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Interleukin-6 receptor pathways in abdominal aortic aneurysm


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Methods

We conducted a systematic review and meta-analysis of studies reporting circulating IL-6 in AAA, and new investigations of the association between a common non-synonymous functional variant (Asp358Ala) in the IL-6R gene (IL6R) and AAA, followed by the analysis of the variant both in vitro and in vivo.

Inflammation may play a role in the development of abdominal aortic aneurysms (AAA). Interleukin-6 (IL-6) signalling through its receptor (IL-6R) is one pathway that could be exploited pharmacologically. We investigated this using a Mendelian randomization approach.

Results

Up to October 2011, we identified seven studies (869 cases, 851 controls). Meta-analysis demonstrated that AAA cases had higher levels of IL-6 than controls [standardized mean difference (SMD) = 0.46 SD, 95% CI = 0.25–0.66, P = 0.001]. Meta-analysis of five studies (4524 cases/15 710 controls) demonstrated that rs7529229 (which tags the non-synonymous variant Asp358Ala, rs2228145) was associated with a lower risk of AAA, per Ala358 allele odds ratio 0.84, 95% CI: 0.80–0.89, P = 2.7 x 10–11. In vitro analyses in lymphoblastoid cell lines demonstrated a reduction in the expression of downstream targets (STAT3, MYC, and ICAM1) in response to IL-6 stimulation in Ala358 carriers.

Conclusions

A Mendelian randomization approach provides robust evidence that signalling via the IL-6R is likely to be a causal pathway in AAA. Drugs that inhibit IL-6R may play a role in AAA management.

Keywords

Abdominal aortic aneurysm • Mendelian randomization • Interleukin-6 • Polymorphism
Introduction

Abdominal aortic aneurysm (AAA) affects ∼2–5% of men and <1% of women aged 65–74 years.1–3 Progressive dilatation of the aorta increases the risk of rupture and surgical intervention is indicated when the diameter reaches 5.5 cm.4 Currently there are no recognized treatments to diminish aneurysm progression at an early stage and a pharmacological treatment that targeted the processes underlying aortic dilatation, as well as vascular disease in general would provide an attractive adjunct to the surveillance and surgery treatment paradigm. However, few if any therapeutic targets have been validated in humans to date.

The major environmental exposures associated with AAA development include male sex, advancing age, cigarette smoking, dyslipidaemia, and hypertension.5 There is also a genetic component to the disease, with twin studies reporting heritability in the region of 70%.6 Two recent genome-wide association studies (GWAS) of AAA have reported robust associations with common variants in DAB2IP and LRP17,8, while the chromosome 9p21.3 locus associated with coronary heart disease (CHD) also shows a strong association with AAA.9 These variants, however, explain only a small proportion of the observed heritability suggesting other contributory genetic mechanisms remain to be discovered.

Interleukin-6 (IL-6) signalling is initiated by binding of IL-6 to its receptor (IL-6R), which forms a dimer with the ubiquitously expressed signal transducer glycoprotein-130 (gp-130). This, in turn leads to the activation of the intracellular receptor-associated kinases and downstream effects via the transcription factor STAT3. Two forms of IL-6 signalling have been described, namely classical and trans-signalling. In classical signalling, IL-6 binds the membrane bound IL-6R (mIL-6R), which is expressed in hepatocytes and cells of the innate immune system. In trans-signalling, IL-6 binds to the circulating soluble IL-6R (sIL-6R), and this complex is capable of binding to gp130 in a wide range of cell types. It has previously been shown that the expression of IL-6 and downstream mediators of IL-6 signalling, such as STAT3, are greater in AAA than in non-aneurysmal aortic tissue.10,11

A common non-synonymous sequence variant in IL6R (p.Asp358Ala, rs2228145, also annotated as rs8192284) results in an increased proteolytic cleavage of the mIL-6R.12 This variant is strongly associated with higher levels of circulating IL-6 levels, but importantly, lower levels of downstream products of IL-6 signalling, such as C-reactive protein and fibrinogen,12–15 suggesting that it acts by the attenuation of signalling via the IL-6 receptor. Furthermore, it was shown that this variant conferred protection against CHD, prompting speculation that targeting the IL-6R in CHD is a potentially novel preventative strategy.16 Whether or not this is the case for AAA has not yet been evaluated.

In the present study, the primary hypothesis is that pro-inflammatory signalling via the IL-6R plays a causal role in AAA development. This was investigated using a Mendelian randomization approach. First, a systematic review and meta-analysis of the published literature was performed in order to establish the relationship between circulating IL-6 levels and AAA. Secondly, novel analysis of the association between the functional Asp358Ala IL6R variant and AAA was performed. Finally, in vitro analyses were used to investigate the mechanism by which this variant could protect from cardiovascular disease.

Methods

Observational association between interleukin-6 and abdominal aortic aneurysms

Studies were identified using a two-stage search strategy following PRISMA guidelines (Supplementary material online). In the first stage, two electronic databases (MEDLINE and EMBASE) were searched and in the second stage articles were identified by manually searching references of articles identified in the first stage and review articles. Authors of large epidemiological studies of AAA were also approached to determine whether they had measures of IL-6 in cases and controls. The MEDLINE database was searched from January 1966 to January 2012, and the EMBASE from 1980 to January 2012. Inclusion criteria were decided by consensus (SCH, RR, MVH, and SEH), and the studies were screened and abstracted in duplicate by SCH and RR. The search strategy is described in detail in the Supplementary material online.

Study populations

Detailed descriptions of the study cohorts and demographic details are presented in the Supplementary material online. Briefly, for the genetic association analyses, we used data from five case–control studies of AAA that have access to genetic information, and are part of an existing collaborative group investigating the genetics of AAA. All studies defined AAA as an infra-renal aortic diameter ≥ 3 cm by ultrasound or computed tomography imaging, or previous AAA rupture/repair. The Aneurysm Consortium (AC) Genome Wide association Study of Abdominal Aortic Aneurysm recruited 1596 cases of AAA from centres across the UK and Western Australia. Control data were taken from the Wellcome Trust Case Control Consortium (n = 5855). The New Zealand study included 1373 individuals with AAA and 718 controls with no previous history of vascular disease. The Secondary Manifestations of ARTerial disease (SMART) study included data from 631 cases of AAA and 6342 AAA free controls recruited from University Medical Center Utrecht, the Netherlands. The Edinburgh Artery Study (EAS) is a prospective population-based cohort that included data from 62 cases of AAA and 819 AAA free controls.17

In the Utrecht Study (separate from the SMART study), 862 individuals with AAA were recruited to the ‘genetics AAA’ study and compared with controls from the ERGO/Rotterdam study (n = 1866).18 Studies were identified as part of established collaborative efforts to understand the genetics of AAA. All studies had full ethical approval.

Genotyping

Participants in the AC AAA GWAS were genotyped using the Illumina 660 k gene-chip, with previously described quality control filters. Individuals in the SMART study were genotyped using KASpar at KBiosciences, UK (www.kbioscience.co.uk). For the New Zealand study, samples were genotyped using the TaqMan allelic discrimination method (using a pre-designed, functionally tested assay Applied Biosystems, assay C_26292282_10). For the Utrecht study, participants were genotyped using the Illumina 610k Chip.

In the SMART study, rs7529229 was genotyped and in the New Zealand study rs4129267 was genotyped. These SNPs are perfect proxies for each other (R² = 1). These SNPs both tag the non-synonymous variant (p.Asp358Ala, rs2228145, R² = 0.97).
disequilibrium between SNPs was assessed using the SNP Annotation and Proxy Search resources (www.broadinstitute.org/mpg/snap/).

**Functional analysis of the p.Asp358Ala (rs2228145) variant**

Epstein Barr Virus-transformed lymphoblastoid cell lines were used based on the genotype, confirmed using Taqman allelic discrimination (Applied Biosystems). After RNA extraction, gene expression was analysed using Taqman Gene Expression Assays designed for targeting STAT3, MYC, ATF3, ICAM1, and BCL3 (Applied Biosystems, Carlsbad, USA) using a 384-well plate format, following the standard protocol provided by Applied Biosystems. Full details of the in vitro methodology are described in the Supplementary material online. These target genes were chosen because they have been shown to be the major downstream target of IL-6—STAT3 signalling.20

**Statistical analysis and power**

To estimate the association between circulating IL-6 and AAA, we performed meta-analysis of summary statistics identified by our systematic review. For each study, we used mean concentrations of IL-6 in cases and controls to determine a standardized mean difference (SMD) and standard error. For studies that reported median concentration levels and inter-quartile ranges (IQR), we took the median to be the mean value and divided the IQR by 1.35 in order to obtain the SDs. Mean differences in each study were pooled using inverse-weighted-fixed-effect meta-analysis. Heterogeneity was assessed using the $I^2$ statistic and Cochrane’s Q. Owing to heterogeneity in units used to measure IL-6, and baseline levels, a SMD was calculated preferentially to a weighted mean difference.

SNP-disease associations in each cohort were determined using logistic regression adjusted for age and gender, under an additive genetic model. Effects sizes are reported as odds ratios (OR) and 95% confidence intervals. Meta-analysis was performed using fixed and random-effects modelling and we measured between study heterogeneity using the $I^2$ statistic. Gene expression was analysed using the Rest Expression Software Tool by MW Pfaffl utilizing a Pair-Wise Fixed Reallocation Randomization Test. With 4523 case and 15 406 controls, there was over 80% to detect modest effects (OR $\approx 1.12$). Concentrations of IL-6 were higher in cases than in controls in all but one of the studies in which no difference was observed.26 This study included only small AAA in the case group (3–5.5 cm), and reported different measurement units to all the other studies (ng/mL vs. pg/mL). Exclusion of this study reduced overall heterogeneity ($I^2 = 56\%$) and increased the statistical significance of the association (P random effects $= 2 \times 10^{-5}$); however, this study did not overly influence the overall effect estimate greatly (sensitivity analysis presented in the Supplementary material online, Figure S2). Possible reasons for heterogeneity may be related to the selection of cases and controls. For example, there is evidence that IL-6 concentrations are correlated with the AAA size$^{22,25,29}$ and subgroups analyses demonstrated that when the analysis was restricted to studies that compared IL-6 in large AAA compared with controls, there was less heterogeneity ($I^2 = 31.5\%$, Figure 1). Furthermore, there was variation in how controls were selected, e.g. from distinct cohorts such as the general population$^{23}$ or from surgical outpatients clinics,26 which is likely to contribute to heterogeneity. There was no evidence of publication bias using Begg’s adjusted rank correlation test (P = 0.7).

**Association of IL6R variants with abdominal aortic aneurysms**

Demographic details of the five case–controls studies are shown in Table 2 and genotyping quality control measures are shown in Table 3. There was a consistent association between the rare allele of rs7529229 (tagging Ala358) and reduced risk of AAA in all cohorts. Meta-analysis, pooling data from 4524 cases and 15 710 controls, demonstrated a per-allele OR of 0.84 (95% CI: 0.80–0.89, $P = 2.7 \times 10^{-11}$; $I^2 = 0\%$ fixed-effect meta-analysis) (Figure 2). In time-to-event analyses of prospective data from the SMART study, using Cox proportional hazard models, adjusted for age and gender, the rare Ala358 allele was also associated with a reduction in the risk of AAA clinical endpoints (AAA rupture, repair; $n_{events} = 223$) (Figure 3, per allele HR: 0.81, 95% CI: 0.67–0.99, $P = 0.043$).

**Functional analyses of the Asp358Ala (rs2228145) IL6R variant**

The aim of these analyses was to determine the downstream effects of the Asp358Ala variant on the expression of IL-6-mediated target genes identified in previous studies,20 namely STAT3, MYC, BCL3, ICAM1, and ATF3.

In lymphoblastoid cells, no difference in the expression of STAT3 targets was observed at baseline (Figure 4A). After IL-6 stimulation, there was a reduction in the expression of STAT3, MYC, ICAM1 in Ala358 homozygotes (CC) compared with Asp358 homozygotes (AA) (Figure 4B). This genotype-dependent difference was abolished by the presence of excess sIL-6R in the media (Figure 4C), suggesting that the effect observed with IL-6...
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Country</th>
<th>Study design</th>
<th>Cases/controls</th>
<th>Case definition</th>
<th>Controls</th>
<th>Diagnosis</th>
<th>IL-6 cases, mean (SD)</th>
<th>IL-6 controls, mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treska</td>
<td>2003</td>
<td>Czech Republic</td>
<td>Case–Control</td>
<td>74/30</td>
<td>Small (&lt;5 cm) = 30 Large (5–8 cm) = 38 Very large (&gt;8 cm) = 22 Ruptured/ symptomatic = 16**</td>
<td>Matched for age and gender No history of atherosclerosis</td>
<td>USS</td>
<td>59.3 (134.8)</td>
<td>6.7 (5.1)</td>
</tr>
<tr>
<td>Fowkes et al.²³</td>
<td>2006</td>
<td>UK</td>
<td>Case–Control</td>
<td>89/98</td>
<td>AP aortic diameter &gt;3 cm, size distribution NR</td>
<td>Normal USS (&lt;3 cm) Matched for age and gender. Nested case–control within the Edinburgh Artery Study³⁸</td>
<td>USS</td>
<td>2.8 (1.62)</td>
<td>1.8 (1.04)</td>
</tr>
<tr>
<td>Dawson et al.²¹</td>
<td>2007</td>
<td>UK</td>
<td>Case–Control</td>
<td>27/15</td>
<td>Large AAA undergoing endovascular repair Undergoing other vascular intervention</td>
<td>Age and sex matched No history of Atherosclerosis/AAA Undergoing routine preventative checks</td>
<td>CT</td>
<td>4.94 (2.49)</td>
<td>2.65 (1.98)</td>
</tr>
<tr>
<td>Flondell-Site</td>
<td>2009</td>
<td>Sweden</td>
<td>Case–Control</td>
<td>360/218</td>
<td>Small (&lt;4.5 cm) = 122 Medium(4.5–5.5 cm) = 108 Large (&gt;5.5 cm) = 130</td>
<td>Matched for age, free from atherosclerosis and AAA. Recruited from screening programme in NZ.</td>
<td>CT/USS</td>
<td>9.1 (31.91)</td>
<td>2.08 (2.90)</td>
</tr>
<tr>
<td>Jones (unpublished)</td>
<td>2012</td>
<td>New Zealand</td>
<td>Case–Control</td>
<td>166/359</td>
<td>All greater than 5 cm, awaiting repair</td>
<td>Matched for age, gender, and race Recruited from surgical clinics</td>
<td>CT/USS</td>
<td>9.1 (6.60)</td>
<td>6.3 (3.93)</td>
</tr>
<tr>
<td>Parry et al.²⁶</td>
<td>2010</td>
<td>UK</td>
<td>Case–Control</td>
<td>75/90</td>
<td>All AAA &lt;5.5 cm</td>
<td>Matched for age, gender, and smoking status Recruited from surgical clinics</td>
<td>USS</td>
<td>3.13 (2.87)</td>
<td>3.14 (2.32)</td>
</tr>
<tr>
<td>Wallinder et al.²⁵</td>
<td>2009</td>
<td>Sweden</td>
<td>Case–Control</td>
<td>78/41</td>
<td>Small (&lt;5 cm) = 38 Awaiting elective repair (&gt;5 cm) = 40</td>
<td>Matched for age, gender, and smoking status Volunteers with infra-renal aortic diameter &lt;3 cm</td>
<td>USS</td>
<td>4.02 (3.79)</td>
<td>2.3 (3.3)</td>
</tr>
</tbody>
</table>

*²⁵Values from ruptures not included in the meta-analysis.
Discussion

In this study, we used an extension of the Mendelian randomisation (MR) paradigm to investigate the potential utility of targeting IL-6 signalling in AAA. In the first stage, we reviewed the observational literature reporting associations between circulating IL-6 and the presence of AAA. Meta-analysis of case–control studies demonstrated that IL-6 was significantly higher in subjects with AAA compared with those without. These data support a hypothesis that IL-6 and associated signalling pathways may play a role in the development of AAA, but it is not possible to directly infer a causal relationship, from observational data associations alone, owing to limitations inherent in such analyses. For example, many of the studies did not adjust for potential confounders such as smoking status, and previously published reports have found that AAAs are a source of circulating IL-6, suggesting that the observed association could at least in part be the result of reverse causation. Furthermore, none of the identified studies was prospective population cohorts, considered the gold standard.
in epidemiological studies and there was some heterogeneity in how cases and controls were selected in the papers identified by the literature search. To address this, we applied the principles of MR for drug target validation by studying the association between a functional non-synonymous variant in \textit{IL6R} and AAA. Genotype is randomly allocated at conception and not subject to reverse causality and therefore represents a useful tool to examine causal relationships in complex diseases phenotypes. The Asp358Ala variant (or close proxy) was selected because it has previously been shown that it has concordant effects as tocilizumab on a range of inflammatory and cardiovascular biomarkers and, therefore, may be considered a useful genetic instrument to investigate the potential for targeting the IL-6R pharmacologically.30 There was a consistent association between the Ala358 variant and a reduced risk of AAA, a finding that suggests targeting the IL-6R is a plausible strategy in AAA. This was a statistically robust association, surpassing threshold levels of significance commonly used in genome-wide studies.

The \textit{IL6R} variants have not been previously identified in two separate GWAS of AAA; however, the largest of the discovery cohorts (\(n_{\text{cases}} = 1866\)) had only 20\% power to detect a variant of this effect size at the significance threshold of \(P < 1 \times 10^{-5}\).

<table>
<thead>
<tr>
<th>Study</th>
<th>Genotyping platform</th>
<th>Minor allele frequency</th>
<th>Call rate (%)</th>
<th>HWE, (P)-value</th>
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</thead>
<tbody>
<tr>
<td>AC</td>
<td>Illumina 670 k Beadchip</td>
<td>0.39</td>
<td>&gt;99</td>
<td>0.28</td>
</tr>
<tr>
<td>New Zealand</td>
<td>ABI Taqman</td>
<td>0.40</td>
<td>&gt;98</td>
<td>0.89</td>
</tr>
<tr>
<td>SMART</td>
<td>Kaspar</td>
<td>0.39</td>
<td>&gt;97</td>
<td>0.77</td>
</tr>
<tr>
<td>EAS</td>
<td>ABI Taqman</td>
<td>0.42</td>
<td>&gt;97</td>
<td>0.08</td>
</tr>
<tr>
<td>UTRECHT</td>
<td>Illumina 610 K</td>
<td>0.38</td>
<td>100</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Figure 2 Association between rs7529229 and abdominal aortic aneurysms following fixed-effect meta-analysis of four case–control studies (4524 cases/15 710 controls). Per allele odds ratio = 0.84 (95\% CI: 0.80–0.89, \(I^2 = 0, P = 2.7 \times 10^{-11}\)).

Figure 3 Time to event analysis curves showing the probability of abdominal aortic aneurysm-related endpoint (Rupture/Endovascular Repair/Open Repair) by genotype at rs7529229 (CC is the rare homozygote) in 7139 individuals from the SMART study, in who there were 223 events. The hazard ratio per C-allele is 0.81, 95\% CI = 0.67–0.99, \(P = 0.043\). The \(P\)-value was calculated using Cox regression, adjusting for age and gender.
used to triage those SNPs to be submitted for analysis in replication studies.

Given the higher levels of circulating IL-6 in patients with AAA, it may seem paradoxical that a variant associated with higher circulating IL-6 is also associated with a lower risk of AAA. However, this variant has previously been associated with reduced concentrations of C-reactive protein and fibrinogen (two downstream markers of IL6R activation), a pattern consistent with a pharmacological blockade of the IL-6 receptor with tocilizumab. Furthermore, it is important to note that this study is not an MR study of circulating IL-6 and AAA, but uses the MR concept to evaluate the potential utility of targeting the IL-6R in AAA disease.

The functional analysis suggests that the Ala358 variant is associated with a reduction in the sensitivity of immune cells to IL-6 in vitro. One possible interpretation of these data is that this variant reduces inflammation in the aortic wall in response to injury, via reduced signalling through the mIL-6R in immune cells recruited to the injury, resulting in a lower risk of AAA development. Although the lymphoblastoid cell line is useful to understand the functional consequences of this variant in vitro, further work on

Figure 4 Gene expression in STAT3 target genes in lymphoblast cell lines of different IL6R rs2228145 (AA = common homozygote; CC, rare homozygote). (A) Basal gene expression in lymphoblast cell lines shows no difference in gene expression between IL6R genotypes. (B) Gene expression following IL-6 stimulation; lower gene expression was observed in all STAT3 target genes in lymphoblasts possessing the C allele. (C) Gene expression following IL-6 stimulation and addition of excess sIL-6R. To examine if the effect seen in (B) was due to receptor shedding, excess soluble IL-6R was added to the stimulated cells, suggesting that the mechanism is of genotype-specific reduction in STAT3 targets is the result of increased shedding of the mIL-6R.
tissue specifically from AAA cases required to understand the in vivo mechanism of action, particularly in understanding the balance of pro- and anti-inflammatory effects on remodelling of the arterial wall in response to damaging environmental exposures that promote vascular injury.

These findings have potentially important translational implications as they support a hypothesis that targeting the IL-6R pharmacologically is a strategy that merits evaluation in AAA. No randomized trials of tocilizumab (a monoclonal antibody to the IL6R used to treat rheumatoid arthritis) have yet examined AAA as an outcome, although two recent open label studies have reported that in patients with rheumatoid arthritis, tocilizumab reduces arterial stiffness,33,35 which suggests that the IL6R blockade has effects on the vasculature. Indeed, a role for tocilizumab in the treatment of other forms of inflammatory vascular disease such as Takayasu’s arteritis has been postulated, although randomized trials in these conditions have not yet been reported.34 Furthermore, there is evidence that targeting inflammatory pathways can both prevent aneurysm formation and regress already established aneurysms33 in murine models of the disease and evidence that tocilizumab acts on this pathway.36 Tocilizumab does, however, have a number of side effects (such as hypersensitivity and respiratory tract infections) and the potential for benefit in patients with AAA would of course need to be balanced against the potential risks.

Although the effect of the IL6R Asp358Ala variant on AAA risk is modest, the potential for clinical benefit from pharmacological intervention at the IL-6R should not be discounted. For example, SNPs in HMGCR (the target for statins) show only a modest (but highly significant) effect on CHD risk, but the role of statins in the prevention of CHD is well established.37,38 The fact that tocilizumab has been shown to be safe in large trials in humans adds further weight to the translational potential of the present findings.

In common with two of the three previously identified well-validated loci associated with risk of AAA the IL6R locus is also associated with CHD.13,16,30,39 The magnitude of the effect identified in the present analysis (OR: 0.86, 95% CI: 0.80–0.89) is, however, greater than that reported for the association with CHD risk (OR: 0.95).30,37 The association with CHD does, however, strengthen the case for targeting the IL-6R therapeutically, as the major non-aneurysm-related cause of death in patients with dilated aortae is cardiovascular disease.40

Limitations

It is important to note the potential limitations of this study. The number of cases and controls identified from the published literature for the case–control analysis of IL-6 levels and AAA is relatively small, but effects were consistent and the overall result statistically robust. We did not have tissue from AAA for expression studies. The in vitro functional studies are in a lymphoblastoid cell-line, which may not mimic the in vivo immune response in the aortic wall. As there was limited phenotypic/risk factor data for some of the cohorts, we were unable to include the IL6R variant in a multivariate regression model including other covariates. However, no convincing effect on phenotypes such as smoking, lipids, and blood pressure has been seen in large-scale association analysis suggesting that the effects are not primarily mediated through, or confounded by these intermediate phenotypes. Finally, we would ideally have a large data set with validated measures of aortic expansion in the context of the IL6R genotype and assess whether or not this could provide clinically useful information to guide surveillance programmes, but this is not available at present.

Conclusion

This study has confirmed that AAA cases have higher concentrations of circulating IL-6 than controls, and provided novel evidence that a common functional variant in IL6R is associated with risk of AAA. This provides evidence that targeting the IL-6R may be a useful and novel strategy in AAA, and supports development of clinical trials in this regard.

Supplementary material

Supplementary material is available at European Heart Journal online.

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