

## ***IFITM3* and susceptibility to respiratory viral infections in the community**

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## ABSTRACT

Interferon-inducible transmembrane proteins 1, 2 and 3 (IFITM1,2 and 3) are viral restriction factors that mediate cellular resistance to several viruses. We have genotyped a possible splice-site altering SNP (rs12252) in the *IFITM3* gene in 34 H1N1 influenza cases with severe pneumonia and over 5000 individuals comprising cases of community-acquired mild lower respiratory tract infection and matched controls of Caucasian ancestry. We found evidence of an association between rs12252 rare allele homozygotes and susceptibility to mild influenza (in patients attending primary care), but could not confirm a previously reported association between this SNP and susceptibility to severe H1N1 infection.

## BACKGROUND

A recent functional genomic screen showed that host interferon-inducible transmembrane proteins 1, 2 and 3 (IFITM1, 2 and 3) block early stages of viral replication and thereby mediate cellular resistance to viruses including influenza, West Nile virus and dengue virus<sup>1</sup>. The mechanism by which *IFITM* proteins inhibit viral infection is not fully understood, but it has been shown, for example, that these proteins prevent the transfer of viral components to the cytosol by blocking membrane fusion<sup>2</sup>. Influenza A virus replication is persistent and associated with increased cell death and exaggerated pro-inflammatory responses in the lungs of mice lacking *Ifitm3*<sup>3</sup>.

The 1000 Genomes project currently lists 15 non-synonymous, 15 synonymous, one stop-site gaining and one possible splice-site altering SNPs in the *IFITM3* gene. The possible splice-site altering SNP (rs12252) is found close to a splice site for one transcript of the *IFITM3* gene<sup>4</sup>. Human genetic studies of the *IFITM3* gene have been limited and no associations have been found with SNPs in the *IFITM3* gene in any published genome-wide association studies, according to the GWAS catalog<sup>5</sup>.

Recently Everitt *et al.*<sup>3</sup> reported a putative association between the C allele in *IFITM3* SNP rs12252 and susceptibility to severe H1N1 or seasonal influenza virus infection in patients from the UK. However, their study included only 53 influenza cases, and the reported association relied upon imputed control genotypes. Small sample sizes are prone to imprecisely estimate genotype frequencies and imputed genotypes may be more inaccurate for rare SNPs such as *IFITM3* rs12252<sup>6</sup> (which has a minor allele frequency of 3% in Europe<sup>4</sup>).

Another study analysed the association between the rs12252 SNP and H1N1 influenza in China, where the frequency of this SNP is much higher<sup>7</sup>. This study found an association between C homozygotes and severe H1N1 infection when compared either to population controls from the 1000 Genomes Project or to mild H1N1 infection cases. This suggests that rs12252 associates with

only severe H1N1 influenza infection. However, despite increased power due to the greater frequency of this SNP in China, interpretation of the results of this study is difficult since sample sizes were small (32 severe and 51 mild cases).

We studied two separate cohorts to examine further this possible association with viral disease. Severe H1N1 influenza cases requiring intensive care unit (ICU) admission for pneumonia (n=34), lower respiratory tract infection (LRTI) cases (n=2730) and healthy controls matched to the LRTI cases (n=2623) were genotyped. The severe H1N1 influenza cases were admitted to ICU with community acquired pneumonia (CAP) and were recruited to the UK based Genomic Advances in Sepsis (GAinS) study<sup>8</sup>. The LRTI cases and healthy controls were recruited from across Europe within the Genomics to combat Resistance against Antibiotics in Community acquired LRTI in Europe (GRACE) study<sup>9</sup>.

## METHODS

### Genotyping methods

Genotyping of rs12252 was performed by restriction fragment length polymorphism (RFLP) using primers CAGGAAAAGGAACTGTTGAGAACC(F) and CTCCTGGAGCCTCCTCTCA(R) in standard PCR conditions. Primers had 3' penultimate base mismatches to IFITM3 to ensure specific amplification of *IFITM3* rather than *IFITM2*. *MscI* (NEB) was used to cut the PCR product in the presence of the wild type T allele. Fragments of length 572, 426 and/or 146bp were visualised on agarose gels, according to the genotype of the sample. All minor homozygotes and heterozygotes were Sanger sequenced in the reverse direction to confirm their genotype, using the same primers.

### Sample collections

The GAinS study recruited adult patients admitted to intensive care units in the UK with community acquired pneumonia or faecal peritonitis<sup>8</sup>. Exclusion criteria for this study were: inclusion in an interventional study of a novel intervention, immunosuppression (known regular systemic

corticosteroid therapy, known regular therapy with other immunosuppressive agent, known to be HIV positive or have acquired immunodeficiency syndrome, neutrophil count less than  $1000\text{mm}^3$  due to any cause – see reference 8 for more details), presence of a directive to withhold or withdraw life sustaining treatment or admission to ICU for palliative care only. Individuals with community-acquired pneumonia, H1N1 infection and self-reported Caucasian ancestry were included in the analysis presented here. All of these patients had chest X-ray evidence of pneumonia. None of these patients suffered from malignant disease or liver cirrhosis. No patient was receiving systemic steroids and no patient had significant pre-morbid exercise restriction. In contrast to the cohort studied by Everitt *et al.*<sup>3</sup>, none of the GAINs patients was pregnant. Only two of these patients had COPD and five had asthma. Superadded/concurrent infections were identified in seven patients (three with *Staphylococcus aureus*, one with *Staphylococcus hominis*, one with *Haemophilus influenzae*, one with *Mycoplasma pneumoniae* and one with respiratory syncytial virus). All patients were mechanically ventilated except for one.

GRACE<sup>9</sup> is a Europe-wide study of LRTI in primary care. Cases and controls used in this study were collected from 14 primary healthcare networks in 11 European countries with cases matched to local controls by age, sex and time of sampling. Subjects of non-European origin were excluded from the analysis. Eligible patients were aged 18 years and over, consulting for the first time with an acute cough (up to 28 days duration) as the main symptom or acute LRTI as the primary diagnosis. None of the CC genotype LRTI patients in this study were hospitalised. In house real-time PCR was performed to diagnose viral infection in three collaborating laboratories based on previous validation of the different nucleic acid amplification methods used in the respective labs<sup>10</sup>.

### Statistical methods

All analyses included 2623 Caucasian European controls from GRACE. Two-tailed Fisher's exact test was used for the H1N1 analyses (SPSS v18). GRACE cases and controls from across Europe were

analysed using logistic regression in PLINK<sup>11,12</sup>, with country as a covariate. All Hardy Weinberg Equilibrium (HWE) p-values were calculated using PLINK<sup>11,12</sup>.

## RESULTS

Association analysis results between our cohort of severe H1N1 cases and a large collection of controls, the largest available collection of directly genotyped Caucasians for rs12252 that we know of, showed no significant association (table 1). We observed no CC homozygotes amongst our cases of severe H1N1 infection and nearly identical frequencies of TC heterozygotes in our cases and controls. However, association analysis between these controls and the combined severe influenza cases (data from our study and Everitt *et al.*<sup>3</sup>) suggests a recessive model of association (table 1), rather than the additive model originally described<sup>3</sup>. Rare homozygotes are driving this association and show an odds ratio (OR) of 23.38, with no association for heterozygotes compared to major homozygotes (OR 1.05, 95% CI 0.48-2.30).

We extended our study to assess the possible role of *IFITM3* rs12252 in susceptibility to mild viral LRTI diagnosed in primary care (n=1248), including patients with rhinovirus (n=498), influenza (n=240), coronavirus (n=169), respiratory syncytial virus (n=116), human metapneumovirus (n=110), parainfluenza virus (n=61), and co-infection with two of these viruses (n=54). Comparing the *IFITM3* rs12252 genotypes of the combined viral primary care cases with those of the controls demonstrated an association for the recessive test (p=0.049; table 1) that was more significant in the subgroup of patients with disease due to influenza virus (p=0.025; table 1). As with severe H1N1, the best fitting model of association was recessive. When non-influenza viral cases were studied, no association was found (table 1). No association was seen in rhinovirus or coronavirus specific analyses either (data not shown). We found no evidence of association between rs12252 and bacterial LRTI (n=379 cases; data not shown).

## CONCLUSIONS

Without directly genotyped controls and large sample sizes, it is not possible to fully define genetic associations. We did not find an association between rs12252 and our H1N1 cases, probably due to small sample size. However, by extending the case series of influenza patients reported by Everitt *et al.*<sup>3</sup> and using our large collection of directly genotyped controls we find a distinct genetic model for severe influenza susceptibility in humans. However, even with the combination of our genotyped H1N1 cases, and the influenza patients of Everitt *et al.*<sup>3</sup> (85% of whom had H1N1), the number of cases in this study is small and the association relies only on three homozygotes genotyped by Everitt *et al.*<sup>3</sup> Not all of these three individuals have undergone population stratification analysis, and given the large differences in the allele frequency of this SNP between populations, population outliers could easily have biased this result. A larger number of H1N1 cases would be needed to better define this association in Europe.

Moreover, by analysing large numbers of cases of community-acquired LRTIs and matched controls we show the same recessive association for mild viral infections. We find the most significant association with susceptibility to mild influenza infection, in contrast to Zhang *et al.*<sup>7</sup>, who found an association with severe but not mild influenza. The interferon-pathway gene *IFITM3* therefore appears to act as a susceptibility locus for influenza infection in humans, encompassing both very mild and life-threatening disease. We found no evidence that this SNP is associated with mild bacterial infection, which is unsurprising since *IFITM3* has not been implicated in the control of bacterial infection. Although *IFITM3* has been shown to mediate infection by multiple viruses, including Influenza A, HIV-1, yellow fever virus and West Nile virus<sup>1,2,13,14,15</sup>, of the pathogens we have studied, the association seemed to be specific for influenza susceptibility. Therefore future studies with other large viral sample sets are necessary to further define this genetic association.

One limitation of our study is that population stratification analysis has not been performed for our samples. Whole-genome genotyping has not been performed to make this possible. Self-reported ancestry has been used to remove non-Caucasians from the analysis.

There have been large numbers of case-control studies of severe viral and bacterial infectious disease which are almost always recruited in a hospital setting. However, there have been very few studies of the genetics of susceptibility to common mild infections that are generally managed in primary care. This is the first large genetic study, of which we are aware, assessing susceptibility to mild lower respiratory infections in a primary care (general practice) setting. Our data suggest that larger studies of mild infection phenotypes in addition to severe cases could be helpful in distinguishing between genetic determinants of initial infection as opposed to severe consequences of infection. Moreover, the large variety of microbial causes of common infections in primary care may allow more efficient identification of genetic loci that impact on risk of infection by multiple pathogens.

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**Table 1: Association results for *IFITM3* SNP rs12252**

Cases	GRACE controls	Everitt <i>et al.</i> <sup>1</sup> influenza cases	GAinS H1N1	GAinS H1N1 and Everitt <i>et al.</i> <sup>3</sup> influenza cases	GRACE mild viral cases	GRACE mild influenza cases	GRACE non- influenza viral cases
n	2623	53	34	87	1248	259	989
HWE	1	0.000048	1	0.0047	0.007	0.12	0.0248
TT (%)	2417 (92.1)	46 (86.8)	31 (91.2)	77 (88.5)	1160 (92.9)	235 (90.7)	925 (93.5)
CT (%)	202 (7.7)	4 (7.4)	3 (8.8)	7 (8)	82 (6.6)	22 (8.5)	60 (6.1)
CC (%)	4 (0.15)	3 (5.6)	0	3 (3.4)	6 (0.48)	2 (0.77)	4 (0.4)
Allelic P		*0.011	0.753	*0.032	0.764	0.154	0.264
Allelic OR		2.498 (1.284- 4.861)	1.107 (0.345- 3.551)	1.936 (1.082- 3.464)	0.960 (0.750- 1.229)	1.358 (0.892- 2.07)	0.8529 (0.6452-1.127)
recessive model P		*2.4x10 <sup>-4</sup>	1	*0.001	*0.049	*0.025	0.150
recessive model		39.29 (8.567-	NA	23.38 (5.152-	3.59 (1.008-	7.126 (1.283-	2.778 (0.6901-

OR		180.137)		106.132)	12.79)	39.58)	11.19)
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HWE: Hardy-Weinberg Equilibrium p-value, OR: Odds Ratio.

\*P-value < 0.05

**Footnote Page**

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