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ARTICLE

Evidence for both copy number and allelic (NA1/NA2) risk at the *FCGR3B* locus in systemic lupus erythematosus

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The Fcγ-receptor locus on chromosome 1q23 shows copy-number variation (CNV), and it has previously been shown that individuals with reduced numbers of copies of the Fcγ-receptor-IIIB gene (*FCGR3B*) have an increased risk of developing systemic lupus erythematosus (SLE). It is not understood whether the association arises from *FCGR3B* (CD16b) itself, is observed because of linkage disequilibrium with actual causal alleles and/or is an effect of CNV on flanking *FCGR* genes. Thus, we extended this previous work by genotyping the *FCGR3B* alleles NA1/NA2 and re-assaying CNV using a paralogue ratio test assay in a family study (365 families). We have developed a novel case/pseudo-control approach to analyse family data, as the phase of copy number (CN) is not known in parents and cannot always be inferred in offspring. The results, obtained by fitting logistic regression models, confirm the association of low CN of *FCGR3B* with SLE (P=0.04). The risk conferred by low copies (<2) was contingent on *FCGR3B* allotype, being greater for deletion of NA1 than the for lower-affinity NA2. The simpler model with just CN was rejected in favour of the biallelic-CN model (P=0.03). We observed a correlation (R^2=0.75, P<0.0001) between *FCGR3B* CNV and neutrophil expression in both healthy controls and patients with SLE. Our results suggest that one mechanism by which CNV at this locus confers disease risk is directly as a result of reduced FcγRIIIb function, either because of reduced expression (related to CNV) or because of reduced affinity for its ligand (NA1/NA2 allotype).
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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease affecting multiple organ systems, which is characterized by the presence of autoreactive B cells and the formation of antibody–antigen immune complexes. The genetic contribution to SLE disease risk is strong;[1–3] however, it is also a genetically complex disease.[1–3] An increased understanding of the structure of the genome is leading to novel discoveries that will help unravel the mechanisms involved.[4]

Structural variation is recognized as a rich source of genetic heterogeneity in the human genome, and copy number variants (CNVs) account for a large part of this diversity.[5–7] About half of the currently identified CNVs encompass genes, thus implicating CNV in disease pathogenesis. Thus far, variation in gene copy number (CN) has been associated with a number of complex inflammatory and infectious disorders,[8] including, although not without some controversy,[9,10] HIV (*CCL3L1*),[11] SLE (*FCGR3B* and complement *C4*)[4,12,13] and Crohn's disease (*IRGM*).[14]

The *FCGR* region on chromosome 1q23.3 shows a complex pattern of CNV and sequence homology. From the five *FCGR* genes within the 172-kb region, three (*FCGR3A, FCGR2C* and *FCGR3B*) have been reported to show CNV[15] although the frequency of CNV at *FCGR3A* is low.

FCGR genes encode functionally diverse Fcγ- receptors, which recognise the Fc portion of immunoglobulin molecules. It is their specificity for different immunoglobulin isotopes and pattern of tissue expression that define the FcγRs. FcγRIIIa, expressed on monocytes, macrophages and NK cells, and FcγRIIIb expressed on neutrophils, are low-affinity activating receptors. This function is known to be altered in SLE patients, which makes the *FCGR* genes key candidates for disease susceptibility. Genetic associations have been reported between SLE and functional polymorphisms at *FCGR2A*, *FCGR2B* and *FCGR3A*.[16–19] Also there have been studies reporting the association of two common allotypes of *FCGR3B* with SLE.[20–22] However, the interpretation of these studies is bedevilled by the confounding effect of CNV of the *FCGR3B* gene.

An association between low *FCGR3B* CN and SLE in a UK case–control study was reported recently.[4] However, there are issues regarding the reliability and accuracy of quantitative PCR (qPCR) to assay CNV,[23] as was used in that study. In addition, it is not understood whether the association arises from *FCGR3B* itself, and/or is an effect of CNV on flanking *FCGR* genes. Thus, we extended this previous work by genotyping the *FCGR3B* alleles NA1/NA2 (HNA1a/HNA1b), and re-assaying CNV, in a larger study using a family-based (365 families) approach, which should be robust to population stratification.

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CNV and relative CNs of the NA1 and NA2 alleles of *FCGR3B* were determined using a paralogue ratio test (PRT) assay.[24,25] PRT is a PCR-based assay,[25,26] which uses the high sequence identity of *FCGR3A* and *FCGR3B* to give accurate CN estimates for both. PRT returns CN measurements with more precision than qPCR because of simultaneous amplification of a target and a control. In addition, CN estimates from PRT are closer to integer values rather than continuous variables as in qPCR, and PRT provides data on the NA1/NA2 allotype, which qPCR does not.

We applied the logistic regression case–control methodology to detect CNV and allotype effects, using an innovative case/pseudo-control approach. In addition, we studied the relationship of both CNV at *FCGR3B* and the NA1/NA2 allotype with other genetic polymorphisms at the *FCGR* locus. Our results support an association between SLE and both CNV at *FCRG3B* and its allotype.

MATERIALS AND METHODS

Study cohort

The association of *FCGR3B* genotype with SLE was studied in 365 UK Caucasian SLE families (365 trios).

All families were recruited through UK rheumatology clinics or by direct patient contact following media publicity. Ethical approval was obtained through MREC98/2/06 and 06/MRE02/9, and all participants gave appropriate informed consent. Each proband met the ACR 1982 revised criteria for diagnosis.[27] Clinical data and sera were collected at a single time point at study enrolment. Disease phenotype varied, but data collection from patients who were not infected (C-reactive protein <15 mg/l) or having a disease flare took place in an outpatient setting. The patients comprised 333 females and 32 males, all Caucasian. Mean age at diagnosis was 38.2 years (SD=9.04 years), with first and fourth quadrants of 32 and 44 years, respectively. Supplementary Table 1 contains more information on the sub-phenotypes.

Paralogue ratio test

CN values were obtained using a PCR-based PRT as described previously.[24,25] The assay was run in duplicate on 96-well plates, each containing seven positive controls of varying CNs. Each experiment was calibrated using linear regression of the expected CN on the observed CN for the positive controls. Plates were repeated if the positive controls gave unexpected results.

Restriction enzyme digest variant ratios

A multiplex PCR was used to amplify two regions, which were then digested with *Taqα1* (New England Biolabs, Ipswich, MA, USA). One restriction enzyme digest variant ratio was used to distinguish between *FCGR3A* and *FCGR3B*, and another to distinguish between NA1 and NA2 allotypes of *FCGR3B* as described previously.[24] VIC (Applied Biosystems, Bedford, MA, USA) fluorescent label was used instead of HEX in the latter assay.

CN estimates

Fluorescent labelled (FAM and HEX) PCR products were pooled together and analysed by electrophoresis on an Applied Biosystems genetic analyser. Genemapper (Applied Biosystems) software was used to visually analyse the results. Products of 67 and 72 bp were amplified from chromosome 1 and chromosome 18, respectively. Peak area ratios were used to estimate a total CN for *FCGR3A* and *FCGR3B* against the non-CNV region on chromosome 18. Mean values were taken from the duplicates and used in further analysis.

Genotype estimates

Fluorescent labelled (FAM and VIC; FAM and HEX) restriction enzyme digest variant ratio products were analysed by electrophoresis on an Applied Biosystems genetic analyser. Genemapper (Applied Biosystems) software was used to visually analyse the results. Peak areas at 134 (digested – *FCGR3A*) and 182 bp (undigested – *FCGR3B*) were used to generate 3A:3B ratios. Peak areas at 174 (digested – NA1) and 209 bp (undigested – NA2) were used to generate NA1:NA2 ratios.

Mean PRT values and both restriction enzyme digest variant ratios were used to estimate the CN values for *FCGR3A* and *FCGR3B,* and NA1/NA2 allotype at the *FCGR3B* locus, given that *FCGR3A* does not vary from the NA1 allotype. For example, a sample with PRT=4, 3A:3B=1 and NA1:NA2=1 would be genotyped as *FCGR3A*=2, *FCGR3B*=2, NA2/NA2.

There were no Mendelian errors observed for any of the 365 families with respect to CN or allotype at *FCGR3B*.

Flow cytometry

The antibodies used in this study were anti-FcγRIII (clone 3G8; BD Biosciences, Oxford, UK), anti-FcγRIII (clone LNK 16, Serotec, Kidlington, UK), anti-FcγRIIIb (Clone 1D3 Serotec), anti-FcγRII (clone FLI8.26; BD Biosciences) and anti-CD19 (BD Biosciences). Isotype controls were IgG2a (clone G155-178), IgG1κ (clone MOPC-21) and IgG2bκ (clone MPC-11). Whole blood aliquots of volume 100 μl were incubated with fluorescent labelled antibodies in the concentrations recommended by the manufacturer, for 15 min at room temperature. All patients gave informed consent for analysis of DNA and blood samples. Erythrocytes were lysed using BD FACS Lysing Solution, and surface expression assessed by flow cytometry. FITC and PE CaliBRITE beads were used to ensure that fluorescence settings on the FACS machine were stable. Granulocytes were selected on the basis of size and granularity. Data were analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Results were analysed using GraphPad Prism 3.0 (GraphPad Software, La Jolla, CA, USA).

FCGR2B I232T genotyping (rs1050501)

Amplification of 40 ng of gDNA was carried out in a final volume of 20 μl, using 0.5U HotStar *Taq* Polymerase (Qiagen, Valencia, CA, USA), forward primer as described previously[28] and reverse primer 5′-GCTTGGGTGGCC CCTGGTTCTCA-3′. Owing to the high degree of homology between *FCGR2B* and *FCGR2C*, the reverse primer is located in intron 6 of *FCGR2B*, which is not present in *FCGR2C*. The conditions for amplification were an initial enzyme activation step of 95 °C for 15 min, followed by 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2.5 min for 35 cycles, and a final 72 °C for 10 min. PCR products were run on a 1.5% agarose gel. ExoSAP-IT (4 μl; GE Healthcare, Buckinghamshire, UK) was added to 10 μl of the PCR product and incubated at 37 °C for 15 min and at 80 °C for 15 min. Products were sequenced on a 3730xl DNA Analyser using forward (5′-TGCCTGTCCTGATGTCTGTC-3′) and reverse (5′-GGGCCAAGTGGAAACTGATA-3′) primers, which are located closer to the I232T polymorphism in exon 5. BioEdit was used to visually analyse the electropherograms.

Statistical analysis

Logistic regression analysis was used to model disease risk as a function of CN and allotype. With regard to CN this is simply an additive model, on the logistic scale, with CN (0–4 in our data) as the explanatory variable.

For the allelic data we developed a novel approach to family analysis. The data for each subject consist of two variables: the quantity of observed NA1 and the quantity of observed NA2 (a three-copy genotype, NA1/NA1/NA2, would be [2 1]). We adopted an approach, that can be regarded as an extension of the transmission disequilibrium test[29] approach. We matched each child with a 'pseudo-control' by deducting the observed quantities for the child from the total observed quantities in the parents. For example, if an affected child had genotype [NA1/NA1/NA2] with parents' genotypes being [NA1/NA2] and [NA1/NA2], then the pseudo-control would be a one-copy genotype NA2 with variables for our analysis of [0 1]. With the assumption of Mendelian inheritance, the matched genotypes [2 1] and [0 1], given parents' genotypes as [1 1] and [1 1], have equal probability of transmission under the null hypothesis that allotype does not affect the disease risk. This example assumes that one parent has a deletion on one chromosome and two copies on the other, with the two-copy haplotype transmitted (see Supplementary Figure 1). The other parent is assumed to have one copy on each chromosome. However, in many cases it is not possible to determine the phase. Nevertheless, a case and pseudo-control can always be made in the same way with equal transmission probabilities under the null. A lack of Mendelian inheritance would not lead to false-positive findings, as (under the null) there is no reason

to believe that *de novo* events would favour increased/decreased NA1 or NA2 in offspring.

We adopted a 'bottom-up' approach to variable selection with regard to testing for CN effect and allelic effects. The CN model was tested first, and then the allelic model was tested against the CN model using a likelihood ratio test.[30]

To determine the correlation between the *FCGR3B* (NA1/NA2) locus and other SNPs, we calculated R^2 between SNP genotypes (0,1,2) and the following variables:

a. For correlation with CN; number of copies.
b. For correlation between alleles; two variables (NA1+NA2; multiple R^2).

We use method (b) to assess the correlation between alleles regardless of CN at *FCGR3B* (this incorporates cases with one copy). For both (a) and (b) we used independent data only (parents only). Significance for correlation between *FCGR3B* and a SNP is assessed with reference to the *P*-value for the regression from where the R^2 arises.

We checked our results against the more standard estimates (D' and R^2) of linkage disequilibrium using just the two-copy individuals, which included complete trios where possible.

To calculate the correlation between the 'null haplotype' (zero copy on a chromosome) for *FCGR3B* and SNPs, we took all individuals with either 0,1 or 2 copies and, taking the null haplotype as the 'minor allele', we coded the genotype as [1 1], [1 2], or [2 2], respectively. This assumes that all two-copy individuals have one copy on each chromosome. Standard measures of linkage disequilibrium such as D' and R^2 will capture any correlation.

The correlation analysis was performed using genotyping for *FCGR2A and FCGR3A* taken from an earlier study,[31] and the genotyping for *FCGR2B* as mentioned above.

The linkage disequilibrium analysis on two-copy individuals and the null-haplotype analysis were done using Haploview. All other analyses were performed using *R*.

RESULTS

We found significant evidence for an association between CN and disease risk (Table 1; P=0.04). The odds ratio of 0.71 implies a protective effect with increased copies (disease risk decreases by 0.71 for each additional copy). Furthermore, we found evidence of an allelic effect over and above the CN effect (P=0.032). Looking at the odds ratios for NA1 and NA2 in Table 1, it is evident that NA1 has a stronger protective effect than NA2 (OR=0.62 and 0.78 for NA1 and NA2, respectively). Assessed on its own merits, without comparison with the CN model, this allelic model is significant (P=0.01, null; genotype has no effect on disease risk). The fit of our allelic model to the data, along with the relative effects of NA1 and NA2, can be seen in Figure 1. The model cuts through the data very closely, and the gradient in the NA1 direction is much steeper than for NA2 (odds of the disease decreases by 0.62 for each copy of NA1 compared with 0.78 for NA2). Frequencies for NA1 and CN can be seen in Table 2.

We found no evidence of an association between CN at *FCGR3A* and disease risk (P=0.46), and no interaction between CN at *3A* and *3B* affecting the disease risk (P=0.89 for interaction effect).

Table 1 Logistic regression results for copy number and allelic models

Model	*Variable*	P-*value*	OR	*LRT P-value*
CN	No. of copies	0.039	0.71 (0.52, 0.98)	
Allelic	NA1	0.011[a]	0.62 (0.44, 0.87)	0.032[a]
	NA2		0.78 (0.56, 1.08)	

All models were subjected to logistic regression with case/control status as the outcome. The 'CN' model has the total number of copies as the explanatory variable. The 'allelic' model has two explanatory variables (number of copies of NA1 and number of copies of NA2) and therefore two parameter estimates and two odd ratios. The 'LRT *P*-value' column indicates the test of the allelic model against the CN model; small *P*-values imply that we reject the CN model in favour of the allelic model.
[a]Indicates significance at the 0.05 level.

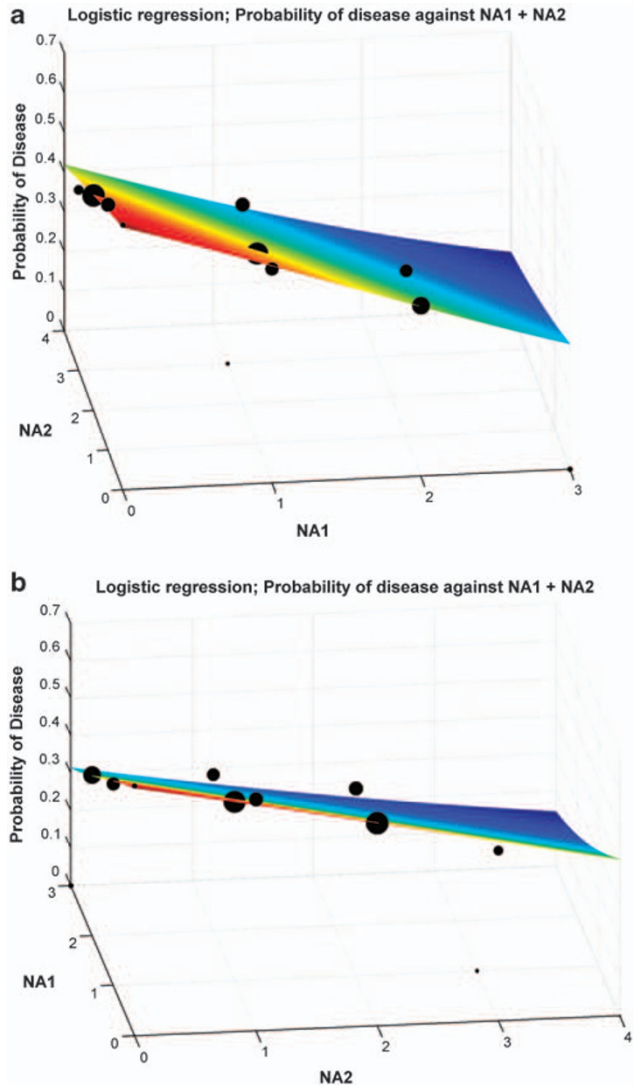

Figure 1 A graphical representation of a multiple logistic regression fit of disease risk against NA1+NA2. This shows how the probability of disease changes over the different combinations of copies of NA1 and NA2. Black spheres represent data (proportion of individuals with disease for given number of NA1 and NA2), whereas the sizes of the spheres are proportional to the number of samples. The coloured plane represents the model (predicted values). Both plots are of the same data, with (**a**) being a rotated view from the *x*-axis (NA1) and (**b**) a reflected view from the NA2 axis.

Table 2 Frequencies of NA1 and total copy number at *FCGR3B*

Variable	*Case*	*Control*	P-*value*
NA1	0.324 (n=723)	0.379 (n=749)	0.027
CN	1.981 (n=365)	2.052 (n=365)	0.037

P-values from *t*-test on equal proportions for NA1 and equal means for CN.

Correlation of *FCGR3B* CN and NA1/NA2 alleles with SNPs associated with SLE

Missense variants in the genes *FCGR2A, FCGR3A* and *FCGR2B* have all been associated with SLE.[18,19,26,32] Previous estimates of LD between *FCGR3B* and other *FCGR* genes would have been inaccurate because of the confounding effect of *FCGR3B* CNV. Thus, we used our current accurate estimate of *FCGR3B* CN state and NA1/NA2 allotype to examine LD relations between *FCGR3B* and other missense polymorphisms that appear to contribute to the risk of SLE potential: *FCGR2A*-H131R, *FCGR3A*-F158V and *FCGR2B*-I232T. Using the data available on the parents from our trio data[31] (n=570), we calculated (multiple) R^2 between the alleles at *FCGR3B* (over all CNs) and the alleles at *FCGR2A*-H131R, *FCGR3A*-F158V and *FCGR2B*-I232T using a regression model, which yielded 0.005 (P=0.40), 0.043 ($P=4.35\times10^{-8}$) and 0.003 (P=0.37), respectively. This agrees very well with the values obtained using a more standard approach (R^2 and D') involving all family data with two-copy individuals (156 families), which can be seen in Figure 2. The estimates of R^2 are small but significant for 3A-F158V, while being extremely small or zero for the others.

The observed correlation between the null haplotype at *FCGR3B* and these SNPs was even smaller (max R^2=0.01, D'=0.56, not included in figure).

FcγRIIIb expression

It has been shown previously in one report that *FCGR3B* CN correlated with cell surface expression.[13] Given the role of the NA1/NA2 allotype in SLE that we have shown in this paper, we sought to establish whether this allotype affected the expression of the gene product. We observed a positive correlation between neutrophil expression of FcγRIIIb (CD16b) as determined by flow cytometry and *FCGR3B* CN in healthy individuals (n=18; R^2=0.75; $P<0.0001$) and individuals with SLE (n=15; R^2=0.73; $P<0.0001$). *FCGR3B* null individuals showed no FcγRIIIb expression (Figure 3, graphs A and B). Little correlation was observed between neutrophil expression of FcγRIIIb and NA1/NA2 allotype in two-copy healthy individuals (n=26) (Figure 3, graph C). CNV at the *FCGR3A* locus was controlled and each individual had CN=2.

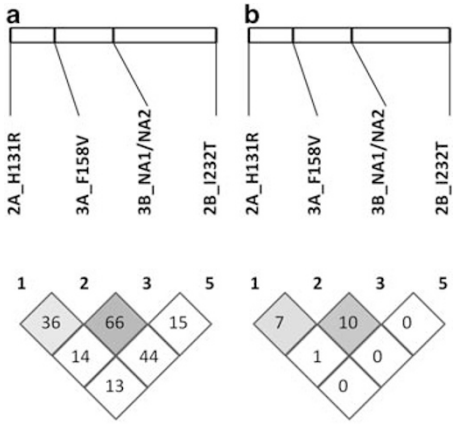

Figure 2 LD plot for surrounding SNPs and two-copy individuals: (**a**) quantification using D', (**b**) quantification using R^2.

DISCUSSION

The results presented here have added to the mounting evidence that a complex and heterogeneous genetic contribution to SLE susceptibility lies within the *FCGR* region. This work confirms earlier studies that used qPCR,[4,13] and improves upon them by using the PRT. This is a more reliable and accurate method to determine CN than qPCR because of simultaneous amplification of a target and a control. The PRT yields CN closer to integer values rather than continuous variables as in qPCR, and simultaneously determines relative CNs of the NA1 and NA2 alleles of *FCGR3B*.

Moreover, our results show that the genetic influence at *FCGR3B* is complex. The risk of autoimmunity is dependent not only on the number of *FCGR3B* genes present in the genome but also on the allelic composition of *FCGR3B*. Specifically, we have shown that the risk of SLE is increased with loss of the higher-affinity[33] NA1 allele compared with the NA2 allele. Rejection of the CN model in favour of the biallelic-CN model is interesting, as this suggests that *FCGR3B* confers risk to SLE as a result of reduced function of FcγRIIIb on neutrophils, which is secondary to a quantitative effect on gene expression, and a qualitative effect on function, which is secondary to NA1/NA2 allelic composition. We have shown that the SLE genetic association exhibited by *FCGR3B* cannot be explained by its correlation with other lupus susceptibility alleles at the *FCGR* locus. A recent study[34] failed to show an association between NA1/NA2 and SLE; however, in the absence of simultaneous estimation of CNV, it is impossible to establish or refute an effect from NA1/NA2.

It is possible that the large deletion that includes *FCGR3B* affects the expression of neighbouring genes at the *FCGR* locus. Given the association of missense alleles at *FCGR2A, FCGR3A* and *FCGR2B*, it is possible that altered expression of any of these genes secondary to the *FCGR3B* deletion event might explain the CN association. However, there are several lines of evidence that indicate that *FCGR3B* itself underlies the genetic association data. First, it was shown previously

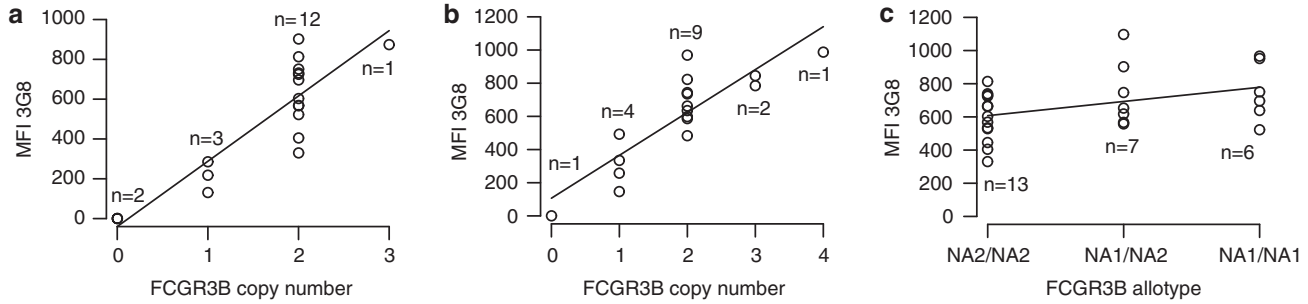

Figure 3 The neutrophil surface expression of FcγRIIIb (CD16, clone 3G8) positively correlates with *FCGR3B* copy number in (**a**) healthy controls (n=18; R^2=0.75; $P<0.0001$) and (**b**) SLE patients (n=15; R^2=0.73; $P<0.0001$). (**c**) Little or no correlation was observed between neutrophil expression of FcγRIIIb and NA1/NA2 allotype in healthy controls with two copies of *FCGR3B* (n=26; R^2=0.16; P=0.06).

that the number of copies of *FCGR3B* correlated with the magnitude of expression of the gene product on neutrophils.[13] We replicated these findings, observing a correlation with CN and neutrophil expression in both healthy controls and patients with SLE (R^2=0.75). Our data showing that NA1 alleles have a greater protective effect in SLE than NA2 alleles further support a role for FcγRIIIb. As NA1 alleles have a higher affinity for ligand than NA2 alleles,[33] loss of NA1 should have a greater functional effect than loss of NA2 alleles. We sought to determine whether NA alleles affected FcγRIIIb expression, and did not observe any marked correlation. This suggests that a functional effect underlies the association found, such as the NA1 allotype harbouring a higher affinity than NA2 for immune complexes containing IgG1 and IgG3.[35]

Our methodology for analysing CNV with family data shows how to make inference on allelic risk when standard methods such as the transmission disequilibrium test cannot be applied. The approach is novel, simple to implement and can be applied using standard statistical software.

The results presented in this paper provide strong evidence that *FCGR3B*, a gene solely expressed on neutrophils, is directly involved in the complex genetic process that leads to SLE. This adds weight to *a priori* beliefs based on the known function of *FCGR* genes, which identify the region as a disease susceptibility locus. Our approach has unravelled the confounding effect of CNV on the *FCGR3B* gene association with SLE and produced evidence of an allelic contribution to disease risk. This raises an important issue of the role of the neutrophil in SLE, a relatively unexplored area of research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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