PEER REVIEW HISTORY

This paper was submitted to the ARD but declined for publication following peer review. The authors addressed the reviewers' comments and submitted the revised paper to BMJ Open where it is re-reviewed and accepted.

ARTICLE DETAILS

TITLE (PROVISIONAL)	Influence of FCGR3A genotype on the therapeutic response to rituximab in rheumatoid arthritis: an observational cohort study
AUTHORS	Kastbom, Alf ; Cöster, Lars; Ärlestig, Lisbeth; Chatzidionysiou, Katerina; van Vollenhoven, Ronald; Padyukov, Leonid; Rantapaa-Dahlqvist, Solbritt; Saevarsdottir, Saedis

VERSION 1 - REVIEW

REVIEWER	Roudier, Jean INSERM UMRs 1097, Immunogenetics of RA
REVIEW RETURNED	24-Apr-2012

GENERAL COMMENTS	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.

REVIEWER	Coenen, Marieke Radboud University Nijmegen Medical Centre, Human Genetics 855
REVIEW RETURNED	02-May-2012

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

REVIEWER	Radstake, Timothy University Medical Center Nijmegen, Rheumatology
REVIEW RETURNED	11-May-2012

GENERAL COMMENTS	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. 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. 3. There need to be corrected for multiple comparison

- The manuscript received a fourth review at the Annals of Rheumatic Disease but the reviewer did not give permission for their comments to be published.

VERSION 1 – AUTHOR RESPONSE

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).

VERSION 2 – REVIEW

REVIEWER	Morgan, Ann University of Leeds, Leeds Institute of Molecular Medicine I have no competing interests
REVIEW RETURNED	29-Jun-2012

GENERAL COMMENTS	The authors have addressed most of the reviewers comments, but there are a number of typographical errors that need correction. However, I have a fundamental problem with the need to accept on

	good faith that an assay is gene specific when the validation of this is not available in the public domain, particularly when this gene is notoriously difficult to genotype. I dont think its acceptable to point to other publications that have also not provided the necessary validation. I think it would be reasonable to undertake direct sequencing using one of the validated assays in 10 individuals of each genotype.

VERSION 2 – AUTHOR RESPONSE

Response to the Reviewer:
- We have now validated the TaqMan assay in 30 samples (10 of each genotype) by comparing the TaqMan results to a previously described direct sequencing assay (Kastbom A, et al. Rheumatology 2005;44:1295-98) which allows differentiation between FCGR3A and FCGR3B. Results were 100% concordant, and this is now included in the manuscript.

- A number of typographical errors have been corrected.

VERSION 3 – REVIEW

REVIEWER	Morgan, Ann University of Leeds, Leeds Institute of Molecular Medicine I have no competing interests
REVIEW RETURNED	10-Aug-2012

GENERAL COMMENTS	I am happy the authors have responded adequately to the previous reviewers comments