

Genetic susceptibility to severe asthma with fungal sensitization

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Summary

Severe asthma is problematic and its pathogenesis poorly understood. Fungal sensitization is common, and many patients with severe asthma with fungal sensitization (SAFS), used to denote this subgroup of asthma, respond to antifungal therapy. We have investigated 325 haplotype-tagging SNPs in 22 candidate genes previously associated with aspergillosis in patients with SAFS, with comparisons in atopic asthmatics and healthy control patients, of whom 47 SAFS, 279 healthy and 152 atopic asthmatic subjects were genotyped successfully. Significant associations with SAFS compared with atopic asthma included Toll-like receptor 3 (TLR3) ($p = .009$), TLR9 ($p = .025$), C-type lectin domain family seven member A (dectin-1) ($p = .043$), interleukin-10 (IL-10) ($p = .0010$), mannose-binding lectin (MBL2) ($p = .007$), CC-chemokine ligand 2 (CCL2) (2 SNPs, $p = .025$ and $.041$), CCL17 ($p = .002$), plasminogen ($p = .049$) and adenosine A2a receptor ($p = .024$). These associations differ from those found in ABPA in asthma, indicative of contrasting disease processes. Additional and broader genetic association studies in SAFS, combined with experimental work, are likely to contribute to our understanding of different phenotypes of problematic asthma.

KEYWORDS

allergy, asthma, disease association, disease association studies, genetics, polymorphism

1 | INTRODUCTION

Asthma leads to an estimated 480,000 deaths annually (Lozano et al., 2012). It is highly problematic for affected patients and their carers and is also costly for health services. Amongst those with asthma, sensitization to fungi (especially *Aspergillus fumigatus*) is associated with more severe disease (O'Driscoll, Hopkinson, & Denning, 2005; O'Driscoll et al., 2009). *Aspergillus fumigatus* is a ubiquitous fungus that is found in the airways of most healthy people as well as those with asthma (Lass-Flörl et al., 1999), yet while most individuals clear inhaled *A. fumigatus* spores without consequence, severe asthmatics can harbour *A.*

fumigatus in their airways and both sensitization and positive cultures are associated with a higher frequency of bronchiectasis, lower lung function and poor asthma control (Denning et al., 2014; Farrant, Brice, Fowler, & Niven, 2016). Asthma patients with severe disease who are sensitized to one or more fungi, but do not meet the diagnostic criteria for allergic bronchopulmonary aspergillosis (ABPA) are classified as having severe asthma with fungal sensitization (SAFS) (Denning, 2006), recently broadened to 'fungal asthma' (Moss, 2014). SAFS is a relatively new classification of allergic asthmatic subjects, but is increasingly recognized (Castanhinha et al., 2015; Denning, 2006; Farrant et al., 2016; Rodrigues et al., 2016).

A diagnosis of SAFS should be considered in patients with severe asthma (British Thoracic Society level 4+), who are sensitized to one or more fungi (as demonstrated by skin prick tests or serum specific IgE), but who have a normal, or near-normal, serum IgE (<1,000 U m/L) and are negative for ABPA (Denning, 2006). It has been suggested that 4%–8% of adult asthmatics will fall into this category (Denning, 2006), with a potential cumulative total of >6.5 million worldwide (Denning, 2015). Although patients with SAFS can be sensitized to any fungus, the majority react to *Aspergillus* species and *A. fumigatus* specifically (Denning, 2006).

It is unclear why a small proportion of patients with asthma develop SAFS while the majority are unaffected by exposure to fungi such as *A. fumigatus*, *Alternaria alternata* and *Candida albicans*. Only one study has investigated genetic association in SAFS (Carvalho et al., 2008); however, many genetic association studies have shown that mutations are associated with susceptibility to other pulmonary diseases caused by *A. fumigatus*, including the closely related disease, allergic bronchopulmonary aspergillosis (ABPA) (Brouard et al., 2005; Overton, Denning, Bowyer, & Simpson, 2016; Vaid et al., 2007).

To increase our knowledge of susceptibility to SAFS, we completed a genetic association study of 22 putative candidate genes for SAFS, focussing on immune genes and those previously associated with other forms of aspergillosis.

2 | METHODS AND METHODS

2.1 | Subjects

SAFS subjects, atopic asthmatic controls and healthy controls were defined by the recruiting physicians according to the criteria in Table 1. We used atopic asthmatics as controls (see Table 1) as all SAFS patients

have asthma, and many are sensitized to non-fungal allergens as well as fungal allergens, which we wanted to control for. Subjects with SAFS were recruited from the tertiary referral clinic at the National Aspergillosis Centre (University Hospital of South Manchester [UHSM], UK) from March 2006 to August 2010. Previously described healthy and asthmatic subjects were used as controls (Langley et al., 2003). These have been used as controls in previous experiments investigating ABPA (Overton et al., 2016) and CCPA (Smith, Hankinson, Simpson, Bowyer, & Denning, 2014; Smith, Hankinson, Simpson, Denning, & Bowyer, 2014). The local research ethics committee (LREC) approved the study, and all subjects gave written informed consent. Statistical analysis for the subject characteristics was completed using GraphPad Prism (Version 5.02; GraphPad Software Inc). Ages, % males and lung function characteristics were compared between the groups using Mann–Whitney U-tests as the data were not normally distributed.

2.2 | DNA extraction

Blood was collected in EDTA-treated blood collection tubes (Becton Dickinson; BD, Oxford, UK). This was centrifuged to separate the plasma and cellular sections and then DNA was then extracted from the cellular section using a phenol–chloroform extraction method. Both the plasma and DNA were stored at –80°C. For the previously recruited subjects, DNA had been collected previously (Marinho, 2010).

2.3 | Gene and SNP selection, genotyping, quality control and data analysis

Twenty-nine candidate genes with immune functions were identified from the literature based on previous associations and biological

TABLE 1 Diagnostic criteria

Disease	Diagnostic criteria
SAFS	<p>All the following are required:</p> <ul style="list-style-type: none"> Severe asthma (BTS level 4+) Serum total IgE <1000 IU/ml Positive SPT or serum specific IgE to one or more fungus; ie. <i>Alternaria</i> spp., <i>Candida albicans</i>, <i>Cladosporium herbarum</i>, <i>Penicillium</i> spp., <i>Saccharomyces cerevisiae</i>, <i>Trichophyton rubrum</i>, <i>Aspergillus</i> spp., and <i>Botrytis cinerea</i>.
Asthmatics not sensitized to fungi (atopic asthmatics)	<p>All the following are required:</p> <ul style="list-style-type: none"> Physician diagnosed asthma No diagnosis of aspergillosis Negative SPT (at 3 mm cut-off) and/or IgE (<0.4) to all fungi tested, including <i>Alternaria alternata</i>, <i>Candida albicans</i>, <i>Cladosporium herbarum</i>, <i>Penicillium notatum</i>, <i>Trichophyton rubrum</i>, <i>A. fumigatus</i>. Either positive SPT (at 3 mm cut-off) and/or IgE (<0.4) other inhalant allergen (eg. mite, cat, dog and grasses). <p>NOTE: Only SPT or IgE need be completed, but if both are done and one is positive this is classed as a positive result.</p>
Healthy control	<p>All the following are required:</p> <ul style="list-style-type: none"> No diagnosis of asthma No diagnosis of aspergillosis Negative SPT (at 3 mm cut-off) and/or IgE (<0.4) to all allergens tested, including mite, cat, dog and grasses Negative SPT (at 3 mm cut-off) and/or IgE (<0.4) to all fungi tested, including <i>Alternaria alternata</i>, <i>Candida albicans</i>, <i>Cladosporium herbarum</i>, <i>Penicillium notatum</i>, <i>Trichophyton rubrum</i>, <i>A. fumigatus</i>. <p>NOTE: Only SPT or IgE need be completed, but if both are done and one is positive this is classed as a positive result.</p>

plausibility for aspergillosis and other fungal infections (Table S1). These included genes involved in immune recognition and response to pathogens, especially those involved in recognition and response to fungus specifically. A total of 325 haplotype-tagging SNPs for the genes of interest were identified using the Genome Variation Server (GVS, <http://gvs.gs.washington.edu/GVS/>) (Table S1). These were usually selected to encompass the entire gene, plus 2500 bp up- and 1500 bp downstream.

Genotyping was completed successfully on 300 SNPs using the Sequenom® MassArray® iPLEX™ Gold system. Quality control was completed and SNPs with Hardy–Weinberg equilibrium $p < .0001$ or call rates $<90\%$ were excluded from the analysis. After this, subjects with call rates $<90\%$ were excluded from the analysis. Genotyping was completed in two batches. Forty-seven SAFS, 279 healthy and 152 atopic asthmatic subjects were genotyped successfully in the first batch and a further 12 atopic asthmatic subjects were genotyped successfully in the second batch. Analysis was completed using SNP and Variation Suite (SVS; version 7.4.3, Golden Helix). Redundant SNPs ($r^2 > 0.80$) were excluded from analysis after evaluation of the LD within our population, as were SNPs that were monomorphic within our population (Table S1). Fisher's exact tests were used to determine association for the remaining 245 SNPs. A p -value of $p < .05$ was considered significant. For SNPs associated with SAFS in the comparison with asthmatic subjects, a genetic association test was completed to identify the p -value for the comparison to healthy subjects. An additional comparison was made between healthy and asthmatic subjects.

3 | RESULTS

3.1 | Characteristics of study participants

The characteristics of the 52 SAFS subjects, 280 healthy subjects and 167 atopic asthmatic subjects recruited for genotyping are shown in Table 2. All are Caucasian. The SAFS patients were older than the asthma patients, (58.6 year vs. 50.1 year, $p = .04$), who were themselves older than the healthy subjects (50.1 year vs. 47.0 year, $p < .0001$). SAFS patients had poorer lung function ($p < .0001$, Table 2).

3.2 | Fungal sensitivity in SAFS

All SAFS patients were tested by specific IgE for sensitivity to *A. fumigatus*, with most (78.8%, 41/52) testing positive. For those not sensitized to *A. fumigatus*, extra testing was completed. Most patients (31/52, 59.6%), including the majority of the non-*A. fumigatus*-sensitized patients, were tested to *Alternaria alternata*, *Candida albicans*, *Cladosporium herbarum*, *Penicillium chrysogenum* and *Trichophyton mentagropyte* in addition to *A. fumigatus*. Patients sensitive to all of these fungi were identified, and many patients were sensitized to multiple fungi (Figure 1). A further nine patients were tested to four of these five fungi, while the remaining 11 patients were tested by specific IgE to fewer specific fungi, at the discretion of the consulting physician and after identification of a sensitizing fungi (usually *A. fumigatus*). Additionally, twenty patients were tested for

TABLE 2 Characteristics of patients and controls recruited

Characteristic	SAFS	Atopic asthma	p-value (SAFS vs. atopic asthma)	Healthy	p-value (SAFS vs. healthy)
n	52	167		280	
Age (year) (median, IQR)	58.6 year (52.9–64.8)	50.1 year (43.6–65.1)	$p = .0435$	47.0 year (44.2–50.7)	$p < .0001$
% male	46.2%	41.3%	n/s	40.4% (113/280)	n/s
Asthma (%)	100% (52/52)	100% (167/167)		0% (0/280)	
Lung function tests (n)	49	165		254	
FEV1%predicted (median, IQR)	66.5 (44.8–74.8)	96.0 (77.5–108.3)	$p < .0001$	107.5 (99.4–117.2)	$p < .0001$
FVC %predicted (median, IQR)	87.3 (68.5–105.0)	108.9 (99.0–118.5)	$p < .0001$	114.7 (105.7–123.9)	$p < .0001$
FEV1/FVC ratio (median, IQR)	62.0 (53.0–67.8)	72.0 (63.0–79.0)	$p < .0001$	79.0 (75.0–83.0)	$p < .0001$
Markers of SAFS					
Total serum IgE (median, IQR) (n)	370 (140–750) (n = 52)	N/A	N/D	N/A	N/D
Positive Aspergillus specific IgE (median, IQR) (n)	35.9% (14/39) (n = 39)	N/A*	N/D	N/A*	N/D

IQR, Interquartile range. N/A, not available. *Diagnostic criteria state that these patients are *Aspergillus* specific IgE or SPT negative. n/s, not significant ($p < .05$). N/D, not done (no data for controls).

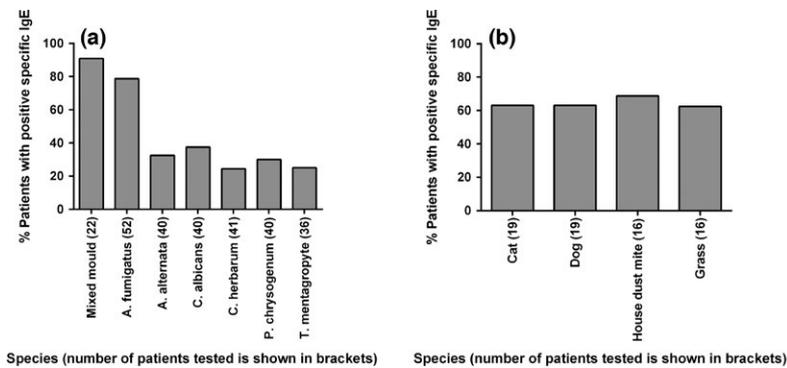


FIGURE 1 SAFS patients sensitivities to fungal (a) and non-fungal (b) allergens. Not all SAFS subjects were tested by specific IgE to all allergens, as this depended on the preferences of the consulting physician. The numbers tested to each allergen are shown in parenthesis

sensitivity to mixed mould, and the majority (90.9%, 20/22) were positive. All positive mixed mould patients were investigated further and sensitivity to a specific fungus was determined; no patient was positive to mixed mould alone. Subjects were only tested for sensitivity to other allergens if this was clinically indicated, but many were found to be positive (Figure 1). All patients were tested for fungal culture on sputum (Langridge, Sheehan, & Denning, 2016), and 21.2% (11/52) were found to be positive. The species cultured were *A. fumigatus* (5/11, 45.5%), *Aspergillus niger* (1/11, 9.1%) and *Penicillium* spp. (4/11, 36.4%); one isolate was not speciated.

3.3 | SNPs in immune genes are associated with SAFS

We initially analysed SNPs for trends towards association with SAFS on the SAFS vs. Healthy model, using a $p < .1$ to identify these trends. Of the 245 SNPs that passed our quality control pipeline, 27 SNPs in 16 immune genes showed a trend towards significance (Table 3). We then identified SNPs associated with SAFS in the SAFS vs. atopic asthma model using a $p < .05$. Ten SNPs in nine immune genes were associated with SAFS in the SAFS vs. asthma comparison (Table 4). These nine genes were Toll-like receptor 3 (TLR3), TLR9, C-type lectin domain family seven member A (CLEC7A, also called DECTIN1), interleukin-10 (IL-10), mannose-binding lectin (MBL, encoded by the gene MBL2), CC-chemokine ligand 2 (CCL2), CCL17, plasminogen (PLG), adenosine A2a receptor (ADORA2A), and the highest associations were with SNPs in CCL17 (rs223827, $p = .002$), MBL2 (rs11003125, $p = .007$), IL-10 (rs1800896, $p = .010$) and TLR3 (rs10025405, $p = .009$). CCL2 contained two SNPs associated with SAFS (rs3760399 and rs2857656). Most associations were with the rare allele or genotype (TLR3 rs10025405, TLR9 rs352140, CLEC7A rs7309123, MBL2 rs11003125, CCL2 rs2857656 and ADORA2A rs2236624).

3.4 | Comparison of SAFS and ABPA genetics identifies many differences

We recently completed studies into genetic susceptibility to ABPA (Overton et al., 2016), investigating the same genes as in the current study of SAFS. A comparison of these results, as well as of the other previous ABPA and SAFS genetics studies that have been completed

in Caucasian subjects, shows that there are major differences between the genetic susceptibility patterns observed for ABPA and SAFS (Table 5). A detailed comparison of the genotype frequencies and p -values in each group confirms this (Table 6). The underlying genetic conditions of these two phenotypes of asthma are clearly different. None of the associations shown in Table 5 or 6 are associated with atopic asthma based on a direct comparison with healthy controls (data not shown).

4 | DISCUSSION

Although various groups including ourselves have investigated genetic susceptibility to different forms of aspergillosis, including the closely related ABPA (Brouard et al., 2005; Overton et al., 2016; Vaid et al., 2007), we believe this is only the second study to investigate genetic susceptibility to SAFS. We have investigated a large set of 22 candidate genes, in which we analysed 245 SNPs. We have identified associations of 11 SNPs in nine genes with SAFS, the highest associations being in CCL17 (rs223827), MBL2 (rs11003125), IL-10 (rs1800896) and TLR3 (rs10025405). CCL2 contained two SNPs associated with SAFS.

CCL17 (also known as TARC) is a Th2-associated chemokine with roles in inflammation, asthma and allergy. It is activated by cytokines such as TNF- α , IL-4 and IL-13, binds to the CC-chemokine receptor 4 (CCR4) and has a variety of functions, including recruitment of Th2 and Treg cells, and prevention of macrophage activation (Hartl, 2009; Katakura, Miyazaki, Kobayashi, Herndon, & Suzuki, 2004; Schuh et al., 2002). This interaction between CCL17, CCR4 and T cells is an important link between innate and acquired immunity (Schuh et al., 2002). In addition, CCL17 may inhibit the expression of the important *A. fumigatus* recognition receptors TLR2 and TLR4.

In our study, SAFS was associated with CCL17 rs223827 (OR 2.93), an intronic SNP and has not been previously associated with disease. Ours is the first study to genotype the CCL17 gene in aspergillosis subjects and the first to find a genetic association with disease, although previous studies have suggested CCL17 as a biomarker of aspergillosis; concentrations of CCL17 in the lung are increased during experimental fungal asthma in mice and during fungal disease in humans (Hartl et al., 2006; Schuh et al., 2002), patients with ABPA have significantly higher serum CCL17 levels than control groups, and these levels can be used to distinguish between

TABLE 3 SNPs showing a trend towards significance ($p < .1$) in the SAFS vs. Healthy model

SNP	Location	Genotype ^a	SAFS	Healthy	Model	SAFS vs. healthy			Risk allele/ genotype
			(<i>n</i> = 47), %	(<i>n</i> = 279), %		odds ratio	95% CI	<i>p</i> - value	
rs2236624 (ADORA2A)	Intronic	TT CT + CC	14.9 85.1	6.5 93.5	Recessive (TT vs. CC, CT)	2.54	1.00, 6.46	.068	TT genotype (rare)
rs7285057 (ADORA2A)	Upstream	CC TC + TT	4.3 95.7	0.4 99.6	Recessive (CC vs. TT, TC)	12.27	1.09, 138.1	.056	CC genotype (rare)
rs2857656 (CCL2)	Promoter	CC GC + GG	14.9 85.1	6.1 93.9	Recessive (CC vs. GG, GC)	2.70	1.05, 6.91	.062	CC genotype (rare)
rs3760399 (CCL2)	5' of gene	GG + AG AA	0.0 100	7.2 92.8	Dominant (AA vs. GG, AG)	N/D	N/D	.092	AA genotype (common)
rs223827 (CCL17)	Intronic	CC + TC TT	42.6 57.4	63.0 37.0	Dominant (TT vs. CC, TC)	2.30	1.49, 5.73	.010	TT genotype (common)
rs11053599 (CLEC7A)	Intronic	AA CA + AA	10.6 89.4	2.9 97.1	Recessive (AA vs. AA, CA)	4.00	1.25, 12.82	.027	AA genotype (rare)
rs7309123 (CLEC7A)	Intronic	GG CG + CC	35.6 64.4	17.9 82.1	Recessive (GG vs. CC, CG)	2.53	1.28, 5.02	.009	GG genotype (rare)
rs10922273 (DENND1B)	Intronic	TT + TC CC	27.7 72.3	12.5 87.5	Dominant (TT, TC vs. CC)	2.67	1.28, 5.54	.013	T allele (rare)
rs6661330 (DENND1B)	Intronic	AA TC + TT	6.4 93.6	0.0 100	Recessive (AA vs. TT, TA)	N/D	N/D	.003	AA genotype (rare)
rs1800896 (IL-10)	Promoter	GG + AG AA	61.7 38.3	76.0 24.0	Dominant (AA vs. GG, AG) ^b	1.96	1.03, 3.76	.048	AA genotype (common)
rs3024509 (IL-10)	Intronic	GG AG + GG	4.3 95.7	0.4 99.6	Recessive (GG vs. AA, AG)	12.36	1.10, 139.10	.056	GG genotype (rare)
rs17879314 (IL-17A)	3' of gene	AA + GA GG	21.3 78.7	35.6 64.4	Dominant (GG vs. AA, GA)	2.05	0.98, 4.29	.066	GG genotype (common)
rs1974226 (IL-17A)	3' UTR	AA GA + GG	10.6 89.4	2.9 97.1	Recessive (AA vs. GG, GA)	4.03	1.26, 12.91	.026	AA genotype (rare)
rs8193036 (IL-17A)	Upstream	CC TC + TT	0.0 100	9.0 91.0	Recessive (TT, TC vs. CC)	N/D	N/D	.034	T allele (common)
rs9395769 (IL-17A)	3' of gene	AA + GA GG	34.0 66.0	20.8 79.2	Dominant (AA, GA vs. GG)	1.97	1.01, 3.84	.059	A allele (rare)
rs11003125 (MBL2)	Promoter	CC + GC GG	72.3 27.7	58.5 41.5	Dominant (CC, GC vs. GG)	1.86	0.94, 3.67	.078	C allele (rare)
rs4252050 (PLG)	5' of gene	GG + AG AA	48.9 51.1	34.8 65.2	Dominant (GG, AG vs. AA)	1.80	0.96, 3.35	.073	G allele (rare)
rs4252200 (PLG)	3' of gene	GG + CG AA	2.1 97.9	11.9 88.1	Dominant (AA vs. GG, AG)	6.20	0.83, 46.44	.041	AA genotype (common)
rs1800469 (TGFB1)	Upstream	AA GA + GG	0.0 100	7.2 92.8	Recessive (AA vs. GG, GA)	N/D	N/D	.092	G allele (common)
rs4833095 (TLR1)	Non-synonymous coding	CC + TC TT	21.3 78.7	37.5 62.5	Dominant (TT vs. CC, TC)	2.22	1.06, 4.66	.032	TT genotype (common)
rs5743595 (TLR1)	Intronic	CC + TC TT	14.9 85.1	30.5 69.5	Dominant (TT vs. CC, TC)	2.50	1.08, 5.81	.035	TT genotype (common)
rs5743708 (TLR2)	Non-synonymous coding	GG + GA AA	12.7 87.2	5.4 94.6	Dominant (GG, GA vs. AA)	2.58	0.95, 7.02	.098	G allele (rare)
rs10025405 (TLR3)	3' of gene	GG + AG AA	80.9 19.1	65.9 34.1	Dominant (GG, AG vs. AA)	2.18	1.01, 4.70	.043	G allele (rare)
rs352140 (TLR9)	Synonymous coding	CC + TC TT	85.1 14.9	71.8 28.2	Dominant (CC, TC vs. TT)	2.24	0.96, 5.21	.072	C allele (rare)
rs11466640 (TLR10)	Intronic	AA + GA GG	17.0 83.0	30.8 69.2	Dominant (GG vs. AA, GA)	2.17	0.97, 4.48	.057	GG genotype (common)

(Continues)

TABLE 3 (Continued)

SNP	Location	Genotype ^a	SAFS	Healthy	Model	SAFS vs. healthy			Risk allele/ genotype
			(n = 47), %	(n = 279), %		odds ratio	95% CI	p- value	
rs4129009 (TLR10)	Non-synonymous coding	CC + TC TT	17.0 83.0	30.8 69.2	Dominant (TT vs. CC, TC)	2.17	0.97, 4.84	.057	TT genotype (common)
rs4711668 (TREM1)	Intronic	TT + TC CC	66.0 34.0	45.7 54.3	Dominant (TT, TC vs. CC)	2.30	1.21, 4.40	.012	T allele (rare)

^aRare genotype is underlined. N/D, Not done: odds ratios could not be calculated because no result for one of the genotypes in one of the groups.

^bThe T/C strand was genotyped (dominant model, TT vs. CC, TC), but the results are reported as G/A because this is the strand that has been genotyped and reported previously. OR, odds ratio; CI, confidence interval.

TABLE 4 SNPs associated with SAFS in the SAFS vs. Atopic Asthma model ($p < .05$)

SNP	Location	Genotype ^a	SAFS	Atopic asthma	Model	SAFS vs. atopic asthma			Risk allele/ genotype
			(n = 47), %	(n = 152) ^b , %		OR	95% CI	p-value	
rs2236624 (ADORA2A)	intronic	TT CT + CC	14.9 85.1	4.6 95.4	Recessive (TT vs. CC, CT)	3.63	1.20, 10.94	.024	TT genotype (rare)
rs2857656 (CCL2)	promoter	CC CG + GG	14.9 85.1	4.6 95.4	Recessive (CC vs. GG, CG)	3.6	1.19, 10.87	.025	CC genotype (rare)
rs3760399 (CCL2)	5' of gene	GG + AG AA	0.0 100	8.6 91.4	Dominant (AA vs. GG, AG)	N/D	N/D	.041	AA genotype (common)
rs223827 (CCL17)	intronic	CC + CT TT	42.6 57.4	68.4 31.6	Dominant (TT vs. CC, CT)	2.93	1.23, 4.31	.002	TT genotype (common)
rs7309123 (CLEC7A)	intronic	GG CG + CC	35.6 64.4	19.9 80.1	Recessive (GG vs. CC, CG)	2.23	1.07, 4.61	.043	GG genotype (rare)
rs1800896 (IL-10)	promoter	GG + AG AA	61.7 38.3	80.9 19.1	Dominant (AA vs. GG, AG) ^c	2.63	1.29, 5.38	.010	AA genotype (common)
rs11003125 (MBL2)	promoter	CC + GC GG	72.3 27.7	49.7 50.3	Dominant (CC, GC vs. GG)	2.65	1.30, 5.41	.007	C allele (rare)
rs4252200 (PLG)	3' of gene	GG + CG AA	2.1 97.9	11.9 88.1	Dominant (AA vs. GG, AG)	6.23	0.81, 47.95	.049	AA genotype (common)
rs10025405 (TLR3)	3' of gene	GG + AG AA	80.9 19.1	59.2 40.8	Dominant (GG, AG vs. AA)	2.91	1.31, 6.44	.009	G allele (rare)
rs352140 (TLR9)	synonymous coding	CC + TC TT	85.1 14.9	67.5 32.5	Dominant (CC, TC vs. TT)	2.74	1.15, 6.57	.025	C allele (rare)

^aRare genotype is underlined.

^bAlthough a further 12 Atopic asthmatic individuals were typed successfully in the second batch, these results are all $n = 152$.

^cThe T/C strand was genotyped (dominant model, TT vs. CC, TC), but the results are reported as G/A because this is the strand that has been genotyped and reported previously. N/D, Not done: odds ratios could not be calculated because no result for one of the genotypes in one of the groups. OR, odds ratio; CI, confidence interval.

subjects colonized with or sensitized to *A. fumigatus* and those with ABPA and peak during exacerbations of ABPA (Hartl et al., 2006). Additionally, studies using mouse models of IA and chronic fungal asthma suggest that CCL17 impairs the pulmonary antifungal response against *A. fumigatus* and results in increased airway inflammation and hyper-responsiveness after exposure to this fungus (Carpenter & Hogaboam, 2005; Schuh et al., 2002).

There are various ways in which CCL17 could contribute to SAFS, including attracting Th2 cells, which can initiate and sustain the allergic response and reduce protective Th1 responses, and inhibiting macrophages, thereby preventing macrophage killing and cytokine

production in response to *A. fumigatus* and other fungi (Hartl, 2009; Katakura et al., 2004; Schuh et al., 2002). CCL17 may also recruit Th17 cells as these cells also express CCR4 (Acosta-Rodriguez et al., 2007). The identification of a SNP in CCL17 associated with SAFS suggests a role for this gene in susceptibility to aspergillosis, in addition to its role as a possible biomarker for diagnosis.

CCL17 is linked to another chemokine, chemokine (C-C motif) ligand 2 (CCL2, MCP-1). Systemic neutralization of CCL17 significantly increases CCL2 levels in the lung (Carpenter & Hogaboam, 2005). CCL2 is a pro-inflammatory cytokine, which is produced by a range

TABLE 5 Comparison of SAFS and ABPA genetics

Gene	SNP	Associated with SAFS	Associated with ABPA
ADORA2A	rs2236624 (intronic)	TT genotype	TT genotype (Overton et al., 2016)
CCL17	rs223827 (intronic)	TT genotype	No association (Overton et al., 2016)
CCL2	rs3760399 (5')	AA genotype	No association (Overton et al., 2016)
	rs2857656 (promoter)	CC genotype	No association (Overton et al., 2016)
CLEC7A	rs7309123 (intronic)	GG genotype	No association (Overton et al., 2016)
	rs11053624 (5')	No association	C allele (Overton et al., 2016)
	rs7959451 (3' UTR)	No association	T allele (Overton et al., 2016)
HLA	HLA type	Not investigated	DR2/DR5 (Chauhan et al., 2000), DRB1*1501 (Chauhan et al., 2000), DRB1*1503 (Chauhan et al., 2000), DR2 (Chauhan et al., 2000), DR4 (Aron et al., 1999), DR7 (Aron et al., 1999), DR5 (Aron et al., 1999; Chauhan et al., 2000)
IL-10	rs1800896 (promoter)	AA genotype	No association with ABPA in asthma (Overton et al., 2016), GG associated with ABPA in CF (Rees et al., 2002)
IL-13	rs20541 (missense)	Not investigated	A allele (Overton et al., 2016)
	rs1800925 (5')	Not investigated	T allele (Overton et al., 2016)
IL-17A	rs3819024 (5')	no association	G allele (Overton et al., 2016)
IL-4R	rs1029489 (3')	Not investigated	A allele (Overton et al., 2016)
	rs6498012 (intronic)	Not investigated	CC genotype (Overton et al., 2016)
	rs1805010	Not investigated	G allele (Knutsen, Kariuki, Consolino, & Warriar, 2006)
MBL2	rs11003125 (promoter)	C allele	No association (Overton et al., 2016)
	rs2099903 (3'UTR)	No association	AA genotype (Overton et al., 2016)
PLAT	rs8178880 (intronic)	no association	AA genotype (Overton et al., 2016)
PLG	rs4252200 (3')	AA genotype	no association (Overton et al., 2016)
	rs4252053 (intronic)	no association	G allele (Overton et al., 2016)
SFTPA2	T1492C	Not investigated	TT genotype (Vaid et al., 2007)
TLR3	rs10025405 (3')	G allele	G allele (Overton et al., 2016)
	rs1879026 (intronic)	no association	GG genotype (Overton et al., 2016)
	rs5743303 (5')	no association	T allele (Overton et al., 2016)
	rs5743305 (5')	no association	TT genotype (Overton et al., 2016)
	rs7668666 (intronic)	no association	A allele (Overton et al., 2016)
TLR9	rs352140 (synonymous coding)	C allele	no association (Overton et al., 2016)
	rs5743836 (upstream)	no association	associated with ABPA in one small study (22 patients, 88 controls) (Carvalho et al., 2008) but not in another larger study (Overton et al., 2016)

Red colour indicates an association with either SAFS or ABPA, green indicates no association or a different association (different allele), blue indicates that this SNP was not investigated.

of cells and acts in a chemo-attractive manner to attract and activate immune and inflammatory cells (Lloyd, 2002; Lu et al., 1998; Traynor et al., 2002). It is also important in the production of Th2 responses (Lloyd, 2002; Lu et al., 1998; Traynor et al., 2002) and appears to have a role in the antifungal response to *A. fumigatus* and in prevention of allergic responses; in non-neutropenic mice sensitized to *A. fumigatus*, overexpression of CCL2 reduces conidial burden, airway inflammation and airway hyper-reactivity after challenge with *A. fumigatus* conidia, while in non-sensitized non-neutropenic mice, neutralization of CCL2 attenuates conidia clearance and increases airway hyper-reactivity, eosinophilia and fibrosis after challenge (Blease et al., 2001). Increased CCL17 levels may reduce CCL2 levels and allow the development of allergic responses to *A. fumigatus*.

Two SNPs in CCL2 were found to be significantly associated with SAFS. The AA genotype of rs3760399 and the CC genotype of rs2857656 were more common in SAFS subjects compared to control groups. rs3760399 and rs2857656 are located 5' of the CCL2 gene and may therefore be in the promoter region and may affect expression; this has been shown for rs2857656 (Guo et al., 2014), where levels of CCL2 were highest in spinal tuberculosis patients who carried the CC genotype. CCL2 levels are increased both in monocytes exposed to *A. fumigatus* conidia, and in models of *A. fumigatus* infection and variations in CCL2 levels appear to affect susceptibility, severity, and allergenicity of murine infection with *A. fumigatus* (Blease et al., 2001; Loeffler et al., 2009); higher levels reduce airway hyper-reactivity and inflammation (Blease et al., 2001). SNPs that affect the expression of

TABLE 6 Detailed comparison for SNPs with different associations in ABPA (Overton et al., 2016) and SAFS

Gene	SNP	Genotype ^a	Atopic asthma (n = 152), %	SAFS (n = 47), %	SAFS p-value	ABPA (Overton et al., 2016) (n = 95), %	ABPA (Overton et al., 2016) p-value
CCL17	rs223827 (intronic)	CC + CT	68.4	42.6	.002	61.1	.236
		TT	31.6	57.4		38.9	
CCL2	rs3760399 (5')	GG + AG	8.6	0.0	.041	5.3	.331
		AA	91.4	100		94.7	
CCL2	rs2857656 (promoter)	CC	4.6	14.9	.025	6.3	.568
		CG + GG	95.4	85.1		93.7	
CLEC7A	rs7309123 (intronic)	GG	19.9	35.6	.043	26.6	.236
		CG + CC	80.1	64.4		73.4	
CLEC7A	rs11053624 (5')	CC + CT	13.2	21.3	.242	24.2	.028
		TT	86.8	29.8		75.8	
CLEC7A	rs7959451 (3' UTR)	TT + CT	21.1	29.8	.237	34.7	.018
		CC	78.9	70.2		65.3	
IL-10	rs1800896 (promoter)	GG + AG	80.9	61.7	.010	73.7	.909
		AA	19.1	38.3		26.3	
IL-17A	rs3819024 (5')	GG + GA	51.3	51.1	1.000	65.3	.032
		AA	48.7	48.9		34.7	
MBL2	rs11003125 (promoter)	CC + GC	49.7	72.3	.007	55.3	.390
		GG	50.3	27.7		44.7	
MBL2	rs2099903 (3' UTR)	AA	3.9	2.1	1.000	11.6	.027
		CC + CA	96.1	10.6		88.4	
PLAT	rs8178880 (intronic)	GG + AG	11.2	10.6	1.000	3.2	.036
		AA	88.8	89.4		96.8	
PLG	rs4252200 (3')	GG + CG	11.9	2.1	.049	9.5	.551
		AA	88.1	97.9		90.5	
PLG	rs4252053 (intronic)	GG + AG	19.7	27.7	.310	32.6	.023
		AA	80.3	72.3		67.4	
TLR3	rs1879026 (intronic)	TT + GT	37.7	25.5	.161	21.1	.007
		GG	62.3	74.5		78.9	
TLR3	rs5743303 (5')	TT + AT	26.3	34.0	.354	41.1	.016
		AA	73.7	66.0		58.9	
TLR3	rs5743305 (5')	AA + TA	64.5	57.4	.393	49.5	.020
		TT	35.5	42.6		50.5	
TLR3	rs7668666 (intronic)	AA + CA	34.9	48.9	.089	48.4	.035
		CC	65.1	51.1		51.6	
TLR9 ^b	rs352140 (synonymous coding)	CC + TC	67.5	85.1	.025	64.2	.590
		TT	32.5	14.9		35.8	

^aRare genotype is underlined.

^brs5743836 was not included as although this was associated with ABPA in one small study (22 patients, 88 controls) (Carvalho et al., 2008) it was not associated in another larger study (Overton et al., 2016). Red background indicates association with the indicated disease. Green background indicates no association with the indicated disease.

CCL2 may therefore affect susceptibility to SAFS. Additionally, the association with the rare CC genotype rs2857656 may be more biologically meaningful than the association with the common AA genotype of rs3760399, as SAFS is a rare disease so association with a rare allele is more expected.

MBL is a collectin that binds to cell wall components of particular pathogens, opsonizing these to enhance phagocytosis, pro-inflammatory cytokine production and activation of the complement cascade (Crosdale, Poulton, Ollier, Thomson, & Denning, 2001; Vaid et al., 2007; van de Wetering, van Golde, & Batenburg, 2004).

Increased levels of MBL are observed during infection and inflammation of the lung (Gomi et al., 2004). The protein functions as an oligomer; in humans, only tetramers and higher-order oligomers are considered functional as only these permit MBL to bind with high avidity (Gomi et al., 2004; Kilpatrick, 2002). Circulating levels of functional MBL vary widely between individuals and are influenced by SNPs in the promoter region, which affect expression, and SNPs in exon 1, which affect oligomerization (Harrison et al., 2012; Kilpatrick, 2002). MBL deficiency is associated with susceptibility, severity or disease progression in many diseases, including CPA (Gomi et al., 2004; Harrison et al., 2012). In our study, one promoter SNP in MBL2 (rs11003125, -550G/C) was significantly associated with SAFS; genotypes containing the rare C allele were significantly more common in the SAFS group compared to the control groups.

RS11003125 has been demonstrated to affect expression of MBL (Garred, Larsen, Seyfarth, Fujita, & Madsen, 2006; Madsen et al., 1995). Haplotypes involving the C allele are associated with reduced circulating MBL levels (Madsen et al., 1995). It is the CG and CC genotypes that are associated with SAFS in the current study, which suggests that reduced levels of MBL in these subjects result in increased susceptibility to disease. This theory is supported by studies demonstrating the importance of MBL in pulmonary host defence to *A. fumigatus* (Dumestre-Perard et al., 2008; Kaur, Gupta, Thiel, Sarma, & Madan, 2007; Lambourne et al., 2009). MBL demonstrates significant binding to *A. fumigatus* in vitro, and this binding enhances conidial phagocytosis by neutrophils and results in activation of the complement cascade (Dumestre-Perard et al., 2008; Kaur et al., 2007). Murine models of IPA show that administration of MBL results in increased inflammatory cytokine production, decreased fungal burden and increased survival (80% in treated, no survivors in untreated) (Kaur et al., 2007). Additionally, significant reductions in MBL serum levels are observed between subjects with IA and controls (Lambourne et al., 2009). This suggests that low levels of MBL reduce the ability of the host to combat infection with *A. fumigatus*, and therefore increase susceptibility to aspergillosis.

However, other studies have demonstrated elevated levels of MBL in ABPA patients compared to controls (Kaur et al., 2006). There are many different factors that affect circulating levels of functional MBL, including an increase in some people with inflammation (Gomi et al., 2004). It may also be that MBL is more important as a modulation gene than as a susceptibility gene and that MBL genotypes affect severity of rather than susceptibility to aspergillosis, with non-functional MBL genotypes associated with worsening breathlessness, and higher MBL levels a marker of more inflammation in CPA and worse respiratory status (Harrison et al., 2012).

RS11003125 has been investigated for association with CPA and allergic aspergillosis previously. This study did not find an association with either disease (Harrison et al., 2012), a result that appears in contrast to our findings but may be due to the fact that the allergic aspergillosis group consisted of a mixed population comprising 28 ABPA patients and seven SAFS patients. This highlights the importance of accurate disease classification and separation of different disease groups.

IL-10 is an anti-inflammatory and immunosuppressive cytokine that is produced by, and acts on, immune cells such as T cells,

neutrophils, monocytes and macrophages (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001). It modulates expression of cytokines, soluble mediators and cell surface molecules by these cells and regulates their recruitment to sites of infection or antigen challenge (Moore et al., 2001; Zuany-Amorim et al., 1995). IL-10 inhibits macrophage activation and pro-inflammatory cytokine and chemokine production by macrophages, T cells and neutrophils (Levitz, Tabuni, Nong, & Golenbock, 1996; Moore et al., 2001; Zuany-Amorim et al., 1995) and promotes a Th2 response by inhibition of the Th1 response (Moore et al., 2001).

IL-10 is produced upon *A. fumigatus* infection in mouse models of IA and appears to be detrimental in this situation; higher levels of IL-10 are associated with increased susceptibility to infection, and neutralization or genetic knockout increases resistance to IA (Cenci et al., 1998; Clemons et al., 2000). However, studies into *A. fumigatus* allergy in mice suggest that IL-10 is important in controlling airway inflammation, modulating the allergic response to *A. fumigatus*, and preventing ABPA-like symptoms. These effects are increased in IL-10^{-/-} mice and are reduced following treatment with IL-10, suggesting that IL-10 is protective in allergy to *A. fumigatus* (Grunig et al., 1997; Mueller et al., 2009). Our findings appear to support this. The AA genotype of the IL-10 -1082(G/A) promoter polymorphism rs1800896 was significantly associated with SAFS in our study. Previous studies have shown that the A allele of this SNP is associated with reduced IL-10 levels compared to the G allele (Rees et al., 2002) and it may be that lower levels of IL-10 prevent the normal control of airway inflammation and allow the development of allergic SAFS symptoms. However, in contrast to this, the opposite, GG genotype of rs1800896, is associated with susceptibility to ABPA in CF (Brouard et al., 2005). This difference in association may result from the fact that ABPA, especially ABPA in CF, is different to SAFS; ABPA patients may have much more colonization and/or mucus production with the fungus (they cough up fungal plugs), while SAFS is more of an allergic asthmatic type disease. It may be that in SAFS the Th2 response is less important and the role of IL-10 in suppression of allergic inflammation is more important in disease development.

TLR3 is one of the recognition receptors involved in the recognition of *A. fumigatus*. In murine infection models, germinating conidia are sensed by epithelial cells through a TLR3/TRIF pathway that results in IDO activation and production of type I IFNs and IL-10, and TLR3^{-/-} mice are highly susceptible to pulmonary aspergillosis and develop high and persistent levels of pathogen-induced inflammation (Carvalho et al., 2012; de Luca et al., 2010). Stimulation of TLR3 with polyinosine-polycytidylic acid (Poly(I:C)) results in impaired conidial phagocytosis and decreased conidiacidal and the hyphal damage activity by neutrophils, but increased reactive oxygen intermediate and myeloperoxidase release in response to both conidia and hyphae (Bellocchio et al., 2004). Stimulation also significantly reduces both fungal growth and inflammation in the lungs of infected mice (Carvalho et al., 2012).

Previous studies investigating SNPs in TLR3 for association with IA found no association (Bochud et al., 2008); however, levels of TLR3 are reduced in macrophages from CPA patients (Smith, Hankinson,

Simpson, Denning et al., 2014) and a recent study has shown that a TLR3 SNP (rs3775296) (which results in a loss-of-function phenotype in DCs) is associated with increased susceptibility to IA in hematopoietic stem cell transplant patients (Carvalho et al., 2012). This SNP is associated with marked decreased TLR3 expression and responsiveness and failure to activate antifungal CD8⁺ T cells (Carvalho et al., 2012). In the current study, TLR3 rs10025405 was associated with SAFS. We found the common AA genotype to be protective against SAFS, and carriers of the rare G allele to be at increased risk of disease. This SNP is intergenic and 3' of the TLR3 gene. Although the role of TLR3 in *Aspergillus* recognition remains poorly defined compared to that of the other TLRs, the association of this SNP with SAFS, and of others with IA, supports this gene as a susceptibility marker for aspergillosis.

Additionally to the genes and SNPs described above, which were highly associated with SAFS ($p < .01$), SNPs in TLR9, PLG, ADORA2A and CLEC7A were also associated with SAFS ($p < .05$).

TLR9 is an endosomal TLR that modulates the inflammatory cytokine response to *A. fumigatus* (Bellocchio et al., 2004; Carvalho et al., 2008; Khan et al., 2016; Ramaprakash, Ito, Standiford, Kunkel, & Hogaboam, 2009). Phagosomal TLR9 expression is controlled by dectin-1 (Khan et al., 2016) and is increased in neutrophils challenged with conidia and hyphae, and induction of TLR9 expression positively correlates with the induction of antifungal effector functions by these cells (Bellocchio et al., 2004). In a mouse model of fungal asthma, TLR9^{-/-} mice exhibited significantly lower airway hyper-responsiveness after challenge with resting conidia compared to the TLR9^{+/+} mice and *A. fumigatus*-sensitized TLR9^{-/-} mice exhibited pulmonary fungal growth up to 28 days after challenge with swollen conidia, an observation that was not found in *A. fumigatus*-sensitized TLR9^{+/+} mice (Ramaprakash et al., 2009). A role for TLR9 in fungal asthma and allergic aspergillosis is supported by the association of a SNP in TLR9 (rs5743836) with ABPA (Carvalho et al., 2008). Although we found no association between this SNP and SAFS, we did find an association with a different SNP (rs352140). The common TT genotype of rs352140 was protective against SAFS, and carriers of the rarer CC or CT genotypes were at increased risk of disease. rs352140 is a synonymous coding SNP (Pro545Pro) that has been previously associated with a variety of diseases, although no reports of association with fungal disease or asthma have been reported. The association of this SNP in TLR9 with SAFS supports TLR9 as important in modulation of the immune response to *A. fumigatus*, particularly in the allergic setting, and in the development of allergic aspergillosis.

Studies have begun to elucidate a role for plasminogen (PLG) in the response to *A. fumigatus*, and in susceptibility to aspergillosis (Behnsen et al., 2008; Loeffler et al., 2009; Zaas et al., 2008). Plasminogen binds to *A. fumigatus* in a dose-dependent manner and can be converted to plasmin while bound (Behnsen et al., 2008). In this context, plasmin can act as a chemo-attractant for monocytes, macrophages and dendritic cells and induces expression of inflammatory cytokines and chemokines by these cells (Li, Laumonier, Syrovets, & Simmet, 2007; Li et al., 2010; Syrovets, Tippler, Rieks, & Simmet, 1997). Variations within the PLG gene may influence the pathogenesis of IA in mice

and one SNP in PLG (rs4252125) has been associated with IA in humans (Behnsen et al., 2008; Zaas et al., 2008). A different PLG SNP (rs4252200) was found to be significantly associated with SAFS in our study; however, as this was an association with the common AA genotype, it may not be biologically meaningful.

ADORA2A is a receptor found on macrophages, T cells and other immune cells. It has anti-inflammatory and immunosuppressive effects, including mediation of apoptosis and suppression of Th1 and Th2 responses, neutrophil chemotaxis and inflammatory cytokine production and may play a critical role in control of inflammation and immunity in infectious disease (Koroskenyi et al., 2011; Lappas, Liu, Linden, Kang, & Malech, 2010; Thiel, Caldwell, & Sitkovsky, 2003). Activation of ADORA2A has been shown to inhibit the development of graft vs. host disease in mice and correlates with reduced T-cell activation, reduced levels of pro-inflammatory cytokines and chemokines and reduced tissue damage (Lappas et al., 2010). ADORA2A is also involved in bronchoconstriction in asthmatic airways (Kim et al., 2009). The anti-inflammatory effects of ADORA2A could be important in the prevention of SAFS and other types of allergic aspergillosis, and it may be that reduced levels of this receptor result in susceptibility. We have found an association of the rare TT genotype of an intronic SNP in ADORA2A (rs2236624) with SAFS. It is as yet unknown what the function of this SNP may be, but alterations in expression or functionality of the ADORA2A protein could affect the anti-inflammatory response and in turn affect susceptibility. The identification of this SNP is therefore an interesting observation and may suggest a role for ADORA2A in the development or prevention of SAFS.

CLEC7A encodes the β -glucan receptor dectin-1. This receptor is found on the surface of macrophages and other immune cells. It binds to *A. fumigatus* and mediates the immune response to β -glucan-containing morphologies of this fungus (swollen conidia, germ tubes and hyphae) (Gersuk, Underhill, Zhu, & Marr, 2006; Hohl et al., 2005; Luther, Torosantucci, Brakhage, Heesemann, & Ebel, 2007; Steele et al., 2005). Dectin-1 has been shown to be involved in phagocytosis, ROS production, cytokine production and recruitment of immune cells (Brown et al., 2003; Cunha et al., 2010; Gersuk et al., 2006; Hohl et al., 2005; Luther et al., 2007; Rivera et al., 2011; Steele et al., 2005). Roles in cytokine production have been demonstrated in macrophages, dendritic cells and neutrophils and dectin-1 may also have a role in mediation of T-cell responses and differentiation of a Th17 response (Cunha et al., 2010; Rivera et al., 2011). In vivo studies have also found increased recruitment of inflammatory cells and increased lung pathology, fungal burdens and mortality in mice lacking dectin-1 (Cunha et al., 2010; Steele et al., 2005; Werner et al., 2009). Dectin-1 is generally seen as protective in aspergillosis, but has been investigated predominantly in the immunocompromised setting. In a recent study, an allergic model of chronic lung exposure to live *A. fumigatus* conidia demonstrated that Dectin-1 recognition of β -glucan led to the induction of multiple pro-inflammatory (IL-1 β and CXCL1) and pro-allergic (Muc5ac, Clca3, CCL17, CCL22 and IL-33) and mediators, which compromised lung function (Lilly et al., 2012).

Previously studies have identified associations between a CLEC7A variant (rs16910526, Y238X) and IA (Chai et al., 2011), as well as other

fungal diseases such as vulvovaginal candidiasis and oral and gastrointestinal *Candida* colonization in haematological stem cell transplant patients (Ferwerda et al., 2009; Plantinga et al., 2009). However, no association was found with candidemia in hospitalized patients (Rosentul et al., 2011) and we found no association with SAFS.

RS16910526 (Y238X) results in an early stop codon and reduced cell surface expression, reduced binding of β -glucan and reduced cytokine production in response to β -glucan, *Candida albicans* and *A. fumigatus* (Cunha et al., 2010; Ferwerda et al., 2009). However, fungal phagocytosis and killing were unaffected (Ferwerda et al., 2009). Previous studies have found the effect of the Y238X mutation to be additive, with heterozygotes showing intermediate levels of expression, binding and cytokine production (Ferwerda et al., 2009), and it is possible that the redundancy of dectin-1 and TLR2 in cytokine production in response to fungi (Brown et al., 2003; Gersuk et al., 2006; Hohl et al., 2005) combined with the additive effect of the mutations means that heterozygote individuals are able to respond efficiently to fungal infection, while GG homozygous individuals are not. Immunocompetence may also have an effect, and it is possible that in immunocompromised subjects such as those investigated in the IA and *Candida* colonization studies (Chai et al., 2011; Plantinga et al., 2009), GC heterozygotes are also susceptible to infection, while in immunocompetent individuals such as those investigated in the current study and in the candidemia study (Rosentul et al., 2011) GC heterozygotes are at no greater risk of infection than controls. It has been shown both in vivo and in vitro that dectin-1 expression increases in response to infection with *A. fumigatus* (Yang et al., 2009), and it may be that this increase in expression limits the effect of the initially low expression, particularly in heterozygotes. This increase in expression in response to *A. fumigatus* infection is inhibited in immunocompromised mice (Yang et al., 2009), which may be another reason for a significant association with IA but not with SAFS.

Although rs16910526 was not associated with SAFS, another SNP in CLEC7A (rs7309123) was associated with SAFS, with carriers of the rare GG genotype being at increased risk of developing disease. This is an intronic SNP that has been previously associated with IA (Sainz et al., 2012). This study found that levels of dectin-1 were lower in individuals carrying the GG genotype compared to carriers of the C allele, and these individuals were at increased risk of IPA (Sainz et al., 2012). Our study found the same association of the GG genotype with disease. As dectin-1 is important in the recognition and response to *A. fumigatus* and may be detrimental in allergic aspergillosis, SNPs that affect the expression or function of this receptor could affect susceptibility to SAFS as well as to IA.

Many of the genes and SNPs identified in our study are closely linked, biologically. In allergic models of *A. fumigatus* exposure, dectin-1 activation can lead to the induction of CCL17 (Lilly et al., 2012), and CCL17 affects CCL2 levels (Carpenter & Hogaboam, 2005). Dectin-1 also controls trafficking of TLR9 to *A. fumigatus* containing phagosomes (Khan et al., 2016), and TLR9 as well as TLR3 are involved in the production of IL-10 (Carvalho et al., 2012; de Luca et al., 2010; Ramaprakash & Hogaboam, 2010). Previous studies have shown how SNPs in different genes such as dectin-1 and CCL2 can have

synergistic effects on susceptibility to aspergillosis (Sainz et al., 2012), and it may be that further analysis will prove that this is the case with SAFS. The pattern of SNP associations we have found is substantially different from that of susceptibility to ABPA, confirming that there are vast differences between these two phenotypes of asthma.

Although the subject numbers used in this study are small compared to those used in large multi-centre GWASs for diseases such as asthma, this is the first sample of SAFS patients investigated, and the numbers of patients used is larger than were used for the initial studies of genetic susceptibility to CCPA and ABPA, where studies reported genetic associations in studies of <30 subjects (Carvalho et al., 2008; Crosdale et al., 2001; Vaid et al., 2007). Our groups also contain well-characterized individuals with well-defined phenotypes and as such allow identification of associations with smaller numbers of subjects than is required for more heterogeneous diseases such as asthma.

The study also has the advantage of being comprehensive in terms of genes investigated. Previous studies investigating genetic association in CCPA, ABPA and SAFS have investigated only a small number of individual SNPs. Previous studies with IA have been more comprehensive and have investigated entire genes, but none have investigated the number or range of genes presented here. As such, this study is a major step forward in identification of candidate genes for SAFS. We have used a candidate gene approach as the alternative GWAS approach would be under-powered with the numbers of patients available.

Selection of appropriate controls in genetic association studies is difficult but important. Both healthy subjects and atopic asthmatics were used as controls in the current study. As all SAFS patients have asthma, comparing with only healthy subjects may identify asthma candidates instead of SAFS candidates. Therefore, in the current study, SAFS subjects were compared to atopic asthmatic controls. An additional comparison to healthy subjects was also included as has been done previously (Carvalho et al., 2008). We acknowledge that our asthmatic controls are slightly younger than our cases, and some could go on to develop SAFS in the following 8 years. However, this difference is small despite being significant (58.6 year vs. 50.1 year) and if anything would reduce rather than increase our power to find associations with SAFS; as such this difference does not invalidate our results. The difference between SAFS and the healthy controls is more pronounced, but we consider the comparison to asthma the principal result and have only used the healthy controls for interest. We consider this first investigation into SAFS genetic susceptibility primarily a hypothesis generating study, and appreciate that future work, including replication, functional work and experiments to confirm that the differences in expression are translated to protein will be required to support our findings.

No functional work was undertaken during the current study. Although it would be interesting to determine the function of some of SNPs identified, this was beyond the remit of the current study. As with any genetic association study, replication of the results observed here will be required to confirm any role for these SNPs in susceptibility to disease. It is important that replicate studies involve suitably large population sizes with the power to identify the associations that

we have found. It is also important that populations are phenotyped comprehensively and that diseases are defined accurately, as using mixed populations of SAFS and ABPA subjects, for example, may reduce the ability to identify associations.

In addition to the identification of SNPs associated with SAFS, we have also investigated susceptibility to atopic asthma by comparing our atopic asthmatic population to the healthy population. This study is small when compared to the large GWASs of asthma, but has identified 20 associated SNPs in 11 genes and may be useful in contributing to our knowledge of asthma genetics.

In conclusion, although our study may be small and requires validation in a larger cohort, our populations are well characterized and our associations are novel; we have identified associations of 10 SNPs in nine genes with SAFS. Most associations were with the rare allele or genotype, which may prove more biologically relevant for susceptibility, as SAFS is a relatively rare disease and association with a common allele may therefore be unlikely. These genes are involved in recognition and response to *A. fumigatus*, and we hope that further work will elucidate roles for these genes in susceptibility to SAFS and allow earlier diagnosis by aiding identification of at risk groups, or even to lead to advanced treatments as our understanding of the disease mechanisms is advanced.

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ETHICS

Subjects with SAFS were recruited from the tertiary referral clinic at the National Aspergillus Centre (University Hospital of South Manchester [UHSM], UK) from March 2006 to August 2010. Previously described healthy and asthmatic subjects were used as controls (Langley et al., 2003). The local research ethics committee (LREC) approved the study and all subjects gave written informed consent.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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SUPPORTING INFORMATION

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