# Effect of FCGR2A and FCGR3A variants on CLL outcome

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**Polymorphisms of activating Fc-γ receptors (FCGRs) on natural killer cells and macrophages result in variable affinity for immunoglobulin G1 monoclonal antibodies and subsequently modulate antibody-dependent cellular cytotoxicity (ADCC) activity. Whether single-nucleotide polymorphisms of FCGRs correlate with survival of chronic lymphocytic leukemia (CLL) patients treated with a monoclonal antibody containing regimen is unclear. We assessed the FCGR3A and FCGR2A genotype of patients enrolled in the REACH trial, where patients received fludarabine and cyclophosphamide (FC) or rituximab plus FC (R-FC). FCGR3A and FCGR2A polymorphisms did not demonstrate prognostic significance in the FC arm (*P* = .42 and *P* = .64, respectively) or R-FC arm (*P* = .41 and *P* = .88, respectively) with respect to progression free survival. Patients with intermediate affinity genotypes (FV and HR) benefited significantly from addition of rituximab (hazard ratio = 0.55 [0.37-0.8 CI]; *P* = .0017 and hazard ratio = 0.63 [0.44-0.9 CI]; *P* = .011, respectively). Similar benefit was suggested for patients with high-affinity VV and HH (hazard ratio = 0.86 [0.4-1.84 CI]; *P* = .7 and hazard ratio = 0.7 [0.41-1.18 CI]; *P* = .18, respectively) and low-affinity FF and RR (hazard ratio = 0.85 [0.56-1.29 CI]; *P* = .44 and hazard ratio = 0.82 [0.47-1.42 CI]; *P* = .48, respectively). Overall, our results suggest that FCGR2A and FCGR3A polymorphisms do not significantly influence the outcomes of relapsed or refractory CLL patients treated with FC or the monoclonal antibody regimen R-FC. (*Blood.* 2010; 116(20):4212-4222)**

## Introduction

Chronic lymphocytic leukemia (CLL), the most common form of adult leukemia in the Western world, has a highly variable clinical outcome and molecular heterogeneity.[1-3] Akin to many other B-cell malignancies, CLL cells express the CD20 surface antigen and can therefore be targeted by anti-CD20 therapy. Rituximab, a monoclonal chimeric anti-CD20 immunoglobulin G1 (IgG1) antibody, has demonstrated significant benefit for patients with follicular non-Hodgkin lymphoma (NHL)[4,5] and diffuse large B-cell lymphoma (DLBCL)[6,7] with respect to progression-free survival (PFS) and overall survival (OS). In addition, chemoimmunotherapy with rituximab has also shown to prolong PFS and overall survival in CLL in untreated and relapsed/refractory patients compared with chemotherapy alone.[8-10]

The mechanisms of action by which monoclonal antibodies may have antitumor effects includes antibody-dependent cellular cytotoxicity (ADCC),[11] complement-dependent cytotoxicity (CDC),[12] and direct proapoptotic effects.[13,14] ADCC is positively regulated by activating FCGRs expressed on natural killer (NK) cells, macrophages, and dendritic cells. A mouse model deficient for the activating FCGR3 locus has been shown to completely diminish the antitumor efficacy of monoclonal antibody therapies trastuzumab and rituximab.[15] Genomic polymorphisms of the FCGR3A and FCGR2A genes resulting in exchanges of amino acids, valine, or phenylalanine at position 158 of FCGR3A and histidine or arginine at position 131 of the FCGR2A, have been shown to influence the affinity of monoclonal antibodies to the FC-receptor on the effector cells.[16] For FCGR3A the VV genotype at position 158 represents the high-affinity form while FV and FF genotype are associated with reduced affinity. For FCGR2A the HH genotype at position 131 represents the higher-affinity form, while HR and RR represent the lower-affinity form.

The clinical association of FCGR single nucleotide polymorphisms (SNPs) on the outcome of patients with CLL and NHL treated with rituximab has previously been investigated. In follicular NHL, patients treated with rituximab as a monotherapy displaying the homozygous (HH or VV genotype) high-affinity variants have demonstrated improved response rates and prolonged time to progression (TTP) compared with the intermediate- and

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low-affinity variants (FV/FF or HR/RR).[17-19] However, long-term follow up reports in follicular NHL including the application of rituximab maintenance therapy have failed to demonstrate significant impact of FCGR SNPs.[20] In CLL with rituximab monotherapy, one study investigated the relationship with FCGR SNPs and overall response rate (ORR) in a small cohort of patients with no apparent association.[21] When rituximab is combined with chemotherapy there has been conflicting data in aggressive and follicular NHL with respect to association with FCGR SNPs and clinical outcome.[22-24] However, in CLL this question has not been addressed in combination with chemotherapy, and, because chemoimmunotherapy is a common treatment option for CLL patients,[25] it becomes imperative for the treatment strategy of CLL to determine whether a subset of patients can be identified that would gain significant benefit and potentially identify patients who may not gain maximal benefit after chemoimmunotherapy. Furthermore, the outcome of such an analysis would aid in the clinical development of molecules that have enhanced ADCC and the design of novel monoclonal antibody therapies for CLL patients.

The aim of this study was to retrospectively analyze FCGR3A and FCGR2A polymorphisms of patients enrolled in the controlled randomized Study of Relapsed Chronic Lymphocytic Leukemia (REACH) trial, where previously treated CLL patients were treated with either standard chemotherapy (FC) or FC plus rituximab (R-FC), and correlate the genotype data of the FCGRs with ORR, PFS, and overall survival (OS).

## Methods

### REACH study design

REACH was an international, multicenter, open-label phase 3 trial that randomized patients with previously treated CLL (1:1) to receive either R-FC or FC alone. The primary objective was to demonstrate superior PFS for R-FC compared with FC alone. The study protocol was approved by institutional review boards at all participating centers, and all patients gave written informed consent in accordance with the Declaration of Helsinki. Details of trial design and eligibility criteria have been described elsewhere.[23] Patients were selected on the availability of DNA and the written informed consent to participate in molecular genetic analyses of peripheral blood samples. Pharmacokinetic samples for measuring serum rituximab concentrations were collected during cycles 1, 3, and 6 from a total of 21 patients who received R-FC.[26]

### Determination of FCGR3A and FCGR2A genotypes

Pretreatment samples for analysis of FCGR3A and FCGR2A polymorphisms were available from 419 of 546 (77%) patients enrolled in the REACH trial selected by the availability of adequate quality and quantity of DNA. Genomic DNA was isolated from whole blood using the MagNa Pure LC DNA Isolation kit 1 (Roche Applied Science). FCGR2A131H/R (rs1801274) and FCGR3A176F/V (rs396991; also referred to as 158F/V by counting from the N-terminus of the mature protein after cleavage of the signal peptide) were genotyped using SYBR Green I (Applied Biosystems), a nonspecific double-stranded DNA intercalating fluorescent dye. To achieve allelic discrimination between wild-type and mutant alleles, 2 physically separate reverse transcription polymerase chain reaction (RT-PCR) reactions (7900HT Sequence Detection System, Applied Biosystems) containing either wild-type or mutant-specific primers were performed. All reactions were carried out in a total volume of 20 μL. Each reaction mixture contained a 5× dilution of SYBR Green I nucleic acid gel stain (Molecular Probes); 1% dimethyl sulfoxide (Sigma-Aldrich); 40μM dATP, dCTP, dGTP, and dTTP; 4.8 U of Delta Z05 Taq DNA polymerase (Roche Molecular Systems); and 20 ng of genomic DNA in 1× PCR buffer (pH 7.5, 10× solution containing 500mM Tris-HCl, 30mM magnesium acetate, and 500mM potassium acetate, all from Sigma-Aldrich). The amplification program consisted of 12 minutes 95°C; followed by 45 cycles of 20 seconds 95°C, 1 minute 61°C for FCGR2A; and 45 cycles of 20 seconds 95°C, 1 minute 58°C for FCGR3A. After amplification, melt analysis was performed by heating the reaction mixture from 60°C to 95°C at the rate of 1.6°C/s. A negative control without DNA template was run with every assay to assess the overall specificity.

Primers were: FCGR2A, forward allele 1: 5′GAAAATCCCAGAAATTCTCCCA3′ at 200nM; forward allele 2: 5′GAAAATCCCAGAAATTCTCCCG3′ at 400nM; reverse common primer: 5′TGGGATGGAGAAGGTGGGATC3′ at 200nM; FCGR3A, forward allele 1: 5′CTACTTCTGCAGGGGGCTTT3′, forward allele 2: 5′CTACTTCTGCAGGGGGCTTG3′, reverse common primer: 5′CAACTCAACTTCCCAGTGTAAT3′, all at 200nM.

### Statistical analysis

Pretreatment clinical features, prognostic markers, response to therapy between treatment groups were compared with demonstrate that patients with available FCGR genotype data are representative of the overall population of the REACH trial. To assess the association of FCGR

**Table 1. Baseline patient characteristics**

| Characteristic | All subjects (n = 419) | FC arm (n = 209) | R-FC arm (n = 210) | *P* |
|---|---|---|---|---|
| Median age, y (range) | 63 (35-83) | 61 (37-81) | 63 (35-83) | .75 |
| **Sex** | | | | .18 |
| Female | 139 (33%) | 76 (36%) | 63 (30%) | |
| Male | 280 (67%) | 133 (64%) | 147 (70%) | |
| **Binet stage** | | | | .83 |
| A | 40 (10%) | 21 (10%) | 19 (9%) | |
| B | 247 (59%) | 120 (57%) | 127 (61%) | |
| C | 132 (31%) | 68 (33%) | 64 (30%) | |
| Del(17p) | 35/415 (8%) | 20/206 (10%) | 15/209 (7%) | .38 |
| Del(11q) | 86/416 (21%) | 47/206 (23%) | 39/210 (19%) | .33 |
| **IgVH status, n** | 410 | 208 | 202 | .54 |
| Mutated | 154 (38%) | 75 (36%) | 79 (39%) | |
| Unmutated | 256 (62%) | 133 (64%) | 123 (61%) | |
| **ZAP70 expression, n** | 368 | 179 | 189 | .92 |
| Negative | 166 (45%) | 80 (46%) | 86 (46%) | |
| Positive | 202 (55%) | 99 (54%) | 103 (54%) | |
| **CD38 expression, n** | 267 | 133 | 134 | .39 |
| Negative | 135 (51%) | 71 (53%) | 64 (48%) | |
| Positive | 132 (49%) | 62 (47%) | 70 (52%) | |

**Table 2. Incidences of FCGR SNPs**

| | All subjects (n = 419) | FC (n = 209) | R-FC (n = 210) | *P* |
|---|---|---|---|---|
| **FCGR2A** | | | | .33 |
| HH | 110 (26%) | 56 (27%) | 54 (26%) | |
| HR | 218 (52%) | 102 (49%) | 116 (55%) | |
| RR | 91 (22%) | 51 (24%) | 40 (19%) | |
| **FCGR3A** | | | | .34 |
| VV | 49 (12%) | 29 (14%) | 20 (10%) | |
| FV | 202 (48%) | 96 (46%) | 106 (50%) | |
| FF | 168 (40%) | 84 (40%) | 84 (40%) | |

genotypes with R-FC therapy, subgroup analysis of PFS, OS, and ORR benefits was performed for FCGR genotype subgroups. In addition, the prognostic and diagnostic values of FCGR genotypes were evaluated by comparing the clinical outcomes (PFS, OS, and ORR) between FCGR genotypes within the FC arm and within the R-FC arm, respectively.

To compare clinical features and outcomes between genotype and/or treatment subgroups, we used Fisher exact tests for binary or categorical variables, Mann-Whitney test for continuous variables, and log-rank tests and Cox regression for PFS, with the median time calculated by Kaplan-Meier analysis. All statistical tests were 2-sided. A *P* value of .05 or less was considered statistically significant.

For the multivariate analysis, a backward selection procedure was used to derive a parsimonious multivariate model for PFS by iteratively removing the least significant variable with $P > .05$ and refitting the Cox proportional hazards model, until all remaining variables in the model were statistically significant ($P < .05$).

Rituximab serum concentrations were analyzed using a linear mixed effects model to incorporate intrapatient variability as random effects. Estimated PK parameters or rituximab concentrations at each time point were compared between FCGR genotypes using Kruskal-Wallis tests.

## Results

FCGR2A and FCGR3A genotyping data were available from 419 of 546 patients enrolled in the REACH trial with 209 within the FC arm and 210 within the R-FC arm. The patient characteristics and pretreatment features are shown in Table 1 and with respect to risk factors such as age, stage, high-risk cytogenetics, IgVH mutational status, and CD38 expression, the 2 treatment arms did not differ significantly. The FCGR2A incidences of the HH, HR, and RR variants were 26%, 52%, and 22%, respectively and for FCGR3A the incidences of the VV, FV, and FF variants were 12%, 48%, and 40%, respectively (Table 2). In the study cohort with available material for FCGR genotyping a significant benefit with respect to PFS was demonstrated for R-FC arm compared with the FC arm (median PFS 28.4 months vs 19.3 months, respectively; hazard ratio 0.69 [0.53-0.9 CI]; $P = .0061$; Figure 1), emphasizing that the study population was representative for the REACH trial.[9]

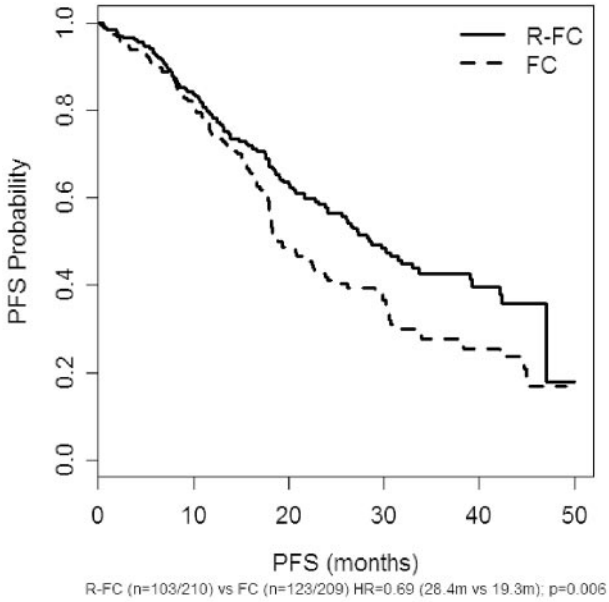


**Figure 1. R-FC versus FC in patients with FCGR2A and FCGR3A genotype data (n = 210 for R-FC and n = 209 for FC).**

To assess the prognostic and diagnostic values of FCGR SNPs in relation to R-FC therapy, we evaluated clinical benefits (R-FC vs FC) within FCGR variant subgroups and also compared clinical outcomes between FCGR variants within the FC and the R-FC treatment arms, respectively. The within-treatment arm analysis addresses whether FCGR SNPs are prognostic, whereas the totality of within-treatment and subgroup analyses informs the diagnostic value of FCGR SNPs specific to rituximab.

**Table 3. Patient outcome with respect to response rates and FCGR2A and FCGR3A genotype**

| | CR | PR | nPR | SD | PD | *P*, CR vs non-CR | *P*, responders (CR/PR/nPR) vs nonresponders |
|---|---|---|---|---|---|---|---|
| **FC** | | | | | | | |
| FCGR2A | | | | | | .14 | .15 |
| HH | 11 (23%) | 13 (28%) | 2 (4%) | 19 (40%) | 2 (4%) | | |
| HR | 13 (15%) | 42 (48%) | 7 (8%) | 20 (23%) | 6 (7%) | | |
| RR | 4 (9%) | 27 (59%) | 2 (4%) | 12 (26%) | 1 (2%) | | |
| FCGR3A | | | | | | .39 | .98 |
| VV | 5 (20%) | 9 (36%) | 3 (12%) | 6 (24%) | 2 (8%) | | |
| FV | 10 (12%) | 40 (47%) | 7 (8%) | 26 (30%) | 3 (3%) | | |
| FF | 13 (19%) | 33 (47%) | 1 (1%) | 19 (27%) | 4 (6%) | | |
| **R-FC** | | | | | | | |
| FCGR2A | | | | | | .86 | .44 |
| HH | 11 (22%) | 26 (52%) | 4 (8%) | 7 (14%) | 2 (4%) | | |
| HR | 25 (25%) | 51 (50%) | 1 (1%) | 21 (21%) | 3 (3%) | | |
| RR | 10 (27%) | 16 (43%) | 0 (0%) | 10 (27%) | 1 (3%) | | |
| FCGR3A | | | | | | .46 | .44 |
| VV | 3 (18%) | 11 (65%) | 1 (6%) | 2 (12%) | 0 (0%) | | |
| FV | 21 (22%) | 48 (51%) | 4 (4%) | 18 (19%) | 4 (4%) | | |
| FF | 22 (29%) | 34 (45%) | 0 (0%) | 18 (24%) | 2 (3%) | | |

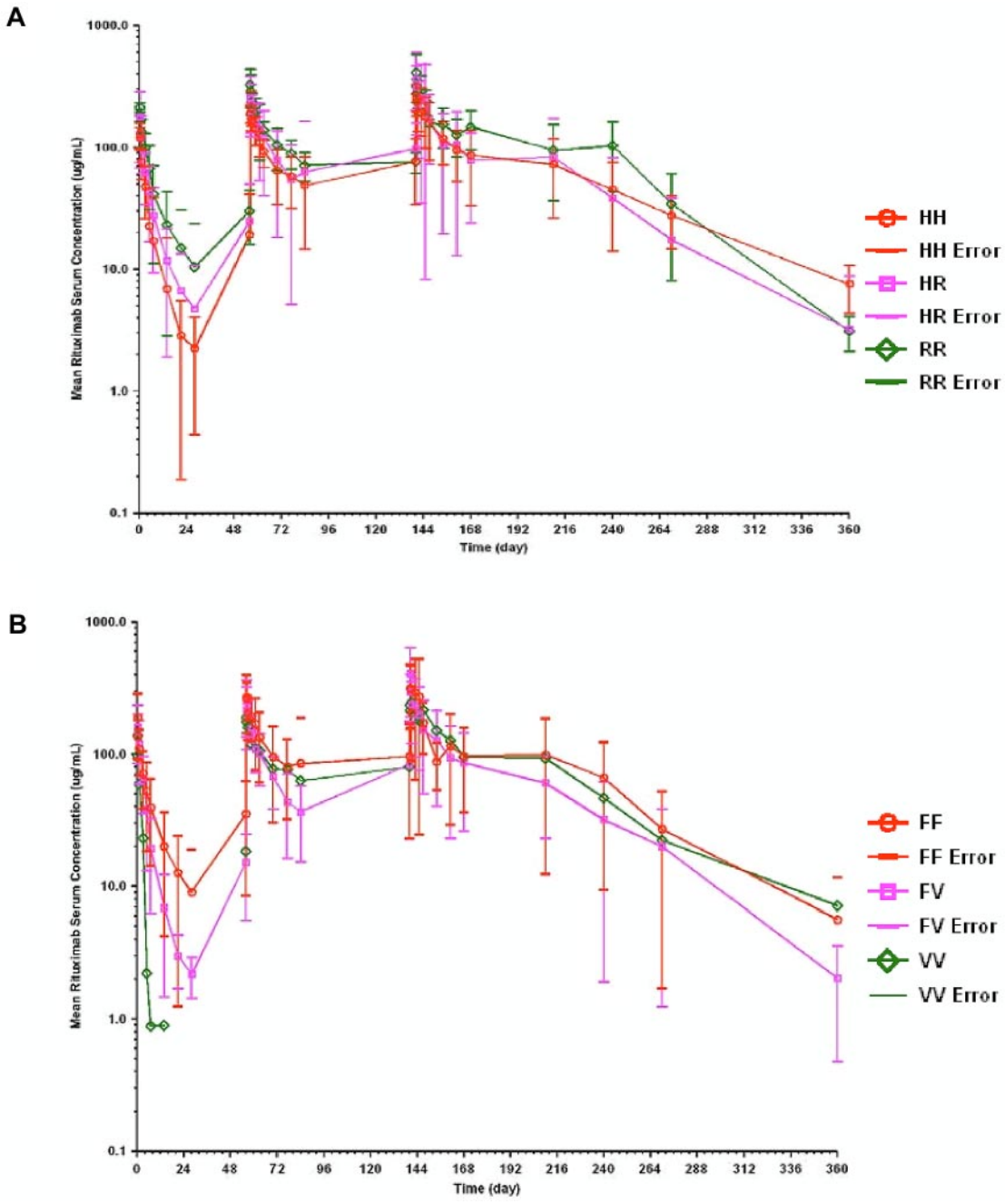


**Figure 2. Effect of FCGR genotypes and rituximab PK.** (A) Arithmetic mean ± SD serum concentration-time profiles of rituximab in patients grouped by FCGR2A genotypes, (B) Arithmetic mean ± SD serum concentration-time profiles of rituximab in patients grouped by FCGR3A genotypes with their polymorphism. Only data for 3 cycles (1, 3, and 6) are displayed from a total of 19 patients.

Initially, we assessed if FCGR SNPs would have any effect on response rates. Within the FC arm and R-FC arm there was no evidence that FCGR2A variants (HH, HR, RR) influenced complete response (CR) rate ($P = .14$ and $P = .86$, respectively) or ORR ($P = .15$;and $P = .44$, respectively). Similarly, we found no evidence of association between FCGR3A variants and CR rate within the FC arm or R-FC arm ($P = .39$, and $P = .46$, respectively) or ORR ($P = .98$ and $P = .44$, respectively; Table 3). In addition, pharmacokinetic results were obtained from 21 patients treated with R-FC; 19 of the 21 patients also have FCGR genotype data. A retrospective analysis was attempted to seek whether there is a correlation between pharmacokinetics of rituximab and FCGR polymorphisms. The rituximab serum concentration-time profiles are presented in Figure 2A-B with derived parameter summarized in Table 4. No significant differences in rituximab pharmacokinetics due to FCGR polymorphisms were observed in this limited dataset of 19 subjects ($P = .3$ for rituximab serum concentration in FCGR2A HRRR versus HH using a linear mixed effects model; $P$ range is .11 to .8 for estimated PK parameters maximum plasma concentration, area under the curve in cycle 1, cycle 3, or cycle 6; statistical test not performed for FCGR3A because there is only 1 patient with VV genotype). Furthermore, we investigated if there was a relationship with FCGR genotypes and infusion related reactions of any grade according to the National Cancer Institute Common Toxicity Criteria version 2.0 within 24 hours of the first rituximab infusion. There were no difference between FCGR2A

**Table 4. Estimated PK parameters of rituximab for patients stratified by FCGR2A (A) and FCGR3A (B) genotypes**

| | | FCGR2A | | | FCGR3A | | |
|---|---|---|---|---|---|---|---|
| **PK parameter** | **Cycle no./dose, mg/m²** | **HH (n = 5)** | **HR (n = 10)** | **RR (n = 4)** | **FV (n = 9)** | **FF (n = 9)** | **VV (n = 1)** |
| **Cmax, μg/mL** | 1/375 | | | | | | |
| Mean (n) | | 134 (5) | 182 (10) | 213 (4) | 178 (9) | 178 (9) | 138 |
| SD | | 26 | 104 | 20 | 65 | 101 | |
| CV% | | 20 | 57 | 9 | 36 | 57 | |
| *P* | | | .11 | | | N/A | |
| **AUC0-last, μg/mL** | | | | | | | |
| Mean (n) | | 9930 (5) | 15 790 (10) | 25 202 (4) | 13 249 (9) | 20 580 (9) | 3900 |
| SD | | 6860 | 8436 | 13 256 | 6093 | 12 140 | |
| CV% | | 69 | 53 | 53 | 46 | 59 | |
| *P* | | | .22 | | | N/A | |
| **Cmax, μg/mL** | 3/500 | | | | | | |
| Mean (n) | | 219 (4) | 272 (9) | 353 (4) | 266 (8) | 302 (8) | 189 |
| SD | | 68 | 113 | 84 | 119 | 101 | |
| CV% | | 31 | 41 | 24 | 42 | 34 | |
| *P* | | | .19 | | | N/A | |
| **AUC0-last, μg/mL** | | | | | | | |
| Mean (n) | | 44 308 (4) | 63 208 (9) | 81 220 (4) | 49 225 (8) | 77 226 (8) | 59 382 |
| SD | | 22 781 | 46 595 | 13 712 | 19 497 | 48 410 | |
| CV% | | 51 | 74 | 17 | 40 | 63 | |
| *P* | | | .35 | | | N/A | |
| **Cmax, μg/mL** | 6/500 | | | | | | |
| Mean (n) | | 267 (3) | 447 (8) | 417 (3) | 424 (7) | 402 (7) | 247 |
| SD | | 69 | 242 | 167 | 213 | 222 | |
| CV% | | 26 | 54 | 40 | 50 | 55 | |
| *P* | | | .29 | | | N/A | |
| **AUC0-last, μg/mL** | | | | | | | |
| Mean (n) | | 84 102 (3) | 97 407 (8) | 93 456 (3) | 93 772 (7) | 92 350 (7) | 101 423 |
| SD | | 33 000 | 69 574 | 56 194 | 56 976 | 68 140 | |
| CV% | | 39 | 71 | 60 | 61 | 74 | |
| *P* | | | .77 | | | N/A | |
| **t, d** | | | | | | | |
| Mean (n) | | 33.3 (3) | 32 (8) | 33.2 (3) | 34.3 (7) | 29.2 (7) | 39.8 |
| SD | | 16.8 | 15.3 | 4.7 | 15.1 | 12.2 | |
| CV% | | 50 | 48 | 14 | 44 | 42 | |
| *P* | | | .77 | | | N/A | |

(44% in HR and RR carriers vs 50% in HH carriers; $P = .5$) and FCGR3A variants (45% in FV and FF carriers vs 50% in VV carriers; $P = .8$).

Because PFS is a more meaningful end point with respect to clinical benefit for CLL and FCGR SNPs genotypes may manifest their differences over an extended duration of time, we next assessed if FCGR SNPs had any effect on PFS within the FC and R-FC arms. Intriguingly, there was no evidence that FCGR2A variants (HH, HR, or RR) influenced PFS (log-rank test: $P = .64$ and $P = .88$; Figure 3A-B) and even when high-affinity variants (HH) were tested versus combination of intermediate- and lower-affinity variant (HR or RR), no significant difference on PFS was detectable in the FC or R-FC arm ($P = .78$ and $P = .75$; Figure 3C,D). Within the FC arm and R-FC arm there was also no evidence that FCGR3A variants (VV, FV, or FF) influenced PFS (log-rank test: $P = .42$ and $P = .41$; Figure 4A-B) nor when high-affinity variants (VV) were tested against lower-affinity variant (FV or FF) in the FC or R-FC arm ($P = .61$ and $P = .26$, respectively; Figure 4C-D). The lack of association with PFS or response rate within the FC arm suggested that FCGR2A and

**Table 5. Clinical benefit (R-FC vs FC) in FCGR2A and FCGR3A variant subgroups**

| | n | | R-FC vs FC | | |
|---|---|---|---|---|---|
| | **FC** | **R-FC** | **PFS hazard ratio (95% CI), *P*, power** | **OS hazard ratio (95% CI), *P*, power** | **ORR % (*P*)** |
| **FCGR2A** | | | | | |
| HH | 56 | 54 | .7 (0.41-1.18), .18, 0.26 | 0.9 (0.39-2.08), .8, 0.04 | 76 vs 46 (.0018) |
| HR or RR | 153 | 156 | 0.68 (0.51-0.93), .015, 0.69 | 0.88 (0.55-1.41), .6, 0.08 | 66 vs 62 (.48) |
| HR | 102 | 116 | 0.63 (0.44-0.9), .012, 0.72 | 0.94 (0.56-1.58), .81, 0.04 | 66 vs 61 (.4) |
| RR | 51 | 40 | 0.82 (0.47-1.42), .48, 0.11 | 0.53 (0.17-1.65), .27, 0.2 | 65 vs 65 (1) |
| **FCGR3A** | | | | | |
| VV | 29 | 20 | 0.86 (0.4-1.84), .7, 0.06 | 0.84 (0.28-2.51), .75, 0.05 | 75 vs 59 (.36) |
| FV or FF | 180 | 190 | 0.68 (0.51-0.9), .0066, 0.78 | 0.94 (0.6-1.46), .77, 0.05 | 68 vs 58 (.052) |
| FV | 96 | 106 | 0.55 (0.37-0.8), .002, 0.88 | 0.71 (0.39-1.28), .25, 0.21 | 69 vs 59 (.19) |
| FF | 84 | 84 | 0.85 (0.56-1.29), .44, 0.12 | 1.33 (0.67-2.63), .42, 0 | 67 vs 56 (.2) |

CI indicates confidence interval.

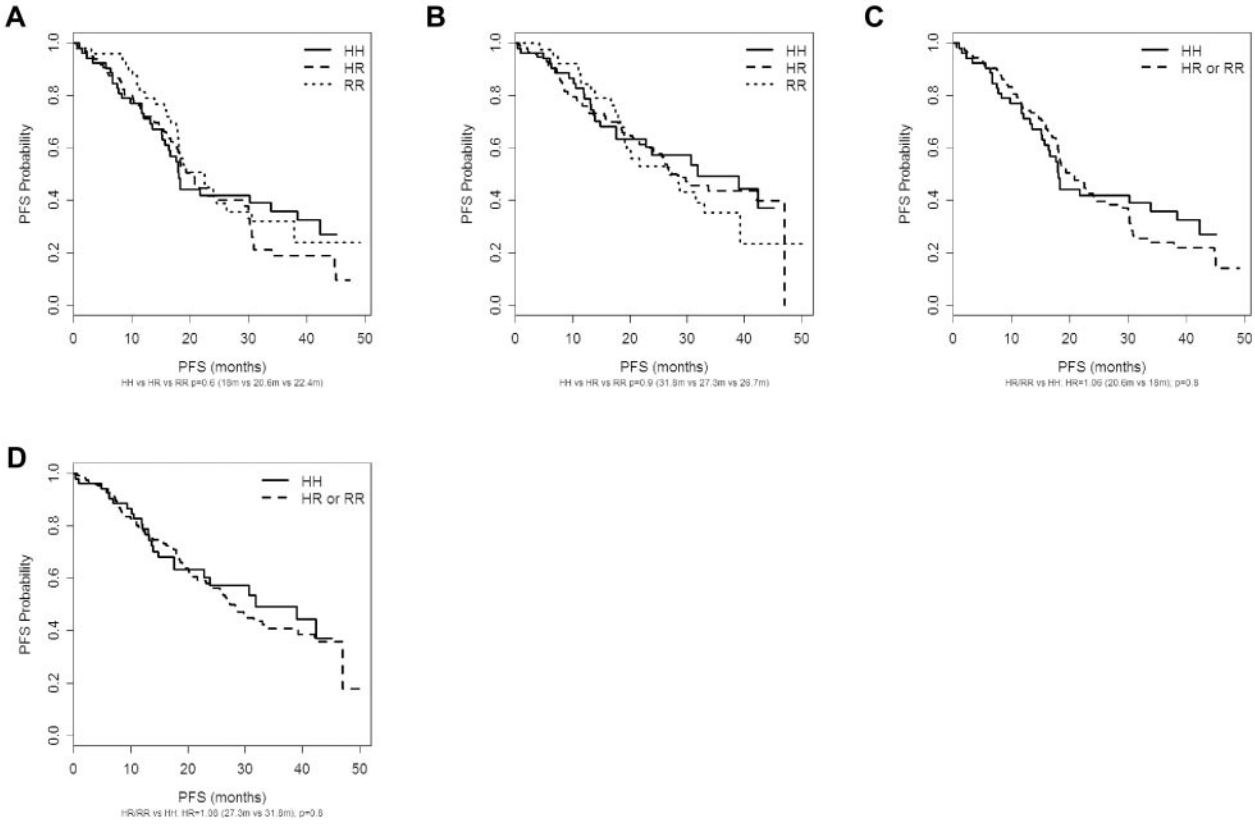


**Figure 3. PFS in patients stratified by FCGR2A genotype.** (A) FC treated patients stratified by FCGR2A genotype. (B) R-FC treated patients stratified by FCGR2A genotype. (C) FC treated patients stratified by FCGR2A genotypes HR/RR vs HH. (D) R-FC treated patients stratified by FCGR2A genotypes HR/RR vs HH.

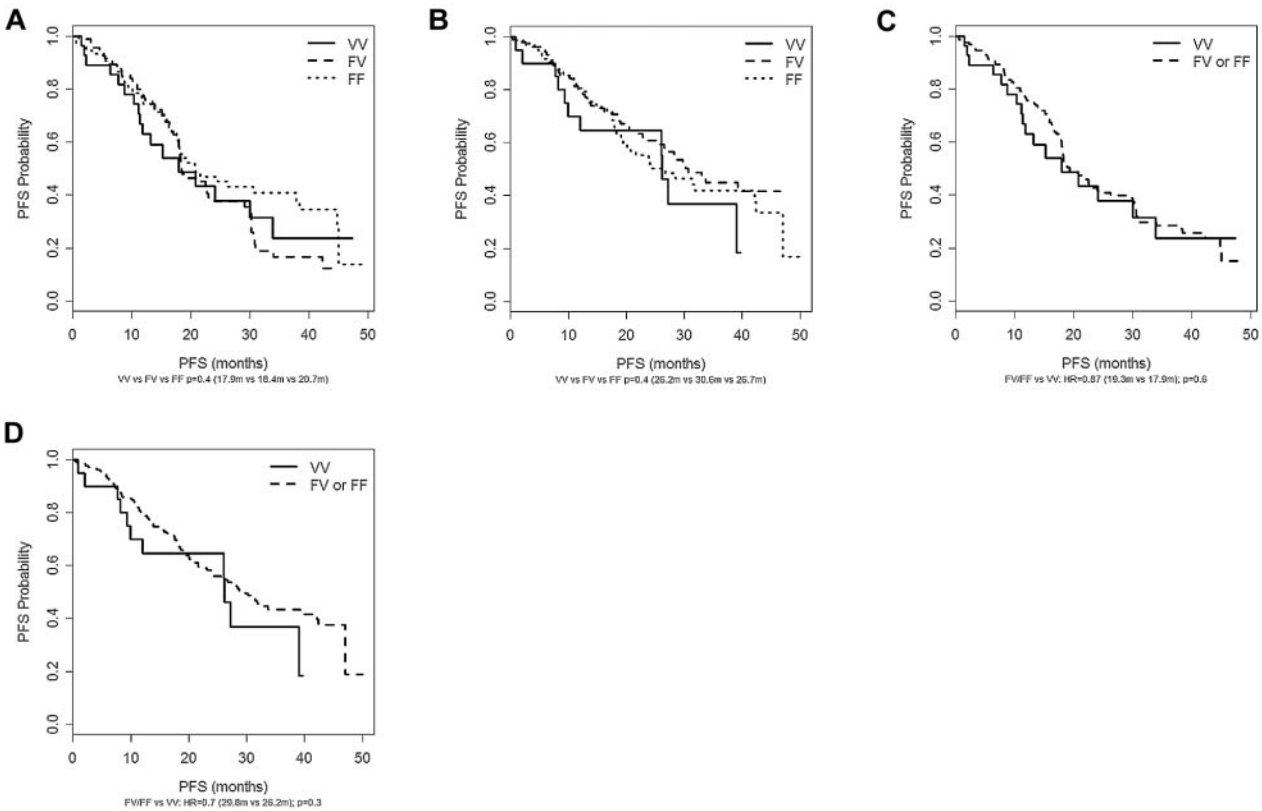


**Figure 4. PFS in patients stratified by FCGR3A genotype.** (A) FC treated patients stratified by FCGR3A genotype. (B) R-FC treated patients stratified by FCGR3A genotype. (C) FC treated patients stratified by FCGR3A genotypes FV/FF vs VV. (D) R-FC treated patients stratified by FCGR3A genotypes FV/FF vs VV.

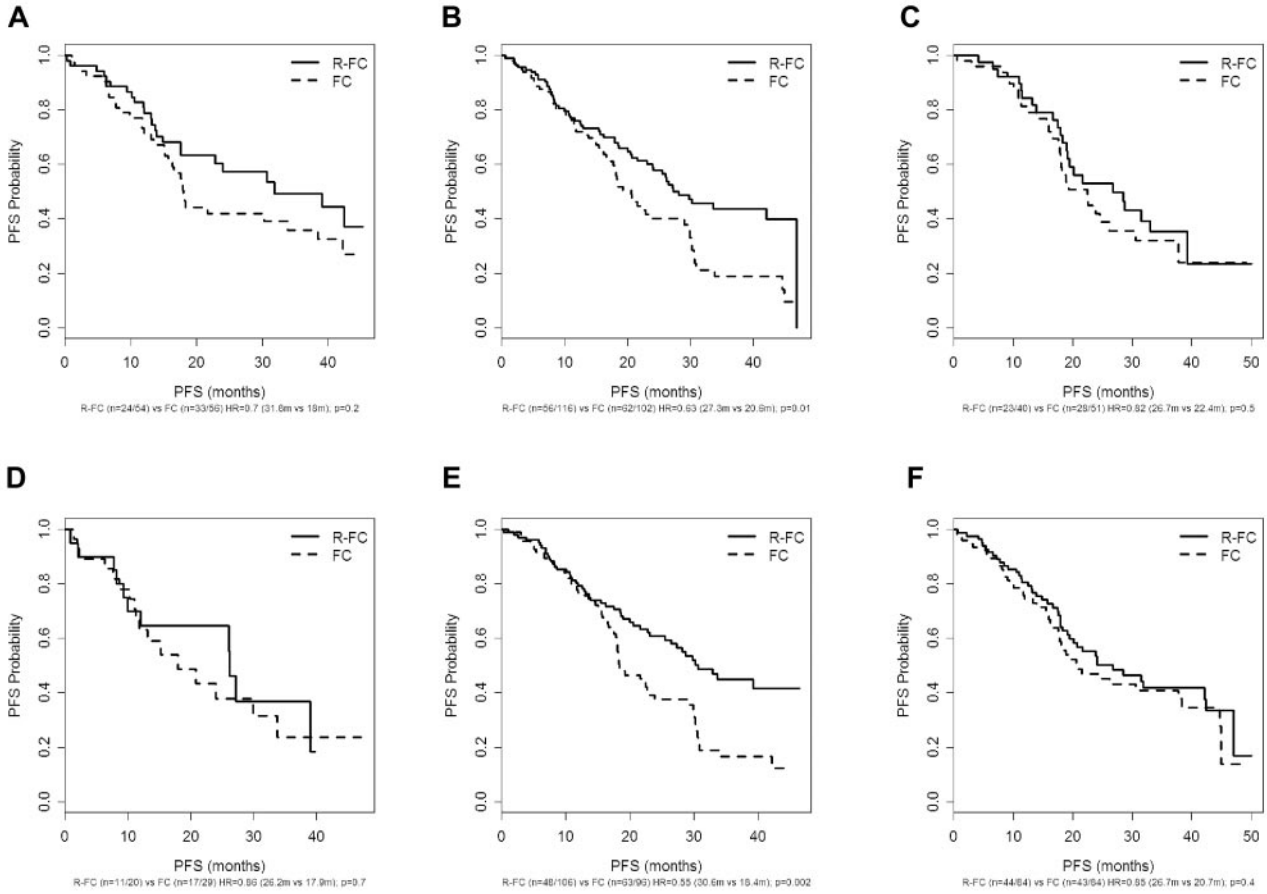


**Figure 5. Treatment comparison of R-FC versus FC.** (A) FCGR2A HH. (B) FCGR2A HR. (C) FCGR2A RR. (D) FCGR3A VV. (E) FCGR3A FV. (F) FCGR3A FF genotype.

FCGR3A variants are not prognostic. Consistently, OS analysis yielded similar results (FCGR2A variants $P = .55$ and $P = .63$ for FC and R-FC arm; FCGR3A variants $P = .18$ and $P = .39$ for FC and R-FC arm, respectively) despite lower event rates (22% in this cohort with available FCGR genotype data) and smaller treatment effect (OS hazard ratio = 0.9, $P = .62$).

Although we did not detect significant differences with respect to FCGR genotype status and clinical outcomes for the FC or R-FC treatment arms, it is imperative to understand whether there were any particular FCGR variant subgroups of patients that had less benefit from rituximab. Subgroup analysis for FCGR variants rituximab showed that addition of rituximab resulted in prolonged PFS across all receptor variants. Median PFS in FCGR2A variants for HH, HR, and RR in the FC arm was 18, 20.6, and 22.4 months, respectively and in the R-FC arm 31.8, 27.3, and 26.7 months, respectively. Median PFS in FCGR3A variants for VV, FV, and FF in the FC arm was 17.9, 18.4, and 20.7 months, respectively and in the R-FC arm 26.2, 30.6, and 26.7 months, respectively. Statistical significance was achieved in the intermediate-affinity variants of the FCGR2A (HR) and FCGR3A (FV) variants (hazard ratio = 0.63 [0.44-0.9 CI], $P = .01$ and hazard ratio = 0.55 [0.37-0.8 CI], $P = .002$, respectively; Table 5 and Figure 5A-F). Patients carrying at least 1 R allele (HR or RR) revealed a similar benefit from the addition of rituximab to FC as the HR carriers alone (hazard ratio = 0.68

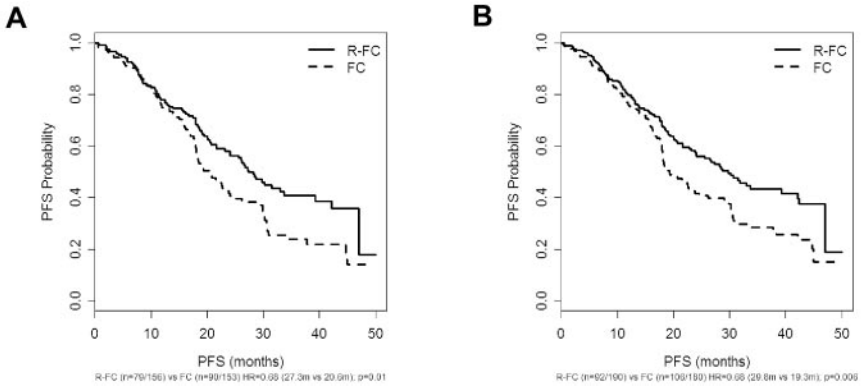


**Figure 6. Treatment comparison of R-FC versus FC.** In patients with (A) FCGR2A HR or RR, and (B) FCGR3A FV or FF genotype.

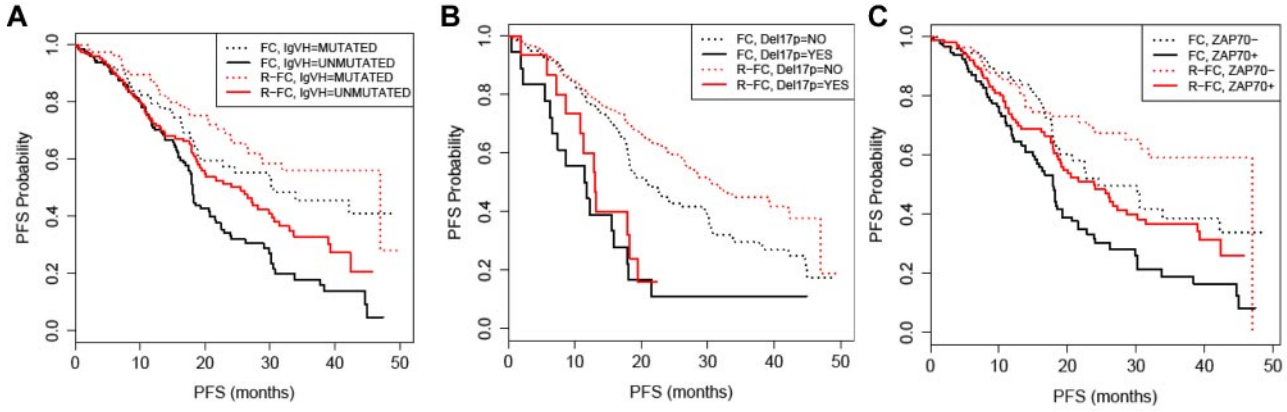


**Figure 7. PFS in patients with FCGR2A and FCGR3A genotype data**. Stratified by treatment and prognostic factors (A) IgVH mutation status, (B) chromosome 17p deletion, and (C) ζ-chain–associated protein kinase 70 expression.

[0.51-0.93 CI], $P = .014$; Figure 6A) and combination of patients carrying at least one F allele (FV or FF) also demonstrated a similar benefit from the addition of rituximab to FC as the FV carriers alone (hazard ratio = 0.68 [0.51-0.9 CI], $P = .0066$; Figure 6B).

We also assessed the robustness of the above findings on FCGR variants when taking into account known prognostic factors for PFS including age, Binet stage, IgVH mutation, genetic aberrations del(17p), del(11q), del(13q), trisomy 12, expression of CD38, and ζ-chain–associated protein kinase 70, in addition to treatment.[27] Table 6 and Figure 7 describe the impact of these risk factors in relation to PFS, OS, and ORR in patients with available FCGR data within the FC and R-FC arm, respectively. The addition of FCGR variants did not provide improvement for a PFS prognostic model including all of the above risk factors. Similarly, FCGR variants were not informative for a parsimonious PFS model derived through a backward selection procedure (described in "Statistical analysis") and included age (hazard ratio = 1.02 [1.01-1.04 CI]; $P = .004$), Binet stage (C vs A/B hazard ratio = 1.81 [1.37-2.39 CI]; $P = .00003$), del(17p) (hazard ratio = 2.49 [1.66-3.74 CI]; $P = .00001$, treatment arm (R-FC vs FC hazard ratio = 0.71 [0.54-0.92 CI]; $P = .01$), immunoglobulin heavy-chain variable region (IgVH; unmutated vs mutated hazard ratio = 2.09 [1.55-2.81 CI]; $P = .000001$), as independent prognostic factors for PFS.

## Discussion

The chimeric anti-CD20 antibody rituximab has demonstrated therapeutic activity in NHL and other mature B-cell neoplasias. The addition of rituximab to chemotherapy resulted in improved cure rates in DLBCL and overall survival benefits for patients with follicular lymphoma CLL if used as upfront treatment. Despite this effective standard of care, the question remains how to better predict treatment. To select therapies for patients who are most likely to derive maximal clinical benefit it is necessary to further understand the mechanisms of action and investigate prognostic and predictive potential of baseline biomarkers based on the biology. Given the proposed role of ADCC mediated by activating FCGR2A and FCGR3A on NK cells, macrophages, and dendritic cells, and the reported impact of gene polymorphisms on the affinity of these receptors to bind IgG1, we genotyped the FCGR3A and FCGR2A genes and the objective of this study was to investigate the influence of FCGR polymorphisms in a large population of well-characterized CLL patients treated within a randomized trial.

To our knowledge the present report is the largest study investigating the effect of FCGR polymorphisms treated within a randomized phase III trial as well as for the first study investigating the relevance of FCGR SNPs for immunochemotherapy in CLL. The clinical outcome of the study cohort with respect to treatment arm (FC vs R-FC) matches the total cohort, showing that addition of rituximab to FC prolongs PFS ($P = .0061$). In addition, the preclinical features and prognostic markers were balanced between the cohorts showing that the study population is representative for the total cohort treated within the REACH trial. In the present study neither FCGR3A nor FCGR2A polymorphisms significantly influenced the response to treatment or PFS of patients treated with FC ($P = .42$ and $P = .64$, respectively) or R-FC ($P = .41$ and $P = .88$, respectively). In addition, none of the genotypes were associated with ORR or OS. In line with this observation, no apparent difference of rituximab serum levels or estimated pharmacokinetic parameters was found among FCGR genotypes in the limited dataset with 19 subjects. With respect to toxicity and adverse events, we also did not find any significant increased risk in the higher-affinity FCGR genotypes for infusion related reactions during and after the first infusion of rituximab. With respect to clinical and prognostic factors the analysis of the CLL cohort analyzed in this study demonstrated no significant differences compared with the parent cohort of the REACH trial.[27]

The present study also analyzed the influence of treatment on PFS within the FCGR variants and revealed that rituximab prolonged PFS across all FCGR variants in a similar magnitude, ranging from 17.9 to 22.4 months in the FC arm to 26.2 to 31.8 months in the R-FC arm. In the intermediate-affinity receptor variants, FV for FCGR3A and HR for FCGR2A, the treatment effect was the most conclusive. For patients carrying the FV variant median PFS was prolonged from 18.4 months in the FC arm to 30.6 months in the R-FC arm ($P = .0017$). For patients carrying the HR variant median PFS was prolonged from 20.6 months in the FC arm to 27.3 months in the R-FC arm ($P = .011$). Due to the lower numbers of patients in the higher-affinity variants (VV and HH) and the subsequently lower statistical power of the analyses, the benefit of adding rituximab to FC was not as conclusive with respect to hazard ratio and $P$ value compared with the lower-affinity variants (Table 4). A trend for benefit was observed nonetheless.

Due to the lower expression of CD20 in CLL[28] compared with other B-cell malignancies like follicular and mantle cell lymphoma, as well as a kinetically unfavorable balance of effector-to-tumor cell ratio, it is conceivable that ADCC may be of lesser importance

**Table 6. Patient outcomes (PFS, OS, and ORR) in relation to known prognostic factors**

| | FC | | | | R-FC | | | |
|---|---|---|---|---|---|---|---|---|
| **Factor** | **n** | **PFS hazard ratio (95% CI), *P*** | **OS hazard ratio (95% CI), *P*** | **ORR % (*P*)** | **n** | **PFS hazard ratio (95% CI), *P*** | **OS hazard ratio (95% CI), *P*** | **ORR % (*P*)** |
| Age ≥ 65 y vs < 65 y | 209 (90 vs 119) | 1.03 (0.72-1.48), .87 | 0.92 (0.51-1.64), .77 | 49 vs 65 (.024) | 210 (88 vs 122) | 2.1 (1.43-3.11), .00018 | 4.71 (2.43-9.12), 4.6e-06 | 62 vs 73 (.13) |
| Binet stage C vs A/B | 209 (68 vs 141) | 2.03 (1.4-2.93), .00018 | 2.01 (1.13-3.57), .018 | 41 vs 66 (.00095) | 210 (64 vs 146) | 1.67 (1.12-2.49), .011 | 1.37 (0.74-2.52), .32 | 55 vs 75 (.0059) |
| IgVH unmutated vs mutated | 208 (133 vs 75) | 2.03 (1.35-3.06), .00072 | 2.47 (1.23-4.97), .011 | 53 vs 65 (.11) | 202 (123 vs 79) | 1.98 (1.28-3.07), .0021 | 2.14 (1.08-4.27), .03 | 63 vs 77 (.044) |
| $CD38^+$ vs $CD38^-$ | 133 (62 vs 71) | 1.47 (0.91-2.35), .11 | 1.9 (0.9-4.01), .092 | 55 vs 63 (.38) | 134 (70 vs 64) | 0.84 (0.51-1.38), .49 | 0.96 (0.49-1.88), .9 | 64 vs 66 (1) |
| $ZAP70^+$ vs $ZAP70^-$ | 179 (99 vs 80) | 1.92 (1.28-2.88), .0017 | 2.13 (1.06-4.27), .034 | 47 vs 69 (.0061) | 189 (103 vs 86) | 1.97 (1.25-3.1), .0037 | 1.5 (0.76-2.95), .24 | 65 vs 72 (.35) |
| Del17p yes vs no | 206 (20 vs 186) | 2.48 (1.46-4.21), .00075 | 3.05 (1.47-6.32), .0027 | 15 vs 63 (5.1e-05) | 209 (15 vs 194) | 3.24 (1.74-6.03), .00021 | 3.02 (1.33-6.85), .0082 | 33 vs 71 (.0067) |
| Del11q yes vs no | 206 (47 vs 159) | 1.52 (1.02, 2.27), .039 | 1.35 (0.72-2.53), .34 | 53 vs 60 (.5) | 210 (39 vs 171) | 0.86 (0.51-1.45), .58 | 1.11 (0.53-2.31), .78 | 62 vs 70 (.34) |
| Del13q yes vs no | 208 (124 vs 84) | 1.1 (.76-1.59), .62 | 1.11 (0.61-2.01), .74 | 59 vs 56 (.77) | 210 (117 vs 93) | 0.96 (0.65-1.42), .83 | 1.03 (0.57-1.87), .92 | 72 vs 65 (.3) |
| Trisomy12 yes vs no | 206 (33 vs 173) | 1.31 (0.8-2.14), .28 | 0.74 (0.29-1.88), .53 | 52 vs 60 (.44) | 210 (24 vs 186) | 1.13 (0.62-2.07), .69 | 1.28 (0.54-3.04), .57 | 62 vs 69 (.49) |

for the efficacy of monoclonal antibody therapy in CLL.[21] However, biochemical studies have shown that ADCC was not increased by rituximab in preclinical models by increasing CD20 levels on the cell surface and activity was maximal at levels of CD20 that occurs on CLL cells.[29] Furthermore, it has been suggested that CDC may also be less likely due to the overexpression of CD55 and CD59 preventing CDC[30]; however, clinical validation of these findings has yet to be reported. It is possible that other factors such as direct signaling and apoptosis may be of more importance in CLL with respect to monoclonal antibody therapy efficacy.[31,32] The current study investigated FCGR SNPs in the context of rituximab given in combination with chemotherapy and it remains a possibility that the addition of immunosuppressive agents like fludarabine and cylophosphamide may have contributed to the diminished role of ADCC in the treatment of CLL. However, preclinical studies have suggested it is also possible that agents such as cyclophosphamide may actually enhance ADCC by up-regulating FCGRs.[33]

Intriguingly, many studies have investigated ways to up-regulate CD20 expression on CLL cells to further enable the ADCC mechanism of action of rituximab and intense research efforts have went into generating a new and improved IgG1 antibodies with increased affinity of the activating FCGRs.[34] Indeed, many of these antibodies have shown impressive in vitro ADCC activity and others have increased apoptosis, CDC, and ADCC to maximize potential benefit for the treatment of NHL and CLL.[35-39] Our data entertain the possibility that ADCC may not be the main mechanism of action for rituximab in the context of immunochemotherapy in CLL and it is hard to ignore the possibility that antibodies with only increased ADCC activity may not have a greater impact than that of rituximab in this setting. Rather, it would appear that it is more likely that antibodies with increased apoptosis activity may result in superior efficacy.

Our observations are in line with other studies investigating the impact of FCGR SNPs in the context of chemoimmunotherapy with rituximab in NHL as well as immunotherapy in CLL.[21] FCGR3A and FCGR2A polymorphisms did not influence survival of DLBCL treated with R-CHOP (rituximab, cyclophosphamide, hydroxydaunorubicin [doxorubicin], vincristine, and prednisone/prednisolone) or follicular NHL treated with CHOP (sequential cyclophosphamide, hydroxydaunorubicin [doxorubicin], vincristine, and prednisone/prednisolone) plus rituximab.[22-24] Similar results were observed with mantle cell lymphoma with CVAD (hypercyclophosphamide, vincristine, doxorubicin, and dexamethasone) plus rituximab.[40] However, a recent report suggested a survival benefit for higher-affinity FCGR3A variants in a cohort of anti-CD20 targeted therapy plus chemotherapy or radioimmunotherapy treated patients with follicular NHL that could not be detected in the chemotherapy alone treated cohort.[41] This may support the specific association of follicular lymphoma and FCGR3A variants as identified with rituximab monotherapy, but these findings need to be confirmed in a homogenously treated patient cohort as part of a randomized trial and a larger patient population.

The present study implies that FCGR SNPs do not influence the outcome of CLL patients treated with either FC or R-FC. Given the overall benefit that has been observed in CLL patients by addition of rituximab to FC this study also supports the use of R-FC as treatment for CLL regardless of patient FCGR genotype. However, due to the low incidence of VV variants a larger cohort may be necessary to prove significant benefit of rituximab in this group. A multivariate analysis revealed that treatment arm was the most powerful independent risk factor. In addition, the major pretreatment clinical features such as age, stage, cytogenetics, and IgVH status remained prognostic factors remained independent risk factors, again confirming the representative character of the study cohort. Overall, the addition of rituximab demonstrated prolonged median PFS across all FCGR variants despite not reaching statistical significance in every subgroup. Selecting CLL patients on the basis of FCGR genotype therefore does not seem warranted given the risk of depriving patients with a chronic disease from a therapy that prolongs their time to progression, time to next treatment and quality of life. Future studies should be directed toward identifying potentially novel predictors of benefit to the R-FC regimen to maximize patient benefit and identify CLL subpopulations that require novel treatment strategies. With the onslaught of novel monoclonal antibodies with enhanced ADCC activity it will be interesting to observe the outcome of patients based on FCGR genotypes; however, a more pressing need exists to identify novel predictors of benefit to R-FC and identify subpopulations that have poorer prognosis and require novel targeted therapeutics akin to that studied in DLBCL with rituximab, cyclophosphamide, hydroxydaunorubicin (doxorubicin), Oncovin (vincristine), and prednisone/prednisolone treatment.[42]

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## Authorship

Contribution: D.D. and M.W. wrote the paper and designed the study; R.-F.Y. and G.D.-N. performed the statistical analyses and had access to the clinical data; O.S. determined the genotypes of each patient; J.Z. contributed to the statistical analysis of the PK data; A. Dufour was responsible for the molecular and genetic data generated within the REACH trial; and T.R., S.I.M., A. Dmoszynska, P.S.-C., K.W., J.L., J.C., B.V.A., L.L., V.A.R., I.B.-B., C.H.G., M.M., and M.K.W. were clinical investigators as part of the REACH trial and submitted samples for the study.

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