available because there were no events; F:  $P=0.1415$ ; I:  $P=0.0405$ ); red/dotted curve versus red/dashed curve (C:  $P=0.5550$ ; F:  $P=0.3584$ ; I:  $P=0.0251$ ); green/dashed curve versus red/dashed curve (C:  $P=0.1034$ ; F:  $P=0.1854$ ; I:  $P=0.0111$ ). M-CLL: patients with chronic lymphocytic leukemia with mutated *IGHV*; U-CLL: patients with chronic lymphocytic leukemia with unmuted *IGHV*.

**Table 2. Multivariable Cox regression analysis for time to treatment and overall survival according to the methylation-based classification adjusted for clinicobiological biomarkers.**

		Time to treatment				Overall survival	
Variable		HR (95% CI)	P	Variable		HR (95% CI)	P
Methylation-based classification	Favorable			Age at diagnosis	≤65 years		
	Poor	8.33 (4.28-16.19)	<0.001		>65 years	3.11 (1.59-6.09)	0.001
Binet stage	A			Methylation-based classification	Favorable		
	B+C	4.87 (2.68-8.85)	<0.001		Poor	1.96 (1.15-3.35)	0.013
Del(13q)	Absent						
	Present	0.52 (0.30-0.91)	0.021				
Del(11q)	Absent						
	Present	0.30 (0.14-0.65)	0.002				

The table displays the final model with variables showing independent prognostic potential with hazard ratios indicating the likelihood of an event given the biomarker status in the specific row. The time to treatment (TTT) model was built on data from 113 patients with chronic lymphocytic leukemia (CLL) among whom 63 started treatment and the overall survival model was built on data from 114 CLL patients of whom 57 died. Adjustment for confounding variables was performed using backward elimination, and *P* values <0.05 were considered statistically significant. The models were built using all standard clinicobiological biomarkers available, including: age at diagnosis (≤65 vs. >65 years), Binet stage (A vs. B+C), sex, ZAP70 expression, CD38 expression, trisomy 12, del(11q), del(13q), *NOTCH1* mutation, and *TP53* aberrations. HR: hazard ratio; CI: confidence interval. OS, overall survival; TTT, time to treatment.

Kaplan-Meier curves in Figure 4C and 4F, respectively. Similarly, the dotted red Kaplan-Meier curves in Figure 4C and 4F for seven M-CLL patients classified by methylation signatures as likely to have a poor prognosis suggest short TTT and OS. Furthermore, the relapse data for seven discrepant patients confirmed that the two U-CLL patients classified as having a favorable prognosis had a significantly different time to relapse than that of the remaining U-CLL patients (Figure 4I, dashed curves; *P*=0.0111), and likewise, the five M-CLL patients classified as having a poor prognosis had a significantly different time to relapse compared to that of the remaining M-CLL patients (Figure 4I, dotted curves; *P*=0.0070). The *IGHV* mutation load was not available for this cohort and we were not able to assess whether the mutation loads of the discrepantly classified patients were close to the *IGHV* mutation cutoff, suggesting a difficulty in classifying those patients similar to those in our cohort.

#### Association of *IGHV* status and methylation-based classification with standard clinicobiological biomarkers of chronic lymphocytic leukemia

In our cohort, we also analyzed the association of standard clinicobiological biomarkers used in CLL prognostication with both methylation-based classification and *IGHV* status. The analysis was based on all variables available for this cohort, including: sex, age, Binet stage, ZAP70 expression, CD38 expression, del(11q), del(13q), trisomy 12, *NOTCH1* mutation, and *TP53* locus aberrations (Online Supplementary Table S1, Online Supplementary Figure S6). Advanced Binet stage, ZAP70 expression, CD38 expression, del(11q), del(13q), and *NOTCH1* mutation were significantly associated with both U-CLL patients (for *IGHV* status stratification) and with poor prognosis patients, according to the methylation-based classification. Furthermore, classification of patients as U-CLL was significantly associated with sex; other biomarkers did not show statistically significant associations with any of the subgroups of patients. The frequency of the biomarkers in the discrepantly stratified patients were too low for definite conclusions to be drawn (Online Supplementary Table S2); however, most of the discrepantly stratified patients had early-stage disease (Binet stage A: 13/17). In univariate Cox regression analyses, the methylation-based classification predicted TTT most

accurately among all standard clinicobiological CLL biomarkers, and only age predicted OS more accurately than did the methylation-based classification (Online Supplementary Table 3). The multivariable models that included all the above biomarkers and were developed using the backward elimination procedure confirmed an independent power of methylation-based classification to predict TTT with a HR=8.33 (95% CI: 4.28-16.19; *P*<0.001) along with Binet stage, del(13q) and del(11q), and OS with a HR=1.96 (95% CI: 1.15-3.35; *P*=0.013) along with age (Table 2). In an identical modeling procedure, *IGHV* mutation status independently predicted TTT with HR=2.35 (95% CI: 1.34-4.15; *P*=0.003) along with Binet stage, *NOTCH1* mutation, and ZAP70 expression, but was not informative regarding OS (Table 3). As the CpG sites in our stratification procedure were selected based on the association between the methylation levels and *IGHV* mutation load (Figure 1A), the multivariable modeling was performed separately for those variables due to the expected intercorrelation.

We also compared the prognosis of patients classified with our procedure with that of the biological subgroups identified by classification procedure recently described by Duran-Ferrer *et al.*<sup>34</sup> This procedure identified: 35 n-CLL, 20 i-CLL and 59 m-CLL with distinct TTT in our cohort (Online Supplementary Figure S9A). All 35 n-CLL predicted to experience poor prognosis were also classified as likely to have a poor prognosis with our classifier. However, our classification procedure stratified 59 m-CLL cases into six with a poor prognosis and 53 with a favorable prognosis. Similarly, 20 i-CLL patients were stratified into 12 with a poor prognosis and eight with a favorable prognosis. The groups identified by our procedure did indeed experience, statistically, significantly different outcomes, as illustrated by the Kaplan-Meier curves in Online Supplementary Figure S9B, C. In the cohort of patients for whom 450k data were available, all 66 n-CLL cases were predicted to experience a poor prognosis according to our classification, and out of 64 m-CLL cases, one was predicted to have a poor prognosis. Of the 29 i-CLL cases, 14 were predicted to have a favorable prognosis while 15 were predicted to have a poor prognosis. The comparison of clinical data for the discrepant cases was not possible as most of their time data were censored.



**Table 3. Multivariable Cox regression analysis for time to treatment and overall survival according to IGHV status adjusted for clinicobiological biomarkers.**

Variable	Time to treatment		P	Variable	Overall survival		P
		HR (95% CI)			HR (95% CI)		
Binet stage	A			Age at diagnosis	≤65 years		
	B+C	4.49 (2.55-7.91)	<0.001		>65 years	3.28 (1.66-6.45)	0.001
NOTCH1 mutation	Absent			ZAP70 expression	Low		
	Present	3.57 (1.20-10.66)	0.022		High	2.04 (1.20-3.47)	0.008
IGHV status	M-CLL						
	U-CLL	2.35 (1.34-4.15)	0.003				
ZAP70 expression	Low						
	High	2.07 (1.19-3.59)	0.009				

The table displays the final model with variables showing independent prognostic potential with hazard ratios indicating the likelihood of an event given the biomarker status in the specific row. The model was built on data from 114 patients with chronic lymphocytic leukemia (CLL) with 64 events. Adjustment for confounding variables was performed using backward elimination, and *P* values <0.05 were considered statistically significant. The models were built using all standard clinicobiological biomarkers available, including: age at diagnosis (≤65 vs. >65 years), Binet stage (A vs. B+C), sex, ZAP70 expression, CD38 expression, trisomy 12, del(11q), del(13q), NOTCH1 mutation, and TP53 aberrations. HR: hazard ratio; CI: confidence interval. M-CLL: chronic lymphocytic leukemia with mutated IGHV; U-CLL: chronic lymphocytic leukemia with unmutated IGHV.

### Polymerase chain reaction validation of microarray data

To follow good laboratory practice, we performed a technical validation of methylation measurements obtained from the microarray analysis in our cohort with methylation-sensitive high-resolution melting. The results obtained corroborated the microarray data (*Online Supplementary Figure S10*).

### Discussion

The initiation of treatment of CLL patients is still based on progression according to clinical symptoms. However, as a substantial group of CLL patients progresses shortly after diagnosis, or rapidly experiences relapse, it is generally acknowledged that some patients may benefit from earlier intervention. IGHV mutation load, together with TP53 aberrations, are currently the most widely adopted prognostic markers in CLL diagnostics; however, molecular biomarkers are not considered in the decision to treat, and the most clinically relevant cutoff for IGHV status is still debated.<sup>29</sup> Moreover, some studies have indicated that TP53 locus aberrations may not be informative for patients with early-stage disease.<sup>11,12</sup>

The prognostic value of methylation changes in CLL has been described;<sup>18,21</sup> however, the clinical utility of methylation signatures directly associated with IGHV mutation load has not yet been studied. Here we investigated the prognostic power of the IGHV-associated methylation changes in CLL and developed a procedure for classifying patients based on those signatures.

We then evaluated the prognostic accuracy of the developed classification procedure and found that it provided a significantly more accurate prediction of TTT and OS than the stratification based on IGHV status alone. Furthermore, we assessed the prognostic validity of classification in an independent cohort of patients;<sup>18,21</sup> despite the fact that the methylation data for this cohort were limited, compared to IGHV status the methylation signatures in the independent CLL cohort also displayed significantly higher prognostic accuracy to predict TTT, OS and relapse. Moreover, the analysis of clinical outcomes in this cohort indicated that considerably longer follow-up (138.0 months vs. 98.9 months in our cohort) further improved the accuracy of the methylation classification (HR=6.03; 95% CI: 2.65-13.74) compared to

that in our cohort (HR=1.82; 95% CI: 1.07-3.09). At the same time, we did not see an improvement of the prognostic value of IGHV status regarding OS with the longer follow-up, which was not informative in either cohort. However, the fact that OS was not informative may be attributed to the specificity of the patients in these two cohorts because IGHV status predicted OS in other studies.<sup>35</sup>

Due to limited data availability, we were not able to evaluate our findings in the context of an already proposed methylation signature-based stratification<sup>21</sup> and CLL-classification indices (such as the CLL-IPI and IPS-E).<sup>11,12</sup> Nevertheless, our data indicate that it is plausible that the performance of biomarker indices that use IGHV mutation status will improve with the implementation of the proposed patients' classification procedure based on methylation changes. It is also important to note that the genome-wide methylation screening technology used in this project has already been proposed for diagnostic use in glioblastoma,<sup>36,37</sup> indicating that, despite its still high cost, this technology is worth considering given the data quality and the amount of data obtained from single experiments.

In summary, our results show that IGHV-associated methylation signatures may be more accurate than IGHV mutation status in predicting CLL patients' outcomes, including the identification of patients with aggressive disease at diagnosis as well as treatment outcomes. Our results also indicate that the prognostic power of biomarker indices including IGHV mutation status can, potentially, be improved with the addition of methylation markers, but this needs to be addressed in further studies.

### Disclosures

No conflicts of interest to disclose.

### Contributions

DH and TKW designed the study; TEK, ML, and KDJ managed the preparation of patients' samples and quality control; JBG performed bisulfite treatment and microarray analysis; DH performed the bioinformatic and statistical analyses assisted by TKW and AS; DH, TKW, AS, and LLH interpreted results; DH designed the methylation-sensitive high-resolution melting assays, and AT performed them; DH, TKW, and LLH wrote the manuscript draft; TKW and LLH supervised the study; LK, ID, TK, TSL, MBM and CGN participated in the collection of clin-

ical samples and clinical data, and standard biomarker analyses. All authors critically reviewed the manuscript and approved the final version.

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### Data-sharing statement

The raw data upon which we built the classification procedure are available in the Online Supplementary File.

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