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Objective—The goal of this study was to investigate the role of complement cascade genes in the pathobiology of human abdominal aortic aneurysms (AAAs).

Methods and Results—Results of a genome-wide microarray expression profiling revealed 3274 differentially expressed genes between aneurysmal and control aortic tissue. Interestingly, 13 genes in the complement cascade were significantly differentially expressed between AAA and the controls. *In silico* analysis of the promoters of the 13 complement cascade genes showed enrichment for transcription factor binding sites for signal transducer and activator of transcription (STAT)5A. Chromatin-immunoprecipitation experiments demonstrated binding of transcription factor STAT5A to the promoters of the majority of the complement cascade genes. Immunohistochemical analysis showed strong staining for C2 in AAA tissues.

Conclusion—These results provide strong evidence that the complement cascade plays a role in human AAA. Based on our microarray studies, the pathway is activated in AAA, particularly via the lectin and classical pathways. The overrepresented binding sites of transcription factor STAT5A in the complement cascade gene promoters suggest a role for STAT5A in the coordinated regulation of complement cascade gene expression. **(*Arterioscler Thromb Vasc Biol*. 2011;31:1653-1660.)**

Key Words: aneurysms ■ gene expression ■ pathology ■ abdominal aortic aneurysm ■ complement cascade

Abdominal aortic aneurysm (AAA), defined as a dilation greater than 3 cm of the infrarenal abdominal aorta, is a complex disease of the aging population.[1] Rupture of AAA is associated with a high mortality rate, making aortic aneurysms the 13th leading cause of death among white males over the age of 65 (WISQARS Leading Causes of Death Reports, 1999 to 2007; http://webappa.cdc.gov/sasweb/ncipc/leadcaus10.html). Characteristics of AAA pathogenesis include inflammation, vascular smooth muscle cell (SMC) apoptosis, oxidative stress, and extracellular matrix degradation.[2] Autoimmunity may also play a role in aneurysm development and progression,[3] with 1 report suggesting increased levels of complement factor 3 (C3) and IgG subclasses in the aneurysmal wall.[4]

Chronic inflammation plays a role in numerous diseases, especially diseases related to aging. The complement system as part of the innate immunity may contribute to many inflammatory diseases such as age-related macular degeneration, arthritis, Parkinson disease, and Alzheimer disease.[5] Complement may also have a potential role in the initiation and progression of aneurysms.[6] Many different types of immune cells have been reported in AAA tissue such as T cells, B cells, macrophages, dendritic cells, mast cells, natural killer cells, and natural killer T cells.[3] The complement maintains a link between the innate and adaptive immune system and is known to directly or indirectly associate with many types of immune cells.[7]

The complement system consists of more than 30 proteins, mainly proteases, which create a cascade when triggered.[7] Composed of 3 pathways, the complement system is a complex primary defense to help humans fight against pathogens. The classical pathway is activated by antibodies bound

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to antigens in immune complexes, whereas the lectin pathway is activated by microbial sugars, such as mannose-binding lectin. The alternative pathway can be activated in the absence of antibodies by the spontaneous hydrolysis of C3.[7,8] All pathways can lead to inflammation, promote antibody production, assist in phagocytosis, and attack cell membranes.[7]

The aim of the current study was to investigate the role of the complement cascade in the pathogenesis of human AAA. Several members of the complement cascade had increased expression at both the mRNA and protein levels in AAA tissue samples. The results, combined with previously published animal studies by other investigators, suggest that the complement cascade is involved in AAA pathogenesis.

Materials and Methods

An expanded Methods section (including Supplemental Tables I to IV and Supplemental Figures I and II) is available in the supplemental materials (available online at http://atvb.ahajournals.org).

Human Samples

Full-thickness aortic wall tissue specimens were collected from patients undergoing AAA repair operations at the Geisinger Medical Center, Danville, PA, or at the Harper University Hospital, Detroit, MI. Nonaneurysmal aortic samples were collected at autopsies. All samples are listed in Supplemental Table I. The collection of the human tissues was approved by the institutional review boards of Geisinger Clinic, Danville, PA, and Wayne State University, Detroit, MI.

RNA Expression Studies

The details on global mRNA expression profiles for aneurysmal and nonaneurysmal human abdominal aorta (Supplemental Table I) have been described previously[9] and the microarray data can be obtained from the Gene Expression Omnibus database (series GSE7084, http://www.ncbi.nlm.nih.gov/geo/). We used in the current study the microarray results only from the Illumina platform. Pathway information was obtained from the Kyoto Encyclopedia of Genes and Genomes.[10] Gene symbols available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) were used.

In Silico Analysis to Identify Transcription Factor Binding Sites

We analyzed the promoter regions of the differentially expressed genes from the complement cascade to identify enriched transcription factor binding sites using a computational approach implemented in Whole Genome rVISTA[11] (http://genome.lbl.gov/vista/index.shtml), a publicly available bioinformatics program.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed with human monocyte cells (THP-1 cell line, catalog no. TIB-202, American Type Culture Collection, Manassas, VA)[12] treated with lipopolysaccharide and interferon (IFN)-γ.[13]

Immunohistochemical Analysis

Control abdominal aorta samples (N=7; donor ages ranging from 44 to 88 years, mean 63.6±15.5 years) were obtained at autopsy (Supplemental Table I). Patient samples (8 AAA patients; ages ranging from 64 to 72 years, mean age 67.9±2.9) were tissues removed from the aneurysmal sac during open surgical repair operations.

Immunostaining was carried out with formalin-fixed paraffin-embedded tissue sections as described previously.[14] The primary antibodies are listed in Supplemental Table III. Double staining was performed with the macrophage/monocyte specific antibody CD68[15] and the antibody against signal transducer and activator of transcription-5A (STAT5A) using EnVision G/2 Doublestain System (Dako, Glostrup, Denmark).

Genetic Association Study

A genetic association study was carried out to test for an association with polymorphisms in the complement cascade genes. A complete list of the 16 polymorphisms used in the final analyses is given in Supplemental Table IV. The DNA samples used for the study consisted of 2 case-control sets: set I had 394 AAA cases and 419 controls; set II consisted of 480 AAA cases and 480 controls, as described previously.[16,17]

Power calculations were performed using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/). We assumed that the polymorphism and the disease locus were in a complete linkage disequilibrium and that they had the same allele frequencies, ie, the polymorphism was the disease locus. Assuming a disease locus with an additive effect and a disease prevalence of 0.02 our initial sample (set I) of 394 cases and 419 controls had an 80% power to detect a susceptibility locus with a genotypic relative risk $\geq$1.2 at a significance level of $P<0.05$ for a single-nucleotide polymorphism (SNP) with a minor allele frequency $\geq$0.2.

Results

The mRNA Expression of Many Complement Cascade Genes Is Altered in AAA

We carried out a microarray-based genome-wide expression profiling of AAA and nonaneurysmal tissue samples previously.[9] The study was based on combined analysis using Affymetrix and Illumina data. The Affymetrix array was used with pooled RNA samples and the Illumina array was used with individual RNA samples. We searched the list of differentially expressed genes for those belonging to the Kyoto Encyclopedia of Genes and Genomes pathway "Complement and Coagulation Cascades" (hsa04610) and found 13 (38%) of the 34 genes of the complement cascade arm of the pathway to have altered expression (Figure 1 and Supplemental Table II). As we describe in the Supplemental Methods, for the current study, we reanalyzed the microarray data using only the Illumina data obtained with individual samples to be able to carry out statistical analyses and assess sample-to-sample variation. Of the 34 complement cascade genes (Figure 1 and Supplemental Table II) probed for with this array, 25 were considered expressed and are shown in the box-and-whiskers plot in Supplemental Figure I. Nine genes (*C4BPA, C4BPB, C8A, C8B, C8G, C9, CR1, CR1L*, and *MBL2*) were scored as not expressed because the signals were not statistically different from background noise at the 99% confidence level (Illumina Beadstudio software detection score <0.99). Five of the 13 differentially expressed complement cascade genes had increased, and 8 had decreased expression in AAA compared with the control tissues (Figure 1, Supplemental Figure I, and Supplemental Table II).

In silico analysis of the promoter regions of the 13 differentially expressed complement cascade genes using Whole Genome rVISTA showed enrichment for binding sites for a transcription factor STAT5A when compared with the entire genome ($-\log_{10} P=2.4$). There were, altogether, 6 binding sites for STAT5A in the set of these 13 genes, whereas there are a total of 2785 binding sites in the entire genome. One binding site for STAT5A was present in the promoters of 4 of the 13 genes (*MASP1*, *SERPING1*, *C7,* and

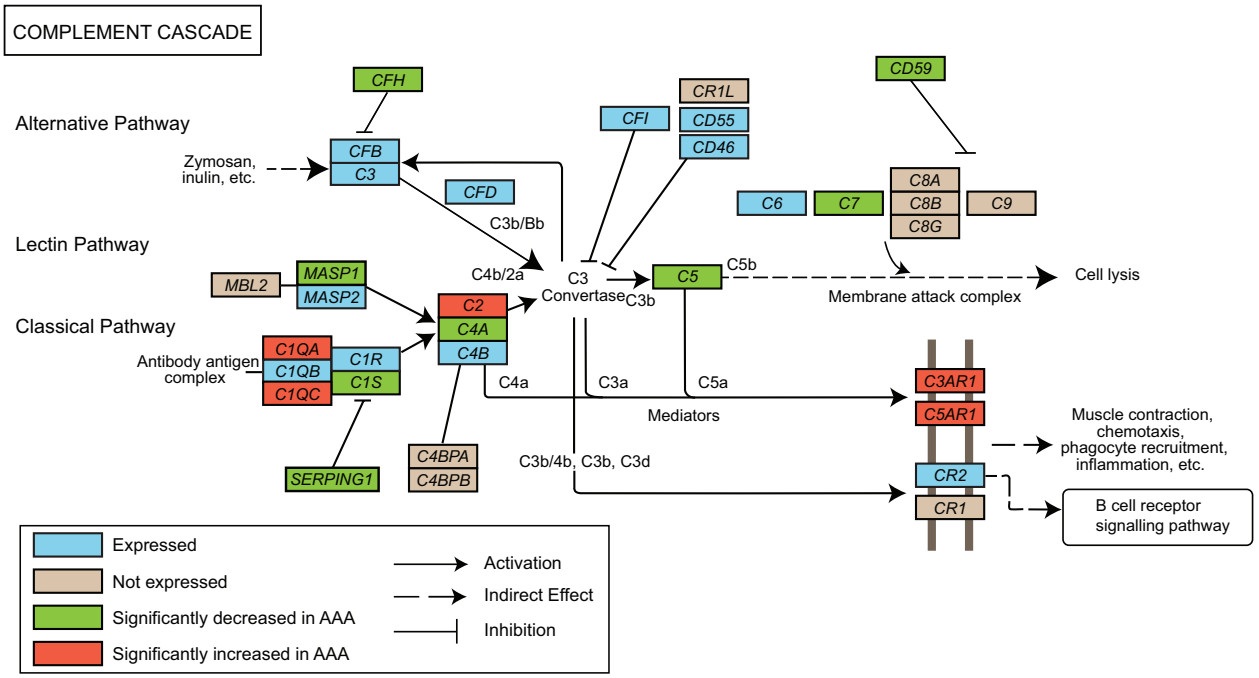

Figure 1. Modified complement and coagulation cascades (hsa04610) pathway from the Kyoto Encyclopedia of Genes and Genomes. Only the complement cascade part of the pathway is shown. Protein symbols have been replaced by gene symbols to reflect gene-centric data. See key for explanation of colors and symbols. Supplemental Table II and Supplemental Figure I provide details on the results. CFB indicates complement factor B; CFH, complement factor H.

C4A), and 2 bindings sites were present in the *C1QC* promoter. It is of interest that 4 (*MASP1*, *SERPING1*, *C7*, and *C4A*) of these 5 genes had decreased expression in AAA tissue and that all but 1 (*MASP1*) are part of the so-called classical pathway of the complement cascade. Based on our microarray expression data, *STAT5A* is expressed in both AAA and nonaneurysmal aorta, with no difference in the mRNA levels (not shown).

To establish whether the *in silico* results reflected true binding, we carried out ChIP followed by quantitative real-time polymerase chain reaction for STAT5A binding on the *C1QC* promoter in a monocyte cell line activated by lipopolysaccharide and IFN-γ to mimic the inflammatory environment present in the aortic wall. Stimulation with IFN-γ was considered relevant to AAA, because mice lacking IFN-γ are resistant to AAA formation in the $CaCl_2$ model,[18] and IFN-γ-producing T cells are present in the blood and aortic wall of most AAA patients.[19] STAT5A protein bound to the *C1QC* promoter at the predicted site with 1000-fold enrichment compared with mock ChIP with mouse IgG.

To identify all the STAT5A binding sites on the promoters of the complement cascade genes, ChIP-chip was carried out using microarrays covering 10 kbp of the promoters of all known genes. STAT5A demonstrated binding to 6297 distinct genes (false discovery rate <0.05), 1095 (17%) of which were differentially expressed in human AAA tissue (431 had increased expression and 664 had decreased expression). In this analysis, we concentrated on the results from the complement cascade genes: strong evidence (false discovery rate <0.05) for STAT5A binding was found on 10 of the 34 complement cascade genes, and moderate evidence ($0.05<$false discovery rate<0.2) on 9 complement cascade genes (Table).

Immunohistochemical Staining Shows Altered Protein Expression of C2

Differences in tissue architecture were evident between AAA and control aortic tissue after hematoxylin–eosin staining of aortic wall samples (not shown). Aneurysmal aortic wall tissue had signs of extracellular matrix degradation, and normal vessel architecture was destroyed. A large amount of extracellular matrix and loss of elastic fibers in the media were consistent with prior observations.[20] AAA tissues also showed neovascularization in the adventitia, media, and intima. Most AAA tissue samples showed an increased number of inflammatory cells in the adventitia and media. Some samples showed peripheral nerves with ganglion cells in the adventitia. Three of 8 AAA samples had thrombus in the lumen, as did 1 of 7 controls (Supplemental Table I). The intima layer was thicker in the AAA samples with a higher grade of atherosclerosis than in the aortic wall of the controls.

Immunohistological staining with antibodies against complement factor B (CFB), C2, complement factor H (CFH), C3, and STAT5A was evaluated in AAA and control tissue. CFB antibody showed no staining in the control aorta or in the AAA tissue (not shown). The fact that this antibody showed positive staining with a tissue array consisting of cancer tissue samples indicated that the lack of staining in aortic wall was not due to low sensitivity of the antibody but rather due to the absence of this antigen in the aortic tissue (ie, true negative result).

The antibody against C2 showed staining in both the AAA and control aortic tissue, with a stronger staining in the AAA

Table. STAT5A Binding on the Promoters of Complement Cascade Genes Based on ChIP-Chip Analysis

Gene Symbol	mRNA Expression in AAA vs Control*	Location of Binding Site†	Significance	
			FDR<0.05 (Strong)	0.05≤FDR<0.2 (Moderate)
C2	Up	Chr. 6: 31971437 to 31971781	0.0287	
C3AR1	Up	Chr. 12: 8110970 to 8111501		0.185
C5AR1	Up	Chr. 19: 52500443 to 52500883	0.0231	
C4A	Down	Chr. 6: 32054233 to 32054607	0.0364	
CD59	Down	Chr. 11: 33701293 to 33701742	0.0117	
C1QB	Expressed	Chr. 1: 22856663 to 22857102	0.0455	
C1R	Expressed	Chr. 12: 7136415 to 7136844		0.126
C3	Expressed	Chr. 19: 6675788 to 6676062	0.0352	
C4B	Expressed	Chr. 6: 32054233 to 32054607	0.0364	
C6	Expressed	Chr. 5: 41248400 to 41248762		0.185
CD46	Expressed	Chr. 1: 205989938 to 205990302		0.056
CD55	Expressed	Chr. 1: 205557019 to 205558372	0.0012	
CFB	Expressed	Chr. 6: 32020752 to 32021211		0.069
MASP2	Expressed	Chr. 1: 11032689 to 11033127		0.104
C4BPB	Not expressed	Chr. 1: 205329267 to 205329800		0.069
C8A	Not expressed	Chr. 1: 57092599 to 57093857	0.0080	
C8B	Not expressed	Chr. 1: 57203403 to 57203849	0.0455	
CR1	Not expressed	Chr. 1: 205733259 to 205733580		0.154
MBL2	Not expressed	Chr. 10: 54200747 to 54201083		0.185

*Results based on microarray analysis.[9] See Figure 1, as well as Supplemental Figure I and Supplemental Table II for details.

†Refers to physical location on the given chromosome (Chr), University of California Santa Cruz Golden Path hg18.

FDR indicates false discovery rate; CFB, complement factor B.

samples (Figures 2 and 3). The control aortic tissue had positive staining mainly around the vessels in the adventitia (Figure 2), but the media layer had only weak staining (Figure 2); in the intima layer (not shown), positive staining was seen only in regions of atherosclerotic changes. The AAA tissue showed a stronger staining for C2 in the adventitial layer (Figure 2), surrounding vessels and neovessels (Figure 3). Infiltrating neutrophils and macrophages were also C2–positive. The media layer had staining in the extracellular regions (Figure 2). The staining in the intima depended on the grade of destruction of the tissue but was always stronger in the AAA samples than in the controls (not shown). Approx-

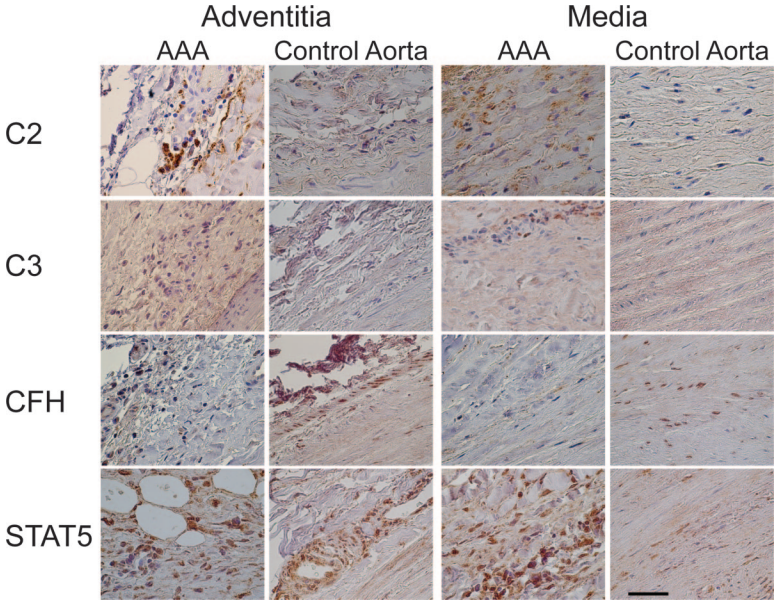

Figure 2. Immunohistochemical staining for C2, C3 (α-chain), complement factor H (CFH), and STAT5A in the adventitia and media of AAA tissue and control aortic tissue. Scale bar=50 μm.

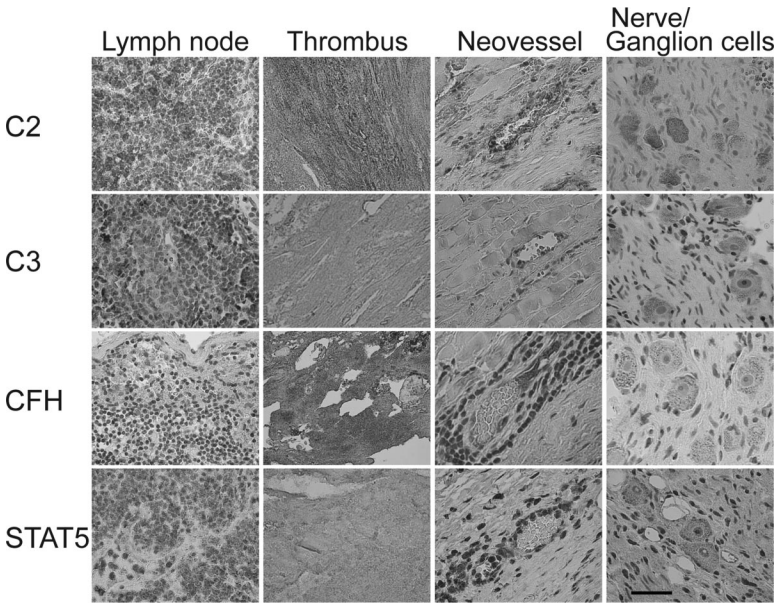

Figure 3. Immunohistochemical images of a lymph node, a thrombus, neovessels, and nerve or ganglion cells in AAA tissue. Antibodies against C2, C3 (α-chain), complement factor H (CFH), and STAT5A were used for staining. Scale bar=50 μm.

imately half of the lymphocytes in lymph nodes showed cytoplasmic staining, and cytoplasm of ganglion cells in the peripheral nerve tissue were positive for C2 (Figure 3). Intraluminal thrombus was also strongly positive for C2 (Figure 3).

The antibody against CFH (Figures 2 and 3) showed positive cytoplasmic and nuclear staining in fibroblasts of the control aortic tissue but not in the AAA tissue (Figure 2). Neutrophils in adventitia of AAA tissue were positive for CFH, whereas the lymphocytes were negative (Figure 3). Ganglion cells were CFH negative, whereas the intraluminal thrombus showed strong staining for CFH in AAA samples (Figure 3).

Antibody against the α-chain of C3b (clone H206) produced only weak staining in the adventitia of the control tissue and in endothelial cells of the vessels, but none in the media; in the intima, staining was evident only in arteriosclerotic plaque lesions. By contrast, the AAA tissue showed an intense extra- and intracellular staining in the adventitia and media, and a weak staining in the intima (not shown). The antibody against the C3 α-chain, C3a (clone H13), showed cytoplasmic staining in infiltrating neutrophils and monocytes in AAA adventitia and media. The intensity of the staining in the media layer was similar in both the AAA and control tissues (Figure 2). In the intima, both control and AAA tissue showed only weak staining (not shown). There was no staining for this antibody in the lymph nodes, thrombus, neovessels, or nerve or ganglion cells (Figure 3).

Both AAA and control samples showed staining for STAT5A in the adventitia and media layers (Figure 2). Cytoplasm of myofibroblasts was positive for STAT5A in both control and AAA tissue (Figure 2). Endothelial cells of the vasa vasorum in both controls and AAA and neovessels in AAA samples showed strong cytoplasmic staining (Figures 2 and 3). Connective tissue in the adventitia of AAA showed STAT5A-positive adipocytes (Figure 2). STAT5A also showed peripheral staining in the ganglion cells and nuclear staining of Schwann cells (Figure 3). Thrombus stained only weakly for STAT5A in AAA samples (Figure 3).

Double staining with antibodies against CD68[15] and STAT5A demonstrated that some of the monocytes and macrophages present in the aortic wall also stained for STAT5A, indicating STAT5A expression (Supplemental Figure II). These findings are consistent with results obtained with human lung tissue, where macrophages showed moderately strong staining for STAT5A (The Human Protein Atlas, www.proteinatlas.org).[21]

The immunohistochemical findings in AAA tissues varied between the AAA samples depending on the grade of destruction of the aortic wall and the extent of inflammation. At least 2 samples gave the results described above. The control aortic tissues showed more consistency between samples taken from different individuals.

No Evidence of Genetic Association Between Complement Cascade Polymorphisms and AAA

In the current study, we also tested the hypothesis that a genetic variant in 1 of the complement cascade genes is associated with AAA and provides genetic susceptibility for AAA. We analyzed all the SNPs associated with age-related macular degeneration[22–24] and additional SNPs to cover the entire pathway (Supplemental Table IV). None of the SNPs showed a deviation from Hardy-Weinberg equilibrium in the control populations. Case-control set I (394 cases and 419 controls) showed a borderline significant association for SNP rs9332739 in *C2*, rs4151667 in *CFB*, and rs1065489 in *CFH*. These associations could not be confirmed in the case-control set II (480 cases and 480 controls) or in the combined analyses with 874 AAA cases and 899 controls. Four SNPs (rs1143664, rs547154, rs9332739, and rs4151667) selected on the basis of prior associations with other diseases, and 1 SNP (rs12146727) selected on the basis of missense substitution, had minor allele frequencies substantially below 0.2 in our study population (Supplemental Table IV). In summary, no associations were found, but the SNPs with low minor

allele frequencies would require larger sample sizes to detect or exclude associations.

Discussion

The exact underlying pathobiology of AAA remains unknown, but several characteristic features have been recognized in the aortic wall including chronic inflammation.[2,3] The complement cascade is at the interface between innate and adaptive immunity by augmenting antibody responses and enhancing immunologic memory.[7] It contributes to opsonization, chemotaxis, and activation of leukocytes and can directly destroy bacteria and cells. In addition, complement factors are part of the disposal of cellular waste by clearing tissue from immune complexes and apoptotic cells.[7]

Our results provide multiple lines of evidence that complement cascade plays an important role in AAA pathogenesis. Based on our microarray studies, the pathway is activated in AAA, particularly via the lectin and classical pathways. First, expression of *C2*, which is at the intersection between the lectin and classical pathways, is elevated. Second, *C1QA* and *C1QC*, which can be activated by an antibody-antigen complex in the first step of the classical pathway, also had increased mRNA expression in AAA. Third, 3 inhibitors of the complement cascade, *CFH* (acting in the alternative pathway by inhibiting CFB activation), *SERPING1* (inhibitor of C1S in the classical pathway), and *CD59* (inhibits the membrane attack complex), all have decreased expression in AAA. Finally the receptors *C3AR* and *C5AR*, located on the cell membrane, have increased expression.

Two previous studies on complement cascade carried out in mouse models also demonstrated a significant role of complement in the development of AAA. In the elastase-induced AAA model with C57BL/6 mice, complete depletion of complement activity protected the mice from AAA development.[25] Complement inhibition done 24 hours before elastase infusion prevented AAA formation and provided evidence that the complement cascade is important in the initiation of AAA. Furthermore, the investigators showed that mice deficient in *Cfb* were resistant to AAA development in the elastase model. In addition, antagonism of C3a also blocked AAA development completely.[25]

In our results, increased gene expression of *C1QA, C1Q,* and *C2,* as well as decreased expression of the inhibitor *SERPING1* in human AAA tissue, suggests that the classical pathway plays a more prominent role in human AAA. The apparent inconsistency between the previously published mouse studies and the current human study could be due to mechanistic differences in the mouse model and human AAA or due to the late-stage disease samples of human AAA tissue studied here, whereas in knockout mice, earlier stages of AAA development were studied.

Of particular interest to AAA is the fact that the classical pathway can be activated independently of antibodies by pentraxins, a family of several pentameric proteins that bind to various lipids.[7] One of the best known pentraxins is the C-reactive protein; serum levels of C-reactive protein have been reported to be elevated in AAA patients,[26] providing another potential mechanism for the increased activity of the complement cascade in AAA.

Only 2 studies were published previously on the complement system in human AAA. The first study showed that C3 measured by ELISA had a 125-fold increase in AAA tissue compared with the control aortic tissue. Additionally IgG1, IgG2, and IgG3 levels, which can initiate the classical pathway, were increased in AAA patients.[4] The fact that C2 mRNA levels are increased in AAA may explain the elevated C3 levels without there being an increased C3 mRNA expression, because C2 is converted to C2a and, with C4b, forms the C3 convertase (C4b2a), which activates C3.[4] C3 has been linked to autoimmune diseases in that C3 activation appears to promote autoimmunity. This was observed in a mouse model of type II collagen-induced arthritis characterized by joint inflammation and destruction.[27] It is possible that complement activation contributes to AAA via a similar mechanism.

The second, more recent study on complement cascade in human AAA was published while the current study was under review and investigated polymorphisms in genes of the alternative pathway in a case-control study of 434 AAA patients and 378 controls.[28] No evidence for association was found, including polymorphism rs2230199 in *C3*, rs17611 in *C5*, rs13157657 in *C7*, and rs4151667 in *CFB*, which were also investigated in the current study.[28]

The components of the early events in complement cascade activation were not the only differentially expressed genes in our microarray study. mRNA levels of the membrane receptors C3AR1 and C5AR1 were also significantly increased in AAA tissue. These receptors interact with the complement cascade components and can induce inflammation,[7] a characteristic feature of the AAA tissue. Both C3AR1 and C5AR1 are G-protein-coupled receptors of the 7-transmembrane type and are similar to chemokine receptors.[7] C3AR1 is present on basophils, mast cells, and SMCs.[7] It is noteworthy that a previous study demonstrated mast cell invasion in AAA tissue[29] and activation of the C3AR1 receptor can induce inflammation and SMC contraction. C5AR1 is expressed on inflammatory cells, such as basophils, mast cells, monocytes, and neutrophils, as well as on endothelial cells and SMCs.[7] Activation of C5AR1 induces inflammation and chemotaxis and increases vascular permeability.[7] The invasion of neutrophils into the AAA tissue has been shown in mouse and human studies.[30,31]

SERPING1, *CFH*, and *CD59*, 3 of the 4 naturally occurring inhibitors in complement cascade, had decreased mRNA levels in AAA tissue on the basis of our microarray results. The importance of CD59 as an inhibitor of the final steps in forming the membrane attack complex was demonstrated in *Cd59*-deficient mice in the angiotensin II–induced mouse model ($mCd59ab^{-/-}/Apoe^{-/-}$) of AAA, which showed accelerated development of AAA.[32]

To elucidate the mechanisms of transcriptional control of complement cascade genes, a search for transcription factor binding on the promoters was carried out *in silico* followed by ChIP-chip analysis. Both methods showed overrepresented binding sites or strong binding of transcription factor STAT5A on the promoters of complement cascade genes.

Interestingly, the *in silico* analysis demonstrated 5 genes with overrepresented STAT5A binding sites, of which 4 belong to the classical pathway. ChIP-chip analysis on monocyte cell cultures showed strong binding of STAT5A on 10 of the 34 complement cascade genes and moderate binding on 9 of the 34 complement genes, consistent with involvement of STAT5A transcription factor in the regulation of these genes. STAT5A was found to be present in both AAA and nonaneurysmal abdominal aorta as mRNA and protein. Double staining showed that some macrophages present in the tissue were positive for STAT5A (Supplemental Figure II). The role of STAT5A in vascular diseases was also investigated in a recently published study carried out in a mouse model.[33] The study used a model in which angiotensin II is infused into *Apoe*$^{-/-}$-deficient mice, and a dominant negative form of monocyte chemoattractant protein, named MCP1-7ND, was administered to these mice.[33] Immunohistochemical staining of phosphorylated STAT5 was decreased in SMCs in the MCP1-7ND group, and these animals tended to have fewer AAAs, although the results were not statistically significant.[33] The authors speculated that the findings suggest that AAA development is due to decreased proliferative and migratory response, but they pointed out that their results were not consistent with another study using a mouse model in which AAAs are induced with calcium chloride.

In humans, the liver is the major source of complement proteins,[34] but other tissues also express complement cascade proteins. Glial cells and neurons have been shown to express complement proteins after stimulation by inflammatory cytokines,[35–37] but their expression has not been described previously in peripheral nerves. In another study, human cerebrovascular SMCs expressed mRNA of many complement cascade genes, including *C1QB, C1R, C1S, C2, C3,* and *C4* from the classical pathway.[38] Our results demonstrated the presence of the complement proteins C2, CFH, C3, and transcription factor STAT5A in the cells of the aortic wall. Although CFB mRNA was expressed in AAA tissues, CFB protein was not detectable in aortic wall tissue. An interesting finding was that ganglion cells in peripheral nerve expressed C2; this might be inflammation induced. Production of CFH in myoblasts has been reported in a previous in vitro study on human myoblast cell lines.[39] CFH protein was present in the cytoplasm of myofibroblasts of the control aortic tissue in our study, but myofibroblasts in AAA wall were negative for CFH. This could be due to downregulation of CFH mRNA in the AAA tissue or due to apoptosis of SMCs.

In conclusion, our results provide strong evidence that the complement cascade plays a role in the pathophysiology of human AAA. Furthermore, the overrepresented binding sites of transcription factor STAT5A on the promoter regions of the complement cascade genes suggest coordinated regulation of their gene expression.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Role of Complement Cascade in Abdominal Aortic Aneurysms

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Supplementary Materials and Methods

Study samples

Full thickness aortic wall tissue specimens were collected from patients undergoing AAA repair operations at the Geisinger Medical Center, Danville, Pennsylvania, USA, or at the Harper University Hospital, Detroit, Michigan, USA. Non-aneurysmal, abdominal, infrarenal aortic samples were collected at autopsies. Samples were stored in RNAlater (Ambion, Austin, TX, USA), or in phosphate-buffered formalin (and embedded in paraffin). All samples are listed in Supplemental Table I. The collection of the human tissues was approved by the Institutional Review Boards of Geisinger Clinic, Danville, Pennsylvania, USA, and Wayne State University, Detroit, Michigan, USA.

As shown in Supplemental Table I we have information of adherent thrombus on the newer tissue samples. Tissue samples taken from patients with a clinical diagnosis of inflammatory AAA were excluded from the study.

Microarray-Based Gene Expression Studies

In our previous study, we used two microarray platforms to generate global mRNA expression profiles for both aneurysmal and non-aneurysmal human abdominal aorta (samples used in these studies are listed in Supplemental Table I). The details on these studies have been described previously,[1] and the microarray data can be obtained at the Gene Expression Omnibus (GEO) database (Series# GSE7084; http://www.ncbi.nlm.nih.gov/geo/). Our previous publication was based on a combined analysis using RNA pools analyzed on Affymetrix arrays and individual RNA samples run on Illumina arrays.[1]

For the current study on complement cascade genes, we re-analyzed the microarray data using only the Illumina data obtained with individual samples (6 AAA cases with a mean age of 67.8±6.6 years; median age: 65.5 years, and 7 controls with a mean age of 65.6±11.2 years; median age: 65 years; Supplemental Table I) to be able to carry out statistical analyses and assess sample-to-sample variation. All samples were collected from the abdominal infrarenal aorta. The analyses were carried out with Illumina Beadstudio software. Detection Score $\geq$ 0.99 in either AAA or control group was used as the criteria for a gene to be considered expressed. Out of the 34 complement cascade genes probed for with this array, 25 were considered expressed based on this criteria and are shown in the box-and-whiskers plot in Supplemental Figure I. Nine genes (*C4BPA, C4BPB, C8A, C8B, C8G, C9, CR1, CR1L* and *MBL2*) were scored as not expressed since the signals were not statistically different from background noise at the 99% confidence level (Illumina Beadstudio software Detection Score < 0.99). Details on the results can be found in Supplemental Table II.

Pathway information was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG).[2] Gene symbols available from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) were used.

In Silico Analysis to Identify Transcription Factor Binding Sites Enriched in Differentially Expressed Complement Cascade Genes

We analyzed the promoter regions of the differentially expressed genes from the complement cascade to identify enriched transcription factor binding sites (TFBSs) using a computational approach implemented in Whole Genome rVISTA[3] (http://genome.lbl.gov/vista/index.shtml), a publicly available bioinformatics program. Whole Genome rVISTA generates a list of TFBSs that are over-represented in the gene set under study when compared to the entire genome. The rVISTA tool identifies TFBSs based on the TRANSFAC® Professional library[4] in interspecies sequence alignments and then determines which of the TFBSs are conserved in order to maximize the identification of functional sites.[5] Here, human sequences from UCSC version hg18, NCBI Build 36.1, and mouse sequences from UCSC version mm8, NCBI Build 36, were aligned. We examined the 5 kb region upstream from the transcription start site of each gene. rVISTA uses the binomial distribution for statistical analyses and a *P*-value cutoff of 0.005 due to the large number of tests performed.

Chromatin Immunoprecipitation

Chromatin-immunoprecipitation (ChIP) was performed using EZ-ChIP Chromatin Immunoprecipitation Kit (catalog #17-371,Millipore, Billerica, MA). Human monocyte cells (THP-1 cell line; catalog #TIB-202, American Type Culture Collection, Manassas, VA)[6] were stimulated for 18 hours using 50% recommended serum, 100 ng/ml LPS (serotype 055: B5, Sigma-Aldrich, St. Louis, MO), and 20 ng/ml IFN-γ (PeproTech Inc., Rocky Hill, NJ).[7] This stimulation protocol has been used previously[7] and was considered relevant to AAA, since mice lacking IFN-γ are resistant to AAA formation in the $CaCl_2$ model[8] and IFN-γ-producing T-cells are present in the blood and aortic wall of most AAA patients.[9] DNA–protein complexes were cross-linked and then sheared to 200–1000 bp using a micro-tip equipped Sonicator 3000 (Misonix Inc., Farmindale, NY). Immunoprecipitation was performed using purified mouse STAT5 antibody (catalog #610191, BD Transduction Laboratories, San Jose, CA), or with control antibodies from the EZ-ChIP kit (anti-RNA polymerase and normal mouse IgG), and ChIP blocked protein G agarose. Proteins were eluted from agarose, crosslinking was reversed, and DNA was purified using spin columns.

ChIP-DNA was amplified using GenomePlex Complete Whole Genome Amplification (WGA) kit (WGA#2, Sigma-Aldrich, St. Louis, MO). Real-time quantitative PCR was carried out using RT2 SYBR Green/Rox qPCR Master Mix and ChampionChIP-qPCR Assay Primers (SABiosciences, Frederick, MD) to gene *C1QC* and control primers to *GAPDH* (housekeeping gene used as a positive control) and IGX1A (intergenic region used as a negative control) according to the manufacturer's recommendations.

Amplified input and chromatin-immunoprecipitated DNA samples were also used in hybridizations with promoter arrays (HG18 Deluxe Promoter HX1 arrays, Roche NimbleGen, Inc., Indianapolis, IN). These arrays contain 2.1 million probes of 50–75mer in size and tile the promoter regions of all known genes covering a genomic region of approximately -7 kbp to +3 kbp for each gene. Labeling, hybridization, washing, and scanning were performed by NimbleGen. Signal intensity data were extracted from scanned images using NimbleScan software. For each probe on the array, log 2 ratios of the Cy5-labeled STAT5 sample versus the Cy3-labeled input DNA sample were calculated. Peaks with false discovery rate (FDR) < 0.05 and 0.05 < FDR < 0.2 are reported.

Immunohistochemical Analysis

Control infrarenal aorta samples (N = 7; donor ages ranging from 44 to 88 years and mean of 63.6±15.5 years) were obtained at autopsy (Supplemental Table I). Patient samples (8 AAA patients; ages ranging from 64 to 72 years, mean age 67.9±2.9) were tissues removed from the aneurysmal sac during open surgical repair operations (Supplemental Table I).

Immunostaining was carried out with formalin-fixed paraffin-embedded tissue sections as described previously.[10] The primary antibodies are listed in Supplemental Table III. A secondary antibody with peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins (Dako, Glostrup, Denmark: K4063) was used and the signal was detected with substrate chromogen solution (Dako, Glostrup, Denmark: K3461/K3469 K3467/K3468 AEC+ Ready-to-use Liquid DAB). Antibodies for each protein were first tested on a tissue known to contain the protein of interest as positive controls. Non-specific mouse IgG antibody in lieu of primary antibody served as a negative control.

Double staining was performed with the macrophage/monocyte specific antibody CD68[11,12] and the antibody against STAT5A to investigate if macrophages/monocytes express STAT5A. The procedure was performed according to the manufacturer's instructions using kit EnVision™ G/2 Doublestain System, Rabbit/Mouse DAB+/Permanent Red (Dako, Glostrup, Denmark: K5261).

For evaluation of the stained slides microscope Nikon OPTIPHOT-2 (Japan) and Nikon Digital Camera DXm1200F (Japan) were used.

Genetic Association Study

A genetic association study was carried out to test for an association with polymorphisms in the complement cascade genes. We tested all the SNPs associated with AMD[13-15] and additional SNPs,

known to be functional or studied in other diseases, to cover the entire pathway. A total of 30 SNPs were attempted in the analyses, but four assays (rs4577202, rs17218697, rs641153, rs11200638) did not perform well, three assays (rs35049192, rs12237774, rs1803261) failed in the design stage at ABI, and seven SNPs (rs17887074, rs11567805, rs34135565, rs34201541, rs2231460, rs11229062, rs28362953) had only one allele (monomorphic) in our population. A complete list of the 16 polymorphisms used in final analyses is in Supplemental Table IV. The DNA samples used for the study consisted of 2 case–control sets: set I had 394 AAA cases and 419 controls that were recruited from Belgium, Canada, and the USA; set II comprised of 480 AAA cases and 480 controls from the USA. These samples have been described previously in other genetic studies of AAA.[16,17]

Genotyping was performed using reagents and TaqMan SNP Genotyping Assay probes from Applied Biosystems (Foster City, CA). Genotype data were first tested for deviation from the Hardy-Weinberg equilibrium (HWE) using an exact test. Allelic association was tested using the general linear mixed model approach implemented in the genetics package of R (http://www.r-project.org/). Uncorrected *P*-values are reported.

Power calculations were performed using the Genetic Power Calculator[18] (http://pngu.mgh.harvard.edu/~purcell/gpc/). We assumed that the polymorphism and the disease locus were in a complete linkage disequilibrium and that they had the same allele frequencies, i.e., the polymorphism was the disease locus. Assuming a disease locus with an additive effect and a disease prevalence of 0.02 our initial sample (set I) of 394 cases and 419 controls had an 80% power to detect a susceptibility locus with a genotypic relative risk ≥ 1.2 at a significance level of $P<0.05$ for a SNP with a minor allele frequency (MAF) ≥ 0.2.

Table I. Human Aortic Tissue Samples Used in Microarray Study,[1] and Immunohistochemical Analyses

Sample Code	Presence of Thrombus	Donor Sex	Donor Age (years)	Cause of Death or Presence of AAA	Microarray Study	IHC
A1-F	NA	F	82	AAA	√	
A2-F	NA	F	68	AAA	√	
A3-F	NA	F	64	AAA	√	
A2-M	NA	M	63	AAA	√	
A3-M	NA	M	67	AAA	√	
A4-M	NA	M	63	AAA	√	
C1-F	NA	F	74	Cancer	√	
C2-F	NA	F	52	Cancer	√	
C3-F	NA	F	84	Aortic arch dissection	√	
C1-M	NA	M	65	Peritonitis	√	
C2-M	NA	M	59	Cancer	√	
C3-M	NA	M	52	Liver cirrhosis	√	
C4-M	NA	M	73	Cancer	√	
GHS00001	NA	M	67	AAA		√
GHS00034	Yes	M	64	AAA		√
WSU052	No	M	70	AAA		√
WSU060	No	M	70	AAA		√
WSU068	Yes	M	72	AAA		√
WSU075	No	M	67	AAA		√
WSU080	No	F	64	AAA		√
WSU081	No	M	69	AAA		√
ME-01-05	No	M	53	Unknown		√
ME-02-05	No	M	78	Cardiovascular		√
ME-05-01	No	F	69	Trauma		√
ME-05-03	Yes	M	54	Cardiovascular		√
ME-05-05	No	F	59	Cardiovascular		√
ME-10-01	No	F	88	Trauma		√
ME-10-03	No	M	44	Overdose		√

Tissue samples were obtained at autopsy or at operation, and were taken from the infrarenal abdominal aorta. All donors and patients were Caucasian.
NA, not available

Table II. mRNA Expression of Complement Cascade Genes in Human Aneurysmal (N = 6) and Non-Aneurysmal (N = 7) Abdominal Infrarenal Aorta Based on a Microarray Study[1]

		Control		AAA			
Symbol	**Gene ID**	**Signal (avg)**	**Detection***	**Signal (avg)**	**Detection***	**DiffScore†**	**Description**
C1QA	712	1881.01	1	4099.82	1	30.07	complement component 1, q subcomponent, A chain
C1QB	713	5991.62	1	8457.79	1	11.1	complement component 1, q subcomponent, B chain
C1QC	714	635.36	1	1808.54	1	26.45	complement component 1, q subcomponent, C chain
C1R	715	658.89	1	361.18	1	-9.33	complement component 1, r subcomponent
C1S	716	4614.07	1	1919.55	1	-42.31	complement component 1, s subcomponent
C2	717	202.17	1	665.29	1	54.86	complement component 2
C3	718	4826.73	1	3996.85	1	-1.96	complement component 3
C3AR1	719	272.35	1	772.85	1	36.85	complement component 3a receptor 1
C4A	720	307.26	1	136.18	1	-27.94	complement component 4A (Rodgers blood group)
C4B	721	142.82	1	57.33	0.99	-8.04	complement component 4B (Childo blood group)
C4BPA	722	7.3	0.83	8.42	0.86	0.68	complement component 4 binding protein, alpha
C4BPB	725	18.42	0.98	9.33	0.88	-5.66	complement component 4 binding protein, beta
C5	727	237.88	1	90.92	0.99	-53.18	complement component 5
C5AR1	728	517.42	1	2029.17	1	40.57	complement component 5a receptor 1
C6	729	143.36	1	109.16	1	-3.19	complement component 6
C7	730	4129.31	1	1933.13	1	-21.33	complement component 7
C8A	731	10.39	0.89	8.37	0.86	-1.35	complement component 8, alpha polypeptide
C8B	732	-0.24	0.43	5.02	0.76	4.83	complement component 8, beta polypeptide
C8G	733	5.95	0.78	16.98	0.97	9.01	complement component 8, gamma polypeptide
C9	735	4.27	0.71	-1.00	0.39	-5.19	complement component 9
CD46	4179	686.46	1	597.68	1	-3.95	CD46 molecule, complement regulatory protein
CD55	1604	748.38	1	1065.49	1	12.69	CD55 molecule, decay accelerating factor for complement (Cromer blood group)
CD59	966	3571.1	1	1842.82	1	-45.08	CD59 molecule, complement regulatory protein
CFB	629	1072.98	1	618.55	1	-10.2	complement factor B
CFD	1675	1916.38	1	1116.43	1	-11.31	complement factor D (adipsin)
CFH	3075	625.45	1	287.41	1	-31.02	complement factor H
CFI	3426	349.61	1	268.72	1	-5.36	complement factor I
CR1	1378	-1.54	0.35	1.29	0.54	2.41	complement component (3b/4b) receptor 1 (Knops blood group)
CR2	1380	-4.32	0.2	132.18	1	7.91	complement component (3d/Epstein Barr virus) receptor 2
MASP1	5648	230.26	1	104.13	1	-18.31	mannan-binding lectin serine peptidase 1 (C4/C2 activating component of Ra-reactive factor)
MASP2	10747	30.08	0.99	25.46	0.99	-1.94	mannan-binding lectin serine peptidase 2
MBL2	4153	-2.12	0.29	-6.92	0.07	-4.48	mannose-binding lectin (protein C) 2, soluble (opsonic defect)
SERPING1	710	4690.86	1	1590.57	1	-48.26	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)

Results are based on re-analysis of Lenk et al. (2007) data.[1] Expression values from Illumina Beadstudio software.

* A detection score of ≥ 0.99 in either control or experimental (AAA) tissue was taken to indicate that the gene was expressed in that tissue.

† The Illumina DiffScore is computed as sign(mean(experiment) − mean(control)) * $-10 * \log_{10}(P)$; therefore, P = 0.05 (nominal) corresponds to sign($\Delta\mu$) * 13.01

Table III. Primary Antibodies Used in Immunohistochemical Staining

Gene Symbol	Antibody name and catalog number	Supplier	Species	ICH Dilution
CFH	Complement Factor H (CFH) Antibody, LS-C36296	LifeSpan BioSciences Inc., Seattle, WA	Mouse monoclonal	1:25
CFB	Complement Factor B (CFB) Antibody, LS-C44981	LifeSpan BioSciences Inc., Seattle, WA	Mouse monoclonal	1:100
C2	Anti-Complement component protein C2, sc-58922	Santa Cruz Biotechnology Inc., Santa Cruz, CA	Mouse monoclonal	1:600
C3[*]	Anti-Complement C3b, 20-511-242249	GenWay Biotech, Inc., San Diego, CA	Mouse monoclonal	1:100
C3[*]	Anti-Complement C3b, alpha, clone H206, CBL189	Millipore Corporation, Billerica, MA	Mouse monoclonal	1:100
C3[*]	Anti- Complement component C3a, clone H13, CBL191[19]	Millipore Corporation, Billerica	Mouse monoclonal	1:100
STAT5A	Anti-Stat5 Antibody, 610191	BD Biosciences, San Jose, CA	Mouse monoclonal	1:50
CD68	Anti-Human CD68, M0876	Dako, Dako, Glostrup, Denmark	Mouse monoclonal	1:51

[*]Only two (CBL189 and CBL191) of the three antibodies recognizing different components of C3 stained the positive controls and were used for staining of aortic samples. The antibody that stained neither the positive control nor the control or AAA aortic tissue was against the β-chain of C3b (#20-511-242249).

Table IV. Polymorphisms Used in This Study

Gene[*]	Gene ID[†]	Chromosomal	Alleles	MAF[‡]	rs number	Function based on	Previous disease
C1QA	712	1p36.3–p341	A/G	0.360	rs172378	Synonymous, G92G	SLE[#]
C1S	716	12p13	A/G	0.069	rs1143664	Synonymous, P389P	SLE[#]
C1S	716	12p13	A/G	0.128	rs12146727	Missense, R119H	
C2	717	6p21.3	A/C	0.077	rs547154	Intron 10	AMD[#]
C2	717	6p21.3	C/G	0.047	rs9332739	Missense, E318D	AMD[#]
C3	718	19p13.3–p13.2	C/G	0.186	rs2230199[§]	Missense, R102G	AMD
C5	727	9q34.1	A/G	0.458	rs17611[§]	Missense, V802I	Liver fibrosis
C5AR1	728	19q13.3–q13.4	C/T	0.275	rs10404456	Upstream (5') of gene	
C5AR1	728	19q13.3–q13.4	A/G	0.255	rs11670330	Intron	
C5AR1	728	19q13.3–q13.4	C/G	0.258	rs4427917	Intron	
C7	730	5p13	A/C	0.249	rs13157656[§]	Missense, T587P	
CFB	629	6p21.3	A/T	0.046	rs4151667[§]	Missense, L9H	AMD[#]
CFH	3075	1q32–q32.1	C/T	0.357	rs1061170	Missense, Y402H	AMD[#]
CFH	3075	1q32–q32.1	G/T	0.160	rs1065489	Missense, E936D	AMD[#]
CFH	3075	1q32–q32.1	A/G	0.360	rs2300430	Intron 7	
CFH	3075	1q32–q32.1	G/T	0.247	rs800292	Missense, V62I	AMD[#]

[*]Gene symbols used are HGNC-approved symbols obtained from www.gene.ucl.ac.uk/nomenclature
[†]Gene ID was obtained from www.ncbi.nih.gov/Gene
[‡]MAF, minor allele frequency in our study population controls.
[§]While the current study was under review, Bradley and co-workers[20] reported a genetic association study on AAA using these polymorphisms.
[#]SLE, systemic lupus erythematosus; AMD, age-related macular degeneration

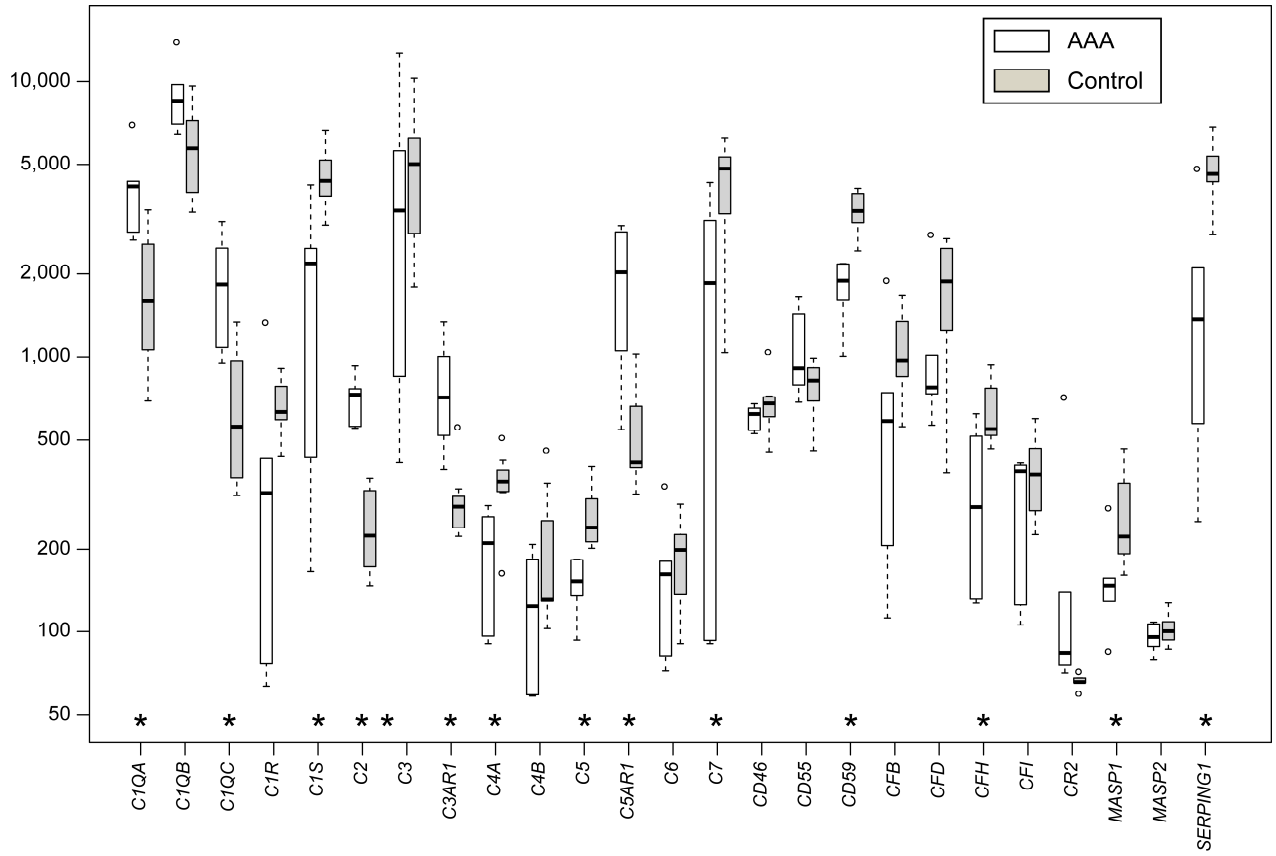

Figure I. Box and whisker plots of mRNA levels for members of the complement cascade. Expression data were based on Lenk et al. (2007) analysis using the expression values from Illumina arrays only with Illumina Beadstudio software.[1] Illumina Beadstudio software Detection Score ≥ 0.99 in either AAA or control group was used as the criteria for a gene to be considered expressed. RNA expression levels in signal intensity units are indicated on the ordinate-axis in logarithmic scale. Open boxes represent AAA patients (*N* = 6) and shaded boxes represent abdominal aorta samples from non-aneurysmal autopsy samples (*N* = 7). Clinical details on the patients can be found in Supplemental Table I. Thick horizontal bars in the boxes indicate median values, boxes indicate interquartile range, whiskers indicate range of non-outlier values, circles indicate outliers less than 3 interquartile range units. Gene symbols available from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) were used. Asterisks indicate statistically significant results after FDR correction. Nine genes (*C4BPA, C4BPB, C8A, C8B, C8G, C9, CR1, CR1L* and *MBL2* in Figure 1) were scored as not expressed since the signals were not statistically different from background noise at the 99% confidence level, and were omitted from this figure. See Supplemental Table II for details on the microarray results.

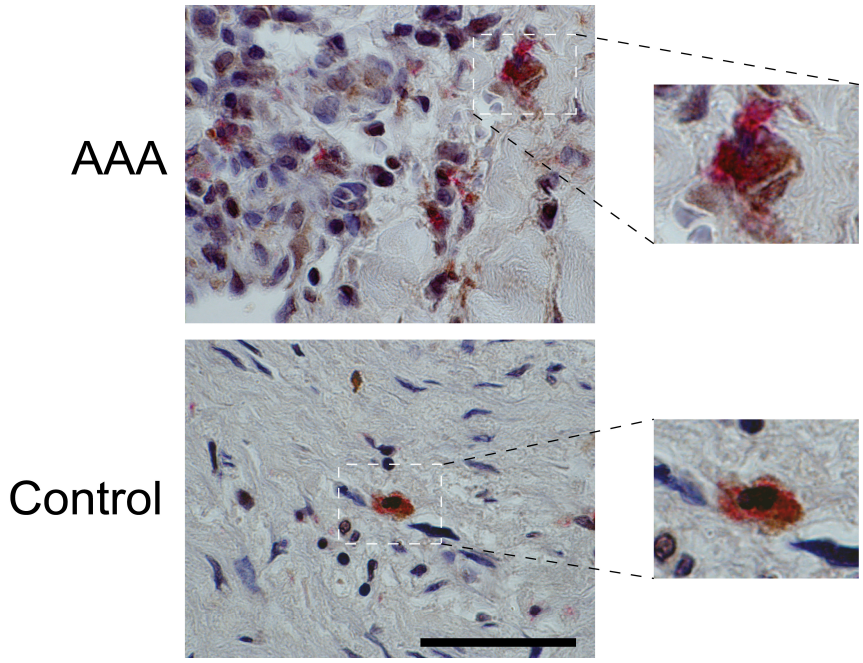

Figure II. Double-staining with antibodies against CD68 (red) and STAT5A (brown) in AAA and control abdominal aortic tissue. CD68 staining identifies monocytes and macrophages.[11] The results demonstrated that some of the monocytes and macrophages present in the aortic wall also stained for STAT5A indicating STAT5A expression. Two-fold enlargements of cells staining for both CD68 and STAT5A are shown at right. Dashed white outline shows the location of main image enlarged at right. Scale bar = 50 μm.

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