Evaluation of serum markers in the LRF CLL4 trial: β2m but not serum free light chains, is an independent marker of overall survival

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Abstract

Chronic lymphocytic leukaemia (CLL) is characterised by heterogeneous clinical behaviour and there is a need for improved biomarkers. The current study evaluated the prognostic significance of serum free light chains (sFLC, kappa and lambda) and other serum markers (β2M, serum thymidine kinase (sTK), soluble CD23 and LDH) together with established biomarkers in 289 patients enrolled into the LRF CLL4 trial. In a multivariable analysis of serum markers alone, higher β2M and Kappa light chains were statistically significant in predicting disease progression and higher β2M, and sTK in predicting mortality. In multivariable analysis for overall survival the following were independently significant: β2M levels, immunoglobulin gene (*IGHV*) mutational status (>98% homology), age, 17p13 deletions (>10%) and CD38 expression. β2M is the only serum marker that retained clear independent value as a biomarker in the LRF CLL4 trial and remains powerfully prognostic requiring evaluation in any future method of risk stratifying patients.

Introduction

Clinical heterogeneity within CLL provides a continuing impetus for the discovery and evaluation of novel biomarkers with prognostic and/or predictive value. Immunogenetic features, especially immunoglobulin gene (IGHV) mutational status, immunophenotypic markers such as CD38, CD49d and ZAP70, recurrent genomic copy number abnormalities and genomic mutations all have prognostic significance in CLL [1-4].

A number of serum factors reflecting one or more CLL-related parameters such as tumour burden and cellular proliferation, activation and/or death have also been shown to correlate with a variety of outcome measures including time to first treatment, treatment response, progression free, and overall survival, and with other serum markers and biomarkers [5]. Both serum Beta 2 microglobulin (β2M)[6] and serum thymidine kinase 1 (sTK) [7-11] are independent markers of progression free and overall survival in trials of chemotherapy and chemo-immunotherapy. Soluble CD23 (sCD23) is associated with shorter PFS and OS in early stage patients [12-16] and the doubling time of sCD23 appears more powerful than sCD23[12]. Serum lactate dehydrogenase (LDH) is an independent predictor of time to first treatment and is associated with shorter PFS, OS and with Richter's transformation [4,17].

Despite the clinical validity of these markers and their relatively low cost, evidence for their clinical utility is less clear. This in part reflects methodological issues, the contribution of factors such as age and renal function to serum levels and whether prognostic significance is based on the evaluation of markers as a continuous variable or as a discontinuous variable with differing cut-offs among studies.

Free immunoglobulin light chains (FLC) are synthesised as a “by-product” of immunoglobulin synthesis. An abnormal serum FLC ratio (κ/λ) reflects clonal tumour production as the excess light chain type corresponds to the surface B cell receptor light chain type, whereas elevated combined FLC concentrations (κ plus λ (cFLC)), in the presence of normal renal function and a normal FLC ratio, is due to polyclonal B-cell activation. Both an abnormal sFLC ratio and raised cFLC are markers of shorter time to first treatment and OS in CLL [18-20] and in a B cell tumour cFLC reflects both polyclonal B cell activation and clonal tumour production. However the prognostic significance of serum free light chains has not been evaluated in the context of a clinical trial. Previous studies have been limited to non-trial patients and therefore have a bias in terms of patient selection and variability in treatments delivered. To address this, we have determined the prognostic significance of serum β2M, sTK, CD23, LDH and free light chains levels, both singly and in combination, in 289 patients with available serum samples who participated in the LRF CLL 4 trial.

Patient and study design

The LRF CLL4 trial randomized 777 previously untreated patients with Binet stage progressive A, B or C disease between January 1999 and October 2004 to receive either chlorambucil, fludarabine or fludarabine and cyclophosphamide. Full details of the design, conduct and outcome of the LRF CLL4 trial have been published [21,22]. Patients analysed in this study are a subset of those 541 randomised from October 2001 onwards and represent all the patients in whom stored serum samples were available (n=289, 53%). Last follow-up was in October 2010 for disease progression (6-9 years after randomisation) and September 2012 for survival (8-11 years after randomisation). In the previous analysis of prognostic factors in this study[22], follow

up was until 31st Oct 2008 and the median follow up for survivors was 68 months so this current study had an additional 2 years follow up for PFS and 4 years for OS. All patients provided written informed consent. The trial was approved by the UK South Thames Multicentre Research Ethics Committee [MREC (1) 98/101] and followed UK Medical Research Council guidelines for good clinical practice.

Laboratory markers

The non-clinical prognostic factors were measured on blood samples taken at the time of trial entry. Details relating to the analysis of the following prognostic markers mutational analysis of immunoglobulin variable region genes (*IGHV*), fluorescence in-situ hybridisation (FISH) for p53, 11q deletion and trisomy 12, evaluation of CD38 and ZAP-70 expression, have been previously described including the rationale for the cut offs used for Zap-70 (10%) and CD38% (7%)[21,22]. Our previous analysis of prognostic factors in the LRF CLL4 trial included two serum markers; serum lactate dehydrogenase and β-2M, both of which were measured by referring centres. In this study,evaluation of β-2M, CD23, serum thymidine kinase and serum free light chains were performed centrally. β-2 microglobulin (B2M) was measured by a latex enhanced immunoturbidimetric method on an Olympus AU640 clinical Chemistry analyzer (Olympus UK, Watford). The assay limit of detection was 0.064 mg/L and assay CVs were 3.8% at 0.8 mg/L, 3.5% at 1.5 mg/L and 3.1% at 2.2 mg/L.

Serum CD23 (sCD23) was measured by a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA, Biosouce, Nivelles, Belgium) performed in accordance with the manufacturer's instructions with the exception that, due to the anticipated higher values in CLL patients, a pre-dilution step was required. The assay limit of detection was 0.15U/ml and assay coefficient of variation (CV) was 9.4 at 2.2 U/ml and 4.2%

at 14.5 U/ml. Serum thymidine kinase activity was determined by a radioenzymatic technique (Prolifigen® TK-REA, Stillwater, MN) according to the manufacturer's instructions. Serum TK activity (U/L) was derived from an appropriate standard curve. Intra-assay and inter-assay CVs were 3.67 and 5.27% respectively.

Serum FLC concentrations were determined nephelometrically (Freelite™, The Binding Site, Birmingham, UK).

Statistical Methods

Serum marker variables are measured on a continuous scale. For clinical usage it might be useful to define a cut-off value for each, essentially dichotomising the scale. To assess the desirability of this approach, each serum marker was divided into quintiles comprising approximately 57 (289/5) patients in each quintile. Then within each quintile the percentage of cases that had progressed by 1 year and by 5 years and were dead at 5 years was determined (death by 1 year only occurred in 9% of patients) The chi-squared test for association was used to assess whether these percentages varied by quintile of serum marker, and this was then broken down into linear and non-linear components to further understand the nature of the association. Progression free survival (PFS) was calculated using time from randomization to progression or death (from any cause) and overall survival was calculated using time from randomization to death (from any cause). Survival times are shown graphically using Kaplan-Meier survival curves.

The associations between survival (PFS and OS) and potential prognostic variables (demographic, genetic and serum markers) measured at baseline were modelled using Cox proportional hazards models. A 2-sided 5% significance level was used. Initially unadjusted analyses were carried out for each variable. Serum marker data

is sometimes divided by a constant (10 or 100) in order to improve readability of the odds ratios.

A 2 phase approach was then used to derive parsimonious models (one for PFS and one for OS) that contained only statistically significant variables. Firstly all the significant variables within each of the 3 categories of demographic, established biomarkers and serum markers were modelled together within each category to determine the most important variables within each category. Forced entry of all variables and forward stepping methods were used, and from these analyses lists of significant variables in each category were derived. Variables that were not on these lists were then added in to the models singly to see if they were now significant. If they were, then they were kept in the model and the process repeated. In the second phase, all the statistically significant demographic, genetic and serum marker variables were brought together into the same model. Those that were not significant were dropped out using a backward stepping approach (though always leaving in treatment), and then not significant variables added in singly to see if they now contributed to the model. Treatment effect, a design variable for the trial from which the data were derived, is included in all these models. The analyses include as many cases as possible, but missing data on individual variables means that the sample size varies slightly depending on what variables are in the model.

Of interest is whether serum markers might usefully replace more expensive genetic markers in helping to assess prognosis in clinical practice. Two models, one containing only serum markers and the other also containing genetic information

were compared using the Akaike Information Criterion. This allowed comparison of 2 different, non-hierarchical models with the same outcome variable. It combines information on how well the model fits the data with information on the complexity of the model. Lower values imply a better model. Data were analysed using IBM SPSS Statistics Version 21.

RESULTS

Characteristics of the 289 patients from the LRF CLL4 trial analysed in this study are shown in Table 1. This cohort of patients is older, but otherwise comparable to the CLL4 trial overall (supplementary data Table 1s) in terms of gender, Binet stage and treatment allocation.

Association between serum markers, divided into quintiles, PFS and OS

The percentage of cases in each quintile that had progressed by 1 year and by 5 years and had died by 5 years is shown in Table 2s. These data suggest that where serum markers are associated with risk of progression or survival, the risk tends to increase as serum marker values increase. The possible exceptions to this were Lambda and the Kappa/Lambda ratio, although there were no consistent patterns across the 3 outcome measures, and p-values were around the 5% level (in the context of multiple significance tests). Given this set of results we felt it preferable to analyse the data as continuous variables rather than trying to dichotomise them.

Univariable analysis of demographic factors, established biomarkers and serum factors.

Table 2 shows unadjusted hazard ratios summarising the associations of progression free survival and overall survival with demographic, established biomarker and serum marker variables.

For demographic variables increasing age and male gender appear to be associated with greater risk of death, though not of disease progression. All the established biomarkers evaluated other than trisomy 12 (ie 11q deletion, no 13q deletion, 17p deletion, unmutated V genes, positive ZAP70, positive CD38 and *TP53* mutation) were associated with increased risk of disease progression and death.

For serum markers, increasing levels of β-2M, LDH, cFLC, kappa light chain, kappa/ lambda ratio (for progression only), CD23 and sTK (for overall mortality only) were associated with poor outcome. Hazard ratios adjusted for the effects of all other serum markers showed that only β-2M, and kappa light chains remained significant for PFS and β-2M, CD23 (in the opposite direction to the unadjusted analysis) and sTK for OS (Table 3).

Multivariable analysis for PFS and OS

The final parsimonious models are shown in Table 4. Significant variables for disease progression included 11q deletion, 17p deletion, P53 mutation, *IGHV* mutation status. For mortality statistically significant variables included age, 13q deletion (associated with better survival), 17p deletion, *IGHV* mutation status, CD38 positivity, β-2M and CD23 (associated with better survival).

Prognostic value of combining serum marker data.

As β-2M and sFLC are relatively inexpensive and readily available in the UK, we addressed the issue of whether the prognostic value of both factors might be comparable to that of established biomarkers. To this end two models were compared: one (model A) incorporating a selection of factors significant in our multivariable analysis and the other (model B) using kappa light chain and β-2M (Table 5) The AIC statistic indicates that model A is superior to model B.

DISCUSSION

The primary aim of this study was to evaluate the prognostic significance of a number of different measures of serum free light chains: kappa/lambda ratio, kappa and lambda levels and combined kappa and lambda levels (cFLC). This was done using both univariable and multivariable analyses, which included age, a series of other serum factors (β2M, sTK, sCD23 and LDH) and established biomarkers, in patients entered into the UK LRF CLL4 phase 3 chemotherapy trial. The rationale for the study was based on the observation that sFLC, measured within 3 - 9 months of diagnosis in predominantly early stage patients, predicts time to first treatment and overall survival but has not been evaluated in patients requiring treatment in a clinical trial setting. We accept that previous studies of sFLC have predominantly examined treatment naïve patients at diagnosis including many patients with non-progressive disease whilst the CLL4 study looks at prognostic variables for patients about to start therapy and hence a different population of patients. Serum markers offer practical advantages over other current biomarkers but their value needs to be proven in the context of large clinical trials against the established biomarkers.

The decision to analyse sFLC and all other serum markers as continuous variables in the study was based on the association of poorer outcomes with rising serum levels when values were divided into quintiles. This is consistent with the results of previous studies and although most utilised a single cut off, the prognostic nomogram developed by Wierda *et al* [23] includes β2M as a continuous variable and the recent scoring system described by Pflug *et al* [1] assigns one point for a β2M of 1.7-3.5mg/L and two points for values above 3.5mg/L. When measures of serum free light chains were evaluated as continuous variables in univariable analysis, increasing levels of cFLC and kappa light chains were associated with a shorter PFS and OS. When adjusted for the effects of all other serum markers, only kappa light chains remained significant for PFS, and this was lost in a multivariable analysis which included demographic and genetic factors. It is unclear why kappa should be more significant than cFLC in this study. cFLC has been shown to be prognostic both in other lymphoid tumours [24,25] but also in a general population without obvious haematological malignancies [26,27]. The increased risk of death in a general population was not restricted to a particular cause of death but may reflect a chronic inflammatory state with polyclonal immunoglobulin production although this is unproven.

In the previous study of prognostic factors in patients entered into the LRF CLL4 trial, a β2M level of greater than 4 mg/L, 11q and 17p deletion, unmutated *IGHV* genes and/or use of the *IGHV3-21* gene, and treatment allocation were independent predictors of progression-free survival whilst 17p deletion, age, β2M level and *IGHV* mutational status and/or *IGHV3-21* usage were independent predictors of overall survival[22]. Similar results were obtained in the current study of 289 patients, with the exception that β2M was no longer an independent predictor of PFS, nor 11q deletion

of OS, while CD38 positivity and the absence of 13q deletion independently predicted shorter OS. Possible factors to account for differences in the prognostic significance of β2M between the two studies include the smaller patient numbers and longer follow up in this study and the evaluation of β2M as a continuous variable rather than using the previous cut off of 4mg/dl. Patients with a creatinine clearance of less than 30 mL/min were excluded from this trial so high β2M levels are unlikely to reflect poor renal function.

We found sTK, sCD23 and LDH to have prognostic significance in univariable analysis. sTK remained significant for OS when adjusted for other serum markers but in contrast to data from the German CLL trials, was not significant in multivariable analysis adjusting for genetic and demographic factors. This may reflect different methodologies as the German studies appear to use either a radioimmunoassay or quantitative immunoassay [1,5].

The univariable analyses suggested an association between increasing CD23 and mortality, but this changed direction once B2M was taken into account in the multivariable analyses (ie an association between decreasing CD23 and mortality), If this finding is genuine it suggests that CD23 values need to be considered in the context of B2M values, and not in isolation. However CD23 was only weakly associated with OS and we did not feel that this would be plausible biologically or compatible with published literature. This result could reflect methodological issues, for example laboratory anomalies with non-linearity at high concentrations, or the need for more complex statistical models and would need replication in a different dataset.

As β2M and sFLC are widely available in the UK we were keen to explore a predictive model incorporating both β2M and kappa FLC. However this was less powerful at predicting both PFS and OS compared to a model incorporating the factors significant in our multivariable analysis. Pflug *et al* [1] also noted that their prognostic model incorporating genomic abnormalities and IGHV mutational status as well as β2M and sTK, was more powerful than the Wierda model [23] which used age, gender, absolute lymphocyte count, Rai stage, number of nodal sites and β2M but no other biomarkers

In summary, we found that β2M but not other serum markers had independent prognostic significance in the UK CLL4 trial and should be evaluated with genetic and immunophenotypic factors in future studies. Serum factors used alone produce an inferior prognostic model and in particular serum free light chains were not independently prognostic in this study. Whether any of these markers will have prognostic value in patients treated with novel non-chemotherapy regimens remains to be determined. Importantly our study does not refute the previous studies showing that sFLC predicts time to first treatment and overall survival in patients with no indication for immediate treatment.

Acknowledgements

GP, SH, DO designed the study, analysed the data and wrote the paper. DC was CI for the original CLL4 study and designed the study. PWT performed all data and statistical analysis and wrote the paper. HP helped write the paper. NM, DA, ZD, DH, JB performed the research.

Table 1: Characteristics of the 289 patients with available serum data in the CLL4 trial.

Variable (N=289 unless otherwise stated)	Descriptive statistics
Gender % male	75% (216)
Age at randomisation (years)	Mean (SD) 65.0 (8.5) Min, Max 38, 84
White blood cells x 10^9/L (N=287)	Median (IQR) 96.1 (94.3) Min, Max 4.6, 541
Haemoglobin g/l	Mean (SD) 117 (23) Min, Max 40, 166
Platelet count x 10^9/L	Median (IQR) 144.0 (87.5) Min, Max 23, 684
Enlarged spleen	57% (165)

Enlarged lymph nodes (a) Site	
Neck	70% (202),
Axillae	71% (206),
Groin	52% (150)
(b) Number of sites	
0	18% (53)
1	14% (40)
2	24% (70)
3	44% (126)
Binet staging (N=287) A	24% (68)
B	45% (130)
C	31% (89)
Treatment Arm Chlorambucil	49% (142)
Fludarabine	26% (76)
Fludarabine and Cyclophosphamide	25% (71)
Clinical response	
Blank	7% (21)

Complete response Nodular partial response None Progressive disease Partial response	12% (35) 20% (59) 15% (44) 7% (20) 38% (110)
OUTCOME VARIABLES	
Progression free survival (PFS) % Progressed at any time % Progressed by 1 year (Kaplan Meier) % Progressed by 5 years (Kaplan Meier) Median time (95% CI) to progression (years)	 90% (260) 31% (SE=3%) 90 events 84% (SE=2%) 243 events 1.9 (95% CI 1.6, 2.3)
Overall survival (OS)% Died at any time % Died by 1 year (Kaplan Meier) % Died by 5 years (Kaplan Meier) % Died by 10 years (Kaplan Meier) Median time (95% CI) to death (years)	 69% (199) 9% (SE=2%) 27 events 46% (SE=3%) 132 events 75% (SE=3%) 197 events 5.8 years (95% CI 5.0, 6.6)
GENETIC VARIABLES	
IGVH (N=270) Mutated Unmutated	 36% (96) 64% (174)
TP 53 deletion (deletion 17p) (N=275)	8% (22)
TP 53 mutation (N=240)	8% (18)

Both TP 53 deletion and TP53 mutation	6% (14)
ATM deletion (deletion 11q) (N=274)	22% (59)
ATM Mutation (N=64)	25% (16)
Both ATM del and ATM mutation	10 patients (only 64 patients had data on mutation status)
Deletion 13q (N=259)	57% (147)
Trisomy 12 (N=259)	21% (55)
ZAP70 (N=249) ZAP70 positive (over 10% cut off)	Median (IQR)10.0 (21.5) Min, Max 0, 93 50% (125)
CD38 (N=255) CD38 positive (over 7% cut off)	Median (IQR) 20.0 (69.0) Min, Max 0, 100 67% (170)
NOTCH1 mutation (N=191)	8% (15)
SF3B1 mutation (N=185)	15% (27)
SERUM MARKERS	
B2M (mg/L)	Median (IQR) 3.87 (2.09) Min, Max 1.28, 15.16
LDH (U/L) (N=262)	Median (IQR) 432.5 (222.7) Min, Max 4.0, 1953.0

Total Serum free light chain (mg/L) (N=286)	Median (IQR) 45.2 (66.4) Min, Max 10.6, 801.1
Kappa (mg/L) (N=287)	Median (IQR) 14.8 (48.5) Min, Max 2.1, 530.8 45% (130/287) had elevated Kappa (>19.4 mg/L)
Lambda (mg/L) (N=286)	Median (IQR) 13.0 (22.3) Min, Max 1.87, 791.7 28% (79/286) had elevated Lambda (> 26.3 mg/L)
Kappa/Lambda ratio (N=286)	Median (IQR) 1.65 (5.37) Min, Max 0.01, 221.0
CD23 (U/ml) (N=287)	Median (IQR) 168 (159) Min, Max 12, 759
STK level (U/L) (N=287)	Median (IQR) 19.8 (41.1) Min, Max 1.6, 258.9

Table 2: Unadjusted hazard ratios for progression free and overall survival for selected demographic, established biomarkers and serum marker variables

	Progression free survival		**Overall survival**	
	Hazard ratio for disease progression (95% CI)	**p-value**	**Hazard ratio for mortality (95% CI)**	**p-value**
(a) Demographic				
Age (yrs)	1.007 (0.992, 1.022)	0.35	1.058 (1.039, 1.077)	p<0.001
Gender F M	 1.0 1.20 (0.91, 1.59)	0.20	 1.0 1.52 (1.08. 2.15)	p=0.02
(a) (b) Established biomarkers				
Trisomy 12 N Y	 1.0 1.19 (0.87, 1.62)	0.27	 1.0 1.37 (0.97, 1.93)	=0.08
11q deletion N Y	 1.0 1.67 (1.24, 2.25)	0.001	 1.0 1.45 (1.04. 2.01)	=0.03
13q deletion N Y	 1.0 0.76 (0.58, 0.98)	0.03	 1.0 0.54 (0.40, 0.72)	<0.001

17p deletion N Y	 1.0 3.79 (2.42, 5.93)	<0.001	 1.0 4.17 (2.63, 6.62)	<0.001
IGVH Mutated Unmutated	 1.0 2.22 (1.67, 2.93)	<0.001	 1.0 2.85 (2.03, 4.01)	<0.001
ZAP70 Negative Positive	 1.0 1.49 (1.14, 1.94)	0.003	 1.0 1.80 (1.32, 2.45)	<0.001
CD38 Negative Positive	 1.0 1.90 (1.43, 2.53)	<0.001	 1.0 2.65 (1.86, 3.79)	<0.001
TP53 mutation N Y	 1.0 8.51 (4.95, 14.61)	<0.001	 1.0 3.35 (2.01, 5.56)	<0.001
SF3B1[a] N Y	 1.0 1.28 (0.84, 1.93)	0.25	 1.0 1.73 (1.12, 2.68)	0.01
(c)Serum markers				
B2M (mg/L)	1.14 (1.08, 1.20)	<0.001	1.24 (1.16, 1.31)	<0.001
LDH/100 (U/L)	1.08 (1.03, 1.14)	0.001	1.13 (1.07, 1.20)	<0.001
Serum free light chain/10	1.011 (1.001, 1.021)	0.037	1.013 (1.002, 1.024)	0.025

(mg/L)				
Kappa/10 (mg/L)	1.030 (1.015, 1.046)	<0.001	1.025 (1.009, 1.041)	0.003
Lambda/10 (mg/L)	0.997 (0.980, 1.014)	0.72	1.001 (0.983, 1.019)	0.91
Kappa/Lambda	1.006 (1.000, 1.012)	0.04	1.002 (0.995, 1.008)	0.59
CD23/100 (U/ml)	1.099 (1.011, 1.195)	0.027	1.102 (1.005, 1.207)	0.039
STK/10 (U/L)	1.023 (0.997, 1.050)	0.09	1.056 (1.026, 1.086)	<0.001

[a]SF3B1 not subsequently used in multivariable analyses as 36% missing data

Table 3: Adjusted hazard ratios for progression free and overall survival for serum marker variables (only statistically significant variables shown)

	Progression free survival		**Overall survival**	
	Hazard ratio for disease progression (95% CI)	**p-value**	**Hazard ratio for mortality (95% CI)**	**p-value**
B2M (mg/L)	1.13 (1.07, 1.20)	<0.001	1.33 (1.23, 1.44)	<0.001
Kappa/10 (mg/L)	1.024 (1.008, 1.039)	0.003	-	
CD23/100 (U/ml)	-		0.839 (0.745, 0.944)	0.003
STK/10 (U/L)	-		1.046 (1.015, 1.078)	0.003

All hazard ratios adjusted for other serum markers and treatment group (a design variable in the CLL4 trial). . Variables that are not significant are not included in the model.

Table 4: Adjusted hazard ratios for progression free and overall survival with demographic, established biomarkers and serum marker variables combined (only statistically significant variables shown)

	Progression free survival n=226		**Overall survival n=224**	
	Hazard ratio for disease progression (95% CI)	**p-value**	**Hazard ratio for mortality (95% CI)**	**p-value**
(a) Demographic				
Age (yrs)			1.063 (1.041, 1.086)	<0.001
(b) Established biomarkers				
11q deletion N Y	 1.0 1.51 (1.08, 2.12)	0.017		
13q deletion N Y			 1.0 0.62 (0.44, 0.87)	0.006
17p deletion N Y	 1.0 2.54 (1.26, 5.13)	0.009	 1.0 4.47 (2.65, 7.53)	<0.001
IGVH Mutated	 1.0	<0.001	 1.0	<0.001

Unmutated	1.92 (1.41, 2.61)		2.12 (1.42, 3.17)	
CD38 Negative Positive			 1.0 1.64 (1.08, 2.49)	0.021
TP53 mutation N Y	 1.0 3.41 (1.56, 7.45)	0.002		
(c) Serum markers				
B2M (mg/L)			1.33 (1.17, 1.51)	<0.001
CD23/100 (U/ml)			0.752 (0.643, 0.880)	<0.001

Hazard ratios adjusted for all other variables in the model and treatment group (a design variable in the CLL4 trial). Variables that are not statistically significant are not included in the model.

Table 5: Comparison of 2 different clinical models for predicting progression free survival and overall survival (n=202). Model A: Measures established biomarkers and B2M to predict outcome. Model B: Measures B2M and serum free light chain variable

Model	**Progression free survival**		**Overall survival**	
	Hazard ratio (95% CI)	**p-value**	**Hazard ratio (95% CI)**	**p-value**
Model A				
11q deletion	1.33 (0.93, 1.91)	0.12	1.15 (0.75, 1.75)	0.53
13q deletion	0.86 (0.62, 1.18)	0.35	0.56 (0.38, 0.82)	0.003
IGVH status	1.77 (1.22, 2.55)	0.002	1.58 (1.01, 2.47)	0.045
CD38 positivity	1.17 (0.83, 1.65)	0.38	1.66 (1.09, 2.54)	0.020
B2M (mg/L)	1.05 (0.96, 1.14)	0.33	1.12 (1.03, 1.23)	0.013
AIC statistic[1]	1641.3		1220.8	
Model B				
B2M (mg/L)	1.09 (1.00, 1.18)	0.044	1.18 (1.08, 1.28)	<0.001
Kappa/10 (mg/L)	1.027 (1.008, 1.047)	0.006	1.013 (0.993, 1.033)	0.21
AIC Statistic	1653.5		1247.2	

Same sample size of n=202 used for all analyses in the table. This excludes patients with TP53 mutation or deletion, and excludes patients from all analyses if they have missing data on any of 11q deletion, 13q deletion, v-gene, CD38, B2M or Kappa.

Supplementary

Table 1S: Comparison of 289 patients with available serum data to 488 patients without available serum data in the CLL4 trial

Variable		No serum data n (%)	Serum data n (%)	p-value (heterogeneity)
Gender	F	131 (27)	73 (25)	0.69
	M	357 (73)	216 (75)	
Treatment allocation	F+Cyclo	125 (26)	71 (25)	0.80
	Chlor	245 (50)	142 (49)	
	Fluda	118 (24)	76 (26)	
Age group	<60	185 (38)	70 (24)	<0.001
	60-	159 (33)	127 (44)	
	70+	144 (30)	92 (32)	

Variable		No serum data n (%)	Serum data n (%)	p-value (heterogeneity)
Binet stage	A	122 (25)	68 (24)	0.86
	B	220 (45)	130 (45)	
	C	143 (30)	89 (31)	

Table 2s: Preliminary serum marker analysis: Progression free survival and overall survival by quintiles of serum marker.

	Quintiles	**Percent progressed at 1 year**	**Percent progressed at 5 years**	**Percent dead at 5 years**
B2M (mg/L)	0 – 2.61	17%	72%	21%
	2.62 – 3.50	25%	83%	32%
	3.51 – 4.30	26%	81%	44%
	4.31 – 5.36	37%	90%	58%
	5.37 +	51%	95%	75%
Chi-squared test				
Overall p		0.001	0.01	<0.001
Trend p		P<0.001	0.001	<0.001
Non-linear p		0.71	0.69	0.91

LDH (U/L)	0 – 303	21%	83%	40%
	304 – 388	31%	69%	38%
	389 – 479	25%	79%	31%
	480 – 571	37%	92%	56%
	572+	46%	92%	63%
Chi-squared test				
Overall p		0.04	0.007	0.004
Trend p		0.005	0.01	0.003
Non-linear p		0.56	0.054	0.09
Total Serum free light chain (mg/L)	0 – 23.86	10%	69%	29%
	23.87 – 37.70	26%	84%	32%
	37.71 – 57.20	21%	88%	50%
	57.21 – 111.03	44%	89%	61%
	111.04+	54%	89%	56%
Chi-squared test				
Overall p		<0.001	0.01	0.001
Trend p		<0.001	0.002	<0.001
Non-linear p		0.27	0.30	0.33

Kappa (mg/L)	0 - 7.40	21%	86%	34%
	7.41 – 12.38	26%	74%	39%
	12.39 – 25.11	22%	81%	36%
	25.12 – 64.84	32%	91%	59%
	64.85+	54%	88%	61%
Chi-squared test				
Overall p		<0.001	0.10	0.003
Trend p		<0.001	0.19	P<0.001
Non-linear p		0.11	0.11	0.36
Lambda (mg/L)	0 – 5.40	26%	74%	40%
	5.41 – 10.52	26%	84%	40%
	10.53 – 17.89	33%	82%	48%
	17.90 – 33.40	21%	86%	40%
	33.41 +	49%	93%	60%
Chi-squared test				
Overall p		0.01	0.10	0.15
Trend p		0.03	0.01	0.06
Non-linear p		0.04	0.74	0.38

Kappa/Lambda	0 – 0.260	35%	90%	45%
ratio	0.261 – 0.650	18%	85%	36%
	0.651 – 2.700	26%	72%	43%
	2.701 – 7.700	31%	84%	50%
	7.701+	45%	88%	54%
Chi-squared test				
Overall p		0.04	0.08	0.41
Trend p		0.12	0.67	0.15
Non-linear p		0.054	0.04	0.60
CD23	0 – 88.92	22%	72%	33%
(U/ml)	88.93 – 145.17	33%	82%	47%
	145.18 – 200.84	28%	86%	45%
	200.85 – 284.62	35%	93%	58%
	284.63+	39%	86%	47%
Chi-squared test				
Overall p		0.36	0.046	0.13
Trend p		0.08	0.01	0.07
Non-linear p		0.73	0.31	0.30

STK	0 – 11.0060	19%	74%	30%
(U/L)	11.0061 – 15.2900	28%	84%	46%
	15.2901 – 25.9560	28%	83%	43%
	25.9561 – 60.5990	44%	91%	49%
	60.5991+	39%	88%	60%
Chi-squared test				
Overall p		0.04	0.13	0.03
Trend p		0.005	0.03	0.003
Non-linear p		0.52	0.56	0.67

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