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

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ABSTRACT

The Fc γ -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 Fc γ -receptor-IIIB gene (*FCGR3B*) have an increased risk of developing SLE. It is not understood if the association arises from *FCGR3B* (CD16b) itself, is observed due to 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 Parologue 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 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 Fc γ RIIb function, either because of

reduced expression (related to CNV) or reduced affinity for its ligand (NA1/NA2 allotype).

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease affecting multiple organ systems, which is characterised by the presence of autoreactive B cells and the formation of antibody-antigen immune complexes (IC). The genetic contribution to SLE disease risk is strong^{1,2,3}, however it is also a genetically complex disease^{1,2,3}. An increased understanding of the structure of the genome is leading to novel discoveries that will help unravel the mechanisms involved⁴.

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^{5,6,7}. About half of the currently identified CNVs encompass genes, hence implicating CNV in disease pathogenesis. Thus far, variation in gene copy number has been associated with a number of complex inflammatory and infectious disorders⁸ including, although not without some controversy^{9,10}, HIV (*CCL3LI*)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 85-Kb region, three have

been reported to show CNV¹⁵; *FCGR3A*, *FCGR2C* and *FCGR3B*, although the frequency of copy number variation at *FCGR3A* is low.

FCGR genes encode functionally diverse Fc γ 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 Fc γ Rs. Fc γ RIIIa, expressed on monocytes, macrophages and NK cells, and Fc γ RIIIb 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 with SLE have been reported between functional polymorphisms in *FCGR2A*, *FCGR2B* and *FCGR3A*^{16,17,18,19}. There have been studies reporting association of two common allotypes of *FCGR3B* with SLE^{20,21,22}. However, the interpretation of these studies is bedevilled by the confounding effect of copy number variation of the *FCGR3B* gene.

An association between low *FCGR3B* copy number and SLE in a UK case control study was recently reported⁴. However there are issues concerning the reliability and accuracy of qPCR to assay CNV²³, as was used in that study. Also it is not understood if 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.

CNV and relative copy numbers of the NA1 and NA2 alleles of *FCGR3B* were determined using a Parologue 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 due to simultaneous amplification of a target and a control. In addition, CN estimates from PRT are close 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 logistic regression case/control methodology to detect CNV and allotype effects, using an innovative case/pseudo control approach. Additionally we studied the relationship between 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.

RESULTS

We found significant evidence for an association between copy number 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 find evidence of an allelic effect over and above the copy number effect ($p\text{-value}= 0.032$). 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 to 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 disease decreases by 0.62 for each copy of NA1 compared to 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 disease risk. ($p=0.89$ for interaction effect).

Correlation of *FCGR3B* copy number and NA1/NA2 alleles with SNPs associated with SLE.

Missense variants in the genes *FCGR2A*, *FCGR3A* and *FCGR2B* have all been associated with SLE^{18,19,26,27}. Previous estimates of linkage disequilibrium between *FCGR3B* and other *FCGR* genes will have been inaccurate because of the confounding effect of *FCGR3B* copy number variation. Thus we used our current accurate estimate of *FCGR3B* copy number state and NA1/NA2 allotype to examine linkage disequilibrium 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 data available on the parents from our trio data²⁸ ($n=570$) we calculated (multiple) R^2 between the alleles at *FCGR3B* (over all copy numbers) and the alleles at *FCGR2A*-H131R, *FCGR3A*-F158V and *FCGR2B*-I232T using a regression model, which returned 0.005 ($p=0.40$), 0.043 ($p=4.35 \times 10^{-8}$) and 0.003 ($p=0.37$), respectively. This agrees very well with a more standard approach (R^2 and D-prime) using 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 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-prime=0.56, not included in figure).

FcγRIIIb Expression

It has previously been shown in one report that *FCGR3B* copy number correlated with cell surface expression¹³. Given the role of the NA1/NA2 allotype in SLE that we have demonstrated in this paper, we sought to establish whether this allotype affected expression of the gene product. We observed a positive correlation between neutrophil expression of FcγRIIIb (CD16b) as determined by flow cytometry and *FCGR3B* copy number 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 (fig. 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) (fig. 3, graph C). CNV at the *FCGR3A* locus was controlled and each individual had CN=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 quantitative genomic PCR^{4,13} and improves upon them by using the paralogue ratio test (PRT). This is a more reliable and accurate method to determine copy number than qPCR due to simultaneous amplification of a target and a control. The PRT returns CN close to integer values rather than continuous

variables in qPCR and simultaneously determines relative copy numbers of the NA1 and NA2 alleles of *FCGR3B*.

Moreover, our results demonstrate that the genetic influence at *FCGR3B* is complex. The risk of autoimmunity is not only dependent on the number of *FCGR3B* genes present in the genome, but is also dependent on the allelic composition of the *FCGR3B*. Specifically we have shown that the risk of SLE is increased with loss of the higher affinity²⁹ NA1 allele compared to 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 demonstrated 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 study³⁰ 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* effects 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 copy number association. However, there are several lines of evidence that indicate that *FCGR3B* itself underlies the genetic association data. Firstly, it was previously shown that the number of copies of *FCGR3B* correlated with magnitude of expression of the gene

product on neutrophils¹³. We replicated these findings, observing a correlation with copy number 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. Since NA1 alleles have a higher affinity for ligand than NA2 alleles²⁹, 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 we 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 (IC) containing IgG1 and IgG3³¹.

Our methodology for analyzing CNV with family data demonstrates how to make inference on allelic risk when standard methods such as TDT 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.

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 MREC98/2/06 and 06/MRE02/9 and all participants gave appropriate informed consent. Each proband met ACR 1982 revised criteria for diagnosis³². Clinical data and sera were collected at a single time-point at study enrolment. Disease phenotype varied, but data collection took place in an outpatient setting from patients who were not infected (C-reactive protein (CRP) < 15mg/l) or having a disease flare. The patients were made up of 333 Females and 32 Males, all Caucasian. Mean age at diagnosis was 38.2 years (standard deviation = 9.04 years), with 1st and 4th quadrant of 32 and 44 years respectively. Supplementary table 2 contains more information on sub-phenotypes.

Parologue Ratio Test (PRT)

Copy number values were obtained using a PCR-based PRT as previous described^{24,25}. The assay was run in duplicate on 96 well plates, each containing seven positive controls of varying copy numbers. 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 (REDVRs)

A multiplex PCR was used to amplify two regions, which were then digested with *TaqI* (New England Biolabs). One REDVR was used to distinguish between *FCGR3A/FCGR3B* and another to distinguish between NA1 and NA2 allotypes of *FCGR3B* as previously described²⁴. VIC (Applied Biosystems) fluorescent label was used instead of HEX in the latter assay.

Copy Number Estimates

Fluorescently 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 67bp and 72bp were amplified from chromosome 1 and chromosome 18 respectively. Peak area ratios were used to estimate a total copy number for *FCGR3A* and *FCGR3B* against the non-copy number variable region on chromosome 18. Mean values were taken from the duplicates and used in further analysis.

Genotype Estimates

Fluorescently labelled (FAM and VIC; FAM and HEX) REDVR 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 134bp (digested - *FCGR3A*) and 182bp (undigested – *FCGR3B*) were used to generate 3A:3B ratios. Peak areas at 174bp (digested – NA1) and 209bp (undigested – NA2) were used to generate NA1:NA2 ratios.

Mean PRT values and both REDVR ratios were used to estimate copy number values for *FCGR3A* and *FCGR3B* and NA1/NA2 allotype at the *FCGR3B* locus, given that *FCGR3A* doesn't vary from the NA1 allotype. For example, a sample with PRT=4; 3A:3B=1; 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-Fc γ RIII (clone 3G8; BD Biosciences), anti-Fc γ RIII (clone LNK 16, Serotec), anti-Fc γ RIIIb (Clone 1D3 Serotec) anti-Fc γ RII (clone FLI8.26; BD Biosciences) and anti-CD19 (BD Biosciences). Isotype controls were IgG2a (clone G155-178), IgG1_κ (clones MOPC-21), and IgG2b_κ (clone MPC-11). 100- μ l aliquots of whole blood were incubated with fluorescently labelled antibodies in concentrations recommended by the manufacturer, for 15 minutes 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 analyzed using FlowJo software (Tree Star, Inc.). Results were analysed using GraphPad Prism 3.0.

***FCGR2B* I232T Genotyping (rs1050501)**

Amplification of 40ng of gDNA was carried out in a final volume of 20 μ L, using 0.5U HotStar *Taq* Polymerase (Qiagen), forward primer as previously described³³ and reverse

primer 5' – GCTTGGGTGGCCCCCTGGTTCTCA -3'. Due 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 minutes, followed by 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2.5 minutes for 35 cycles and a final 72°C for 10 minutes. PCR products were run on a 1.5% agarose gel. 4µL of ExoSAP-IT (GE Healthcare) were added to 10µL of PCR product and incubated at 37°C for 15 minutes and at 80°C for 15 minutes. Products were sequenced on a 3730xl DNA Analyzer using forward (5'-TGCCTGTCCTGATGTCTGTC-3') and reverse (5' – GGGCCAAGTGGAACTGATA – 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 copy number and allotype. With regard to copy number this is simply an additive model, on the logistic scale, with copy number (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 consists 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 take an approach which can be regarded as an extension of the transmission disequilibrium test³⁴ (TDT) approach. We match each child with a 'pseudo control' by deducting the observed quantities for the child from the total observed 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 [1 1] and [1 1], have equal probability of transmission under the null hypothesis that allotype does not affect 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 copy number effect and allelic effects. The copy number model was tested for first, and then the allelic model was tested against the copy number model using a likelihood ratio test³⁵.

To determine 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 copy number; Number of copies.
- b) For correlation between alleles; Two variables (NA1 + NA2 ; Multiple R^2).

We use method (b) to assess correlation between alleles regardless of copy number at *FCGR3B* (this incorporates cases with 1 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-prime and R^2) of linkage disequilibrium (LD) using just the 2-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 LD such as D-prime and R^2 will capture any correlation.

The correlation analysis was performed using genotyping for *FCGR2A* and *FCGR3A* taken from an earlier study²⁸ and the genotyping for *FCGR2B* mentioned above.

The LD analysis on 2-copy individuals and the null haplotype analysis made use of Haploview. All other analyses were performed using R.

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LEGENDS TO FIGURES

Figure 1

A graphical representation of a multiple logistic regression fit of disease risk against NA1 + NA2. This displays 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), while 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) rotated to a view from the x-axis (NA1) and (B) reflected view from the NA2 axis.

Figure 2

LD plot for surrounding SNPs and 2-copy individuals; panel A quantification using D-prime, panel B quantified using R^2 .

Figure 3

The neutrophil surface expression of Fc γ RIIIb (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 Fc γ RIIIb and NA1/NA2 allotype in healthy controls with two copies of *FCGR3B* (n=26; $R^2 = 0.16$; $p = 0.06$)

TABLES

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

Table 1 Logistic regression results for copy number and allelic models.

All models are logistic regression with case/control status as the outcome. ‘CN’ model has the total number of copies as the explanatory variable. ‘Allelic’ model has two explanatory variables (number of copies of NA1 and number of copies of NA2) and therefore two parameter estimates and two odds ratios. The ‘LRT p-val’ column indicates the test of the allelic model against the CN model; small p-values imply we reject the CN model in favour of the allelic model. * Indicates significance at the 0.05 level.

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

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

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

FIGURES

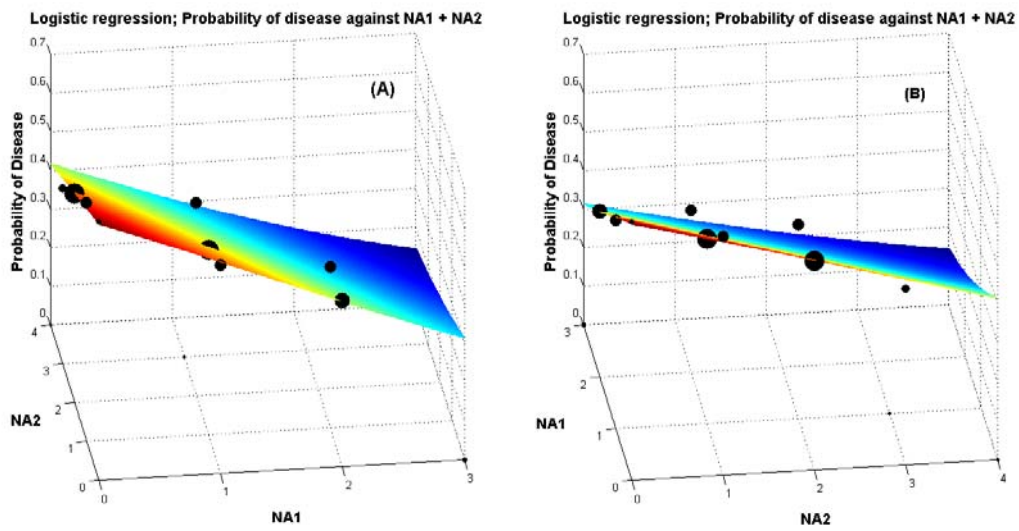
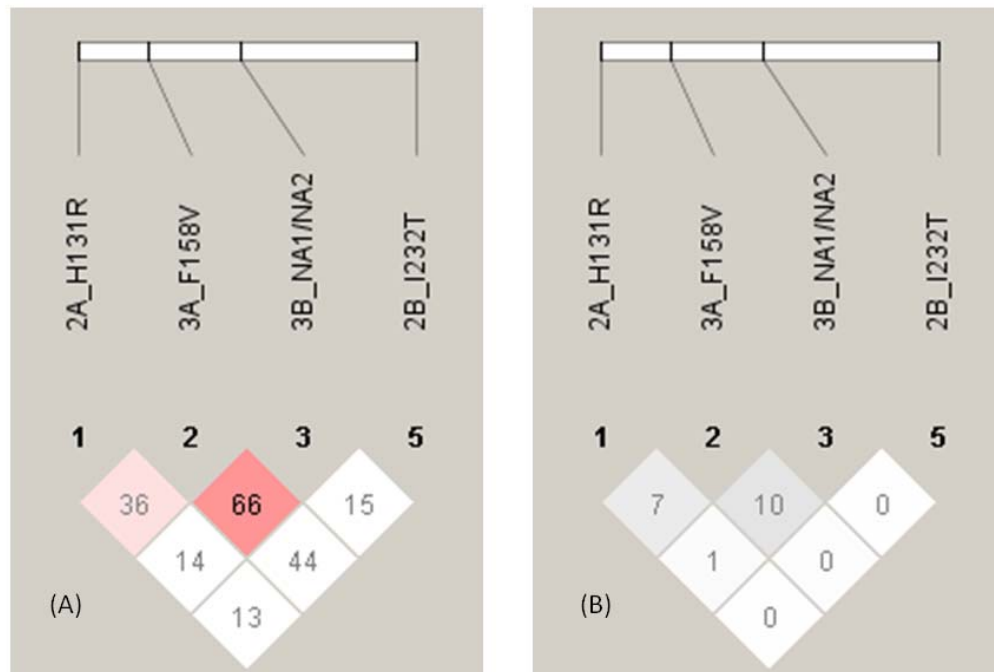
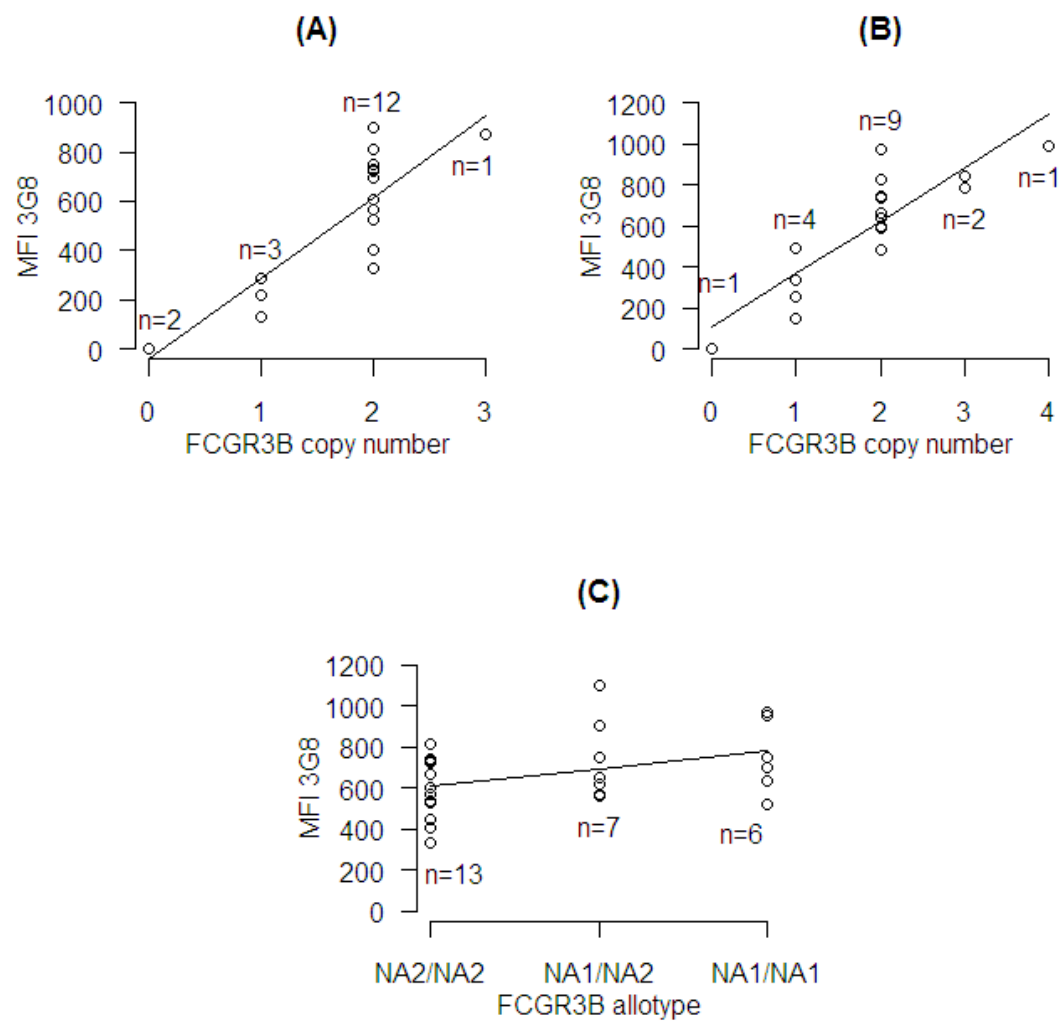
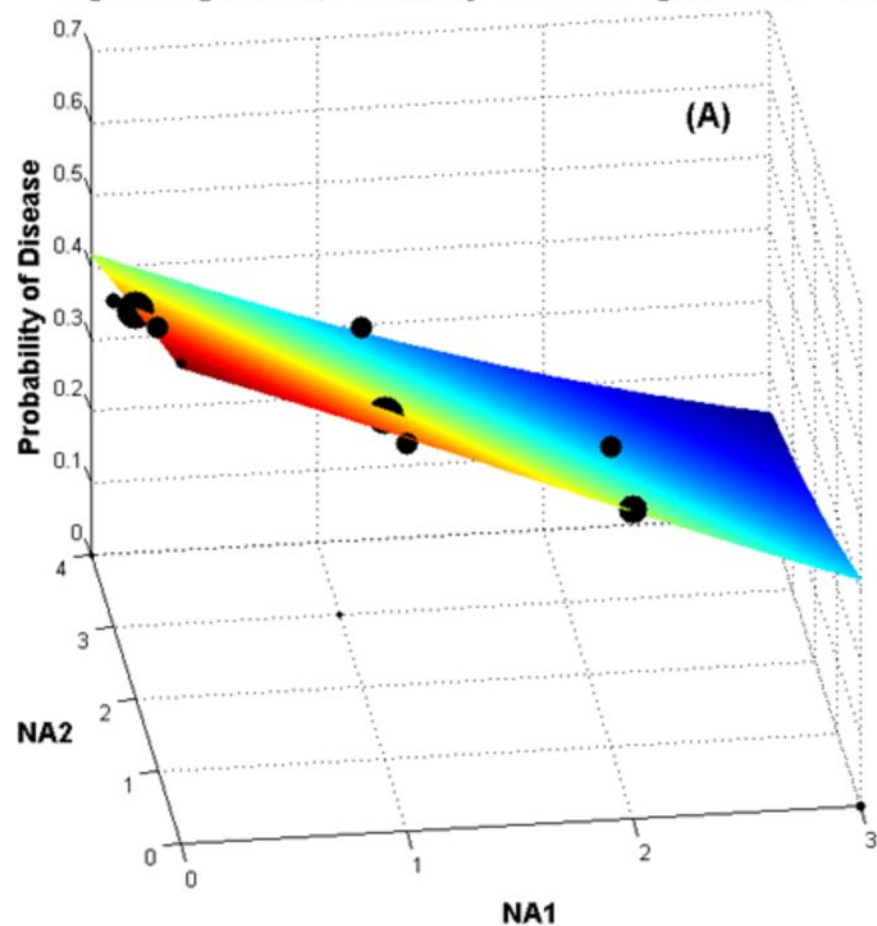


Figure 1

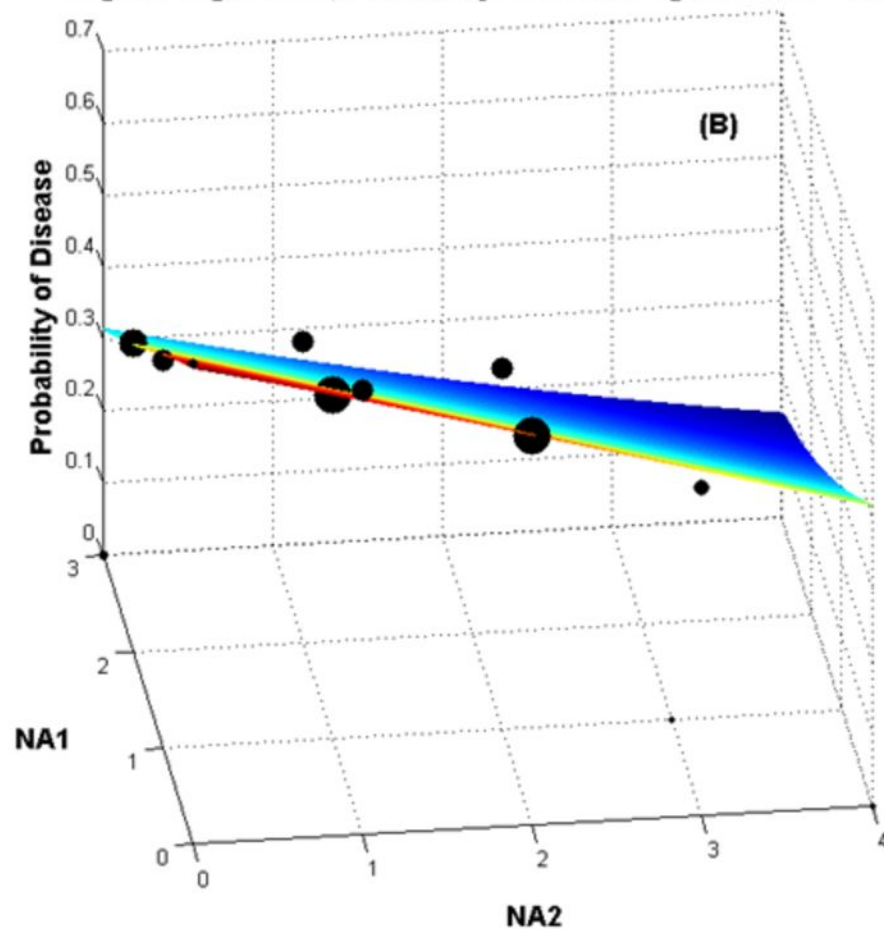
**Figure 2**

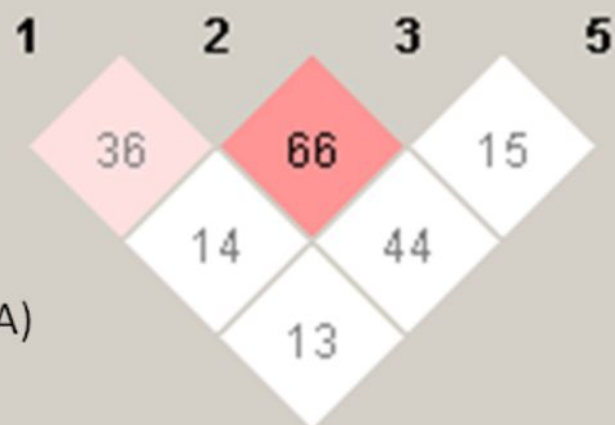
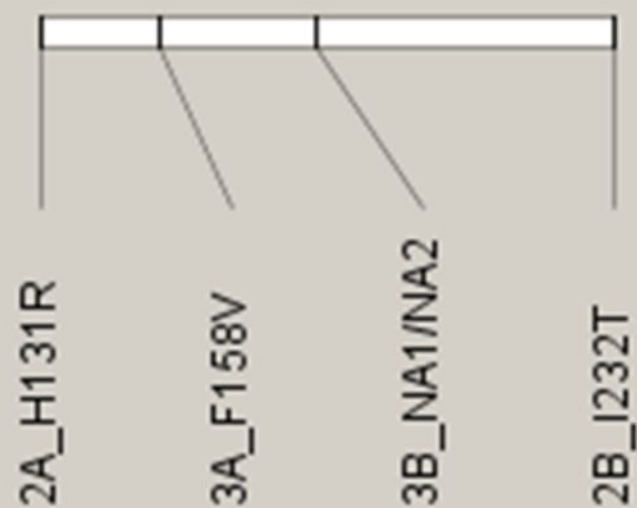
**Figure 3**

Logistic regression; Probability of disease against NA1 + NA2

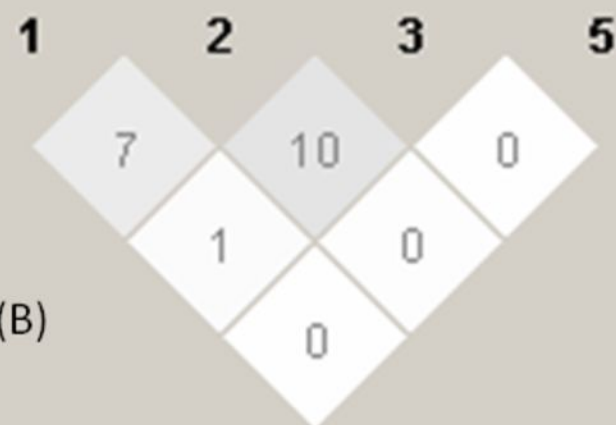
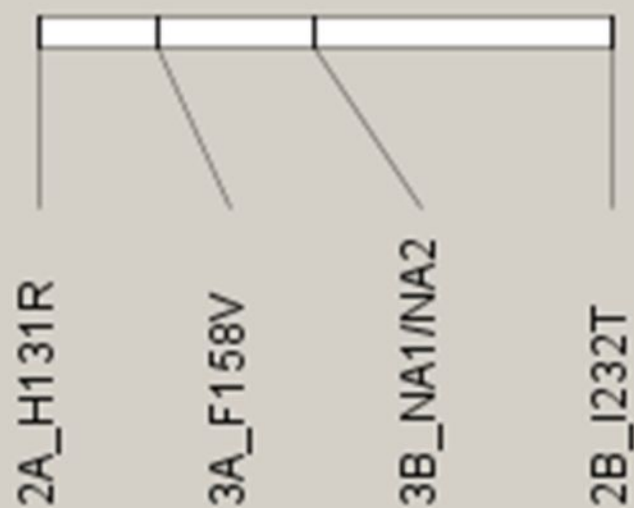


Logistic regression; Probability of disease against NA1 + NA2





(A)



(B)

