The role of cytokine gene polymorphisms in the pathogenesis of abdominal aortic aneurysms: A case-control study

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Background: **Cytokines are the primary mediators of inflammation and also influence matrix metalloproteinase expression, both of which are important in development of abdominal aortic aneurysm (AAA). A significant, but as yet unknown, familial factor contributes to the pathogenesis of AAA. Many cytokine genes contain polymorphic sites, some of which affect cytokine production in vitro. Cytokine gene polymorphisms may therefore influence the pathogenesis of AAA. The purpose of this study was to determine whether there is any association between cytokine gene polymorphisms and AAA.**
Methods and Results: **This case-control study comprised 100 patients with AAA and 100 age-matched and sex-matched control subjects. For each case and control subject in the study, genotypes at the following cytokine gene polymorphic loci were determined: interleukin (IL)–1β +3953, IL-6 −174, IL-10 −1082, IL-10 −592, and tumor necrosis factors-α −308. Allele and genotype frequencies were compared between AAA and control groups, and odds ratios (OR) were calculated for the presence of AAA with each allele at each locus examined as risk factors. The IL-10 −1082 A allele was significantly more common in the AAA group than the control group ($P = .03$). The OR for the IL-10 −1082 A allele as a risk factor for AAA was 1.8 (95% confidence interval, 0.9-3.6).**
Discussion: **These associations suggest a significant role for IL-10 in the pathogenesis of AAA. This association of AAA with the IL-10 −1082 A allele is also biologically plausible; the IL-10 −1082 A allele is associated with low IL-10 secretion, and it may be that AAA develops in patients who are unable to mount the same anti-inflammatory response as those who do not have AAA. (J Vasc Surg 2003;37:999-1005.)**

The precise cause of abdominal aortic aneurysm (AAA) is unknown. The principal structural proteins in arterial walls are collagen and elastin. Aortic wall samples from AAA show marked loss of elastin compared with normal aortic wall,[1] and this loss of mural elastin has been suggested as the primary event in aneurysm formation.[2] While the total amount of collagen in AAA walls is usually preserved,[3] there is increased collagen turnover in AAA.[4] The turnover of extracellular matrix proteins such as elastin and collagen is regulated by matrix metalloproteinases (MMPs), and elevated levels of MMPs have been detected in AAA tissue compared with normal aortic wall tissue.[5,6] An important source of MMPs in AAA tissue are tissue macrophages,[7] which form part of a chronic cellular inflammatory infiltrate that is a characteristic of AAA aortic wall.[8,9] The production of MMPs and tissue inhibitors of metalloproteinases is in part regulated by cytokines,[10,11] which also have a role in chronic inflammation.

Cytokines are the principal mediators of inflammation. Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are proinflammatory cytokines produced by macrophages and other cells early in the inflammatory response. They cause endothelial and neutrophil activation and stimulate further cytokine release by inflammatory cells.[12,13] IL-6 is another proinflammatory cytokine that is produced by nucleated cells after stimulation by other cytokines. IL-10 is a potent anti-inflammatory cytokine. It inhibits macrophage function[14] and acts indirectly on T lymphocytes by interfering with the antigen presenting cell signals to the T cells.[15] It reduces proinflammatory cytokine production by macrophages, neutrophils, and T cells.

Many cytokine genes contain polymorphic sites, and although more than 900 such polymorphisms have been identified, only a small minority of these polymorphisms affect cytokine transcription in vitro.[16] For example, the IL-10 gene contains three polymorphic sites, at the −1082, −819, and −592 positions in relation to the start codon.[17] The bases present at these three sites are either guanine or adenine (−1082), cytosine or thymine (−819), and cytosine or adenine (−592). The polymorphisms at −819 and −592 are linked, only −819/−592 cytosine-cytosine (CC) or thymine-adenine (TA) haplotypes existing. The presence of an adenine base at −1082 results in an approximately 25% reduction in IL-10 production by maximally stimulated peripheral blood lymphocytes,[18] and the −1082/−819/−592 guanine-cytosine-cytosine haplotype is also associated in vitro with higher IL-10 production than any other haplotype.[18] For the IL-6 −174 single nucleotide polymorphism, where there is either a guanine (G) or cytosine (C) base at this site, the G allele results in plasma IL-6 levels two to three times higher in healthy

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Table I. Frequency of risk factors in case and control groups for subjects for whom data were recorded

	AAA (n = 91)	*%*	*Control (n = 100)*	*%*	*P**
Smoking history	84	92	73	73	<.001
Current smoker	24	26	23	23	.62
Hyperlipidaemia	20	22	14	14	.19
Previous CVA	2	2	6	6	.28
Angina	16	18	16	16	.85
Hypertension	55	60	36	36	<.001
Previous MI	20	22	12	12	.08
Diabetes mellitus	6	7	15	15	.07

AAA, abdominal aortic aneurysm; *CVA*, cerebrovascular accident; *MI*, myocardial infarction.
*Fisher's exact test.

subjects.[19] At the TNF-α −308 polymorphic site the presence of an adenine (A) base, substituted for the more common guanine base, is associated with TNF-α gene transcription six to nine times higher in human B cells virally transfected with this polymorphism,[20] and a thymine (T) base at the IL-1β +3953 polymorphic site (more commonly cytosine) is associated with higher IL-1β production by isolated peripheral blood monocytes in response to lipopolysaccharide stimulation.[21]

AAA have a significant genetic component in their cause; 11% to 20% of patients with AAA have a family history of AAA.[22,23] The screen-detected prevalence of AAA in siblings of patients with AAA has been estimated at 15% to 36%, compared with the population prevalence of between 2% and 8%.[24,25] Given the role of cytokines in mediating inflammation and MMP production, it is feasible that cytokine gene polymorphisms may be responsible for this genetic component in the pathogenesis of AAA. The purpose of this study was to determine whether there is any association between AAA and the following cytokine gene polymorphisms: TNF-α −308, IL-1β +3953, IL-6 −174, IL-10 −1082, IL-10 −592, and IL-10 −1082/−819/−592 haplotype.

METHODS

Selection of cases and control subjects. Cases consisted of 100 consecutive patients who consented to take part in the study, who were admitted to either the Leicester Royal Infirmary or the Leicester General Hospital for open AAA repair. Patients admitted for elective surgery and those admitted for either emergency repair of symptomatic nonruptured AAA or emergency repair of ruptured AAA were included. All patients were white Europeans. All patients who underwent elective surgery had AAA greater than 5.5 cm in diameter. Sex-matched and age-matched (±5 years) white control subjects were recruited from patients attending vascular outpatient clinics and those admitted for surgery at the Leicester General Hospital. In control subjects, when aortic diameter had not been assessed either radiologically or at surgery, clinical examination was used to exclude those with incidental AAA. The presence or absence of the following cardiovascular risk factors were recorded for both patients and control subjects unless these could not be accurately determined (eg, unconscious patient undergoing surgery to repair ruptured AAA who subsequently did not survive without regaining consciousness): smoking history, current smoker, hyperlipidemia, previous cerebrovascular accident, angina, hypertension, previous myocardial infarction, and diabetes mellitus. Patients were defined as having hyperlipidemia or hypertension if they were receiving pharmacologic treatment for these conditions at recruitment into the study. The study was approved by the Leicestershire regional ethics committee, and all patients, or in the case of unconscious patients their relatives, gave informed consent to participate in the study.

Cytokine polymorphism genotyping. Peripheral blood was drawn from each patient (at induction of anaesthesia) or control subject into sterile collection tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and centrifuged at 770g for 10 minutes. The buffy coat was drawn off, and DNA was extracted with proteinase K digestion (Sigma, Poole, UK) with subsequent salt extraction and ethanol precipitation.[26] DNA was quantified with a fluorometer (Dynaquant 200; Hoefer Pharmacia Biotech, San Francisco, Calif).

Genotypes were determined for the following loci by induced heteroduplex genotyping: IL-1β +3953, IL-6 −174, IL-10 −1082, IL-10 −592 and TNF-α −308. This method has been validated in our laboratory with both the polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) test (IL-1β +3953, IL-6 −174) and the PCR–single stranded polypeptide (SSP) test (IL-10 −1082, IL-10 −592 and TNF-α −308), and results were consistent with these more traditional methods. Primers and heteroduplex generators (Oswel DNA, Southampton, England) used were as described by Morse et al.[27] PCR mixes (25μL) contained 2.5 μL of either DNA (100 ng/μL) or heteroduplex generator (0.5 ng/μL), 200 μmol of each deoxynucleotide triphosphate (Helena Biosciences, Sunderland, UK), 0.5 μmol of each forward and reverse primer, 0.25 units of *Taq* polymerase (Helena Biosciences), 1× *Taq* polymerase buffer (Helena Biosciences), and magnesium chloride, as follows: TNF-α −308 and IL-10 −1082, 2.5 mmol; IL-10 −592, 2.0 mmol/L; IL-1β +3953 and IL-6 −174, 1.5 mmol. PCR parameters, optimized for a Helena Biosciences Phoenix thermal cycler were initial denaturation at 95° C for 5 minutes for all loci, followed by 30 cycles (25 for TNF-α −308) of denaturation at 95° C for 1 minute; annealing at 59° C (TNF-α −308), 60° C (IL-1β +3953 and IL-6 −174), 57° C (IL-10 −1082), or 62° C (IL-10 −592) for 1 minute; and extension at 72° C for 1 minute, with a final extension at 72° C for 5 minutes.

After the initial PCR step, equal volumes (10 μL) of genomic DNA and heteroduplex generator amplicons were mixed, heated to 95° C for 5 minutes, then allowed to cool to 45° C over 25 minutes to allow DNA heteroduplex formation. Heteroduplexes were stained with SYBR I green (BioWhittaker Molecular Applications, Rockland, Me) and

Table II. Control group: Allele frequency, observed and expected genotype frequency,* and comparison between observed and expected genotype frequency

Locus	*Allele*	*n*	*Allele frequency (%)*	*Genotype*	*Observed frequency (%)*	*Expected frequency (%)*	χ^2 *(2 df)*	P
TNFα −308	G	158	79	GG	63	62		
				GA	32	33	0.02	.99
	A	42	21	AA	5	5		
IL-1β +3953	C	144	72	CC	55	52		
				CT	34	40	1.04	.59
	T	56	28	TT	11	8		
IL-6 −174	G	113	56.5	GG	28	32		
				GC	57	49	1.34	.51
	C	87	43.5	CC	15	19		
IL-10 −1082	G	106	53	GG	29	28		
				GA	48	50	0.08	.96
	A	94	47	AA	23	22		
IL-10 −592	C	157	78.5	CC	62	62		
				CA	33	34	0.13	.94
	A	43	21.5	AA	5	4		
IL-10 haplotype	GCC	106	53	GCC/GCC	29	28		
	ACC	51	25.5	GCC/ACC	25	27		
	ATA	43	21.5	GCC/ATA	23	23	0.43†	.99
				ACC/ACC	8	6		
				ACC/ATA	10	11		
				ATA/ATA	5	5		

TNF, tumor necrosis factor; *IL*, interleukin; *G*, guanine; *A*, adenine; *C*, cytosine; *T*, thymine; *df*, degrees of freedom.
*Calculated with Hardy-Weinberg equation.
†5 df.

resolved on 15% Tris-Borate EDTA polyacrylamide minigels (27.5:1 acrylamide:bis; Bio-Rad, Hemel Hempstead, UK) run for 3 hours at a constant 100 V. Gels were visualised with a dark reader.

Statistical analysis. The assumption of Hardy-Weinberg equilibrium was tested by comparing observed genotype frequency in patients and control subjects with their expected frequency at equilibrium based on the observed allele prevalences. Comparison was based on the χ^2 test. Comparisons between patient and control group risk factor, allele, genotype, and haplotype frequency were made with Fisher exact or χ^2 tests as appropriate. Mantel-Haenszel matched analysis of patients and control subjects was performed with the presence of each allele at each locus as "exposures."

RESULTS

Demographics. All patients and control subjecs were matched precisely for sex; 80 subjects in each group were men (80%). Median age of the control group was 73 years (range, 48-87 years), compared with 72 years (range, 48-85 years) in the patient group ($P = .49$, Mann-Whitney *U* test). Mean difference in age between patients and control subjects was 0.6 years (range, −4 to +5 years). Complete risk factor data were available for 91 patients (91%) and all control subjects. The frequency of risk factors in each groups is shown in Table I.

Allele and genotype frequency. Allele frequency and observed and expected genotype frequency, the latter calculated assuming Hardy-Weinberg equilibrium, are shown in Table II (control group) and Table III (AAA group). Observed genotypic distributions in both patients and control subjects were consistent with the assumption of Hardy-Weinberg equilibrium. The IL-10 −1082 A allele was significantly more common in the AAA group compared with the control group ($P = .03$, Fisher exact test) (Table IV). The AAA group also had a lower frequency of the IL-10 GCC haplotype (0.415) compared with the control group (0.53), but this did not reach statistical significance ($P = .07$, $\chi^2 = 5.32$, 2 degrees of freedom [df]). There was no significant difference in allele frequency between the AAA and control groups for TNF-α −308, IL-1β +3953, IL-6 −174, or IL-10 −592 loci (Table IV).

IL-1β +3953 genotype frequency was significantly different between the control and AAA groups ($P = .04$, $\chi^2 = 6.67$, 2 df), with the TT genotype more common in the control group than in the AAA group. IL-10 −1082 AA genotype was more common in the AAA group than in the control group, but not significantly so ($P = .07$, $\chi^2 = 5.264$, 2 df). There was little difference in genotype frequency for the other loci studied (Table V). IL-10 −1082/−819/−592 haplotypes were assumed from the allele frequency identified at the −1082 and −592 positions (−819

Table III. AAA group: Allele frequency, observed and expected genotype frequency,* and comparison between observed and expected genotype frequency

Locus	*Allele*	*n*	*Allele frequency (%)*	*Genotype*	*Observed frequency (%)*	*Expected frequency (%)*	χ^2 *(2 df)*	P
TNFα −308	G	158	79	GG	64	62		
				GA	30	33	0.27	.8
	A	42	21	AA	6	5		
IL-1β +3953	C	158	79	CC	60	62		
				CT	38	33	1.67	.4
	T	42	21	TT	2	5		
IL-6 −174	G	114	57	GG	33	32		
				GC	48	49	0.02	.9
	C	86	43	CC	19	19		
IL-10 −1082	G	83	41.5	GG	17	17		
				GA	49	49	0.00	1.0
	A	117	58.5	AA	34	34		
IL-10 −592	C	146	73	CC	55	53		
				CA	36	40	0.50	0.7
	A	54	27	AA	9	7		
IL-10 haplotype	GCC	83	41.5	GCC/GCC	17	17		
	ACC	63	31.5	GCC/ACC	26	26		
	ATA	54	27	GCC/ATA	23	23	0.97†	.9
				ACC/ACC	12	10		
				ACC/ATA	13	17		
				ATA/ATA	9	7		

TNF, Tumor necrosis factor; *IL*, interleukin; *G*, guanine; *A*, adenine; *C*, cytosine; *T*, thymine; *df*, degrees of freedom.
*Calculated with Hardy-Weinberg equation.
†5 df.

Table IV. Comparison of allele frequency in control and case groups

		Control		*AAA*		
Locus	*Allele*	*n*	*Frequency (%)*	*n*	*Frequency (%)*	P*
TNFα −308	G	158	79	158	79	1.00
	A	42	21	42	21	
IL-1β +3953	C	144	72	158	79	.13
	T	56	28	42	21	
IL-6 −174	G	113	56.5	114	57	.92
	C	87	43.5	86	43	
IL-10 −1082	G	106	53	83	41.5	.03
	A	94	47	117	58.5	
IL-10 −592	C	157	78.5	146	73	.24
	A	43	21.5	54	27	

TNF, Tumor necrosis factor; *IL*, interleukin; *G*, guanine; *A*, adenine; *C*, cytosine; *T*, thymine.
*Fisher exact test.

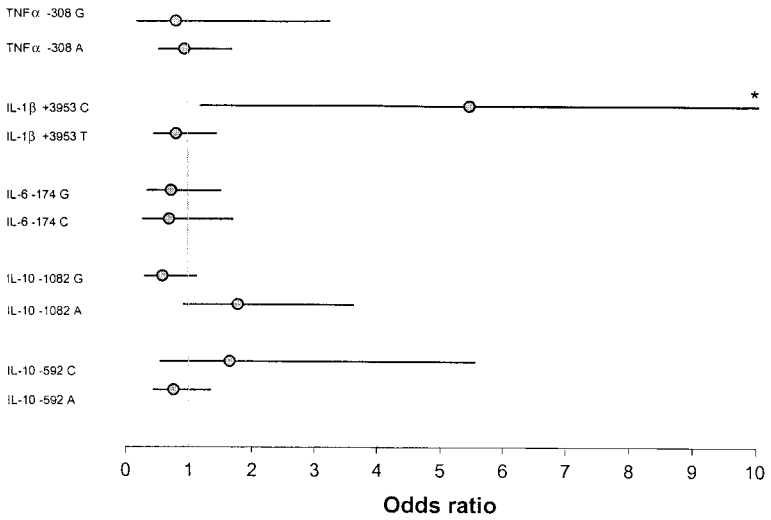
and −592 alleles are linked). There was no difference in the combinations of IL-10 haplotypes between the AAA group and the control group (Table VI); however, the GCC/GCC genotype, associated with higher IL-10 secretion than any other combination of haplotypes, was more than one and a half times as common in the control group than in the AAA group (29% vs 17%, respectively; $P = .06$, Fisher exact test).

Matched case-control analysis. The OR and 95% confidence intervals (CI), evaluating the presence of each allele studied as a risk factor for being a case, are shown in

Table V. Case and control genotype frequency for five loci studied

Locus	*Genotype*	*Control (n)*	*AAA (n)*	χ^2 *(2 df)*	P
TNFα −308	GG	63	64		
	GA	32	30	0.16	.92
	AA	5	6		
IL-1β +3953	CC	55	60		
	CT	34	38	6.67	.04
	TT	11	2		
IL-6 −174	GG	28	33		
	GC	57	48	1.65	.44
	CC	15	19		
IL-10 −1082	GG	29	17		
	GA	48	49	5.26	.07
	AA	23	34		
IL-10 −592	CC	62	55		
	CA	33	36	1.69	.43
	AA	5	9		

TNF, tumor necrosis factor; *IL*, interleukin; *G*, guanine; *A*, adenine; *C*, cytosine; *T*, thymine.



Matched analysis odds ratios and their 95% confidence intervals for each genetic locus as a risk for AAA. *IL-1 +3953 C confidence interval truncated; true value, 1.20-51.07. *TNF*, Tumor necrosis factor; *IL*, interleukin; *G*, guanine; *A*, adenine; *C*, cytosine; *T*, thymine.

the Figure. OR was obtained with matched-pair analysis of each case with its matched control. Alleles at three loci had OR greater than 1: IL-1β +3953 C (OR, 5.50; 95% CI, 1.20-51.07); IL-10 −1082 A (OR, 1.80; 95% CI, 0.92-3.64); IL-10 −592 C (OR, 1.67; 95% CI, 0.55-5.58). While the IL-1β +3953 C allele was the only one of these 95% CIs to exclude 1, only 2 patients and 11 controls were not "exposed" to this risk factor.

DISCUSSION

Our data demonstrate that in patients with AAA the IL-10 −1082 A allele is significantly more common than in age-matched and sex-matched control subjects. IL-10 A allele frequency in the control group was also similar to that observed by other authors in other groups of white persons,[18,28] meaning that this is not likely to be due to chance. The IL-10 −1082/−819/−592 GCC/GCC genotype was more common in the control group than in the AAA group. The distribution of IL-1β +3953 genotypes was significantly different in patients with AAA compared with the control group. The OR for having AAA was 5.5 in those with an IL-1β +3953 C allele, with a 95% CI that excluded a value of 1, and 1.8 in those with an IL-10 −1082 A allele.

Table VI. IL-10 −1082/−819/−592 haplotype and genotype frequency for control and case groups

		Control		*AAA*				
		n	*Frequency (%)*	*n*	*Frequency (%)*	χ^2	*df*	P
IL-10 haplotype	GCC	106	53	83	41.5			
	ACC	51	25.5	63	31.5	5.31	2	.07
	ATA	43	21.5	54	27			
IL-10 genotype	GCC/GCC	29	29	17	17			
	GCC/ACC	25	25	26	26			
	GCC/ATA	23	23	23	23	5.48	5	.36
	ACC/ACC	8	8	12	12			
	ACC/ATA	10	10	13	13			
	ATA/ATA	5	5	9	9			

IL, Interleukin; *G*, guanine; *C*, cytosine; *A*, adenine; *T*, thymine; *df*, degrees of freedom.

We do not suggest that either of these polymorphisms should be viewed as a consistent genetic marker for AAA, because many patients with AAA in this study did not possess the alleles associated with AAA. Rather, this study strongly suggests a prime etiologic role for cytokines in the pathogenesis of AAA. The IL-10 −1082 A allele, which was more common in the AAA group, is associated with decreased IL-10 production in in vitro studies, and the IL-10 −1082/−819/−592 GCC/GCC genotype, which was more common in the control group, is associated with high IL-10 production.[18] Since IL-10 is predominantly an anti-inflammatory cytokine, it may be that patients who produce less IL-10 cannot limit the inflammatory response to any particular stimulus and thus the inflammatory cascades may persist longer in these patients, predisposing them to AAA formation. Also, IL-10 may be acting directly to limit the action of MMPs. In animal and laboratory studies IL-10 inhibited production of MMPs.[28-30]

The findings regarding the IL-1β +3953 polymorphism must be examined with caution because of the small number of patients in both the AAA and control groups who had either a T allele or a TT genotype. The IL-1β allele frequency observed in both the control and AAA groups is similar to that in other studies, and the genotype frequency in the control group is similar to that observed in other groups of healthy white persons. However, the genotype frequency in the AAA group is different from that noted in these studies,[31] which suggests that this difference may be true. The finding that the IL-1β C allele was associated with AAA was surprising. IL-1β is a proinflammatory cytokine, and the C allele is associated with low IL-1β production in in vitro studies.[22] It would therefore be expected that high secretor status for IL-1β would predispose to AAA formation. However, clinical findings contrary to those demonstrated in basic laboratory studies have been found,[32] and it has been suggested that this effect may be due to either different cells (which may exhibit different responses between genotypes from those studied in vitro) being responsible for in vivo cytokine production or different responses to physiologic stimulation rather than that to single supramaximal artificial stimuli as used in in vitro experiments. It is not clear whether our findings are the result of this type of phenomenon or due to statistical chance.

The cytokine genes examined were chosen in part because they all affect cytokine secretion in vitro, but also because these four genes are located on separate autosomes and the confounding effect of genetic linkage between these genes is not present (TNF-α, chromosome 6; IL-1β, chromosome 2; IL-6, chromosome 7; IL-10, chromosome 1). However, the two IL-10 loci examined are clearly closely linked, with only three haplotypes found in white persons. The −1082 A allele can be inherited with either an A or C allele at −592, which occurs evenly, and the −1082 G allele is only inherited with a C allele at −592. The IL-10 −1082 polymorphism affects cytokine secretion independently of the alleles present at the −819/−592 positions. The −592 locus only affects cytokine secretion as part of the IL-10 haplotype, with the GCC/GCC genotype showing higher secretion than any other combination of haplotypes. However, since the A allele was more common in the AAA group and the GCC/GCC genotype more common in the control group, this supports rather than conflicts with the theory that reduced IL-10 production has a role in AAA formation.

The limitations of this study lie principally in the control group. The method used to recruit controls in a case-control study is a critical part of the study design.[33] A possible control group for this study would have been patients undergoing the same diagnostic process as was used to identify the cases but who were subsequently found not to have AAA. This was explored during the design of this study but found to be logistically impossible in the time frame allotted for the study. The control group was recruited from hospital areas where the cases had originated from, ie, vascular outpatient departments and surgical wards, to get the closest possible match with the cases. While this method of control selection contributes to selection bias,[34] that allele and genotype frequency in the control group was similar to that found in other studies of white Europeans[28] suggests that this bias is minimal in this study.

A further limitation in the control group was that their aortic diameter was not formally measured. Many, however, had undergone laparotomy, or preoperative or outpatient ultrasound or computed tomography, and all had been examined clinically for the presence of AAA. It is still possible that

some control subjects had occult AAA. This is unlikely to have affected the findings of this study. The prevalence of AAA in the case group was 100% and in the control group was likely to be less than the prevalence in the general population (3%-8%[25,26]). Thus the confounding effect of the presence of a few AAA in the control group is likely similarly small. However, this also means that the differences in allele frequency may actually be an underestimate of the true population difference between those with AAA and those without, with those control subjects with occult AAA contributing to the allele frequency in the control group.

With the relatively small number of patients in this study, we were unable to examine the role of epistasis, and larger studies may be able to examine the interactions between these and other genetic loci. Further research should first be directed to confirm these findings in an independent study, preferably with a more robust control group. If the association between the IL-10 −1082 A allele is confirmed, a prospective cohort study would be required to prove a causal relationship between AAA and this genetic locus. The insights that this study gives for the role of cytokines in the pathogenesis of AAA may not merely be furthering understanding of this complex process but may also suggest a possible therapeutic option for small AAA. IL-10 therapy has been successful in some chronic inflammatory diseases, with a good side-effect profile,[35] and IL-10 could therefore potentially be used to treat small AAA.

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