CHAPTER 5

Contribution of Fcγ receptor IIIA gene 158V/F polymorphism and copy number variation to the risk of ACPA-positive rheumatoid arthritis

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ABSTRACT

Objectives: Fcγ receptors (FcγRs) are potent immune-modulators. FcγRs-genes encompass a complex region, polymorphic by both single nucleotide polymorphisms (SNPs) and copy number variation (CNV). The genetic complexity of FcγRs-genes, combined with the heterogeneity of rheumatoid arthritis (RA) may have caused inconsistent findings in previous studies on FcγR-SNPs in RA. Since there is increasing evidence that anti-citrullinated peptide autoantibodies (ACPA) positive RA and ACPA-negative RA have different genetic background, we investigated whether FcγRIIIA158V/F SNP differently associates with ACPA-positive and ACPA-negative RA. Moreover, this study is also the first to assess whether CNV of FcγRIIIA-gene affects the FcγRIIIA158V/F SNP genotyping and if CNV of FcγRIIIA-gene confers risk to RA.

Methods: This study comprises 945 RA patients and 388 healthy controls, all Dutch Caucasians. FcγRIIIA158V/F SNP was genotyped using Sequenom. The CNV of FcγRIIIA-gene was determined in 369 RA patients and 240 controls using Multiplex Ligation-dependent Probe Amplification (MLPA). Associations between the genotypes and RA were analysed stratifying for the presence/absence of ACPA and the presence/absence of CNV of the FcγRIIIA-gene.

Results: The FcγRIIIA-158V variant was associated with susceptibility to ACPA-positive RA (OR=1.3, 95%CI 1.01-1.6, p=0.034). In patients without CNV this association was also present (OR=1.6, 95%CI 1.2-2.4, p=0.005). FcγRIIIA-gene showed CNV that was not significantly different between patients and controls.

Conclusion: The FcγRIIIA-158V allele confers risk to ACPA-positive RA, before and after correcting for the presence of CNV. Although the FcγRIIIA-gene shows CNV, this was not associated with higher risk of RA.

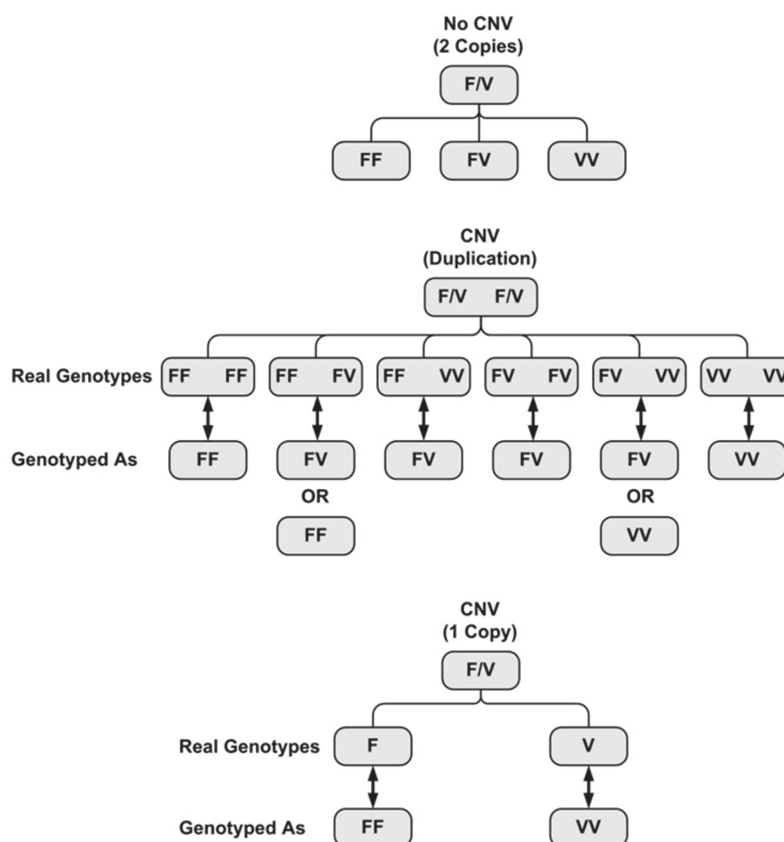
INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease for which the aetiology and pathogenesis remain largely unclear. One of the characteristic features of part of the RA patients is the expression of auto-antibodies such as rheumatoid factors (RF) and ACPA [1]. Multiple genetic risk factors have been unequivocally shown to predispose for ACPA-positive RA but not for ACPA-negative RA, like *HLA* shared epitope (*SE*) [2], *PTPN22* [3], and recently *TRAF-C5* [4]. Also the results of HLA-association studies and genome wide SNP scans revealed that ACPA-positive RA has a different genetic background than ACPA-negative disease [5-7]. This emphasizes the need to systemically study genetic risk factors in ACPA-positive and in ACPA-negative RA separately.

The Fcγ receptors (FcγRs) play a crucial role in immunity by linking the IgG antibody mediated responses with cellular effector and regulatory functions [8]. FcγRIIIA is expressed by natural killer (NK) cells, macrophages [9] and a subset of T lymphocytes [10]. Additionally, this intermediate-affinity FcγR is believed to play a pivotal role in the clearance of immune complexes [11].

These receptors are encoded by genes clustered on the long arm of chromosome 1 (1q21-q24) in a complex region showing extensive nucleotide sequence homology that resulted from duplication and recombination events which occurred in this cluster during the evolution [12]. In addition, copy number variation (CNV) has been shown to be present in this region in several large scale whole genome studies and focused studies [13-16]. The presence of common CNVs can cause false SNP genotyping results. **Figure 1** summarizes the possible effects of CNV on SNP genotyping. A higher copy number (CN) may falsely enrich the heterozygotes, while the presence of a lower CN (a single copy) may falsely enrich the homozygotes (hemizygosity as one allele is absent). The subsequent skewing of genotypes may lead them to fail Hardy-Weinberg equation (HWE) and may blur the association of the studied SNPs with disease susceptibility. It may also limit the ability of the genome-wide SNP association studies to detect disease associated SNPs in regions with CNV [17]. Such genetic complexity renders successful genotyping of different SNPs in that region using classical methods notoriously difficult.

Figure 1: Possible effect of copy number variation (CNV) on single nucleotide polymorphism (SNP) genotyping. This figure illustrates how the presence of CNV can cause false SNP genotyping results. A high copy number falsely enriches the heterozygotes, while the presence of low copy number falsely enriches the homozygotes. In this figure the use of F and V characters was only for illustration purpose but this kind of erroneous genotyping result can occur in every genetic region that harbours both SNPs and CNV.



The presence of such a genetic complexity in the FcγR region, combined with the heterogeneity of RA, might be the cause of inconsistent findings in previous studies on FcγR SNPs in relation to RA. In particular, the functionally relevant, FcγRIIIA 158V/F polymorphism (rs396991) had been extensively studied in RA case-control studies, revealing remarkably contradicting results. The 158V allele was found to be associated with RA susceptibility in many studies [18-21], in another study the 158F allele was associated with RA [22], whereas in other studies no association with RA was observed [23-28]. These contradicting results can in part be caused by methodological difficulties due to the extreme homology to *FcγRIIIB* [29] but difficulties in genotyping due to the presence of CNV as well as the heterogeneity of RA regarding the ACPA status are likely causes that have never been addressed.

Given the important role of FcγRIIIA in auto-immunity, we specifically wanted to study the association of FcγRIIIA 158V/F polymorphism with ACPA-positive RA. The ACPA-

negative RA group was studied as well. Additionally, we investigated whether the presence of CNV of *FcγRIIIA* gene has any effect on the association between the FcγRIIIA 158V/F SNP and RA and also if the presence of CNV of the *FcγRIIIA* gene itself associates with susceptibility to RA.

PATIENTS AND METHODS

Subjects

Nine hundred and foirty-five Dutch Caucasian individuals with RA, all of whom fulfilled the American College of Rheumatology (ACR) classification criteria for RA were studied and described elsewhere [30-32]. Controls were 388 unrelated Dutch Caucasians with no history of RA [33]. For both patients and controls an informed written consent according to the Declaration of Helsinki was obtained. The Commissie Medische Ethiek, the Leiden institutional review board, approved all protocols.

ACPA status was available for 619 patients, and was positive in 58.8% (N=364) of cases. Rheumatoid factor (RF) status was available for 899 patients, and was positive in 64.9% (N=583) of cases. Shared epitope (SE) status was available for 610 patients and was positive in 70.2% (N=428) of cases. Serum ACPA was determined by ELISA (CCP2, Immunoscan RA Mark 2, Euro-diagnostica, Arnhem, the Netherlands and Axis-Shield, Dundee, UK) and the cut-off level for ACPA positivity was set at 25 arbitrary units (AU), according to the manufacturer's instructions.

Genotyping

FcγRIIIA 158V/F (rs396991) was genotyped using the MassArray matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry, according to the protocols recommended by the manufacturer (Sequenom, San Diego, California, USA). The sequences of PCR primers used in the assay were (ACGTTGGATGTTCACAGTCTCTGAAGACAC) and (ACGTTGGATGAAGCCACACTCAAAGACAGC) and the sequence of the extension primer was (ggagACTTCTGCAGGGGGCTT). SpectroCaller software supplied by the manufacturer was used to automatically call the genotypes. All doubtful calls were rechecked, and after manually evaluating their spectra, they were either accepted or recalled, and if still

doubtful the calls were rejected. Ten per cent of samples were genotyped in duplo. The error rate of genotyping was 0%.

CNV

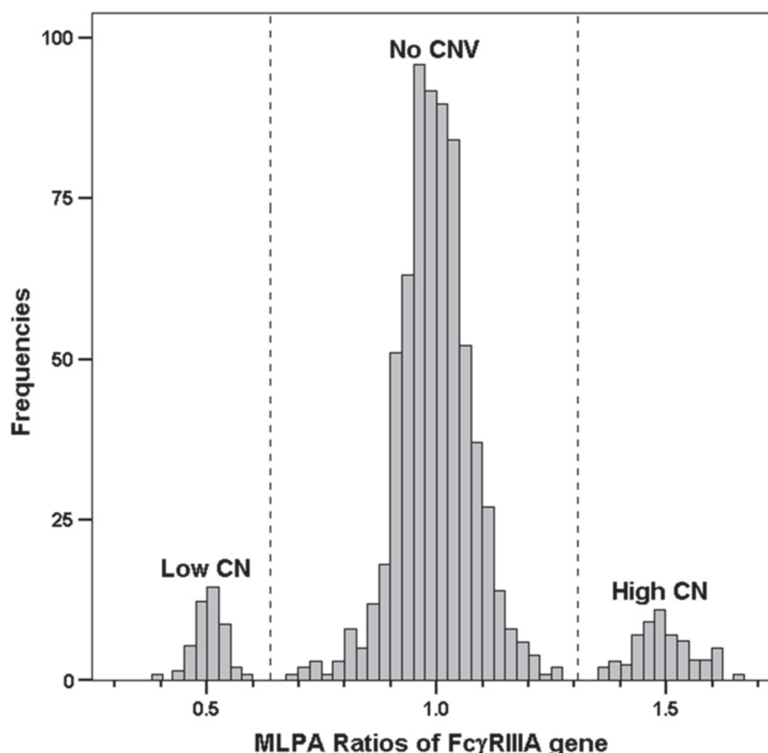
The CNV status of *FcγRIIIA* gene was assessed using Multiplex Ligation-dependent Probe Amplification (MLPA); which is a sensitive method for copy number quantification [34]. MLPA probe design and assay were performed as described by White *et al*. [35]. The MLPA probe sequences used were (GACTCCCACCTTGAATCTCATCCCCAGGGTCTCA) and (CTGTCCCATTCTTGGTGCTGGGTGGATCTAAATCCAGG). Because a relatively large amount of DNA is needed for MLPA (in our experiment 125 ng DNA per sample to get accurate and reliable results), enough DNA was not available from all the RA patients and controls genotyped for the FcγRIIIA 158V/F SNP. Additionally not all the DNA samples used for SNP genotyping were extracted using the same method. According to the manufacturer protocols, the usage of DNA samples extracted using different methods may influence the MLPA results. The presence of remnants of phenol in phenol-extracted DNA can inhibit MLPA-PCR and impede ligase enzyme activity and the use of old magnetic particles in automated DNA extraction devices may result in incomplete sample denaturation, subsequently influencing MLPA results and rendering them incomparable. Therefore DNA samples that were extracted using phenol were not used for MLPA. Consequently, the MLPA was performed on 456 RA patients and 285 controls for whom we had enough DNA that was extracted using the same method.

Statistical analysis

The χ^2 test with 2 degrees of freedom (Epi Info v6, CDC, Atlanta, Georgia, USA) was used to compare the relation between genotypes and ACPA+ve and ACPA-ve RA. The MLPA results were analysed as described by White *et al.* [36]. The height of each probe-specific peak was divided by the sum of three control peaks to give a ratio. The median ratio for *FcγRIIIA* across all samples within an assay was calculated and used to normalize the ratios around a value of 1. The normalized ratio for each individual was calculated and plotted in a scatter plot (**Figure 2**). Subgroups corresponding to different *FcγRIIIA* gene copy numbers were defined by eye and confirmed by cluster analysis (using R statistical software version 2.5.0),

and are delineated by vertical dotted lines. To minimize the possibility of mis-genotyping of FcγRIIIA158V/F polymorphism that can be caused by CNV (**Figure 1**), we performed the analysis on the subgroup of individuals with no CNV (The middle cluster in **Figure 2**), thus excluding genotypes from samples with either high or low copy number (the first and the third clusters in **Figure 2**). P-values were considered statistically significant if < 0.05.

Figure 2: Copy number variation (CNV) of the FcγRIIIA gene. Cluster 1 represent samples with a low copy number and cluster 3 represents samples with a high copy number of FcγRIIIA compared with cluster 2 that represents samples without CNV of the FcγRIIIA gene. MLPA, multiplex ligation-dependent probe amplification.



RESULTS

This study included 945 RA patients and 388 healthy controls. The genotype frequencies of the FcγRIIIA 158V/F SNP in RA patients and controls are shown in **Table 1**. The genotype frequencies of FcγRIIIA-158V/F polymorphism (rs396991) were in accordance with HWE (P-value is 0.6 in cases and 0.4 in controls). No statistically significant differences in the genotype or allele frequencies between RA patients and controls were observed. However after stratifying for ACPA status, the 158VV genotype was more frequent in ACPA positive RA patients compared to controls (P=0.05, OR=1.5, 95%CI 0.99-2.27). Similarly, the frequency of the 158V allele in the ACPA positive RA group (N=358) was higher compared

to controls (P=0.034, OR=1.3, 95%CI 1.01-1.55). No differences were found in the ACPA negative group (N=252) (**Table 1**).

Table 1: Comparison of the FcγRIIIA 158V/F genotype and allele frequencies in patients with rheumatoid arthritis (RA) and controls

		Genotypes			MAF			VV vs FV+FF	
	Total No	FF No (%)	FV No (%)	VV No (%)	%	OR (95% CI)	p Value	OR (95% CI)	p Value
Controls	388	148 (38.1)	189 (48.7)	51 (13.2)	37.5	_	_	_	_
All patients with RA	945	353 (37.3)	442 (46.8)	150 (15.9)	39.3	1.1 (0.9 to 1.28)	0.4	1.3 (0.87 to 1.78)	0.2
ACPA-positive RA	358	117 (32.7)	175 (48.9)	66 (18.4)	42.9	1.3 (1.01 to 1.55)	0.034	1.5 (0.99 to 2.27)	0.05
ACPA-negative RA	252	103 (40.9)	117 (46.4)	32 (12.7)	35.9	0.9 (0.73 to 1.19)	0.6	0.9 (0.58 to 1.58)	0.9

OR and p for each row represent comparison with the control group as reference ACPA, anti-citrullinated peptide antibodies; MAF, minor allele frequency.

Table 2: Copy Number Variation of the FcγRIIIA gene

	Total	Low CNV No (%)	Most common No (%)	High CNV No (%)	p Value*
Controls	285	11 (3.9)	258 (90.5)	16 (5.6)	_
Patients with RA	456	8 (1.8)	426 (93.4)	22 (4.8)	0.2
ACPA-positive	148	4 (2.7)	135 (91.2)	9 (6.1)	0.8
ACPA-negative	110	2 (1.8)	102 (92.7)	6 (5.5)	0.6

p Value compared with controls.
ACPA, anti-citrullinated peptide antibodies; CNV, copy number variation; RA, rheumatoid arthritis.

The *FcγRIIIA* gene shows CNV in 6.6% of RA patients and in 9.5% of controls (**Table 2**). Since the presence of CNV might lead to skewing of the genotype frequencies by causing genotyping errors (**Figure 1**), we assessed whether CNV of the *FcγRIIIA* gene has an influence on the association of FcγRIIIA-158V/F polymorphism and RA. Therefore, we determined the association between FcγRIIIA 158V/F and ACPA+ve and ACPA-ve RA in subjects with no CNV of *FcγRIIIA* gene. Genotypes from individuals with no CNV were selected (cluster 2 in **Figure 2**), thus excluding samples that showed either low or high CN of *FcγRIIIA* gene (cluster 1 & 3 respectively in **Figure 2**). Without stratifying for ACPA, the 158VV genotype was significantly more frequent in RA patients compared to controls (17.2 vs 11.7 respectively, P=0.05, OR=1.6, 95%CI 0.97-2.6), but the frequency of the 158V allele was not significantly higher in RA patients compared to controls. Subsequently, stratifying for ACPA status showed an increased risk of RA as the 158VV genotype was more frequent in

the ACPA positive RA patients compared to controls (21.5 vs 11.7 respectively, P=0.009, OR= 2.1, 95%CI 1.2-3.8) also the presence of the 158V allele is associated with ACPA positive RA (P=0.039, OR=1.4, 95% CI 1-1.9). No association was found in the ACPA negative group (**Table 3**). Comparing the data without and with stratification for CNV (**Table 1** and **3** respectively) reveals that almost similar results were observed for the effect of the 158V allele on the risk of ACPA positive RA. In contrast the odds ratio for the effect of the 158VV genotype on the risk of RA became higher after correcting for the presence of CNV, although the confidence intervals were overlapping.

Table 3: Comparison of the FcγRIIIA 158V/F genotype and allele frequencies in patients with rheumatoid arthritis (RA) and controls with no copy number variation

		Genotypes			MAF			VV vs FV+FF	
	Total No	FF No (%)	FV No (%)	VV No (%)	%	OR (95% CI)	p Value	OR (95% CI)	p Value
Controls	258	94 (36.4)	134 (51.9)	30 (11.7)	37.6	_	_	_	_
All patients with RA	426	162 (38)	191 (44.8)	73 (17.2)	39.6	1.1 (0.9 to 1.4)	0.5	1.6 (0.97 to 2.6)	0.05
ACPA-positive RA	135	42 (31.1)	64 (47.4)	29 (21.5)	45.2	1.4 (1 to 1.9)	0.039	2.1 (1.2 to 3.8)	0.009
ACPA-negative RA	102	42 (41.1)	43 (42.2)	17 (16.7)	37.7	1 (0.7 to 1.4)	0.97	1.5 (0.8 to 3)	0.2

OR and p for each row represent comparison with the control group as reference.
The patients with RA and controls chosen for analysis in this table showed no evidence of CNV based on MLPA results (cluster 2)
ACPA, anti-citrullinated peptide antibodies; CNV, copy number variation; MAF, minor allele frequency; MLPA, multiplex ligation-dependent probe amplification

All the subjects identified as having low copy number (a single copy) were genotyped as homozygous for either the 158V or the 158F alleles (as suggested by **Figure 1**).

The third aim of this study was to investigate whether the difference in *FcγRIIIA* gene copy number confers risk to RA. The distribution of CNV was not significantly different between patients and controls with and without stratifying for ACPA status (**Table 2**).

DISCUSSION

In the current study we investigated the association between the FcγRIIIA 158V/F SNP and ACPA-positive as well as ACPA-negative RA and explored the effect of CNV of *FcγRIIIA* gene on the association of FcγRIIIA 158V/F SNP with RA. We observed that the association

between the FcγRIIIA-158V allele and RA is refined to the ACPA-positive group. In addition, after correction for the effect of the presence of CNV on genotypes, the strength of the association was slightly increased. To our knowledge this is the first study to consider the effect of disease heterogeneity (presence/absence of ACPA) and genetic heterogeneity (effect of CNV on SNP genotyping) on the association between FcγRIIIA-158V/F polymorphism and RA.

The FcγRIIIA is expressed on NK cells and on macrophages, the expression by macrophages is limited to only a few tissues which correlate with the sites of pathology seen in patients with rheumatoid arthritis (synovium, dermis under stress, lungs, pericardium and liver) [37]. The FcγRIIIA expression on NK cells and the number of FcγRIIIA-IgG binding sites per NK cell correlates with the antibody-dependent cell-mediated cytotoxicity (ADCC) function of these cells [38]. The presence of the FcγRIIIA 158V/F SNP, which is a T to G substitution at nucleotide 559 in *FcγRIIIA* gene that results in a switch from phenylalanine to valine at amino acid position 158 in the immunoglobulin binding domain, has functional consequences. It was shown that this 158V/F SNP affects the binding affinity of FcγRs to IgG: the 158V allele is associated with higher NK cells IgG binding affinity compared to the 158F allele, with a gene dosage effect [39]. In addition IgG stimulation of NK cells from 158VV individuals resulted in higher Ca2+ influx, higher concentrations of interleukin-2 (IL2) receptor (CD25) expression and reduced survival of NK cells after activation induced cell death when compared with 158FV or 158FF individuals [40].

So far, the results of the published studies concerning the FcγRIIIA-158V/F SNP in RA vary markedly. They differ in the presence or absence of its association with RA as well as in the allele frequencies within similar ethnic populations. The results of these studies are summarised in **Table 4**. In our study, the genotype and minor allele frequency (MAF) in controls and RA patients were almost identical to those previously reported in Dutch Caucasians [23].

Table 4: The FcγRIIIA 158V/F polymorphism association with rheumatoid arthritis (RA) in different studies

		Counts		MAF%		Alleles		VV vs FV+FF	
Study	Ethnicity	RA	Controls	RA	Controls	p Value	OR (95% CI)	p Value	OR (95% CI)
V allele associated with RA									
Morganet al[18]	UK	141	124	37	28	0.028	1.51 (1.03 to 2.21)	0.134	1.8 (0.78 to 4.18)
Morganet al[18]	India	108	113	36	27	0.05	1.5 (0.98 to 2.28)	0.55	1.34 (0.46 to 3.91)
Morganet al[19]	UK	828	581	35	31	0.02	1.21 (1.03 to 1.42)	0.029	1.45 (1.02 to 2.07)
Kastbomet al[20]	Sweden	181	362	38	31	0.033	1.33 (1 to 1.75)	0.064	1.67 (0.93 to 2.99)
F allele associated with RA									
Nietoet al[22]	Spain	117	142	32	41	0.039	0.68 (0.47 to 1)	0.77	0.9 (0.41 to 1.95)
No association with RA									
Matsumotoet al[1]	Japan	187	158	28	24	0.19	1.25 (0.88 to 1.79)	0.36	1.52 (0.58 to 4.08)
Milicic et al[27]	UK	401	420	35	34	0.5	1.07 (0.87 to 1.32)	0.1	1.46 (0.9 to 2.37)
Milicic et al[27]	India	63	92	23	33	0.054	0.6 (0.35 to 1.04)	0.024	0.2 (0.03 to 0.98)
Kyogokuet al[28]	Japan	382	303	27	30	0.15	0.84 (0.66 to 1.07)	0.55	0.84 (0.47 to 1.52)
Alizadehet al[23]	Netherlands	601	1326	39	37	0.22	1.09 (0.95 to 1.26)	0.68	1.06 (0.8 to 1.4)
Brunet al[24]	Norway	112	89	38	34	0.38	1.2 (0.78 to 1.85)	0.91	0.96 (0.42 to 2.21)
Stewart-Akerset al[25]	USA	145	105	35	31	0.38	1.18 (0.8 to 1.76)	0.32	1.46 (0.65 to 3.3)
Chenet al[26]	Taiwan	212	371	37	35	0.55	1.08 (0.83 to 1.39)	0.28	1.3 (0.78 to 2.17)

MAF, minor allele frequency.

It was suggested that these contradicting results originated from methodological difficulties, due to the extreme homology of *FcγRIIIA* to *FcγRIIIB* gene that might lead to falsely detecting an *FcγRIIIB* sequence as the FcγRIIIA-158V variant leading to false over-presentation of the 158V allele [29]. As indicated, there are two additional explanations that may have been overlooked. First, we now provide evidence that the FcγRIIIA-158VV genotype associates with ACPA positive subset of RA. A previous meta-analysis [23] showed an odds ratio of 1.3 for the FcγRIIIA-158VV genotype to increase the risk of RA; the percentage of ACPA positive RA patients in those studies is unknown. Our data suggest the need for an additional meta-analysis in ACPA positive RA specifically. Second, the presence of CNV in this gene cluster may previously have led to skewing of the genotype frequencies, subsequently affecting disease associations. Since the frequency of CNV was reported to vary significantly in different ethnic populations [36], CNV may be another cause of the different allele frequencies of FcγRIIIA-158V/F polymorphism and different associations with RA observed in different populations.

In our study, after controlling for the *FcγRIIIA* gene copy number, the association between RA patients in general (without considering the ACPA status) and presence of the 158VV genotype became borderline significant (P=0.05, OR=1.6 95% CI 0.97-2.6 vs P=0.2, OR=1.3 95% CI 0.87-1.78 without controlling for the CNV). Similarly in the ACPA positive group before correcting for the presence of CNV, presence of the 158VV genotype was associated with RA susceptibility with a borderline significant P-value (0.05) and an odds ratio of 1.5 (95% CI 0.99-2.27). In the ACPA positive group without CNV this association had an odds ratio of 2.1 (95% CI 1.2-3.8). Although these confidence intervals are overlapping and although the presence of CNV of *FcγRIIIA* didn't significantly change the genotype frequencies (probably because the frequency of CNV was relatively low in comparison to the 158V allele), correction for the presence of CNV affected the association between the 158VV genotype and RA. To our opinion, these data underline the need to take the CNV into consideration while performing analysis on SNPs.

The CNV of other FcγRs genes has been shown to associate with susceptibility to several auto-immune diseases such as lupus nephritis [16] and idiopathic thrombocytopenic purpura (ITP) [41]. The present study evaluated CNV in the *FcγRIIIA* gene. We confirmed the presence of CNV in the *FcγRIIIA* gene with a frequency of 9.5% in healthy controls and 6.6% in RA patients. A comparable frequency of CNV was recently reported in another study with a smaller sample size (116 ITP patients and 100 healthy controls) [41]. We did not find an association between CNV of *FcγRIIIA* gene and susceptibility to RA. Though, because of the low frequency of CNV in this gene, we were underpowered to formally conclude that CNV of *FcγRIIIA* gene is not associated with RA susceptibility.

In conclusion, the *FcγRIIIA*-gene shows CNV which was not differently distributed between RA patients and healthy controls. The analysis of association between the FcγRIIIA-158V/F polymorphism with RA, stratified for ACPA status and CNV of the *FcγRIIIA*-gene, revealed that the FcγRIIIA-158VV genotype confers risk to ACPA positive RA. The fact that a SNP in the FcγRs genes associates with ACPA positive RA points to the relevance of antibodies in the pathophysiology of ACPA+ve RA.

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