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Complete List of Authors:	Kastbom, Alf; Rheumatology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Department of Rheumatology in Ostergotland, County Council of Ostergotland Cöster, Lars; Rheumatology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Department of Rheumatology in Ostergotland, County Council of Ostergotland Ärlestig, Lisbeth; Umeå University, Rheumatology Chatzidionysiou, Katerina; Karolinska, Rheumatology van Vollenhoven, Ronald; Karolinska University Hospital, Rheumatology Padyukov, Leonid; Karolinska Institutet, Rheumatology Unit, Department of Medicine Rantapaa-Dahlqvist, Solbritt; University Hospital, Rheumatology Saevarsdottir, Saedis; Medicine, Rheumatology
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FCGR3A genotype and the therapeutic response to rituximab in patients with rheumatoid arthritis

Alf Kastbom[1], Lars Cöster[1], Lisbeth Ärlestig[2], Aikaterini Chatzidionysiou[3,4], Ronald van Vollenhoven [3,4], Leonid Padyukov[3], Solbritt Rantapää-Dahlqvist[2], Saedis Saevarsdottir[3]

1. Rheumatology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Department of Rheumatology in Östergötland, County Council of Östergötland, Linköping, Sweden.
2. Department of Public Health and Clinical Medicine/Rheumatology, Umeå University, Umeå, Sweden
3. Rheumatology Unit, Department of Medicine, Karolinska University Hospital and Karolinska Institute, Stockholm, Sweden.
4. Unit for Clinical Therapy Research of Inflammatory Diseases, Department of Medicine, Karolinska Institute, Stockholm, Sweden.

Corresponding Author:

Dr. Alf Kastbom

Department of Clinical and Experimental Medicine, Rheumatology

Linköping University

SE-581 85 Linköping

Sweden

alf.kastbom@liu.se

Key words: Fc gamma receptor, Rheumatoid arthritis, Rituximab, Single nucleotide polymorphism, Therapy response.

Abstract

Objectives: To determine whether a polymorphism in the Fcγ receptor type IIIA (*FCGR3A*-F158V), influencing IgG binding affinity, relates to the therapeutic efficacy of rituximab in rheumatoid arthritis (RA) patients.

Methods: Patients with established RA starting rituximab as part of routine care (n=177; 145 females and 32 males) were evaluated according to EULAR response criteria dichotomised into responders and non-responders. *FCGR3A*-F158V genotype was determined by a commercially available TaqMan assay.

Results: The frequency of responders differed significantly across *FCGR3A* genotypes (*P*=0.017 in a 3x2 contingency table). Heterozygous patients showed the highest response rate at 83%, as compared to patients carrying 158FF (68%) or 158VV (56%) (p=0.028 and p=0.016, respectively). Among 158VV patients, response rates differed between male and female patients (p=0.036), but not among 158FF or 158VF patients (p=0.72 and p=0.46, respectively).

Conclusions: Therapeutic efficacy of rituximab in RA patients is influenced by *FCGR3A* genotype, with the highest response rates found among heterozygous patients. This may suggest that different rituximab mechanisms of action in RA are optimally balanced in *FCGR3A*-158VF patients. Similar to the previously described associations with RA susceptibility and disease course, the impact of 158VV on rituximab response may be influenced by sex.

Introduction

The growing arsenal of biologic agents available in rheumatoid arthritis (RA) pharmacotherapy increases the demand for predictors of therapeutic responses and/or side effects. Human Fcγ receptors mediate effector functions of IgG antibodies and may modulate the therapeutic efficacy of all biologic substances containing IgG-Fc parts. Hence, a functional single nucleotide polymorphism in *FCGR3A (FCGR3A*-F158V*,* rs396991*)*, influencing IgG binding affinity to the activating Fcγ receptor type IIIA (FcγRIIIA) [1], has been associated with the therapeutic efficacy of rituximab (RTX), *i.e.* an anti-CD20 B cell depleting IgG1 monoclonal antibody, in B cell malignancies [2, 3]. RTX-coated B cells may be eliminated by several mechanisms; complement-dependent cytotoxicity, antibody-dependent cytoxocity (ADCC) by Natural Killer (NK) cells, and/or phagocytosis by FcγR-bearing cells. Although the relevance in humans has not been established, mice lacking proper complement or NK-cell functions are equally well B-cell depleted as native animals, thus pointing towards a major role of FcγR-bearing macrophages [4]. It has been convincingly shown that RTX binds with higher affinity to 158V in an allele-dose dependent manner and that ADCC by NK cells are affected accordingly [5]. Data regarding *FCGR3A* genotype and RTX efficacy in RA has been lacking until recently, when Ruyssen-Witrand *et al* reported an association between carriage of the high-affinity binding valine (V) allele of *FCGR3A* and higher response rates to RTX [6].

Numerous case-control studies have been performed with regard to *FCGR3A*-F158V and RA susceptibility. Although initial studies showed remarkably discordant results, subsequent investigations have shown an association between homozygousity of the high-affinity allele (158VV) and an increased risk of RA in Europeans [7-10]. In studies presenting data stratified for sex, the increased risk conferred by the 158VV genotype only attributes to the male

population [7, 10]. In line with this, male patients with early RA carrying 158V have a more severe disease course whereas, intriguingly, in female patients the opposite is seen [7]. The biological basis for this sex difference is yet to be elucidated, but it has been shown that estrogen influences both FcγRIIIA expression and FcγRIIIA-mediated release of TNF and IL-1β from monocytic cells [11].

The current study was conducted to explore the influence of *FCGR3A* genotype on RTX efficacy in RA patients.

Patients & Methods

Patients with established RA (n=177; 145 females and 32 males) who started rituximab (Mabthera) as part of routine care at three rheumatology clinics in Sweden (Karolinska University Hospital, Solna; Linköping University Hospital, Linköping; and Umeå University Hospital, Umeå) were included in the study. Baseline characteristics and concurrent medication are shown in table 1. The therapeutic response was assessed by the EULAR response criteria after 3-6 months [12]. DNA was extracted from whole blood by standard techniques and *FCGR3A*-F158V was genotyped by a commercially available TaqMan assay (Applied Biosystems, ID C_25815666_10).

Statistical analysis

Response rates were compared by chi square test across *FCGR3A* genotypes, and by Fisher´s exact test between sexes. Baseline characteristics were tested by chi square test for categorical

variables and by one-way ANOVA for continuous variables. Two-sided p-values <0.05 were considered significant. Analyses were performed using SPSS version 19.

Ethical considerations

Informed consent was obtained from all patients according to the Declaration of Helsinki. The study protocol was approved by the regional ethics committees in Linköping, Stockholm, and Umeå.

Results

Overall, 130 patients (73%) achieved a moderate or good EULAR response, whereas 47 (27%) were non-responders. The distribution of *FCGR3A* genotypes was 81 (46%) 158FF, 78 (44%) 158VF, and 18 (10%) 158VV, which is in agreement with previous findings in Swedish RA populations [7, 13], and in Hardy-Weinberg equilibrium (p>0.95). The proportion of EULAR good responders did not differ significantly across *FCGR3A* genotypes (21%, 26%, and 28% for 158FF, 158VF, and 158VV, respectively). However, the proportion of good and moderate responders together, compared to non-responders, was significantly different across *FCGR3A* genotypes (p=0.017 in a 3x2 contingency table), where heterozygous patients showed the highest response rate (65 out of 78 patients, 83%), as compared to 55/81 (68%) patients carrying 158FF (OR 2.36, 95% CI 1.04-5.41, p=0.028), and 10/18 (56%) 158VV patients (OR 4.0, 95%CI 1.16-13.9, p=0.016)(figure 1). The frequency of responders was not significantly different between 158FF and 158VV (OR 1.69 (95% CI 0.53-5.37), p=0.4). Baseline characteristics (table 1) revealed no significant differences across *FCGR3A* genotypes. Experiencing a therapeutic response was more common among

rheumatoid factor (RF) positive cases as compared to RF-negative cases (OR 2.38 (1.05-5.39), p=0.038), and in the RF-positive group, response rates remained significantly different across *FCGR3A* genotype (p=0.004), but did not reach statistical significance among RF-negative cases (p=0.056). After stratifying for sex, response rates among female patients remained different in 158VF as compared to 158FF (p=0.047) and 158VV (p=0.001), but this was not seen in the smaller group of males (p>0.4) (figure 1). Furthermore, among 158VV patients, men had significantly higher response rates than women (100% *vs* 39%, respectively, p=0.036), while therapeutic response among 158FF or 158VF patients did not differ between men and women (p=0.72 and p=0.46, respectively).

Discussion

Although an influence of *FCGR3A*-F158V on RTX efficacy was in line with our hypothesis, the lack of a clear allele-dose effect points towards a more complex role of *FCGR3A* in RTX therapy of RA than anticipated. Based on previous reports of increased RTX-induced ADCC [5], more pronounced peripheral B-cell depletion [14], better clinical outcomes in B-cell malignancies [2, 3], we expected any difference to appear in favor of the 158VV genotype. Also, a recent study on 111 RA patients described an association between the 158V allele and response to RTX [6]. Our study is, to our knowledge, the largest on this topic to date, and we surprisingly found a significantly larger proportion of responders among *FCGR3A*-F158V heterozygous patients, a finding to which there is no immediate explanation from previous experimental work. Regarding the *in vivo* situation little is known, however, as the mechanisms whereby RTX reduces signs and symptoms of RA remain incompletely understood. The initial view that the disease-modifying action of RTX depends on depletion

of B cells and the eventual disappearance of pathogenic autoantibodies is contradicted by several observations. For instance, although circulating autoantibodies may indeed decline, they seldom disappear [15], and both non-circulating B cells and autoantibody production in the synovium are clearly less affected by RTX than their circulating counterparts [16]. Other proposals for RTX mode of action in RA include the immune complex decoy hypothesis, suggesting that RTX-opsonized B cells keep disease-promoting FcγR-expressing phagocytic cells busy eliminating B cells instead of perpetuating synovial inflammation [17]. In this context, one would expect that the more pronounced activation of *FCGR3A*-158VV macrophages would render them less prone to divert from the immune complex driven rheumatoid inflammation in the synovium, and hence corresponding to a worse therapeutic outcome in 158VV patients as seen in the current study. Alternatively, *FCGR3A*-158VV individuals could, as compared to *FCGR3A*-158FF, handle RTX-opsonized B cells more efficiently and may thereby, to a greater extent and more rapidly, return from this drug-induced diversion. Our finding of a significantly higher response rate among heterozygous patients could possibly point towards several mechanisms being involved in RTX action and that, in RA, these are most optimally balanced in individuals with the intermediate binding variant of FcγRIIIA, *i.e. FCGR3A*-158VF.

A previous study on RA patients reported that the proportion of responders among 158VF patients was significantly higher compared to 158FF cases, but that the proportion of responders among 158VV were similar to 158VF, albeit failing to reach statistical significance when compared to 158VF or 158FF [6]. Merging the response data from the two studies yields a significantly better response rate among 158VF as compared to 158FF, (OR 2.82 (95% CI 1.44-5.56), p= 0.001), whereas the 158VV group does not differ significantly

from neither 158VF (OR 0.41 (0.15-1.15), p= 0.067) nor 158FF (OR 1.17 (95% CI 0.46-2.99), p=0.83).

Shortcomings of the current study include the fact that therapeutic response was assessed with up to 3 months variation between patients. However, a separate analysis of patients with response assessed at 6 month (n=108) yielded similar results, making a major impact of follow-up time-point unlikely. Further, the relatively limited number of male patients calls for cautious interpretation of the findings regarding sex differences. Still, we believe the 158VV sex difference is of interest, as no tendencies towards sex differences were found among 158FF and 158VF patients, despite being substantially larger groups. Also, there are previously described sex dependent associations of 158VV in RA [7, 10].

Based on currently available data, we conclude that *FCGR3A-F158V* heterozygousity is associated with a better response to RTX in RA patients as compared to homozygousity of the low-affinity F allele. Data regarding patients carrying two high-affinity alleles, 158VV, remains inconclusive and needs to be resolved before *FCGR3A*-F158V may become clinically relevant as a predictor of RTX response in RA.

Conflicts of interests

RFvV has received research support and honoraria from Roche. None of the other authors declare any conflicts of interest

Funding

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Table 1. Baseline characteristics of the 177 RA patients according to *FCGR3A* genotype.

	158VV (n=18)	**158VF (n=78)**	**158FF (n=81)**	**Total (n=177)**
Mean (SD) disease duration, years	11 (6)	15 (10)	14 (12)	14 (11)
Females, n (%)	13 (72)	61 (78)	71 (88)	145 (82)
Rheumatoid factor positive, n (%)	12 (67)	62 (81)	63 (78)	137 (78)
Baseline DAS28, mean (SD)	5.2 (1.0)	5.5 (1.1)	5.5 (1.2)	5.5 (1.1)
Concurrent DMARD therapy, n (%)	15 (83)	48 (62)	59 (73)	122 (69)
Oral glucocorticoid therapy, n (%) *	13 (72)	55 (72) #	48 (64) ##	116 (69) ###
Number of previous TNF inhibitors, mean (SD)	1.4 (1.2)	1.3 (1.1)	1.2 (1.1)	1.3 (1.1)

* Data available on: # 76/78 patients, ## 75/81 patients, ### 169/177 patients.

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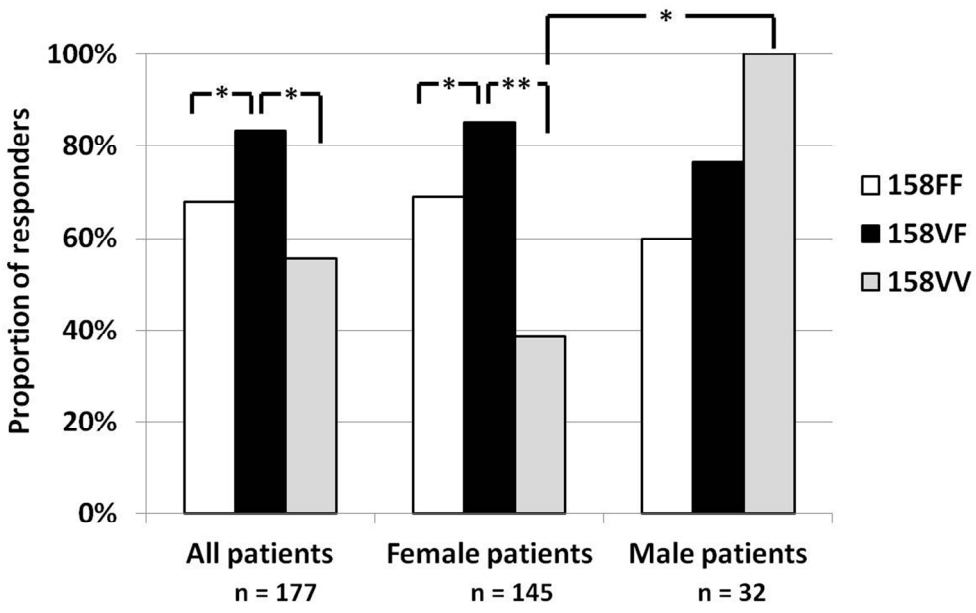
Figure legends

Figure 1.

Proportion of rituximab responders in relation to *FCGR3A* genotype in RA patients.

Significant differences are indicated * $p < 0.05$, ** $p < 0.01$.

Figure 1.



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STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Reviewer: 1
Comments to the Author
The significance of this large study is limited by the fact that rituximab was not administered according to a precise scheme to treat the patients: in some patients it was a first line treatment, in others a second line....
Then, the time of evaluation of response was not the same between patients...
This type of study is difficult and should be run prospectively, according to a well defined plan, every patient receiving rituxan in first line, according to an identical procedure, and being evaluated at a precise time after infusion.

We agree that a prospective randomised trial would be the most stringent way to assess the FCGR3A impact on rituximab efficacy. However, any clinically meaningful influence of FCGR3A would, in our opinion, appear with this 'real-life' approach. Regarding the time points of response assessment, results remained similar when analysing only response after 6 months (n=108). Therefore, we assume the impact of follow-up timing to be limited in this dataset.

Reviewer: 2
Comments to the Author
The authors investigated whether an associaiton exists between a genetic variant in FCGR3A and the response to rituximab in patients with rheumatoid arthritis. The biological link between RTX treatment outcome and FCGR3A validates the selection of the gene. The authors conclude that patients heterozygous (VF) for the variant have a better response to treatment compared to the patients homozygous for the variant (VV or FF). The paper is well written and the methods are appropriate.

The number of patients included in the study (n=177) is very small. This makes it difficult to draw firm conclusions concerning the observed association. It is therefore highly recommended to include a replication cohort in this study. To prove that the observed association is true.
The patient group carrying the VV genotype is very small (n=18) compared to the other genotype groups It might be that the difference in response observed between the VV and VF group disappears or strenghtens when a larger group is analysed. This should be addressed in more detail in the discussion.

Of course we agree that a larger patient material would have been preferable! However, we think that our report is justified in light of the opposing results obtained in the smaller French study recently published in ARD Ruyssen-Witrand *et al*. Ann Rheum Dis 2012;71:875-7. A sentence regarding the small VV group, and the discordance to the data of Ruyssen-Witrand *et al* has now been introduced in the discussion (page 9)

Due to the small study population I think it is not valid to perform stratified analysis (e.g. based on sex and RF factor) as the subgroups analysed become very small. For instance the association analysis of the male subgroup only includes 32 patients, in addition the genotypes observed in this subgroup is different from those in the females (in the females/total group the VF and FF genotype groups are almost of equal size whereas males carry more often the VF genotype). In the discussion it is indicated that the results should be taken with caution but I think genetic association analysis should not be performed with such small groups.

Our opinion remains that these findings are of interest, given the previous finding sex differences found regarding FCGR3A and RA. Since RF is a known predictor of rituximab response in RA it was included in the analysis, but could be excluded if this is judged to improve the quality of our manuscript.

Depicting the numbers for the genotype groups in figure 1 will make the figure easier to interpret.

We actually believe that changing bars from representing percentages to actual numbers of patients will instead hamper the ability to visual comparison between genotype groups. We are, however, happy to provide numbers for each genotype group below the x axis instead of 177, 145, and 32 as it now stands.

In the discussion the authors compare their data with an earlier study addressing the same question. A meta-analysis is performed indicating that the VF patients show a better treatment response. Though this analysis does not confirm the observed difference between the VF and VV genotypes. In the Swedish population the OR is 4.0 whereas the OR in the meta-analysis is 0.41. This lack of replication and more importantly the change in the direction of the association is not addressed at all in the paragraph including the meta-analysis , only in the final paragraph a remark is placed concerning the VV genotype. These observations could be addressed in more detail in the discussion.

A remark regarding the discordance to Ruyssen-Witrand *et al* has been added in the discussion (page 9)

Reviewer: 3
Comments to the Author
This paper is well written and this is an important area of investigation.
I have one major area of concern with regards to this study, which should be addressed:
FCGR3A and FCGR3B are highly homologous and obtaining accurate genotyping by standard allele specific methods has proven to be problematic for most groups working in this field. The authors have therefore chosen to use a commercial assay that has not been subject to validation within the public domain. The primer sequences are not included in the manuscript and I believe that this assay warrants validation by direct sequencing on the DNA samples used in the study, since the quality of DNA can have major influences over genotyping accuracy at this locus. This is particularly important given the heterozygous effect observed with RTX response.

This is a very important remark by the reviewer. The genotype frequencies in the current study are in line with previous Swedish reports and this is now clarified in the manuscript. If the FCGR3B “allele” was detected in the assay, an increased proportion of V alleles would have been seen. Moreover, this Taqman assay has been widely used and we cannot find any indications that genotype frequencies assessed by this method deviate from what would be expected from the background populations. Although the manufacturer will not release primer sequences, we have been reassured by the company that the probe sequence is FCGR3A specific. Thus, any primer cross reactivity to FCGR3B would only result in decreased detection signals due to a lower number of FCGR3A amplicons, but would not result in FCGR3B “signaling”. Low detection signals have not been a problem during FCGR3A genotyping in our lab, probably because primers are in enough excess in the assay.

Furthermore, there has been no mention of the known FCGR3A CNV. What is the prevalence of this CNV in the Swedish population? Could this explain the heterozygous effect?

According to a previous study, approximately 95% of Swedish individuals carry 2 copies of FCGR3A (Niederer HA et al: Copy number, linkage disequilibrium and disease association in the FCGR locus. Hum Mol Genet 2010;19:3282–94). We find it hard to believe that this underlies the heterozygous effect. A remark about this has been added in the discussion (page 9)

Minor comments:
What was the power of this study since the numbers remain low for a pharmacogenetic study.
Were the p values adjusted for the number of analyses performed?
Please see answer to reviewer 4, point 3.
Clinical response was assessed between 3-6 months, which is a wide interval for a treatment that requires retreatment every 6 months. This does not appear to have been considered, evaluated or adjusted for in the analyses if necessary.

As stated in the discussion, results remained similar when analysing only the patients with data from 6 months follow-up (n=108). Therefore, we assume that the impact of follow-up timing is limited in this dataset.

Whilst the discussion was well written, the mechanism of the heterozygous effect received a disproportionately large word count compared to discussion of the limitations of the study, for which potential genotyping error, low statistical power and large numbers of statistical tests are prominent.

A couple of remarks have been added in the discussion (p.9) regarding the limitations of the study.

Reviewer: 4
Comments to the Author
Kastbom and colleagues attempted to decipher the possible value of FcGRIIIa genotyping in predicting the response to rituximab. Although such studies are of importance for the possible fine tuning of treatments, there are several concerns with this paper that deserve the attention of the authors.

1 The number of patients (177) is very low for these kind of studies. There is a big concern for the possible role of Type I / Type II errors.

We agree that a larger patient material would have been preferable! Still, this is the largest study available on this topic.

2. Looking at the role of FcGRIIIa the authors are trying to assess whether a primary immune mediated response is associated with a clinical response, the DAS28. I have severe problems with this strategy. BAsed on what is mentioned above, wouldn't it be better to relate the genotyping to the absolute response in DAS rather that the EULAR defined respondership which was designed to follow therapy only. Maybe than, differences become more clearer.

This is an important remark by the reviewer. We did analyse absolute DAS28 changes, and found very similar results. This is now pointed out in the results section.

3. There need to be corrected for multiple comparison

We disagree with the reviewer on this point. We do not see the need for statistical correction regarding our à-priori decided main question, *i.e.* whether one genotype associates with one outcome (therapeutic response) and one stratification (sex).

FCGR3A genotype and the therapeutic response to rituximab in patients with rheumatoid arthritis

Alf Kastbom[1], Lars Cöster[1], Lisbeth Ärlestig[2], Aikaterini Chatzidionysiou[3,4], Ronald van Vollenhoven [3,4], Leonid Padyukov[3], Solbritt Rantapää-Dahlqvist[2], Saedis Saevarsdottir[3]

1. Rheumatology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Department of Rheumatology in Östergötland, County Council of Östergötland, Linköping, Sweden.
2. Department of Public Health and Clinical Medicine/Rheumatology, Umeå University, Umeå, Sweden
3. Rheumatology Unit, Department of Medicine, Karolinska University Hospital and Karolinska Institute, Stockholm, Sweden.
4. Unit for Clinical Therapy Research of Inflammatory Diseases, Department of Medicine, Karolinska Institute, Stockholm, Sweden.

Corresponding Author:

Dr. Alf Kastbom

Department of Clinical and Experimental Medicine, Rheumatology

Linköping University

SE-581 85 Linköping

Sweden

alf.kastbom@liu.se

Key words: Fc gamma receptor, Rheumatoid arthritis, Rituximab, Single nucleotide polymorphism, Therapy response.

Article focus

- A functional polymorphism in the gene encoding Fcγ receptor type IIIA (FCGR3A) influences the outcome of B cell-depleting therapy with rituximab in malignancies.
- Although rituximab is frequently used for the therapy of severe RA, studies on RA patients have been lacking.
- We wished to determine if FCGR3A-F158V genotype associates with rituximab efficacy in RA.

Key Messages:

- FCGR3A heterozygous patients experienced significantly higher response rates than 158FF and 158VV.
- The results are discordant to a similar recently published study based on 111 RA patients
- There are indications of a sex-specific effect of the 158VV genotype, as have been previously described regarding RA susceptibility and disease course.

Strenghts and limitations:

- Although limited by the small numbers of males and 158VV patients, this is until now the largest published study on FCGR3A and rituximab in RA patients.
- Differences could have been attenuated by the 'real-life' approach, *i.e.* that therapy was not administered by a standardised scheme, and the time to evaluation varied between patients.

Abstract

Objectives: To determine whether a polymorphism in the Fcγ receptor type IIIA (*FCGR3A*-F158V), influencing IgG binding affinity, relates to the therapeutic efficacy of rituximab in rheumatoid arthritis (RA) patients.

Design: Cohort study

Setting: Three university hospital rheumatology units in Sweden.

Participants: Patients with established RA (n=177; 145 females and 32 males) who started rituximab (Mabthera) as part of routine care.

Primary outcome measures: Response to rituximab therapy in relation to FCGR3A genotype, including stratification for sex.

~~***Methods:*** Patients with established RA starting rituximab as part of routine care (n=177; 145 females and 32 males) were evaluated according to EULAR response criteria dichotomised into responders and non-responders. *FCGR3A*-F158V genotype was determined by a commercially available TaqMan assay.~~

Results: The frequency of responders differed significantly across *FCGR3A* genotypes (*P*=0.017 in a 3x2 contingency table). Heterozygous patients showed the highest response rate at 83%, as compared to patients carrying 158FF (68%) or 158VV (56%) (p=0.028 and p=0.016, respectively). Among 158VV patients, response rates differed between male and female patients (p=0.036), but not among 158FF or 158VF patients (p=0.72 and p=0.46, respectively).

Conclusions: Therapeutic efficacy of rituximab in RA patients is influenced by *FCGR3A* genotype, with the highest response rates found among heterozygous patients. This may suggest that different rituximab mechanisms of action in RA are optimally balanced in *FCGR3A*-158VF patients. Similar to the previously described associations with RA susceptibility and disease course, the impact of 158VV on rituximab response may be influenced by sex.

Introduction

The growing arsenal of biologic agents available in rheumatoid arthritis (RA) pharmacotherapy increases the demand for predictors of therapeutic responses and/or side effects. Human Fcγ receptors mediate effector functions of IgG antibodies and may modulate the therapeutic efficacy of all biologic substances containing IgG-Fc parts. Hence, a functional single nucleotide polymorphism in *FCGR3A (FCGR3A*-F158V*,* rs396991*)*, influencing IgG binding affinity to the activating Fcγ receptor type IIIA (FcγRIIIA) [1], has been associated with the therapeutic efficacy of rituximab (RTX), *i.e.* an anti-CD20 B cell depleting IgG1 monoclonal antibody, in B cell malignancies [2, 3]. RTX-coated B cells may be eliminated by several mechanisms; complement-dependent cytotoxicity, antibody-dependent cytoxocity (ADCC) by Natural Killer (NK) cells, and/or phagocytosis by FcγR-bearing cells. Although the relevance in humans has not been established, mice lacking proper complement or NK-cell functions are equally well B-cell depleted as native animals, thus pointing towards a major role of FcγR-bearing macrophages [4]. It has been convincingly shown that RTX binds with higher affinity to 158V in an allele-dose dependent manner and that ADCC by NK cells are affected accordingly [5]. Data regarding *FCGR3A* genotype and RTX efficacy in RA has been lacking until recently, when Ruyssen-Witrand *et al* reported an association between carriage of the high-affinity binding valine (V) allele of *FCGR3A* and higher response rates to RTX [6].

Numerous case-control studies have been performed with regard to *FCGR3A*-F158V and RA susceptibility. Although initial studies showed remarkably discordant results, subsequent investigations have shown an association between homozygousity of the high-affinity allele (158VV) and an increased risk of RA in Europeans [7-10]. In studies presenting data stratified for sex, the increased risk conferred by the 158VV genotype only attributes to the male

population [7, 10]. In line with this, male patients with early RA carrying 158V have a more severe disease course whereas, intriguingly, in female patients the opposite is seen [7]. The biological basis for this sex difference is yet to be elucidated, but it has been shown that estrogen influences both FcγRIIIA expression and FcγRIIIA-mediated release of TNF and IL-1β from monocytic cells [11].

The current study was conducted to explore the influence of *FCGR3A* genotype on RTX efficacy in RA patients.

Patients & Methods

Patients with established RA (n=177; 145 females and 32 males) who started rituximab (Mabthera) as part of routine care at three rheumatology clinics in Sweden (Karolinska University Hospital, Solna; Linköping University Hospital, Linköping; and Umeå University Hospital, Umeå) were included in the study. Baseline characteristics and concurrent medication are shown in table 1. The therapeutic response was assessed by the EULAR response criteria after 3-6 months [12]. DNA was extracted from whole blood by standard techniques and *FCGR3A*-F158V was genotyped by a commercially available TaqMan assay (Applied Biosystems, ID C_25815666_10).

Statistical analysis

Response rates were compared by chi square test across *FCGR3A* genotypes, and by Fisher´s exact test between sexes. Baseline characteristics were tested by chi square test for categorical

variables and by one-way ANOVA for continuous variables. Two-sided p-values <0.05 were considered significant. Analyses were performed using SPSS version 19.

Ethical considerations

Informed consent was obtained from all patients according to the Declaration of Helsinki. The study protocol was approved by the regional ethics committees in Linköping, Stockholm, and Umeå.

Results

Overall, 130 patients (73%) achieved a moderate or good EULAR response, whereas 47 (27%) were non-responders. The distribution of *FCGR3A* genotypes was 81 (46%) 158FF, 78 (44%) 158VF, and 18 (10%) 158VV, which is in agreement with previous findings in Swedish RA populations [7, 13], and in Hardy-Weinberg equilibrium (p>0.95). The proportion of EULAR good responders did not differ significantly across *FCGR3A* genotypes (21%, 26%, and 28% for 158FF, 158VF, and 158VV, respectively). However, the proportion of good and moderate responders together, compared to non-responders, was significantly different across *FCGR3A* genotypes (p=0.017 in a 3x2 contingency table), where heterozygous patients showed the highest response rate (65 out of 78 patients, 83%), as compared to 55/81 (68%) patients carrying 158FF (OR 2.36, 95% CI 1.04-5.41, p=0.028), and 10/18 (56%) 158VV patients (OR 4.0, 95%CI 1.16-13.9, p=0.016)(figure 1). The frequency of responders was not significantly different between 158FF and 158VV (OR 1.69 (95% CI 0.53-5.37), p=0.4). Baseline characteristics (table 1) revealed no significant differences across *FCGR3A* genotypes. Experiencing a therapeutic response was more common among

rheumatoid factor (RF) positive cases as compared to RF-negative cases (OR 2.38 (1.05-5.39), p=0.038), and in the RF-positive group, response rates remained significantly different across *FCGR3A* genotype (p=0.004), but did not reach statistical significance among RF-negative cases (p=0.056). After stratifying for sex, response rates among female patients remained different in 158VF as compared to 158FF (p=0.047) and 158VV (p=0.001), but this was not seen in the smaller group of males (p>0.4) (figure 1). Furthermore, among 158VV patients, men had significantly higher response rates than women (100% *vs* 39%, respectively, p=0.036), while therapeutic response among 158FF or 158VF patients did not differ between men and women (p=0.72 and p=0.46, respectively). Absolute changes in DAS28 in relation to FCGR3A yielded similar results as for categorical EULAR responses (data not shown).

Discussion

Although an influence of *FCGR3A*-F158V on RTX efficacy was in line with our hypothesis, the lack of a clear allele-dose effect points towards a more complex role of *FCGR3A* in RTX therapy of RA than anticipated. Based on previous reports of increased RTX-induced ADCC [5], more pronounced peripheral B-cell depletion [14], better clinical outcomes in B-cell malignancies [2, 3], we expected any difference to appear in favor of the 158VV genotype. Also, a recent study on 111 RA patients described an association between the 158V allele and response to RTX [6]. Our study is, to our knowledge, the largest on this topic to date, and we surprisingly found a significantly larger proportion of responders among *FCGR3A*-F158V heterozygous patients, a finding to which there is no immediate explanation from previous experimental work. Regarding the *in vivo* situation little is known, however, as the mechanisms whereby RTX reduces signs and symptoms of RA remain incompletely

understood. The initial view that the disease-modifying action of RTX depends on depletion of B cells and the eventual disappearance of pathogenic autoantibodies is contradicted by several observations. For instance, although circulating autoantibodies may indeed decline, they seldom disappear [15], and both non-circulating B cells and autoantibody production in the synovium are clearly less affected by RTX than their circulating counterparts [16]. Other proposals for RTX mode of action in RA include the immune complex decoy hypothesis, suggesting that RTX-opsonized B cells keep disease-promoting FcγR-expressing phagocytic cells busy eliminating B cells instead of perpetuating synovial inflammation [17]. In this context, one would expect that the more pronounced activation of *FCGR3A*-158VV macrophages would render them less prone to divert from the immune complex driven rheumatoid inflammation in the synovium, and hence corresponding to a worse therapeutic outcome in 158VV patients as seen in the current study. Alternatively, *FCGR3A*-158VV individuals could, as compared to *FCGR3A*-158FF, handle RTX-opsonized B cells more efficiently and may thereby, to a greater extent and more rapidly, return from this drug-induced diversion. Our finding of a significantly higher response rate among heterozygous patients could possibly point towards several mechanisms being involved in RTX action and that, in RA, these are most optimally balanced in individuals with the intermediate binding variant of FcγRIIIA, *i.e. FCGR3A*-158VF.

A previous study on RA patients reported that the proportion of responders among 158VF patients was significantly higher compared to 158FF cases, but that the proportion of responders among 158VV were similar to 158VF, albeit failing to reach statistical significance when compared to 158VF or 158FF [6]. Merging the response data from the two studies yields a significantly better response rate among 158VF as compared to 158FF, (OR 2.82 (95% CI 1.44-5.56), p= 0.001), whereas the 158VV group does not differ significantly

from neither 158VF (OR 0.41 (0.15-1.15), p= 0.067) nor 158FF (OR 1.17 (95% CI 0.46-2.99), p=0.83).

Shortcomings of the current study include the fact that therapeutic response was assessed with up to 3 months variation between patients. However, a separate analysis of patients with response assessed at 6 month (n=108) yielded similar results, making a major impact of follow-up time-point unlikely. Our finding that 158VV patients less frequently experience response to RTX is discordant with the findings of Ruyssen-Witrand *et al*, and the limited number of 158VV patients (in both studies) adds uncertainty to firm conclusions regarding this particular genotype. Further, the relatively limited number of male patients calls for cautious interpretation of the findings regarding sex differences. Still, we believe the 158VV sex difference is of interest, as no tendencies towards sex differences were found among 158FF and 158VF patients, despite being substantially larger groups. Also, there are previously described sex dependent associations of 158VV in RA [7, 10]. The FCGR locus is subject to copy number variation (CNV), and in the current study CN of FCGR3A was not investigated. However, the the functional consequences of FCGR3A CNV remains unknown, and a previous study showed that only 5% of Swedish individuals carry ≠2 copies of FCGR3A [18].

Based on currently available data, we conclude that *FCGR3A-F158V* heterozygousity is associated with a better response to RTX in RA patients as compared to homozygousity of the low-affinity F allele. Data regarding patients carrying two high-affinity alleles, 158VV, remains inconclusive and needs to be resolved before *FCGR3A*-F158V may become clinically relevant as a predictor of RTX response in RA.

Conflicts of interests

RFvV has received research support and honoraria from Roche. None of the other authors declare any conflicts of interest

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Table 1. Baseline characteristics of the 177 RA patients according to *FCGR3A* genotype.

	158VV (n=18)	**158VF (n=78)**	**158FF (n=81)**	**Total (n=177)**
Mean (SD) disease duration, years	11 (6)	15 (10)	14 (12)	14 (11)
Females, n (%)	13 (72)	61 (78)	71 (88)	145 (82)
Rheumatoid factor positive, n (%)	12 (67)	62 (81)	63 (78)	137 (78)
Baseline DAS28, mean (SD)	5.2 (1.0)	5.5 (1.1)	5.5 (1.2)	5.5 (1.1)
Concurrent DMARD therapy, n (%)	15 (83)	48 (62)	59 (73)	122 (69)
Oral glucocorticoid therapy, n (%) *	13 (72)	55 (72) #	48 (64) ##	116 (69) ###
Number of previous TNF inhibitors, mean (SD)	1.4 (1.2)	1.3 (1.1)	1.2 (1.1)	1.3 (1.1)

* Data available on: # 76/78 patients, ## 75/81 patients, ### 169/177 patients.

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Figure legends

Figure 1.

Proportion of rituximab responders in relation to *FCGR3A* genotype in RA patients.

Significant differences are indicated * $p < 0.05$, ** $p < 0.01$.

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Complete List of Authors:	Kastbom, Alf; Rheumatology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Department of Rheumatology in Ostergotland, County Council of Ostergotland Cöster, Lars; Rheumatology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Department of Rheumatology in Ostergotland, County Council of Ostergotland Ärlestig, Lisbeth; Department of Public Health and Clinical Medicine/Rheumatology, Umeå University Chatzidionysiou, Katerina; Rheumatology Unit, Department of Medicine, Karolinska University Hospital and Karolinska Institute van Vollenhoven, Ronald; Rheumatology Unit, Department of Medicine, Karolinska University Hospital and Karolinska Institute Padyukov, Leonid; Rheumatology Unit, Department of Medicine, Karolinska University Hospital and Karolinska Institute Rantapaa-Dahlqvist, Solbritt; Department of Public Health and Clinical Medicine/Rheumatology, Umeå University Saevarsdottir, Saedis; Rheumatology Unit, Department of Medicine, Karolinska University Hospital and Karolinska Institute
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1. Rheumatology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Department of Rheumatology in Östergötland, County Council of Östergötland, Linköping, Sweden.
2. Department of Public Health and Clinical Medicine/Rheumatology, Umeå University, Umeå, Sweden
3. Rheumatology Unit, Department of Medicine, Karolinska University Hospital and Karolinska Institute, Stockholm, Sweden.
4. Unit for Clinical Therapy Research of Inflammatory Diseases, Department of Medicine, Karolinska Institute, Stockholm, Sweden.

Corresponding Author:

Dr. Alf Kastbom

Department of Clinical and Experimental Medicine, Rheumatology

Linköping University

SE-581 85 Linköping

Sweden

alf.kastbom@liu.se

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Response rates were compared by chi square test across *FCGR3A* genotypes, and by Fisher´s exact test between sexes. Baseline characteristics were tested by chi square test for categorical variables and by one-way ANOVA for continuous variables. Two-sided p-values <0.05 were considered significant. Analyses were performed using SPSS version 19.

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across *FCGR3A* genotype (p=0.004), but did not reach statistical significance among RF-negative cases (p=0.056). After stratifying for sex, response rates among female patients remained different in 158VF as compared to 158FF (p=0.047) and 158VV (p=0.001), but this was not seen in the smaller group of males (p>0.4) (figure 1). Furthermore, among 158VV patients, men had significantly higher response rates than women (100% *vs* 39%, respectively, p=0.036), while therapeutic response among 158FF or 158VF patients did not differ between men and women (p=0.72 and p=0.46, respectively). Absolute changes in DAS28 in relation to *FCGR3A* yielded similar results as for categorical EULAR responses (data not shown).

Discussion

Although an influence of *FCGR3A*-F158V on RTX efficacy was in line with our hypothesis, the lack of a clear allele-dose effect points towards a more complex role of *FCGR3A* in RTX therapy of RA than anticipated. Based on previous reports of increased RTX-induced ADCC [5], more pronounced peripheral B-cell depletion [14], better clinical outcomes in B-cell malignancies [2, 3], we expected any difference to appear in favor of the 158VV genotype. Also, a recent study on 111 RA patients described an association between the 158V allele and response to RTX [6]. Our study is, to our knowledge, the largest on this topic to date, and we surprisingly found a significantly larger proportion of responders among *FCGR3A*-F158V heterozygous patients, a finding to which there is no immediate explanation from previous experimental work. Regarding the *in vivo* situation little is known, however, as the mechanisms whereby RTX reduces signs and symptoms of RA remain incompletely understood. The initial view that the disease-modifying action of RTX depends on depletion of B cells and the eventual disappearance of pathogenic autoantibodies is contradicted by

several observations. For instance, although circulating autoantibodies may indeed decline, they seldom disappear [15], and both non-circulating B cells and autoantibody production in the synovium are clearly less affected by RTX than their circulating counterparts [16]. Other proposals for RTX mode of action in RA include the immune complex decoy hypothesis, suggesting that RTX-opsonized B cells keep disease-promoting FcγR-expressing phagocytic cells busy eliminating B cells instead of perpetuating synovial inflammation [17]. In this context, one would expect that the more pronounced activation of *FCGR3A*-158VV macrophages would render them less prone to divert from the immune complex driven rheumatoid inflammation in the synovium, and hence corresponding to a worse therapeutic outcome in 158VV patients as seen in the current study. Alternatively, *FCGR3A*-158VV individuals could, as compared to *FCGR3A*-158FF, handle RTX-opsonized B cells more efficiently and may thereby, to a greater extent and more rapidly, return from this drug-induced diversion. Our finding of a significantly higher response rate among heterozygous patients could possibly point towards several mechanisms being involved in RTX action and that, in RA, these are most optimally balanced in individuals with the intermediate binding variant of FcγRIIIA, *i.e. FCGR3A*-158VF.

A previous study on RA patients reported that the proportion of responders among 158VF patients was significantly higher compared to 158FF cases, but that the proportion of responders among 158VV were similar to 158VF, albeit failing to reach statistical significance when compared to 158VF or 158FF [6]. Merging the response data from the two studies yields a significantly better response rate among 158VF as compared to 158FF, (OR 2.82 (95% CI 1.44-5.56), p= 0.001), whereas the 158VV group does not differ significantly from neither 158VF (OR 0.41 (0.15-1.15), p= 0.067) nor 158FF (OR 1.17 (95% CI 0.46-2.99), p=0.83).

Shortcomings of the current study include the fact that therapeutic response was assessed with up to 3 months variation between patients. However, a separate analysis of patients with response assessed at 6 month (n=108) yielded similar results, making a major impact of follow-up time-point unlikely. Our finding that 158VV patients less frequently experience response to RTX is discordant with the findings of Ruyssen-Witrand *et al*, and the limited number of 158VV patients (in both studies) adds uncertainty to firm conclusions regarding this particular genotype. Further, the relatively limited number of male patients calls for cautious interpretation of the findings regarding sex differences. Still, we believe the 158VV sex difference is of interest, as no tendencies towards sex differences were found among 158FF and 158VF patients, despite being substantially larger groups. Also, there are previously described sex dependent associations of 158VV in RA [7, 10]. The *FCGR* locus is subject to copy number variation (CNV), and in the current study CN of *FCGR3A* was not investigated. However, the the functional consequences of *FCGR3A* CNV remains unknown, and a previous study showed that only 5% of Swedish individuals carry ≠2 copies of *FCGR3A* [18].

Based on currently available data, we conclude that *FCGR3A-F158V* heterozygousity is associated with a better response to RTX in RA patients as compared to homozygousity of the low-affinity F allele. Data regarding patients carrying two high-affinity alleles, 158VV, remains inconclusive and needs to be resolved before *FCGR3A*-F158V may become clinically relevant as a predictor of RTX response in RA.

Conflicts of interests

RFvV has received research support and honoraria from Roche. None of the other authors declare any conflicts of interest

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Contributorship Statement

AK were involved in conception and design of the study, acquisition of patient data, genotyping, and drafted the paper. LÄ and LP were involved in design of the study, genotyping, and interpretation of results. LC, AC, RvV, SR-D, and SS were involved in design of the study, and acquisition of patient data, and interpretation of result. All authors revised the draft paper.

Data Sharing Statement

There is no additional data available

Table 1. Baseline characteristics of the 177 RA patients according to *FCGR3A* genotype.

	158VV (n=18)	**158VF (n=78)**	**158FF (n=81)**	**Total (n=177)**
Mean (SD) disease duration, years	11 (6)	15 (10)	14 (12)	14 (11)
Females, n (%)	13 (72)	61 (78)	71 (88)	145 (82)
Rheumatoid factor positive, n (%)	12 (67)	62 (81)	63 (78)	137 (78)
Baseline DAS28, mean (SD)	5.2 (1.0)	5.5 (1.1)	5.5 (1.2)	5.5 (1.1)
Concurrent DMARD therapy, n (%)	15 (83)	48 (62)	59 (73)	122 (69)
Oral glucocorticoid therapy, n (%) *	13 (72)	55 (72) #	48 (64) ##	116 (69) ###
Number of previous TNF inhibitors, mean (SD)	1.4 (1.2)	1.3 (1.1)	1.2 (1.1)	1.3 (1.1)

* Data available on: # 76/78 patients, ## 75/81 patients, ### 169/177 patients.

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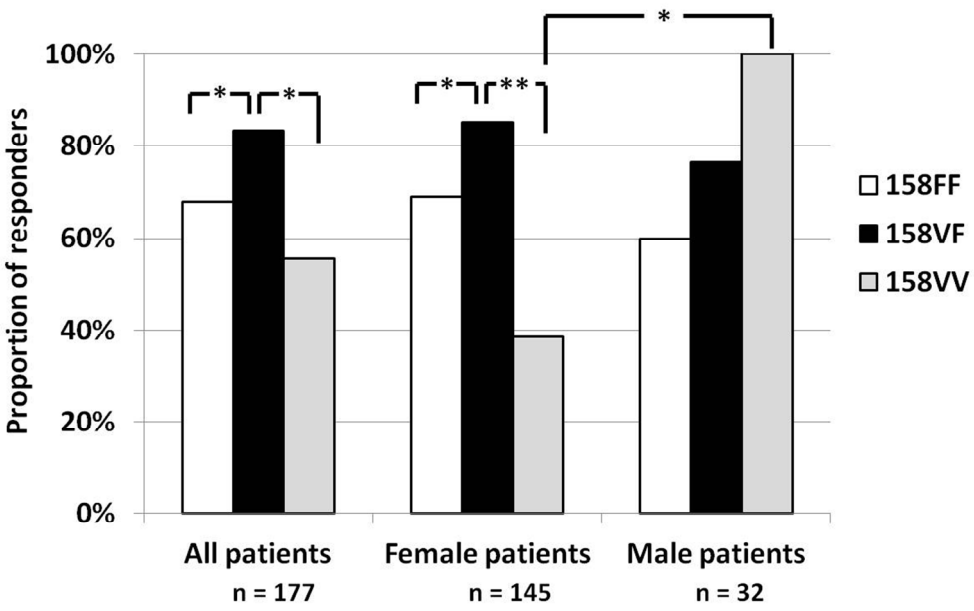
Figure legends

Figure 1.

Proportion of rituximab responders in relation to *FCGR3A* genotype in RA patients.

Significant differences are indicated * $p < 0.05$, ** $p < 0.01$.

Figure 1.



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STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.