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ORIGINAL ARTICLE

Serum amyloid A levels are associated with polymorphic variants in the *serum amyloid A 1* and *2* genes

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Abstract

Background Serum amyloid A (SAA) is secreted by liver hepatocytes in response to increased inflammation whereupon it associates with high-density lipoprotein (HDL) and alters the protein and lipid composition of HDL negating some of its anti-atherogenic properties.

Aims To identify variants within the *SAA* gene that may be associated with SAA levels and/or cardiovascular disease (CVD).

Methods We identified exonic variants within the *SAA* genes by deoxyribonucleic acid (DNA) Sanger sequencing. We tested the association between *SAA* variants and serum SAA levels in 246 individuals with and without CVD.

Results Increased SAA was associated with rs2468844 (beta [β] = 1.73; confidence intervals [CI], 1.14–1.75; $p = 0.01$), rs1136747 (β = 1.53 (CI, 1.11–1.73); $p = 0.01$) and rs149926073 (β = 3.37 (CI, 1.70–4.00); $p = 0.02$), while rs1136745 was significantly associated with decreased SAA levels (β = 0.70 (CI, 0.53–0.94); $p = 0.02$). Homozygous individuals with the *SAA1.3* haplotype had significantly lower levels of SAA compared with those with *SAA1.1* or *SAA1.5* (β = 0.43 (CI, 0.22–0.85); $p = 0.02$) while *SAA1.3/1.5* heterozygotes had significantly higher SAA levels compared with those homozygous for *SAA1.1* (β = 2.58 (CI, 1.19–5.57); $p = 0.02$).

Conclusions We have identified novel genetic variants in the *SAA* genes associated with SAA levels, a biomarker of inflammation and chronic disease. The utility of SAA as a biomarker for inflammation and chronic disease may be influenced by underlying genetic variation in baseline levels.

Keywords Biomarker · Cardiovascular disease (CVD) · Genetic variants · Serum amyloid A (SAA)

Introduction

Chronic diseases such as diabetes mellitus and cardiovascular disease (CVD) are increasing global health concerns [1, 2] that require concerted preventative efforts coupled with development of effective treatment options [3]. In addition to the more established CVD risk factors, such as smoking, diabetes, hypertension, and dyslipidaemia, it is becoming clearer that chronic inflammation also plays a significant role in the development of atherosclerosis [4].

Serum amyloid A (SAA) protein concentrations increase up to 1000-fold in response to infection, injury and inflammation [5–8]. SAA is secreted by liver hepatocytes or by macrophages, vascular endothelial cells and adipocytes [5–8]. SAA is an amphipathic alpha-helical apolipoprotein (apo) [9] involved in the mobilisation of cholesterol for tissue repair and regeneration and undertakes a "housekeeping" role in normal tissues [10, 11]. However, there is increasing evidence that implicates SAA in the pathological processes of multiple chronic diseases [12]. When released, SAA readily associates with high-density lipoprotein (HDL), becoming the major carrier of this protein in the circulation [13, 14]. HDL is traditionally considered to be atheroprotective; however, evidence

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suggests its association with SAA causes a change in the protein and lipid composition of HDL which negates some of its anti-atherogenic properties as it transitions to a pro-atherogenic state [13, 15, 16]. SAA has been detected in foam cells within atherosclerotic lesions and may lead to plaque instability [6, 17–19].

There are four human *SAA* genes (*SAA1-4*) within a 150-kb region on the short arm of chromosome 11 [20]. *SAA1* and *SAA2* encode acute phase proteins (ASAA) that are released in response to inflammatory stimuli, *SAA3* is a pseudogene, and *SAA4* is constitutively expressed [21]. Several variants in *SAA1* and *SAA2* have been previously reported in association with SAA levels, CVD, and carotid intima media thickness (cIMT) risk [22–24].

Furthermore, *SAA1* has several transcripts, (SAA1.1, 1.2, 1.3, 1.4, and 1.5) defined by two non-synonymous single nucleotide polymorphisms (SNPs) in exon 3 at positions 2995C/T (rs1136743) and 3010C/T (rs1136747). These genotypic combinations define three haplotypes that correspond to *SAA1.1* (52Val, 57Ala), *SAA1.3* (52Ala, 57Ala), and *SAA1.5* (52Ala, 57Val) [25–27]. Previous studies have reported association of these haplotypes with amyloidosis and rheumatoid arthritis [25, 26, 28–31]. *SAA1.5* has been shown to have a higher binding affinity for HDL and a slower clearance rate from the circulation, in comparison to *SAA1.1* and *SAA1.3* [32, 33].

We sought to identify coding variants associated with SAA levels in *SAA1*, *SAA2*, and *SAA4* in a well-characterised cohort of individuals, with and without CVD.

Materials and methods

Study participants

Study participants were recruited following attendance at the nuclear cardiology and renal clinics at the Royal Victoria and Belfast City Hospitals, between October 2015 and February 2017.

Evaluation of cardiovascular outcomes

CVD status was determined on the basis of a myocardial perfusion scan or by a previous diagnosis of angina or stroke. The test was interpreted by a consultant cardiologist or an associate specialist, and the presence or absence of myocardial ischaemia or infarction was noted. The degree of image abnormality was rated using a semi-quantitative model comprising 20 myocardial segments each scored from 0 (normal) to 4 (severely abnormal). The score for each segment was summed to give an overall total; summed scores greater than 2 were designated as abnormal and represented significant myocardial ischaemia or infarction, thus indicating an underlying diagnosis of coronary artery disease (CAD). Summed scores of 2 or less were deemed normal and not in keeping with flow-limiting CAD. The difference between summed stress and rest scores was designated the summed difference score (SDS) and reflected the burden of myocardial ischaemia detected. Thus, individuals with a summed score of less than 2 were allocated into the "no CVD" status group, and those with a score greater than 2 into the "CVD" status group.

Isolation of HDL_2 and HDL_3 from serum

HDL_2 and HDL_3 were isolated from freshly thawed serum by rapid ultracentrifugation at 100,000 rpm according to the method of McPherson et al. [34]. This was a three-step procedure, taking 6 h in total. Firstly, crude HDL was isolated from serum by rapid flotation and sedimentation followed by isolation of HDL_2 and HDL_3 via two rapid flotation steps. Lipoproteins were stored immediately at − 80 °C until required for analysis.

Measurement of serum amyloid A

SAA levels were measured in serum samples isolated from whole blood following centrifugation at 3000 rpm at 4 °C for 10 min using an enzyme-linked immunosorbent assay (ELISA, Invitrogen™ Human SAA kit KHA0011C, CA, USA) using a Grifols Triturus automated ELISA system (Vicopisano, Italy) as per the manufacturer's instructions. The coefficients of variation for SAA were 2.8% (interspecific) and 8.0% (intraspecific).

Genotyping

DNA was amplified by polymerase chain reaction (PCR) using oligonucleotide primers and annealing conditions listed in the supplementary information (supplementary Table 1) using Taq PCR mastermix kit (Qiagen, Hilden, Germany). PCR clean-up was conducted using Exoprostar-1 step mix as per manufacturer's instructions (GE Healthcare Life Sciences, Little Chalfont, UK) and Sanger cycle sequencing using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Ethanol precipitation and DNA sequencing were completed by Genomic Core Services, Queen's University Belfast, UK.

Statistical analysis

The chi-square and one-way analysis of variance (ANOVA) tests for trend were used to investigate the differences in qualitative (CVD status) and quantitative (serum SAA levels) traits, respectively. Regression analysis was used to adjust for the potential confounders (SPSS, version 21, SPSS, Inc., Chicago, IL).

Results

Subject characteristics

A total of 252 participants were recruited to the study; however, serum and DNA samples were only available for 246 participants; these were split into two categories, no CVD ($n = 100$) and CVD ($n = 146$) (Table 1). A significant but modest correlation between SAA with age and estimated glomerular filtration rate (eGFR) ($r = 0.15$, $p = 0.03$ and $r = -0.19$, $p < 0.001$, respectively) was detected. Females tended to have higher serum SAA levels than males (30 mg/L (25, 36) vs. 19 mg/L (16, 23); $p < 0.001$).

Preliminary screening

Preliminary screening of DNA from 46 individuals included 23 individuals with the lowest levels of SAA (< 10 mg/L) and 23 individuals with the highest SAA levels (> 70 mg/L). PCR and DNA sequence analysis of all 12 exonic regions (4 exons from *SAA1*, *SAA2*, and *SAA4*) was undertaken to identify SNPs with a minor allele frequency (MAF) greater than 5% in association with SAA levels. Several SNPs were identified in *SAA1* exons 1, 3, and 4 and also *SAA2* exon 4 (data not provided). No SNPs with a MAF > 5% were identified in *SAA1* exon 2, *SAA2* exons 1, 2, and 3 or any exons of *SAA4*. As such, DNA sequence analysis was restricted to *SAA1* exons 1, 3, and 4 and *SAA2* exon 4 in the remaining 200 study participants.

Variants associated with serum amyloid A levels

Nineteen SNPs were identified in exons 1, 3, and 4 of *SAA1* and exon 4 of *SAA2* in the 246 study participants. Of these, three SNPs were significantly associated with higher levels of SAA (rs149926073: β = 3.37; CI, 1.70–4.00; $p = 0.02$;

Table 1 Subject characteristics for controls and cases of cardiovascular disease

	Control ($n = 150$)	CVD ($n = 102$)	p value
Age (years)	65 (10)	66 (9)	0.52
Males, *n* (%)	83 (55)	60 (59)	0.34
Diabetes, number with diabetes (%)	55 (37)	40 (39)	0.39
DBP (mmHg)	81 (81)	81 (81)	0.76
SBP (mmHg)	132 (118, 148)	135 (123, 151)	0.31
MABP (mmHg)	98 (90, 106)	98 (90, 108)	0.75
HDL cholesterol (mmol/L)	1.3 (1.0, 1.6)	1.2 (1.0, 1.5)	0.64
LDL cholesterol (mmol/L)	2.2 (1.7, 2.9)	2.0 (1.5, 2.6)	0.07
Total cholesterol (mmol/L)	3.8 (3.0, 4.8)	3.6 (3.1, 4.3)	0.10
Troponin (ng/L)	9.9 (5.0, 16.0)	10.5 (5.8, 15.7)	0.72
HbA1c (mmol/mol)	49 (39, 62)	51(38, 69)	0.35
CRP (mg/L)	2.91 (1.22, 5.35)	2.82 (0.95, 4.70)	0.86
eGFR (mL/min/1.73 m2)	97(75, 116)	95 (71, 110)	0.70
Weight (kg)	87 (78, 100)	87 (76, 102)	0.84
Demi span (cm)	80 (80)	81 (81)	0.30
Anti-platelet, *n* (%)	46 (38)	56 (55)	< 0.001*
Anti-coagulant, *n* (%)	27 (19)	20 (20)	0.49
Anti-oestrogen, *n* (%)	1 (0.7)	1 (1.0)	0.65
Anti-convulsants, *n* (%)	9 (6.2)	11 (11)	0.13
Anti-hypertensives, *n* (%)	53 (37)	32 (32)	0.26
Hypoglycaemics, *n* (%)	38 (26)	32 (32)	0.21
Statins, *n* (%)	95 (66)	78 (77)	0.04*
Corticosteroids, *n* (%)	28 (19)	15 (15)	0.24
Beta-blockers, *n* (%)	43 (30)	49 (49)	< 0.001*
ACE inhibitors, *n* (%)	38 (26)	42 (42)	< 0.001*
Calcium channel blocker, *n* (%)	33 (22.8)	23 (22.5)	0.55
Nitrates, *n* (%)	4 (2.8)	27 (26.7)	< 0.001*
Diuretics, *n* (%)	36 (24.8)	29 (28.7)	0.30
NSAIDs, *n* (%)	7 (4.9)	6 (6.0)	0.46

Where * indicates significance, $p \leq 0.05$

Table 2 Linear regression analysis of single nucleotide polymorphisms associated with serum amyloid A levels ($n = 246$)

Exon	SNP	Minor allele	MAF	βeta (95% CI)	p value
SAA1 exon 1	rs11024595	T	0.26	0.73 (0.61–1.01)	0.06
	rs2445166	A	0.10	0.72 (0.51–1.00)	0.09
	rs11545466	G	0.07	0.83 (0.56–1.18)	0.27
	rs549103464	Insertion	0.07	1.07 (0.87–1.25)	0.65
SAA1 exon 3	rs11024597	T	0.34	0.97 (0.81–1.19)	0.83
	rs149926073	A	< 0.01	3.37 (1.70–4.00)	0.02*
	rs1136743	C	0.32	3.55 (0.89–1.32)	0.40
	rs11545468	C	0.08	0.82 (0.55–1.13)	0.20
	rs1136745	C	0.16	0.70 (0.53–0.94)	0.02*
	rs1136747	T	0.24	1.53 (1.11–1.73)	0.01*
SAA1 exon 4	rs15790	T	0.08	1.28 (0.94–1.80)	0.11
	rs145680768	C	< 0.01	0.95 (0.31–2.33)	0.75
	rs12218	T	0.46	1.22 (0.93–1.41)	0.19
	rs1059571	G	< 0.01	0.73 (0.19–0.97)	0.32
SAA2 exon 4	rs11540206	A	< 0.01	1.14 (0.61–3.79)	0.37
	rs149402852	A	< 0.01	0.83 (0.28–1.33)	0.21
	rs116861605	A	0.01	2.69 (0.68–2.16)	0.51
	rs2468844	G	0.18	1.73 (1.14–1.75)	0.01*

Given the skewed distributions, variables were log transformed; data in the table shows anti-logged values. Where * indicates a significant result $p \leq 0.05$

rs1136747: β = 1.53; CI, 1.11–1.73; $p = 0.01$; and rs2468844: β = 1.73; CI, 1.14–1.75; $p = 0.01$) and one was associated with lower levels of SAA (rs1136745: β = 0.70; CI, 0.53–0.94; $p = 0.02$) (Table 2).

In a linear regression analysis, three of the four SNPs remained significantly associated with SAA: rs1136745 (β = 0.65; CI, 0.48–0.24; $p = 0.01$), rs2468844 (β = 1.43; CI, 1.13–1.79; $p < 0.001$), and rs1136747 (β = 1.43; CI, 1.13–1.80; $p < 0.001$) following adjustment for age, gender, eGFR, and the presence of diabetes (Table 3). All four SNPs were shown to exert independent effects following inclusion within a single linear regression model together with gender, diabetes, eGFR, and age: rs1136747 (β = 1.30; CI, 1.35–1.65; $p = 0.03$); rs149926073 (β = 2.66; CI, 1.11–6.33; $p = 0.03$), rs2468844 (β = 1.32; CI, 1.55–1.67; $p = 0.02$), and rs1136745 (β = 0.72; CI, 0.54–0.74; $p = 0.03$), with all SNPs remaining significantly associated with SAA levels ($p < 0.05$; Table 3).

Table 3 Adjusted linear regression analysis of variants associated with serum amyloid A levels

SNP	Beta (95% CI)[1]	p value[1]	Beta (95% CI)[2]	p value [2]
rs1136745	0.65 (0.48–0.24)	0.01	0.72 (0.54–0.74)	0.03
rs2468844	1.43 (1.13–1.79)	< 0.001	1.32 (1.55–1.67)	0.02
rs149926073	1.53 (0.70–3.37)	0.29	2.66 (1.11–6.33)	0.03
rs1136747	1.43 (1.13–1.80)	< 0.001	1.30 (1.35–1.65)	0.03

Given the skewed distributions, variables were log transformed; data in the table shows anti-logged values. Beta values[1] and p^1 represent results of SNPs in the adjusted model alone, adjusting for gender, age, estimated glomerular filtration rate (eGFR), and diabetes. Beta values[2] and p^2 represent all four SNPs together in an adjusted model, adjusted for gender, age, eGFR, and diabetes

Associations of genetic variants with cardiovascular disease status

No significant associations between CVD status and SAA variants were detected ($p > 0.05$; Table 4).

Serum amyloid A 1 haplotypes

Genotypic combinations of rs1136743 and rs1136747 defined three haplotypes previously reported in association with SAA: *SAA1.1* (52Val, 57Ala), *SAA1.3* (52Ala, 57Ala), and *SAA1.5* (52Ala, 57Val). Between-group comparisons of haplotype combinations and SAA and CVD status showed *SAA1.3* homozygotes had significantly lower SAA than *SAA1.1* homozygotes (9.1 mg/L (4.9, 19) vs. 21 mg/L (11, 45); $p = 0.02$, Table 5, Fig. 1). *SAA1.5* homozygotes had higher SAA concentrations than those homozygous for *SAA1.1*, although this failed to reach significance ($p > 0.05$). Heterozygous individuals with *SAA1.3/1.5* had significantly higher SAA levels than *SAA1.1/1.1* homozygotes (55 mg/L (17, 87) vs. 21 mg/L (11, 45); $p = 0.02$, Table 5, Fig. 1), which remained significant following adjustment for age, gender, eGFR, and diabetes.

Between-group comparisons of haplotype combinations and HDL_2SAA showed that *SAA1.3* homozygotes had significantly

Table 4 Regression analysis of *serum amyloid A* single nucleotide polymorphisms in CVD ($n = 100$) and controls ($n = 146$)

SNP	Minor allele	Controls, *n* (%)	CVD, *n* (%)	Odds ratio (95% CI)	*p* value
rs11024595	T	49 (17)	33 (16)	0.94 (0.59–1.63)	0.98
rs2445166	A	26 (9)	15 (7)	0.80 (0.40–1.61)	0.54
rs11545466	G	18 (6)	11 (5)	0.89 (0.42–1.89)	0.74
rs549103464	Insertion	18 (6)	12 (6)	1.00 (0.70–1.43)	0.99
rs11024597	T	99 (33)	61 (30)	0.95 (0.65–1.38)	0.78
rs1136743	C	94 (31)	59 (29)	0.94 (0.64–1.39)	0.76
rs11545468	C	19(6)	19 (9)	1.71 (0.85–3.44)	0.13
rs1136745	C	42 (14)	31 (15)	1.23 (0.70–2.16)	0.47
rs15790	T	22 (7)	16 (8)	1.03 (0.54–2.00)	0.93
rs145680768	C	4 (1)	0 (0)	0.00 (0.00)	1.00
rs12218	T	122 (41)	88 (43)	1.06 (0.71–1.60)	0.77
rs1059571	G	6 (2)	0 (0)	0.00 (0.00)	1.00
rs112509629	T	0 (0)	5 (2)	0.00 (0.00)	1.00
rs11540206	A	1 (0.3)	4 (2)	6.07 (0.67–55.1)	0.11
rs149402852	A	4 (1)	3 (1)	1.10 (0.24–5.03)	0.90
rs116861605	A	7 (2)	6 (3)	1.27 (0.41–3.91)	0.68
rs2468844	G	60 (20)	27 (13)	0.65 (0.41–1.04)	0.07
rs149926073	A	4 (1)	3 (1)	1.15 (0.51–5.26)	0.86
rs1136747	T	68 (23)	41 (20)	0.89 (0.57–1.40)	0.63

lower HDL_2SAA than *SAA1.1* homozygotes (0.25 mg/L (0.11, 0.57) vs. 0.70 mg/L (0.26, 2.50); $p = 0.04$), which remained significant following adjustment for age, gender, eGFR, and diabetes (beta estimate = 0.56; 95% confidence intervals, 0.26–0.88; $p = 0.02$, Table 6). Heterozygous individuals with *SAA1.3/1.5* had significantly higher HDL_2SAA levels than *SAA1.1/1.1* homozygotes (4.06 mg/L (2.78, 6.00) vs. 0.70 mg/L (0.26, 2.50); $p < 0.01$, Table 6), which remained significant following adjustment for age, gender, eGFR, and diabetes (beta estimate = 2.03; 95% confidence intervals, 1.47–6.27; $p < 0.001$, Table 6).

Between-group comparisons of haplotype combinations and HDL_3SAA showed that *SAA1.3* homozygotes had significantly lower HDL_3SAA than *SAA1.1* homozygotes (4.0 mg/L (2.0, 7.0) vs. 10 mg/L (5.0, 24); $p = 0.01$), which remained significant following adjustment for age, gender, eGFR, and diabetes (beta estimate = 0.48; 95% confidence intervals, 0.12–0.60; $p < 0.001$, Table 6). Individuals heterozygous for *SAA1.3/1.5* had significantly higher HDL_3SAA levels than *SAA1.1/1.1* homozygotes (43 mg/L (26, 60) vs. 10 mg/L (5.0, 24); $p < 0.001$, Table 6), which remained significant following adjustment for age, gender, eGFR, and diabetes (beta estimate = 2.15; 95% confidence intervals, 2.05–14.7; $p < 0.001$, Table 6).

No significant associations between *SAA1* haplotypes and CVD status were detected in either unadjusted or in analyses adjusted for lipid levels, blood pressure, age, and diabetes ($p > 0.05$) (Table 7).

Table 5 Difference in serum amyloid A levels between *serum amyloid A 1* haplotypes ($n = 246$)

Haplotype	*n* (%)	Mean serum SAA, mg/L (IQR)	Beta (95% CI)[1]	*p* value[1]	Beta (95% CI)[2]	*p* value[2]
1.1/1.1	109 (46)	21 (11, 45)		Reference		Reference
1.1/1.3	19 (8)	16 (3.7, 73)	0.74 (0.45–1.21)	0.23	0.80 (0.48–1.33)	0.40
1.1/1.5	80 (34)	26 (15, 70)	1.25 (0.93–1.67)	0.14	1.32 (0.79–1.79)	0.07
1.3/1.3	9 (4)	9.1 (4.9, 19)	0.43 (0.22–0.85)	0.02*	0.34 (0.17–0.69)	< 0.001*
1.3/1.5	7 (3)	55 (17, 87)	2.58 (1.19–5.57)	0.02*	2.48 (1.16–5.32)	0.02*
1.5/1.5	11 (5)	32 (11, 72)	1.51 (0.81–2.82)	0.19	1.50 (0.79–2.83)	0.21

Given the skewed distribution, SAA was log transformed; data in the table shows anti-logged values; results are expressed as geometric means (interquartile range (IQR)). Where * indicates a significant result $p \leq 0.05$. All haplotype combinations were compared to the reference 1.1/1.1 haplotype, where *p* value[1] reflects the unadjusted model; *p* value[2] reflects the adjusted model (age, gender, estimated glomerular filtration rate (eGFR), and diabetes)

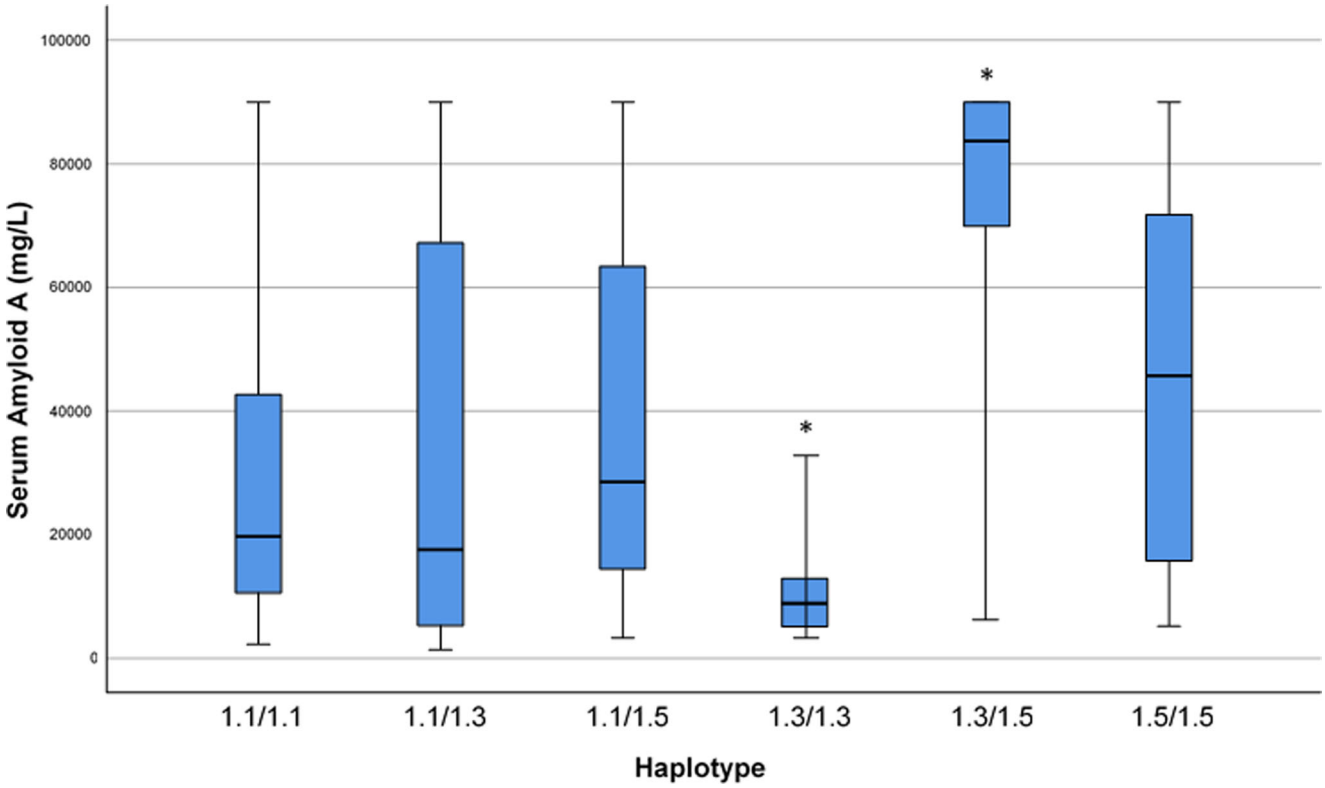

Fig. 1 Variation in serum amyloid A levels by haplotype (n = 246) showing median and interquartile range. Between-group comparisons of haplotype combinations and SAA levels showed *SAA1.3* homozygotes had significantly lower SAA than *SAA1.1* homozygotes (*p = 0.02). Heterozygous individuals with *SAA1.3/1.5* had significantly higher SAA levels than *SAA1.1/1.1* homozygotes (*p = 0.02)

Table 6 Differences in SAA levels in HDL_2 and HDL_3 fractions between serum amyloid A 1 haplotypes (n = 246)

Haplotype	n (%)	Mean SAA mg/L (IQR)	Beta estimate (95% CI)[1]	p value[1]	Beta estimate (95% CI)[2]	p value[2]
HDL_2SAA						
1.1/1.1	109 (46)	0.70 (0.26, 2.50)	Reference		Reference	
1.1/1.3	19 (8)	0.60 (0.21, 1.58)	0.91 (0.75, 1.20)	0.66	0.79 (0.60, 1.17)	0.80
1.1/1.5	80 (34)	0.80 (0.31, 2.50)	1.12 (0.70, 2.05)	0.50	1.23 (0.77, 2.54)	0.27
1.3/1.3	9 (4)	0.25 (0.11, 0.57)	0.64 (0.33, 0.97)	0.04*	0.56 (0.26, 0.88)	0.02*
1.3/1.5	7 (3)	4.06 (2.78, 6.00)	1.94 (1.46, 5.10)	< 0.001*	2.03 (1.47, 6.27)	< 0.001*
1.5/1.5	11 (5)	0.60 (0.27, 1.61)	0.93 (0.31, 2.29)	0.74	0.84 (0.22, 2.09)	0.49
HDL_3SAA						
1.1/1.1	109 (46)	10 (5.0, 24)	Reference		Reference	
1.1/1.3	19 (8)	10 (3.0, 33)	0.99 (0.73, 1.36)	0.96	1.06 (0.74, 1.49)	0.79
1.1/1.5	80 (34)	12 (6.0, 29)	1.17 (0.69, 2.87)	0.34	1.25 (0.74, 3.53)	0.23
1.3/1.3	9 (4)	4.0 (2.0, 7.0)	0.58 (0.18, 0.79)	0.01*	0.48 (0.12, 0.60)	< 0.001*
1.3/1.5	7 (3)	43 (26, 60)	2.11 (2.04, 11.1)	< 0.001*	2.15 (2.05, 14.7)	< 0.001*
1.5/1.5	11 (5)	0.7 (0.3, 2.5)	1.35 (0.69, 10.3)	0.16	1.34 (0.56, 12.3)	0.22

Given the skewed distribution, SAA was log transformed; data in the table shows anti-logged values; results are expressed as geometric means (interquartile range (IQR)). Where * indicates statistical significance ($p \leq 0.05$). All haplotype combinations were compared to the reference 1.1/1.1 haplotype, where p value[1] reflects the unadjusted model; p value[2] reflects the adjusted model (age, gender, estimated glomerular filtration rate (eGFR), and diabetes)

Table 7 *Serum amyloid A* 1 haplotype associations by CVD status, in CVD ($n = 146$) and controls ($n = 100$)

Genotype	Controls, *n* (%)	Cases, *n* (%)	Odds ratio (95% CI)[1]	*p* value[1]	Odds ratio (95% CI)[2]	*p* value[2]
1.1/1.1	64 (45)	45 (48)	Reference		Reference	
1.1/1.3	12 (9)	7 (8)	0.83 (0.30–2.27)	0.72	0.87 (0.28–2.73)	0.82
1.1/1.5	50 (35)	30 (32)	0.53 (0.47–1.54)	0.60	0.90 (0.46–1.75)	0.76
1.3/1.3	4 (3)	5 (5)	1.78 (0.45–6.99)	0.41	1.72 (0.34–8.67)	0.51
1.3/1.5	6 (4)	1 (1)	0.24 (0.03–2.04)	0.19	0.23 (0.02–2.26)	0.21
1.5/1.5	6 (4)	5 (5)	1.19 (0.34–4.12)	0.80	0.96 (0.23–3.91)	0.96
1.1 allele	190 (63)	127 (68)	Reference		Reference	
1.3 allele	26 (18)	18 (10)	0.97 (0.51–1.83)	0.97	0.82	0.93
1.5 allele	68 (48)	41 (22)	1.11 (0.71–1.74)	0.65	0.89	0.48

Beta[1] and *p* value[1] indicate an unadjusted analysis; beta[2] and *p* value[2] indicate an adjusted (lipid levels, blood pressure, age, and diabetes) analysis

Discussion

We investigated associations between *SAA* SNPs and SAA levels and CVD status. Our data identified several novel associations between genetic variants in both *SAA1* exon 3 and *SAA2* exon 4 and SAA levels, as well as significant association with SAA levels between previously characterised *SAA1* haplotypes. *SAA1* and *SAA2* both encode for ASAA, which is an important mediator in the inflammatory response, with both inflammation and increased SAA levels previously implicated in the pathogenesis of atherosclerosis [8].

We identified novel and previously reported variants in *SAA1* exons 1, 3, and 4 and *SAA2* exon 4 making these regions genetically informative with regard to SAA levels. To our knowledge, the association between rs1136745 and SAA has not been reported previously. Interestingly, rs1136745 was associated with lower SAA levels ($p = 0.02$), suggesting a potential protective effect of this genetic variant in limiting the increase in SAA levels in response to inflammation and reducing the subsequent atherosclerosis risk [35].

Two SNPS within exon 3 of *SAA1* (rs149926073 and rs1136747) and rs2468844 in exon 4 of *SAA2* were significantly associated with increased SAA levels. To our knowledge, none of these SNPs have been previously reported in association with SAA. However, rs2468844 has been reported in association with significant reductions of serum HDL-C and increased cIMT but not ischemic stroke [23, 36]. Xie and colleagues (2010) suggested that the *SAA* gene could directly influence the risk of developing cIMT, independent of changes in HDL levels and other determinants of CVD risk. If these assumptions are correct, it is possible that this may be mediated via increased SAA levels which ultimately could lead to diminished HDL function and increased risk of atherosclerosis. In our study, rs149926073 and rs1136747 were not associated with CVD status ($p > 0.05$), while rs2468844 just failed to reach the significance threshold ($p = 0.07$), although study power or inequitable phenotypic comparisons may have confounded these findings.

To evaluate the independent effects of these SNPs on SAA levels, we included all four in a single regression model together with other potential confounders such as gender, diabetes, eGFR, and age. All four SNPs (rs1136745, rs1136747, rs149926073, and rs2468844) remained significantly associated with SAA levels supporting their independent contributions to genetic risk ($p < 0.05$, Table 3).

Our study also examined previously defined haplotypic structure across *SAA1*. Individuals homozygous for *SAA1.3* had significantly lower levels of SAA compared with those homozygous for *SAA1.1* ($p = 0.02$) in support of reported associations between SAA1.3 and its reduced affinity for HDL [32]. Previous reports also identified a link between SAA1.5 and elevated SAA levels, suggesting SAA1.5 has a higher affinity for HDL and while we did report higher SAA in individuals homozygous for *SAA1.5* in our study, this failed to reach statistical significance [32, 33, 37].

We have previously shown that levels of serum SAA are highly correlated with HDL_2SAA and HDL_3SAA levels [15]; therefore, we sought to determine if the observations found between SAA haplotypes and serum SAA levels were also represented in the HDL sub-fractions. In line with the findings for serum SAA, heterozygous individuals for the *1.3/1.5* haplotype had significantly higher SAA levels than individuals homozygous for the *1.1* haplotype for both HDL_2 and HDL_3 SAA ($p < 0.01$). This was unsurprising, given serum SAA levels have been shown to correlate with HDL SAA levels and may suggest that individuals with the *SAA 1.5* allele have HDL particles with a higher affinity for SAA. We and others have previously reported that increased SAA levels within HDL fractions may compromise the functionality of the HDL particle reducing its cardioprotective properties [15, 16].

Although several studies have previously reported associations between the *SAA1.5* haplotype with amyloidosis and rheumatoid arthritis, to our knowledge, none have reported associations with CVD and we also failed to find any evidence in our study [25, 26]. Nevertheless, elevated SAA levels associated with CVD may suggest that individuals homozygous

for *SAA1.3* may have a reduced risk of developing CVD compared with those with the *SAA1.1* haplotype.

SAA levels were not associated with any *SAA4* genetic variants in support of previous reports, which is perhaps unsurprising given SAA4 is a constitutively expressed protein possibly modulated through mechanisms independent of SAA1 and SAA2 [38]. SAA4 shares only 50% homology with ASAA, and the *SAA4* gene does not contain the promotor motif CTGGGA, or the NF-IL6 binding site, commonly found in acute phase proteins and the gene possesses only a truncated NF-κB recognition sequence (GACTTT), which may explain why *SAA4* expression is not increased during an inflammatory response [39].

Conclusion

We have identified several novel as well as previously reported SNPs in *SAA* genes and correlated *SAA* genotypes with serum SAA levels before and after adjustment for potential confounding variables. The correlation between SAA levels and *SAA* genotypes is of interest given individual genetic background is likely to modulate release of SAA into the circulation in response to increased inflammation associated with many chronic diseases. The utility of which SAA as a potential biomarker is modified by the genetic variability in SAA response to inflammation.

Limitations

Although the study was well powered, there was insufficient sample size to detect low-frequency genetic variants < 5%, which may have exerted moderate effect sizes. This study focused on the genetic variants within a European population and as such, geographic variation may limit the generalisability of these findings to other populations. There are some limitations to using myocardial perfusion imaging for patient phenotyping. It should be recognised that a myocardial perfusion test allocates patients into CVD groups based on the presence of flow-limiting coronary artery disease (i.e. ischaemia) or myocardial infarction. Some patients designated as having no CVD may have coronary atheroma at an early stage but without functional consequences.

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Analysis and interpretation of data: KG, GM
Drafting of manuscript: KG, APM, MH, GM
Critical revision: KG, MH, APM, GM

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Office for Research Ethics Committees Northern Ireland (14/NI/1132). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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