The absolute percent deviation of *IGHV* mutation rather than a 98% cut-off predicts survival of chronic lymphocytic leukaemia patients treated with fludarabine, cyclophosphamide and rituximab

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Summary

The degree of somatic hypermutation, determined as percent deviation of immunoglobulin heavy chain gene variable region sequence from the germline (*IGHV*%), is an important prognostic factor in chronic lymphocytic leukaemia (CLL). Currently, a cut-off of 2% deviation or 98% sequence identity to germline in *IGHV* sequence is routinely used to dichotomize CLL patients into mutated and unmutated groups. Because dissimilar *IGHV*% cut-offs of 1% to 5% were

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identified in different studies, we wondered whether no cut-off should be applied and *IGHV*% treated as a continuous variable. We analysed the significance of *IGHV*% in 203 CLL patients enrolled on the original frontline fludarabine, cyclophosphamide and rituximab (FCR) trial with a median of 10 years follow-up. Using the Cox Proportional Hazard model, *IGHV*% was identified as a continuous variable that is significantly associated with progression-free (PFS) and overall survival (OS) (P<0.001). Furthermore, we validated this finding in 323 patients treated with FCR off-protocol and in the total cohort (n=535). Multivariate analysis revealed a continuous trend. Higher *IGHV*% levels were incrementally associated with favorable PFS and OS in both FCR-treated cohorts (P<0.001, both cohorts). Taken together, our data suggest that *IGHV*% is a continuous variable in CLL patients treated with FCR.

Keywords

CLL; FCR; *IGHV* gene; immunoglobulin heavy chain gene

Introduction

The mutation status of the immunoglobulin heavy chain variable region gene (*IGHV*) is an established prognostic factor in patients with chronic lymphocytic leukaemia (CLL). Fais et al (1998) identified the presence of somatic hypermutation (SHM) in CLL cells and the prognostic significance of the percent of SHM deviation from the germline sequence (*IGHV* %) in patients with CLL was described the following year (Damle, et al 1999, Hamblin, et al 1999). In recent years a cut-off value of 2% deviation from the germline sequence of *IGHV* (i.e. 98% identity) has been commonly used in clinical practice and, according to the current dogma, CLL patients with   2% deviation, or otherwise   98% identity, are considered 'mutated' (M-CLL), whereas CLL patients with <2% *IGHV*% deviation or >98% identity are considered 'unmutated' (U-CLL).(Chiorazzi 2012) The use of a 2% cut-off to classify patients into M-CLL and U-CLL was based on a handful of studies.(Hamblin, *et al* 2008, Oscier, *et al* 2010, Tobin, *et al* 2005) Although other *IGHV*% cut-offs, such as <1% vs 1–3% vs >3%(Davis, et al 2016), 3%(Krober, et al 2002) and 5%(Lin, et al 2002), have been studied, a 2% cut-off has been regularly used in clinical practice. All of the above studies that aimed to identify an *IGHV*% cut-off, except one (Davis, et al 2016), analysed a relatively small number of patients who were not uniformly treated and/or had a relatively short follow-up. Using a maximally selected log-rank statistical analysis, Krober et al (2002) another study demonstrated a 3% cut-off, and Tobin, et al (2005) used the Youden index method to identify a similar cut-off. Conversely, Lin et al (2002) suggested that a >5% cut-off is most appropriate because normal CD5+ B cells have <5% *IGHV*% deviation compared to the germline *IGHV* sequence.(Fais, et al 1998) Other reports pointed out that assessment of *IGHV*% levels could be affected by polymorphisms that mimic SHM(Davis, et al 2003, Matsuda, et al 1993), the choice of SHM assessment technique and the choice polymerase chain reaction (PCR) primers(Ghia, et al 2007, Xochelli, et al 2015).

Because different groups established a variety of *IGHV*% cut-off points, ranging from 1% to 5%, we wondered whether there is no universal cut-off and the *IGHV*% is a continuous prognostic variable. Furthermore, it is important that in a chronic disease, such as CLL, the

prognostic impact of any marker should be based on an analysis with a very long follow-up and large number of patients rather than a short follow-up and limited number of patients. Therefore, to test our hypothesis, we analysed a large cohort of patients with a long follow-up who were treated with the same regimen and whose *IGHV* mutation status was assessed using the same methodology. We analysed the prognostic impact of *IGHV*% in 203 patients treated on the original frontline fludarabine, cyclophosphamide and rituximab (FCR) protocol (the FCR-300 trial)(Thompson, et al 2016) in whom the median follow-up was 10 years, and in 332 patients treated with FCR off protocol(Chihara, et al 2016).

Patients and Methods

We analysed the data of 203 patients enrolled on the FCR-300 clinical trial (n=300). Only patients who underwent SHM testing at initial diagnosis were included in this analysis. In addition, we analysed data of 332 off-protocol patients who were treated with FCR-based regimens(Chihara, et al 2016). Treatments administered in the off-protocol group were FCR (n=213), FCR with high dose rituximab (n=47), FCR with granulocyte-macrophage colony-stimulating factor (GM-CSF; n=50) and FCR with granulocyte colony-stimulating factor (n=22). Although *IGHV* mutation status is now considered relevant in patients with *IGHV3-21* mutation with stereotypy subset 2 (Baliakas, *et al* 2015), we did not have information on the stereotypy status for our cohort; therefore all patients with *IGHV*3-21 were excluded (Tobin, et al 2003). The standard laboratory workup of the patients enrolled in these clinical trials preceded the currently accepted routine karyotype analysis, fluorescence *in situ* hybridisation (FISH) analysis, CD38 assessment, ZAP70 protein expression, next generation sequencing and stereotypy status. Therefore, these important data could not be included in our analysis. All patients provided written informed consent, all of which have been approved by the institutional review board.

The SHM testing method was as follows: stored bone marrow or peripheral blood samples from patients which contained at least 10% CD19+/CD5+ CLL cells, as estimated by flow cytometry, were analysed. RNA extraction was followed by cDNA synthesis using random hexamers. Multiplex PCR amplification was performed using the VH leader forward primers and two sets of reverse primers: CH and JH. The primer sequences were:

Forward Primers (VH Leader Primers)

VH1 leader (VH1-F) 5′ CCA TGG ACT GSA YYT GGA GVR TC 3′

VH2 leader (VH2-F) 5′ ATG GAC ACA CTT TGC TMC ACR CTC 3′

VH3 leader (VH3-F) 5′ CCA TGG AGT TKG GRC TBH GCT GG 3′

VH4 leader (VH4-F) 5′ ACA TGA AAC AYC TGT GGT TCT TCC 3′

VH5 leader (VH5-F) 5′ ATG GGG TCA ACC GCC ATC CT 3′

VH6 leader (VH6-F) 5′ ATG TCT GTC TCC TTC CTC ATC 3′

Reverse Primers

CH   5′ - TTG GGG CGG ATG CAC T- 3′

JH   5′ -AAC TGA GGA GAC GGT GAC C- 3′

PCR amplification conditions were as follows: incubation at 96°C for 1 min, followed by cDNA amplification for 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. PCR products were purified and sequenced. Given that our assay is RNA-based, only productive rearrangements were expected to be amplified. The degree of SHM was quantified by comparing the resulting *IGHV* sequence with the consensus germline sequence in the IMGT/V-QUEST database (http://www.imgt.org) and the percent change of base pairs was calculated. For the calculation, the entire *IGHV* sequence that includes the complementarity determining region 3 (CDR3) was compared with germline sequence. Sequences less than 150 base pairs in length or without the authentic CDR3 sequence were regarded inadequate for assessment. Ideally, ~300 b.p. sequence is expected, and was achieved in majority of the cases. As these cases were tested as a part of clinical assay, we have minimum reporting criteria set, which included 150 b.p.

Overall survival (OS) was calculated as the number of years from the date of diagnosis to the date of death or last follow-up, and progression-free survival (PFS) was calculated as the number of years from the date of start of treatment to date of last follow-up or disease progression. The Kaplan-Meier product limit method was used to estimate the median survival (OS and PFS). Cox proportional hazard regression models were used to identify any association between covariates and survival (OS and PFS). Classification and regression tree (CART) analysis using the martingale residuals of the Cox models were used to determine the functional form of *IGHV*% that was included in the multivariate analyses. To validate the significance of *IGHV*% as a continuous variable, we also analysed the *IGHV*% after combining the two groups (combined group; n=535). Statistical analysis was performed using the Stata/SE version 14.2 statistical software (Stata Corp. LP, College Station, TX).

Results

The clinical characteristics of 203 patients treated with the original FCR-300 protocol and of 332 patients treated with FCR off protocol (FCR-control group) are summarized in Table I. The median follow-up of patients was 10.7 years (0.1–15.5) in the original FCR-300 trial and 5.6 years (0.2–12.1) in the FCR control group. There were no significant differences in the baseline clinical features of the patients in the two cohorts. Mean number of treatment cycles was 6 (range 1–6) in both cohorts.

Cox proportional hazard models were used to determine the association between *IGHV*%, as a continuous variable, and survival (PFS and OS) in the FCR-300 trial cohort (Figure 1A, B). The *IGHV*% deviation was significantly associated with PFS (hazard ratio (HR) 95%; confidence interval (CI): 0.81 (0.76–0.87); P<0.001) and with OS (HR: 95%; CI: 0.84 (0.78–0.91); P<0.001), suggesting that, as the percent deviation of *IGHV* from the germline sequence increases, the risk of disease progression or death significantly decreases. The CI of 0.81 represents a decrease in the rate of progression of 19% for every 1% increase in the *IGHV*% (Figure 1A, B). In order to further validate this observation, we analysed the survival of patients treated with FCR off-protocol (n=332) (Figure 1C, D). Similar to patients treated on the FCR-300 study, there was a significant association between PFS (HR:

0.84; CI: (0.79–0.90); P<0.001) and OS (HR: 95%; CI: 0.90 (0.82–0.99); P<0.039) and *IGHV*% deviation in the FCR control group. Similarly, when we evaluated all 535 patients of the two groups and performed the same analysis (Figure 1E, F) we found that a sustained continuum increase in *IGHV*% deviation significantly correlated with PFS and OS.

We then performed univariate and multivariate analyses for PFS and OS using the *IGHV*% deviation value as a continuous variable rather than the conventionally used cut-off value of 2% (Tables II and III, respectively). The variables that were significantly associated with a better PFS and OS in the univariate analysis were lower β2 microglobulin levels, younger age, lower lactate dehydrogenase levels, undetectable minimal residual disease as assessed by flow cytometry, a higher number of FCR cycles, and a lower white blood cell count (WBC). Multivariate analysis identified *IGHV*% as being significantly associated with PFS. Other factors significantly associated with longer PFS were number of treatment cycles (4–6) and WBC <200 × 10^9/l (Table II). Similarly, multivariate analysis identified *IGHV*% as being significantly associated with OS. Other factors significantly associated with longer OS were the number of treatment cycles (4–6) and age <65 years (Table III).

Because there was a significant difference in the median follow-up and the overall survival curves in the original FCR and the off-protocol FCR groups (Figure 1 B, D), we assessed the causes of death and the first salvage treatments administered to the patients of the two groups. One hundred and four deaths were recorded among the 203 patients of the original FCR-300 cohort: 46 patients died of disease progression, 14 of transformation, 13 from complications of stem cell transplantation (SCT), 11 from solid tumours, 9 from an infection while in remission, 8 from infection during disease progression and 3 from haematological neoplasms. Among the 332 patients of the FCR control group, which had a median follow-up of 5.6 years, 64 deaths were recorded: 21 patients died from other neoplasms (9 solid tumours and 12 haematological malignancies), 15 from disease progression, 8 from disease transformation, 7 from various infections, 4 from pre-existing comorbidities, 4 from complications of SCT and 5 patients died of unknown causes.

In the original FCR-300 trial, 124 relapsed patients received salvage treatment, of which 32 received FCR, 23 miscellaneous chemotherapy regimens, 19 alemtuzumab-based regimens, 16 rituximab with GM-CSF, 10 ibrutinib-based regimens, 10 lenalidomide based regimens, 4 received R-CHOP (rituximab + cyclophosphamide, doxorubicin, vincristine and prednisolone), 4 hyper-CVAD (hyperfractionated cyclophosphamide, doxorubicin, vincristine and dexamethasone + methotrexate and cytarabine) and 6 were treated with radioimmunotherapy. In the FCR control group, 123 patients received salvage treatment. Forty-two patients received an ibrutinib-based regimen, 15 ofatumumab-based regimens, 14 lenalidomide with rituximab, 10 with bendamustine with rituximab and 10 with rituximab and high dose steroids, 8 received FCR and 8 hyper CVAD, 7 were treated with OFAR (oxaliplatin, fludarabine, cytarabine and rituximab), 3 with alemtuzumab, 3 received FBR (fludarabine, bendamustine, rituximab), 2 venetoclax, and one patient was treated with idelalisib. Overall, the FCR off-protocol patients had access to newer, more effective salvage regimens.

Discussion

In this analysis, we assessed the prognostic significance of *IGHV* mutation status by analysing the absolute value of *IGHV*% on PFS and OS in a large cohort of patients treated with frontline FCR and followed for up to 16 years. Our analysis revealed that a sustained increase in absolute *IGHV*% deviation is significantly associated with longer PFS and OS. The higher the *IGHV*% deviation, the better the PFS and OS, whereas the lower the *IGHV* %, the higher the risk of disease progression and death. Similar results were obtained in a separate cohort of 323 CLL patients treated with FCR or FCR-based regimens. However, the overall survival rates of patients with lower *IGHV*% in this cohort were greater (Figure 1D) than those of patients enrolled in the original FCR trial (Figure 1B). These differences could be attributed to a shorter follow-up in the off-protocol cohort and - that a higher proportion of the off-protocol relapsed patients were treated with more effective novel salvage regimens. These salvage therapies were not available at that time for most relapsed patients enrolled in the original FCR-300 trial, most of which were treated with less effective salvage regimens.

Our data suggest that the absolute level of *IGHV*% deviation rather than a 2% cut-off or 98% cut off for sequence identity is a better and more reliable predictor of treatment outcome. Given that the PFS of patients with high *IGHV*% deviation followed for up to 16 years might be as high as 70%, the absolute *IGHV*% rather than the 2% cut-off should be taken into consideration when considering frontline FCR treatment. Nevertheless, although analyses of the two different cohorts yielded similar results, confirmation from large cohorts of FCR-treated CLL patients from other institutions is needed. Furthermore, because of a lack of data on routine karyotype analysis, FISH analysis, next-generation sequencing studies and stereotypy status for these patients, we could not perform a correlative analysis to compare our data with other studies showing that cytogenetic aberrations (del11q and del17p) significantly correlated with *IGHV* mutation status in CLL patients (Gladstone, *et al* 2012). On the same note, one study reported that patients with unmutated *IGHV* and sole del13q abnormality (n=13) had significantly shorter cumulative probability of treatment as compared to mutated *IGHV* and sole del13q abnormality (n=34), however the OS was not significantly different (Gladstone, *et al* 2011).

One should keep in mind that the 2% *IGHV*% deviation cut-off dichotomizing CLL patients into M-CLL (good) and U-CLL (bad), previously reported in patients receiving chemotherapy, might fail to predict survival outcomes in patients treated with novel, non-haemotoxic agents. For example, initial results from the ibrutinib trial(O'Brien, et al 2016) showed that dichotomizing patients into M-CLL and U-CLL is probably irrelevant in patients treated with this agent. Whether assessment of *IGHV*% as a continuous variable might become a useful prognostic indicator in ibrutinib- or in venetoclax-treated patients remains to be determined. Lin et al (2002) suggested that CLL should be regarded as a disease with a continuous variable phenotype. Our findings support this hypothesis because they suggest that as the percent deviation of *IGHV*% increases, CLL cells become more susceptible to DNA damaging agents.

Another potential explanation for our results could be derived from previous reports demonstrating a robust correlation between the birth rates of CLL cells, *IGHV*% and disease course (Murphy, *et al* 2017, van Gent, *et al* 2008). In addition, antigen-driven CLLs, i.e., those with significantly higher mutation % deviation, had a better survival then antigen-naïve non-significantly mutated *IGHV* and unmutated *IGHV*% (Degan, *et al* 2004). Furthermore, because the telomere length is longer in patients with higher *IGHV*% deviation from the germline sequence, these patients have lower proliferation rates and hence a longer survival. In one study (Grabowski, *et al* 2005), patients with mutated *IGHV* with shorter telomere lengths were found to have a survival similar to patients with unmutated *IGHV*. Within the mutated subgroup, patients who responded to CpG-oligodeoxynucleotide (CpG-ODN) stimulation had an inferior outcome compared to those who did not proliferate, i.e., those with a higher *IGHV*% (Tarnani, *et al* 2010). Overall, these findings suggest that the higher the *IGHV*% deviation, the more the cells are anergic to the B-cell receptor (BCR) trigger, have longer telomere lengths, are less responsive to CpG-ODN stimulation, have lower proliferative rates and therefore exhibit longer patient survival.

In summary, we demonstrated that CLL patients' survival is impacted by a continuous increment of *IGHV*% deviation. The higher the percent deviation, the better is treatment outcome in patients treated with FCR. Therefore, the prognostic relevance of *IGHV*% as a continuous, rather than a dichotomized, variable should be further investigated.

Acknowledgments

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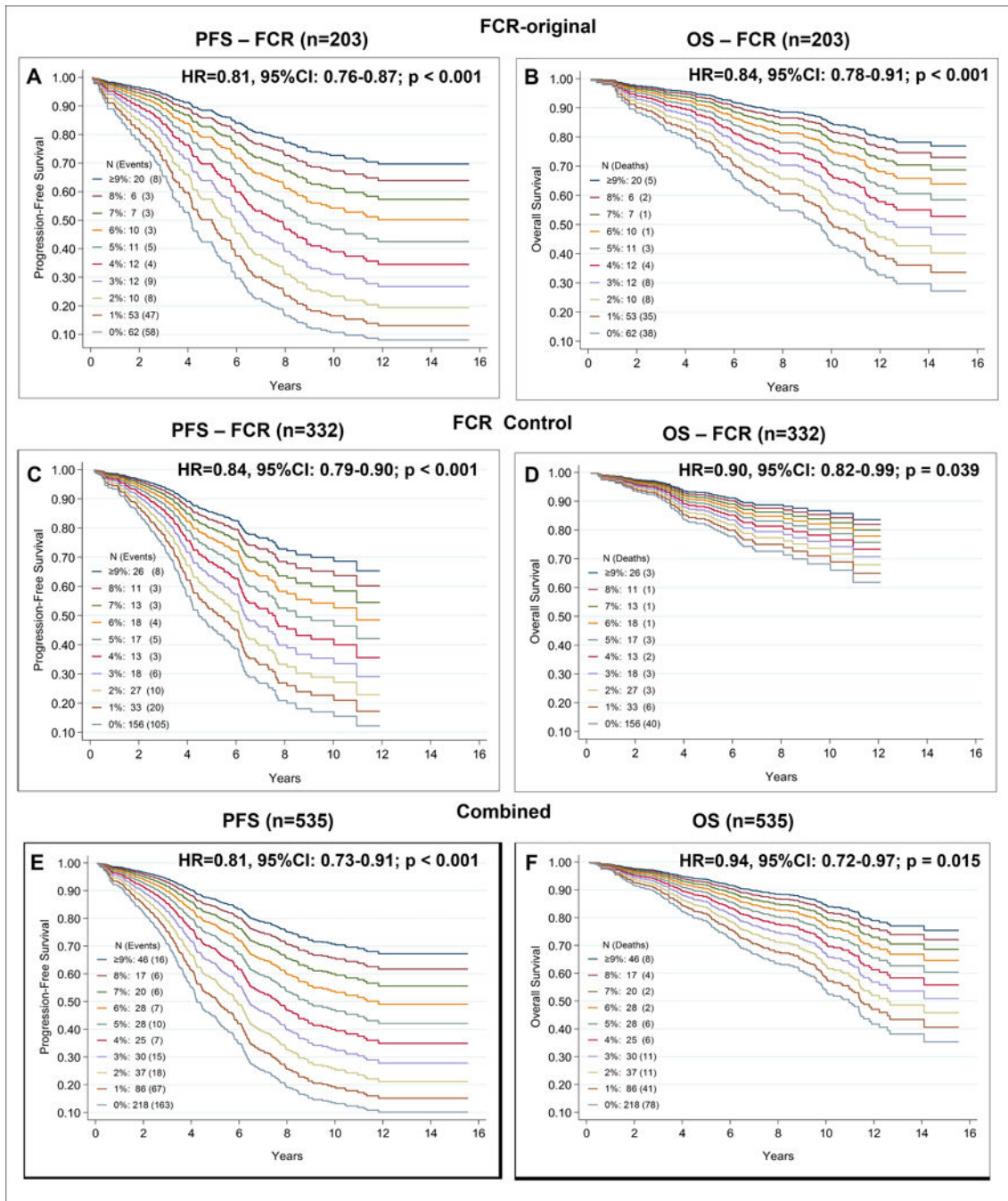
Figure 1. A sustained increase in the percentage deviation of *IGHV* mutations is significantly associated with a lower risk of PFS and OS

The number of patients and the events in each cohort are depicted in the figure. Univariate analysis identified a significant statistically association between *IGHV* mutation from the germline sequence and survival (progression-free and overall survival) (A–B) Survival functions were determined after the univariate Cox proportional hazard model was fitted using the continuous *IGHV*% deviation as a continuous variable **A)** PFS in patients treated on the original FCR-300 clinical trial (FCR-original, n=203; median follow-up 10.7 years).

The estimated five-year survival was 44%, 45%, 40%, 50%, 83%, 82%, 90%, 86%, 83% and 84% at 0, 1, 2, 3, 4, 5, 6, 7, 8 and ≥9% of *IGHV*% deviation, corresponding to various cutoff points for *IGHV* mutation % deviation (0-0.9, 1-1.9, 2-2.9, 3-3.9 and ≥4% and so on) or otherwise called as (100-99.1, 99-98.1, 98-97.1, 97-96.1 and ≤96%) percentage identity, respectively (HR: 95%; CI: 0.81 (0.76–0.87); P <0.001). **(B)** OS in patients treated on the original FCR-300 clinical trial. The estimated five-year survival was 87%, 70%, 60%, 67%, 92%, 100%, 90%, 100%, 83% and 80% at 0, 1, 2, 3, 4, 5, 6, 7, 8 and ≥9% of *IGHV*%, respectively (HR: 95%; CI: 0.84 (0.78–0.91); P < 0.001). Similar analyses of PFS and OS in patients treated with FCR off-protocol (FCR Control, n=332; median follow up of 5.6 years) is depicted in **C** and **D**. **(C)** PFS in patients treated with FCR-based regimens off-protocol. The estimated five-year survival was 43%, 42%, 84%, 77%, 80%, 78%, 88%, 83%, 71% and 70% at 0, 1, 2, 3, 4, 5, 6, 7, 8 and ≥9% of *IGHV*%, respectively (HR: 95%; CI: 0.84 (0.79–0.90); P<0.001). **(D)** OS in patients treated with FCR-based regimens off-protocol. Estimated five-year survival was 81%, 83%, 96%, 85%, 80%, 93%, 100%, 93%, 82% and 87% at 0, 1, 2, 3, 4, 5, 6, 7, 8 and ≥9% of *IGHV*%, respectively (HR: 95%; CI: 0.90 (0.82–0.99); P=0.039. **(E)** PFS in the combined cohorts (i.e., patients treated on the FCR-300 clinical trial and patients treated with FCR-based regimens off-protocol). The estimated five-year survival was 43%, 44%, 71%, 65%, 82%, 79%, 89%, 84%, 76% and 74% at 0, 1, 2, 3, 4, 5, 6, 7, 8 and ≥9% of *IGHV*%, respectively HR: 95%; CI 0.81 (0.73–0.91); P<0.001). **F)** OS in the combined cohorts (i.e., patients treated on the FCR-300 clinical trial and patients treated with FCR-based regimens off protocol). The estimated five-year survival was 82%, 75%, 86%, 76%, 86%, 96%, 96%, 95%, 82% and 88% at 0, 1, 2, 3, 4, 5, 6, 7, 8 and ≥9% of *IGHV*%, respectively (HR: 95%; CI: 0.84 (0.72–0.97); P =0.015).

95% CI: 95% confidence interval; FCR: fludarabine, cyclophosphamide and rituximab; HR: Hazard ratio; OS: overall survival; PFS: progression-free survival

Table I

Baseline characteristics of patients with CLL treated with frontline FCR FCR-300 clinical trial and FCR-based therapies (off-protocol)

Characteristic	Measure/Category	FCR-300 clinical trial (n=203)	FCR-based therapy (control group) (n=332)
Age, years	**Median (range)**	57 (17–85)	58 (28–84)
Age (65 years)	**n (%)**	49 (24)	68 (21)
Gender	**Male/Female (n)**	151/52	220/112
Rai stage	**(0, 1, 2/3, 4) n (%)**	134 (66)/69 (34)	217 (65)/115 (35)
WBC, ×10⁹/l	**Median (range)**	81.5 (2.1–407.8)	84.2 (1.4–541)
Hb, g/l	**Median (range)**	125 (61–187)	125 (23–165)
Platelet count, ×10⁹/l	**Median (range)**	156 (34–406)	147 (10–420)
β2M (mg/l)	**(</ 4 mg/l) n (%)**	116 (57)/85 (42)	220 (67)/111 (33)
Number of cycles	**1–3** **4–5** **6**	22 30 159	77 84 232
Progressed	**n (%)**	132 (65)	158 (48)
Died	**n (%)**	105 (52)	64 (20)
Follow-up, years	**Median (range)**	10.7 (0.1–15.5)	5.6 (0.2–12.1)

β2M; β2 microglobulin; CLL: chronic lymphocytic leukaemia; FCR: fludarabine, cyclophosphamide and rituximab; Hb: haemoglobin concentration; WBC: white blood cell count.

Table II

Univariate and multivariate analyses of PFS of CLL patients treated with frontline FCR

FCR-300 Original	N	Events	HR	Univariate 95% CI	P-value	Multivariate (continuous) HR	95% CI	P-value
IGHV%	203	148	0.81	(0.76–0.87)	<0.001	0.80	(0.75–0.86)	<0.001
β2M (mg/l)	201	146	1.17	(1.09–1.27)	<0.001			
WBC ×10^9/l	203	148	1.00	(1.00–1.01)	0.002			
Cycles (n)	203	148	0.71	(0.62–0.80)	<0.001			
Age 65 years								
No	154	106						
Yes	49	42	1.69	(1.18–2.42)	0.004			
β2M 4 mg/l								
No	116	74						
Yes	85	72	2.02	(1.46–2.80)	<0.001			
WBC 200 ×10^9/l								
No	190	132						
Yes	21	19	2.45	(1.50–3.98)	<0.001	2.30	(1.40–3.77)	0.001
Cycles								
1–3	22	21						
4–5	27	19	0.36	(0.19–0.68)	<0.001	0.28	(0.15–0.52)	<0.001
6	154	108	0.29	(0.18–0.47)	<0.001	0.24	(0.15–0.39)	<0.001

Other variables that were significantly associated with increased risk of progression events in the univariate analysis but not in the multivariate models (and therefore not included in this table) were increased age, high β2 microglobin, elevated lactate dehydrogenase levels, and detectable minimal residual disease (not shown).

95% CI: 95% confidence interval; β2M: β2 microglobulin; CLL: chronic lymphocytic leukaemia; FCR: fludarabine, cyclophosphamide and rituximab; HR: Hazard ratio; PFS: progression-free survival; WBC: white blood cell count.

Table III

Univariate and multivariate analyses of OS of CLL patients treated with frontline FCR

FCR on clinical trial – FCR-300	N	Events	Univariate			Multivariate (continuous)		
			HR	95% CI	P-value	HR	95% CI	P-value
IGHV%	203	105	0.84	(0.78–0.91)	<0.001	0.82	(0.76–0.90)	<0.001
β2M (mg/l)	201	103	1.24	(1.15–1.34)	<0.001			
Cycles (n)	203	105	0.66	(0.58–0.75)	<0.001			
Age ≥ 65 years								
No	154	66						
Yes	49	39	2.92	(1.96–4.35)	<0.001	1.92	(1.23–3.01)	0.004
β2M ≥ 4 mg/l								
No	116	45						
Yes	85	58	2.51	(1.70–3.71)	<0.001			
Cycles								
1–3	22	19						
4–5	27	18	0.52	(0. 27–0.99)	0.04	0.48	(0.25–0.94)	0.03
6	154	68	0.23	(0.14–0.38)	<0.001	0.27	(0.15–0.48)	<0.001

Other variables that were significantly associated with increased risk of death in univariate analysis but not in multivariate models were age >65 years, high β2M, elevated lactate dehydrogenase levels, ZAP70 positivity and undetectable minimal residual disease (not shown)

95% CI: 95% confidence interval; β2M; β2 microglobulin; CLL: chronic lymphocytic leukaemia; FCR: fludarabine, cyclophosphamide and rituximab; HR: Hazard ratio; OS: overall survival.