Contribution of NOTCH1 genetic variants to bicuspid aortic valve and other congenital lesions


ABSTRACT
Introduction Bicuspid aortic valve (BAV) affects 1% of the general population. NOTCH1 was the first gene associated with BAV. The proportion of familial and sporadic BAV disease attributed to NOTCH1 mutations has not been estimated.
Aim The aim of our study was to provide an estimate of familial and sporadic BAV disease attributable to NOTCH1 mutations.
Methods The population of our study consisted of participants of the University of Leicester Bicuspid aortic valve genetic research—8 pedigrees with multiple affected family members and 381 sporadic patients. All subjects underwent NOTCH1 sequencing. A systematic literature search was performed in the NCBI PubMed database to identify publications reporting NOTCH1 sequencing in context of congenital heart disease.
Results NOTCH1 sequencing in 36 subjects from 8 pedigrees identified one variant c.873C>G/p.Tyr291* meeting the American College of Medical Genetics and Genomics criteria for pathogenicity. No pathogenic or likely pathogenic NOTCH1 variants were identified in 381 sporadic patients. Literature review identified 64 relevant publication reporting NOTCH1 sequencing in 528 pedigrees and 9449 sporadic subjects. After excluding families with syndromic disease pathogenic NOTCH1 variants were detected in 9/435 (2.1%; 95% CI: 0.7% to 3.4%) of pedigrees and between 0.05% (95% CI: 0.005% to 0.10%) and 0.08% (95% CI: 0.02% to 0.13%) of sporadic patients. Incomplete penetrance of definitively pathogenic NOTCH1 mutations was observed in almost half of reported pedigrees.
Conclusions Pathogenic and likely pathogenic NOTCH1 genetic variants explain 2% of familial and <0.1% of sporadic BAV disease and are more likely to associate with tetralogy of Fallot and hypoplastic left heart.

INTRODUCTION
Bicuspid aortic valve (BAV) is the most common valvular congenital heart defect, affecting 1% of the general population. BAV commonly associates with coarctation of aorta (CoA) and ventricular septal defect (VSD). BAV can lead to clinical complications including aortic valve disease, thoracic aortic aneurysm (TAA) and infective endocarditis. BAV clusters within families following an autosomal dominant pattern of inheritance and its heritability has been estimated between 47% and 89%.

NOTCH1 (MIM# 190198) was the first gene associated with inherited aortic valve disease. Garg et al described two pedigrees, where predominantly left-sided, cardiac lesions (including BAV) co-segregated with damaging NOTCH1 mutations. Subsequently, NOTCH1 mutations were associated with other left (eg, CoA, hypoplastic left heart syndrome (HLHS)) and right-sided (eg, pulmonary stenosis, pulmonary atresia) congenital defects as well as tetralogy of Fallot (ToF) and Adams-Oliver syndrome (AOS).

The report of Garg et al nurtured further sequencing studies. Mohamed et al reported two likely pathogenic mutations in a sample of 48 patients with BAV. McKeller et al reported missense NOTCH1 variants in 10.4% of patients with concomitant BAV and TAA, whereas McBride et al found potentially damaging NOTCH1 mutations in 6.6% of subjects with left ventricular outflow tract disease. The uncertain functional status of identified genetic variants informed further sequencing studies in patients with ‘more severe’ phenotypes including CoA, and BAV associated with aortic root phenotype. Finally, variable analytical approaches were applied including analysis of NOTCH1 variants under recessive model of inheritance, sex-specific analysis or burden testing. The use of various phenotype definitions, research approaches and analytical methods made interpretation of these results difficult and the proportion of BAV disease attributed to NOTCH1 mutations has not been estimated. This lack of knowledge may hinder use of NOTCH1 sequencing for the purpose of genetic counselling.

To provide an accurate estimate of familial and sporadic BAV disease attributed to pathogenic NOTCH1 mutations, we performed sequencing of NOTCH1 in a cohort of familial and sporadic cases of BAV as well as a systematic review of the pathogenicity of previously reported variants.
AIM AND HYPOTHESIS
We hypothesised that the burden of BAV disease due to NOTCH1 mutations had been overestimated. The main aim of our study was to provide an estimate of familial and sporadic BAV disease attributable to NOTCH1 mutations. The secondary aim was to identify a pattern of association of NOTCH1 mutations with congenital cardiac lesions. The tertiary aim was a critical review of the available literature and an overview of the contribution of NOTCH1 variants in congenital heart disease.

METHODS
Study participants
The population of our study were participants of the University of Leicester Bicuspid aORtic vaLVe gEnetic research (BRAVE)—an ongoing, multicentre recruitment of patients with BAV disease. Participants have been identified by review of clinic and discharge letters. Diagnosis of BAV was confirmed by review of cardiac MRI (cMRI), trans-oesophageal echocardiography (TOE) or unequivocal transthoracic echocardiography. Where possible, intra-operative description of valve morphology was obtained. Demographic and clinical data were collected using purposefully designed questionnaires (REDCap). Individuals with positive familial history were offered cascade echocardiographic screening.

Laboratory processing of blood
A venous blood sample (30 mL) was obtained from each participant for the purpose of DNA extraction (QiaSymphony DSP DNA Midi kit/automatic device). All samples were assessed for purity by checking 260/280 nm and 260/230 nm absorbance ratios and diluted to a concentration of 100 ng/µL.

Exome sequencing in pedigrees
DNA samples from subjects with familial BAV underwent whole exome sequencing (WES) (BGI, China). Library capture was performed using BGI Exome (9M) capture kit. Exome sequencing was performed using the Illumina HiSeq platform. Sequence reads for each sample were aligned to the reference genome (hg19) using Burrows-Wheeler Aligner V0.7.15. Variant calling was carried out using the HaplotypeCaller of GATK (V.3.6) (Broad Institute).

NOTCH1 sequencing in sporadic subjects
DNA samples from patients with sporadic BAV underwent targeted NOTCH1 sequencing (Source BioScience, UK). Libraries were prepared using the KAPA Hyperplus kit (Roche), hybridised using the SeqCap Hybridisation kit (Roche) and sequenced with the Illumina MiSeq.

Variant analysis
Variant calls were filtered according to quality control metrics: quality by depth, Fisher strand, RMSMapping quality and Read position rank sum (testing for distance from end of read). Annotation was carried out using Ensembl Variant Effect Predictor and included assignment of amino acid changes, gnomAD allele frequency and the Combined Annotation Dependent Depletion (CADD) functional prediction score.

NOTCH1 variant burden testing
A NOTCH1 variant burden testing was performed following the protocol described by Gillis et al (see online supplemental materials). The allele counts from sequencing of sporadic patients recruited to BRAVE study were compared with the counts obtained from the control population of gnomAD (gnomAD database V2.1.1). Both datasets were filtered to include variants with a minor allele frequency (MAF) of <0.0001 and/or MAF frequency of <0.001 and a CADD score of >20.0. Allele counts in the control dataset were scaled to the mean total allele count. Fisher’s exact test was used to assess for enrichment of variants in the cases over the controls.

Literature search
A systematic literature search was performed in the NCBI PubMed database using “NOTCH1 aortic valve”, “NOTCH1 mutation” and “NOTCH1 variant” terms. The search included all manuscripts published before 31 October 2020 reporting NOTCH1 sequencing in context of congenital heart disease where BAV can be a phenotypic feature. No age or ethnicity exclusion criteria were applied as BAV is an inherited condition and affects all ethnicities. Only original papers written in English and containing information on sequencing of NOTCH1 variants were considered. The compiled bibliography of all papers identified as suitable was further reviewed to identify other qualifying papers.

All reported NOTCH1 variants were recorded and annotated for gnomAD MAF. Variants with MAF <0.001 were assessed for pathogenicity in accordance with the American College of Medical Genetics and Genomics (ACMG) criteria using VarSome, ClinVar and review of the source publications.

For the purpose of summary analysis, the subjects of each of the studies were divided into familial and sporadic cases. Familial cases were defined as affected probands, who had at least one objectively confirmed, affected first-degree or second-degree relative. Sporadic cases were defined as individual patients with no reported first-degree or second-degree affected relatives. Detailed information on criteria used for classification of familial and sporadic patients are provided in the online supplemental materials.

RESULTS
Patient characteristics
The group of familial BAV disease comprised 8 families and included 36 individuals (21 affected and 15 unaffected). Clinical phenotypic information and family trees are presented in figure 1 and table 1.

Of the 21 affected subjects, 19 (90%) had an unequivocal diagnosis of BAV and 2 (9.5%) subjects (figure 1, pedigree B) had a diagnosis of aortic stenosis (AS) but were counted as affected due to the autosomal dominant transmission of a pathogenic NOTCH1 mutation. In all pedigrees, the inheritance pattern was consistent with an autosomal dominant transmission. Among affected subjects, 14 of 21 individuals (67%) were male. Information about aortic valve cusp fusion pattern was available for 13 of 19 individuals (68%). Right-coronary and left-coronary cusp fusion pattern was observed in 10 (52%) individuals (table 1). TAA was diagnosed in 11 (52%) participants (8 males and 3 females). Two had CoA and one subjects had a VSD (figure 1).

The subgroup of individuals with sporadic BAV comprised 381 subjects. In 176 individuals (46%), the diagnosis of BAV was made using cMRI, in 55 (14%) by means of TOE, 103 (28%) patients were diagnosed by means of transthoracic echocardiogram and in 12 (3%) using a CT-aortogram; the remaining patients were diagnosed based on surgical description of the valve. Altogether, surgical description of the valve was available for 129 patients (34%). BAV with fusion of the right-coronary
and left-coronary cusps was present in 231 (61%) patients, 68 subjects (18%) had a right-coronary and non-coronary cusp leaflet fusion and 12 had left-coronary and non-coronary leaflet fusion; in the remaining 70 subjects the pattern could not be determined (table 1). There was a predominance of males among the sporadic subjects (n=277, 73%) with male-to-female ratio of 2.7. The prevalence of associated phenotypes was: TAA (defined as aortic diameter ≥40 mm at any point of the aorta or previous TAA surgery) n=137 (35%), CoA n=57 (15%) and VSD n=15 (4%) (table 1).

Exome sequencing of pedigrees
WES in 36 subjects from 8 pedigrees revealed 13 NOTCH1 genetic variants (11 intronic variants, 1 synonymous variant and 1 nonsense variant) segregating with disease (table 2). Of these variants, only c.873C>G/p.Tyr291* in pedigree B (figure 1) met the ACMG criteria for pathogenicity. We previously reported this pedigree with more detailed phenotypic description.23

Sequencing of NOTCH1 in sporadic cases
Sequencing of NOTCH1 in individuals with sporadic BAV disease and filtering to a MAF frequency of <0.001 and CADD score >20 or MAF <0.0001 identified nine variants (table 3). None of the variants met ACMG criteria for classification as pathogenic. Rare variant burden testing compared with gnomAD controls16 showed no significant difference (9/762 alleles in cases compared with 4376/225,370 alleles in controls; p=0.15).

Literature review
A literature search in the PubMed database using the terms “NOTCH1 aortic valve”, “NOTCH1 mutation” and “NOTCH1 variant” performed on 31 October 2020 returned 106,175 and 206 publications, respectively. There were 1858 unique references of which 1759 were rejected based on the title and the remaining assessed based on the abstract. Fifty-four manuscripts met the inclusion criteria and 10 publications were identified through review of bibliography (figure 2 and online supplemental table 1).

Twenty-five studies focused on sequencing of sporadic subjects, 19 studies sequenced individuals from pedigrees with multiple affected individuals and 15 studies used a mixed design of sequencing both extended pedigrees as well as sporadic subjects. Altogether, the publications reported NOTCH1 sequencing in subjects from 528 different pedigrees and 4669 sporadic cases.
In addition, four studies focused on investigating de novo mutations by sequencing trios of affected proband and unaffected parents (4780 trios) (online supplemental table 1; no: 56, 58, 62, 63).

The included manuscripts reported pathogenic and likely pathogenic NOTCH1 genetic variants in 89 individual patients carrying 74 unique genetic variants (online supplemental table 2 and figure 1). Of the recurring variants, only rs774966208 (c.578G>C, p.Gly193Ala) and rs1228192276 (c.428C>T, p.Pro143Leu) were reported in more than two individuals and both were assigned likely pathogenic ACMG status. Variant rs774966208 was reported in five patients with TOF and likely pathogenic status. Variant rs1228192276 was reported in three patients with TOF and in five patients with TOF and likely pathogenic status. Variant rs774966208 was reported in five patients with TOF and five patients with TOF and likely pathogenic status. Variant rs1228192276 was reported in three patients with TOF and three patients with TOF and likely pathogenic status. Variant rs774966208 was reported in five patients with TOF and five patients with TOF and likely pathogenic status. Variant rs1228192276 was reported in three patients with TOF and three patients with TOF and likely pathogenic status.

Table 1: Clinical characteristics of patients

<table>
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<tr>
<th>Variant ID</th>
<th>Pedigree ID</th>
<th>Location</th>
<th>MAF (gnomAD)</th>
<th>Function</th>
<th>Pathogenicity class</th>
</tr>
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<tbody>
<tr>
<td>NA</td>
<td>B</td>
<td>p.Tyr291* c.873C&gt;G</td>
<td>–</td>
<td>Stop gained</td>
<td>Pathogenic</td>
</tr>
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<td>rs2229975</td>
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<td>p.Pro284Pro c.852G&gt;A</td>
<td>0.1335</td>
<td>Synonymous variant</td>
<td>Benign</td>
</tr>
<tr>
<td>rs3812605</td>
<td>H</td>
<td>c.3171+220A&gt;G</td>
<td>0.6629</td>
<td>Intron variant</td>
<td>Uncertain significance</td>
</tr>
<tr>
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<tr>
<td>rs4880100</td>
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<td>Intron variant</td>
<td>Benign</td>
</tr>
<tr>
<td>rs10781498</td>
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<td>c.1555+102C&gt;T</td>
<td>0.4246</td>
<td>Intron variant</td>
<td>Benign</td>
</tr>
<tr>
<td>rs11145767</td>
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<td>Intron variant</td>
<td>Benign</td>
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<tr>
<td>rs3125009</td>
<td>B</td>
<td>c.1100-140G&gt;A</td>
<td>0.4267</td>
<td>Intron variant</td>
<td>Benign</td>
</tr>
</tbody>
</table>

BAV, bicuspid aortic valve; MAF, minor allele frequency; NA, not available.

Table 2: Genetic variants co-segregating with affection status within the pedigrees with familial form of BAV disease

<table>
<thead>
<tr>
<th>Variant ID</th>
<th>Pedigree ID</th>
<th>Location</th>
<th>MAF (gnomAD)</th>
<th>Function</th>
<th>Pathogenicity class</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
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<td>D</td>
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<td>Intron variant</td>
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</tr>
</tbody>
</table>

Analysis of the phenotypic spectrum observed within the 28 affected pedigrees (harbouring pathogenic and likely pathogenic variants) revealed that in the majority NOTCH1 mutations were associated with a wide range of congenital heart diseases including complex lesions such as TOF, truncus arteriosus or HLHS (online supplemental table 1; no: 1, 7, 17, 19, 24, 41, 50). In seven of these pedigrees, at least one individual was affected by TOF, in seven other by HLHS and two families had individuals with tricuspid valve atresia resulting in hypoplastic right ventricle. Phenotypic information were not available for three pedigrees. Only in nine pedigrees were the pathogenic and likely pathogenic NOTCH1 variants associated with simple congenital phenotypes like isolated BAV, VSD or CoA (figure 2).

Of the 25 affected pedigrees (non-syndromic disease) harbouring definitely pathogenic mutations (stop mutations, frame shift, splice variants leading to loss of whole exomes), complete penetrance was observed in 11 pedigrees, incomplete penetrance was observed in 10 pedigrees (38 affected subject of 56 confirmed carriers of mutations in 10 pedigrees); detailed information/family trees were not available for 4 pedigrees.

Among the 40 sporadic patients harbouring pathogenic and likely pathogenic NOTCH1 mutations, 25 had TOF and 8 had HLHS (online supplemental table 1; no: 5, 17, 25, 35, 36, 42, 62, 63). Detailed phenotypic information was not provided for two individuals. In only five sporadic participants were the NOTCH1 pathogenic or likely pathogenic mutations reported in context of isolated congenital aortic valve or aortic disease: one patient had congenital AS, two patients BAV associated with TAA, one patient CoA and one patient had subvalvular AS with hypoplastic aorta and CoA (online supplemental table 1; no: 42, 43, 51, 63) (figure 2).

After excluding families with syndromic disease, pathogenic and likely pathogenic NOTCH1 variants were detected in 28/435 (6.4%; 95% CI: 4.1% to 8.7%) of pedigrees with a wide spectrum of congenital cardiac lesions. Only in nine pedigrees, 9/435 (2.1%; 95% CI: 0.7% to 3.4%) NOTCH1 pathogenic and likely pathogenic variants were associated with BAV and/or other simple lesions like isolated VSD or CoA.

Among non-syndromic sporadic cases pathogenic and likely pathogenic, NOTCH1 mutations were responsible for 0.4% (95% CI: 0.3% to 0.6%) of the disease (40/9318). However, of the 40 patients 33 had either TOF or HLHS and a detailed
Congenital heart disease

Table 3  NOTCH1 genetic variants (MAF frequency of <0.001 and CADD score >20 or MAF <0.0001) identified among 381 patients with sporadic BAV disease

<table>
<thead>
<tr>
<th>Variant ID</th>
<th>Location</th>
<th>MAF (gnomAD)</th>
<th>Coding change</th>
<th>Protein change</th>
<th>CADD</th>
<th>Pathogenicity class</th>
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<td>rs199652954</td>
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<td>0.000007</td>
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<td>rs368400902</td>
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<td>0.000003</td>
<td>c.3887G&gt;A</td>
<td>p.Ang1296His</td>
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<td>Likely benign</td>
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<td>–</td>
<td>c.4834G&gt;A</td>
<td>p.Gly1612Ser</td>
<td>23.6</td>
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<td>p.Ala105Gly</td>
<td>18.97</td>
<td>Uncertain significance</td>
</tr>
</tbody>
</table>

BAV, bicuspid aortic valve; MAF, minor allele frequency.

phenotype was missing for two individuals. Therefore, the actual prevalence of NOTCH1 pathogenic or likely pathogenic variants associated with sporadic BAV disease or BAV associated with simple lesions like isolated CoA or VSD was between 0.05% (95% CI: 0.005% to 0.10%; 5/9318) and 0.08% (95% CI: 0.02% to 0.13%; 7/9318), respectively.

DISCUSSION

In this study, we present results of NOTCH1 sequencing of 8 pedigrees with familial BAV and 381 patients with sporadic BAV as well as results of a systematic review of NOTCH1 sequencing in 528 pedigrees with familial congenital cardiac lesions and 9449 sporadic cases of congenital heart disease. The main finding from our study is that the pathogenic and likely pathogenic NOTCH1 genetic variants explain only a small proportion of familial (2%) and sporadic (<0.10%) BAV disease. In non-syndromic familial and sporadic forms, NOTCH1 mutations are more commonly associated with more complex congenital phenotypes and ToF and HLHS in particular.

WES of eight extended pedigrees recruited by our group identified a single NOTCH1 pathogenic variant.23 There was a significant phenotypic heterogeneity within the carriers of the mutation with the phenotypes including BAV, AS of a trileaflet valve, TAA and VSD.23 We did not identify any pathogenic or likely pathogenic NOTCH1 genetic variants in 381 participants with sporadic BAV. In the majority of BRAVE study participants, BAV was diagnosed using high-resolution imaging modalities (cMRI, TOE and intra-operative inspection of valve). We performed cascade echocardiographic screening in cases suspected of familial transmission. In this way, we have ensured accurate distinction between familial and sporadic cases. We have applied stringent and uniform criteria to categorise subjects reported in the literature.

Our findings are consistent with previous attempts of sequencing NOTCH1 variants in large populations of individuals with BAV disease. Variant burden testing of NOTCH1 in 441 patients with BAV and TAA compared with ExAC controls showed enrichment in controls rather than cases.16 Burden testing performed in 60 patients with hereditary TAA associated with BAV compared with European controls found no difference in the proportion of qualifying NOTCH1 variants.24

The second important finding form our study concerns the frequency of incomplete penetrance of NOTCH1 mutations. This phenomenon was observed in almost half of the families

Figure 2  Study design and key findings. BAV, bicuspid aortic valve; BRAVE, Bicuspid aoRtic vaLe gEnetic research; CoA, coarctation of aorta; VSD, ventricular septal defect.
carrying definitely pathogenic NOTCH1 mutations and almost one-third of carriers of pathogenic or likely pathogenic variants had no clinical phenotype.

The results of our study indicate that sequencing of NOTCH1 should be considered in familial and sporadic cases of AOS and sporadic and familial cases of ToF and HLHS, however, only around 6% of these will yield a positive result. NOTCH1 sequencing may be considered in pedigrees with BAV and associated simple congenital phenotypes (CoA and VSD) with an expected yield of 2%. Caution has to be used when applying variant filtering in extended pedigrees to allow for incomplete penetrance. Finally, sequencing of NOTCH1 in sporadic subjects with isolated BAV with or without TAA is not likely to identify pathogenic NOTCH1 variants.

Our data have to be interpreted in context of possible methodological biases. Our analysis of NOTCH1 mutation frequency is based on the number of variants that we annotated as pathogenic. However, a relatively large proportion of variants were classified as being of uncertain significance based on available evidence and contribution of these variants to disease cannot be excluded. Also, the segregation analysis of the four variants of unknown significance detected in the sporadic patients from BRAVE study was not possible as their relatives had not been recruited to the study.

The proportion of pedigrees with familial form of BAV disease in which NOTCH1 nonsense, frameshift and splice variants were reported is likely to be overestimated due to non-reporting bias. It is possible that many more pedigrees with familial BAV had undergone sequencing for NOTCH1 genetic variants but results of these efforts remain unpublished if no causative genetic variants were identified.

Sporadic patients, who reported no familial history of the disease, have not undergone cascade echocardiographic screening. This could result in non-detection of familial cases in our sporadic cohort.

Majority of the reported familial and sporadic patients, including the BRAVE study cohort, were of white ethnic origin. In summary, the large sequencing effort in pedigrees and sporadic subjects with BAV combined with systematic literature review showed that NOTCH1 mutations can be found in around 6% of families of white ethnic origin, with multiple congenital cardiac defects (including BAV) and around 2% of pedigrees with isolated familial BAV and/or CoA/VSD. Pathogenic NOTCH1 variants explain <0.10% of isolated sporadic BAV cases of white ethnic origin. Further research in population of other ethnicities is necessary to confirm these estimates. Our study also indicates that further research is necessary to explain the incomplete penetrance of NOTCH1 mutations.

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Contributors RMD, NJS, TRW and APB designed the project. RMD is responsible for the overall content as guarantor. RMD, SEH, POJ and TRW performed the sequencing and calculations obtained from literature review. SEH performed bioinformatics work on the sequencing data. RMD, KS, MS, SLH, DSq, DSj, KL, PS, NW, AC, ME, SC, GJS and APB were involved in recruitment and phenotyping of patients. MA and SK were involved in processing of biological samples. NJS, TRW and APB supervised the findings of this work. VB provided advice on statistical calculations. All authors discussed the results and contributed to the final manuscript.

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