

# Microarray Analysis of Human Abdominal Aortic Aneurysm With Emphasis on Cardiovascular Genes Revealed Differentially Expressed Genes

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## Abstract

**Background/Aim:** We examined gene expression profiles in abdominal aortic aneurysm (AAA) lesions vs. normal aortas by cDNA microarray and real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).

**Materials and Methods:** Phosphorus (<sup>32</sup>P)-labeled cDNA from AAA specimens (mean AAA size 6.65 cm) and normal aortas were hybridized with a 588-gene microarray primarily of the cardiovascular system. The results were validated by qRT-PCR.

**Results:** A total of 35 out of the 588 genes were differentially expressed, with either log<sub>2</sub> ratio of AAAs/controls ≥1 (upregulated; 20 genes) or ≤-1 (downregulated; 15 genes) in AAA lesions vs. normal aorta, and 25 of these were significantly different (71%). Expression of matrix metalloproteinase 9, TIMP metalloproteinase inhibitor 3, collagen

*continued*

Presented in part at the Annual Meeting of the American Association of Immunologists, Honolulu, HI (Virtual Meeting), May 8-12, 2020.



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Received July 12, 2025 | Revised January 16, 2026 | Accepted February 6, 2026



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type I  $\alpha$  1 chain (*COL1A1*), *COL6A3*, *COL15A1*, intercellular adhesion molecule 1 (*ICAM1*), *ICAM2*, decorin, endoglin, apolipoprotein D (*APOD*), *APOE*, phospholipid transfer protein, calcium and integrin binding 1 (*CIB1*), phospholipase A2 group IIA, von Willebrand factor, serpin family B member 6 (*SERPINB6*), urokinase-type plasminogen activator, H19, C-C motif chemokine ligand 2, and platelet-derived growth factor receptor beta was upregulated in AAA vs. normal aorta. Expression of collagen type IV  $\alpha$  4 chain (*COL4A4*), *COL11A2*, gap junction protein  $\alpha$  1 (*GJA1*), biglycan, integrin subunit  $\alpha$  8, galectin-1, low-density lipoprotein receptor-related protein 1, acetyl-CoA acyltransferase, serpin family E member 1, melanoma cellular adhesion molecule, sodium channel epithelial 1 subunit beta (*SCNN1B*), natriuretic peptide receptor 1 (*NPR1*), superoxide dismutase 3, actinin  $\alpha$  1 (*ACTN1*) and cardiac phospholamban (*PLN*) was downregulated.

**Conclusion:** Eleven genes differentially expressed ( $p \leq 0.05$ ) in AAA lesions vs. normal aortas were not reported previously: upregulated: *COL6A3*, *COL15A1*, *ICAM2*, *APOD*, *CIB* and *SERPINB6*; downregulated: *GJA1*, *SCNN1B*, *NPR1*, *ACTN1* and *PLN*. Remaining results confirmed previous reports regarding 21 genes differentially expressed in AAA. qRT-PCR results were in general in agreement with microarray results.

**Keywords:** Abdominal aortic aneurysm, microarray, qRT-PCR, gene expression.

## Introduction

Abdominal aortic aneurysm (AAA) is an immunological disease with a strong genetic component (1-6). Three percent of individuals aged 60 years or older are diagnosed annually with AAA, which in the US is the 13th cause of death of men and women irrespectively of age (7-9). AAA is responsible for 1-2% of the deaths of males 65 years old or older. In 2013 AAA was responsible for 152,000 deaths worldwide (7) and remained at the same levels in 2021 (153,927 deaths worldwide) (8). Mortality of ruptured AAA is very high, in the range of 85-90%, making it the 15th biggest killer overall and the 10th biggest killer for men aged 55 years and older in the US (9).

AAA is a complex multifactorial disease (1-6), characterized by aortic dilation, remodeling and enlargement of the abdominal aorta with an arterial diameter  $>3$  cm or  $>50\%$  of the diameter of normal arteries. Genetic and environmental factors are involved in this disease [reviewed in (2)]. Substantial evidence has accumulated demonstrating that AAA is likely an autoimmune disease (1-6, 10), including:

(i) The presence of mononuclear cell infiltrates in AAA lesions consisting of T-, B- and natural killer cells (11, 12), as

well as antigen-presenting cells in close proximity of CD4<sup>+</sup>, CD8<sup>+</sup> and B-cells (13). These cells express early (CD69), intermediate (CD25, CD38) and late human leukocyte antigen (HLA) class II, CD45RO activation antigens (10).

(ii) Substantial proportions of identical  $\alpha$ - and  $\beta$ -chain T-cell receptor (TCR) transcripts have been identified in AAA lesions (10, 14, 15), demonstrating the presence of clonally expanded  $\alpha\beta$ TCR<sup>+</sup> T-cells. These clonal expansions strongly suggest that AAA is a specific antigen-driven T-cell disease (10, 14, 15) and can be explained only by proliferation and clonal expansion of T-cell clones in response to self- or nonself antigen(s) that they recognize, which remain to be identified [reviewed in (16)].

(iii) An association of AAA with certain HLA class I (HLA-A2 and HLA-61) and HLA class II (HLA-DRB1\*02 and HLA-DRB1\*04) has been reported (17-20).

(iv) T-Helper 1 (Th1) cytokines are responsible in part for the destruction of the aortic wall in AAA (21, 22). However, T-helper 2 (Th2) cytokines have been also reported in AAA lesions (23).

(v) IgG autoantibodies isolated from AAA lesions recognize antigens expressed in normal aorta (24).

(vi) Putative AAA self and nonself (microbial) antigens have been reported and many play a critical role in

the pathogenesis of the disease. Self antigens include elastin and elastin fragments, microbial-associated glycoprotein-36, carbonic anhydrase, oxidized low-density lipoprotein and collagen type I and III [reviewed in (1, 25)]. Nonself antigens, of microbial origin, have been also reported and include *Chlamydia pneumoniae*, *Treponema palladium* and cytomegalovirus [reviewed in (1, 25)]. Molecular mimicry may be also involved in the pathogenesis of AAA (26).

(vii) The frequency, suppressor activity and the levels of expression of forkhead box P3 transcripts and protein are significantly decreased in peripheral blood T-cells from patients with AAA vs. donors of normal aorta (27, 28).

Antigen-induced activated and proliferating T-cells, monocyte/macrophages, natural killer cells, B-cells, neutrophils, mast cells and possibly other infiltrating cells, as well as endothelial cells, vascular smooth muscle cells, adventitial fibroblasts and other cell types in AAA lesions produce increased amounts of molecules involved in proteolysis, and in particular enzymes degrading elastin and collagen, including matrix metalloproteinases (MMPs) and TIMP metalloproteinase inhibitors (TIMPs) (29, 30), cysteine and aspartic proteases and their inhibitors, molecules involved in immune function and inflammation, including pro-inflammatory and other cytokines and chemokines and molecules involved in apoptosis, neovascularization, lipid metabolism and other functions (31-44). This cascade of events resulting in the production of these molecules, together with the direct cell-mediated action of effector T-cells and the presence of autoantibodies, appear to be the primary mechanisms for the propagation of AAA, the destruction of the integrity of the aortic wall, and the growth of the aneurysm related to fatty acid metabolism (29-44).

Microarray approaches are best suited to study these molecules and the mechanisms involved because of their ability to quantitate many transcripts at the same time (33-44). We employed cDNA cardiovascular microarrays to study and compare the expression of 588 genes in AAA lesions and normal aortic specimens, with the objective of identifying genes whose expression is altered in AAA.

Table I. Characteristics of the patients with abdominal aortic aneurysm (AAA)\*. All patients were Caucasian males.

Patient	Age, years	AAA size, cm	HTN	COPD	TOB	CHOL	DM	Other
AAA02	65	5.8	Yes	Yes	Yes	Yes	No	CAD
AAA07	71	5.5	No	No	No	Yes	Yes	AOVR
AAA09	78	7.4	Yes	Yes	No	No	No	CAD/CRI
AAA10	78	7.9	No	No	No	Yes	Yes	CAD
AAA12	77	UN	UN	UN	UN	UN	UN	UN
Mean	73.8	6.65						

AOVR: Aortic valve replacement; CAD: coronary artery disease; CHOL: high cholesterol; COPD: chronic obstructive pulmonary disease; CRI: chronic renal insufficiency; DM: *diabetes mellitus*; HTN: hypertension; TOB: current or ex-tobacco smoker; UN: unknown. \*Reproduced in part, with the permission of The American Association of Immunologists, Inc., from Lu et al. (14). Copyright 2014.

## Materials and Methods

**Patients.** The patient population consisted of patients who presented for and underwent elective open repair of an uncomplicated infrarenal AAA. All consented to analysis of tissue samples of aortic wall obtained specifically for analysis for this study. No patient had or had undergone treatment for any type of autoimmune disease. The characteristics of these patients (n=5) are described in Table I. These patients were male, with a mean age of 73.8 years. AAA predominantly affects elderly males [reviewed in (1, 2, 8, 9)]. As normal controls, grossly normal infrarenal abdominal aortic specimens, without visible evidence of atherosclerosis, were used and were obtained at autopsy from individuals of the same age group who died from nonvascular diseases. Control abdominal aortic specimens, obtained at autopsies (35, 45-50), or from cadavers or transplant donors (36, 37), have been used in many studies. Adherent blood clots were stripped away from the aortic wall from these specimens prior to use. This study was carried out in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board of Advocate Lutheran General Hospital, Park Ridge, IL, USA; IRB protocol approval number 3116. Written informed consent was obtained from all patients with AAA who were recruited in the

study. The study was also examined at Temple University Hospital, and was found to be exempt from the need for approval.

*RNA preparation.* All aortic specimens were divided into two portions. One was snap-frozen in liquid nitrogen after procurement and stored at  $-80^{\circ}\text{C}$ . RNA was extracted within 2 weeks and cDNA was prepared within a month. cDNA was stored at  $-80^{\circ}\text{C}$ , and the microarray experiments using the synthesized cDNA were carried out within 2 years from the collection of AAA specimens. The other portion was embedded in optimal cutting temperature medium for histology and immunohistochemistry studies. Immunohistochemistry using a panel of antilymphocyte monoclonal antibodies was carried out as described elsewhere (51, 52) and the results have been reported elsewhere (10, 14). Total RNA was prepared from fresh (or freshly cryopreserved) AAA tissue from patients with AAA and normal control aortas, using a guanidinium thiocyanate solution (Stratagene, La Jolla, CA, USA) as recommended by the manufacturer; and then was treated with DNase (Atlas Pure Total RNA labeling system; Clontech Laboratories, Inc., Mountain View, CA, USA) to remove possible genomic DNA contamination.

*Microarray.* A cDNA microarray of 588 genes, Atlas Human Cardiovascular Array kit, comprised primarily of molecules of the cardiovascular system and including nine common house-keeping genes, was obtained from Clontech Laboratories, Inc. Please see the Supplementary Material for a complete list of microarray genes.

*Probe synthesis and microarray preparation.* Radiolabeled cDNA was prepared using reagents obtained from the Atlas Human Cardiovascular Array kit (Clontech Laboratories, Inc.), following the protocol provided by the manufacturer with minor modifications. Briefly, 5  $\mu\text{g}$  of total RNA from each of the aorta specimens were incubated with 2  $\mu\text{l}$  of coding sequence primer mix (0.02 mM; Clontech Laboratories, Inc.), and denatured at  $70^{\circ}\text{C}$  for 6 min. Then, 300 units of Superscript II RNase H-reverse transcriptase (Invitrogen, Waltham, MA, USA) and

deoxyadenosine triphosphate labeled with  $^{32}\text{P}$  (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 0.1 M dithiothreitol and 5 $\times$  buffer were added in the mixture. After incubation at  $42^{\circ}\text{C}$  for 1 h, the reaction was terminated by adding 10 $\times$  Termination Mix (Clontech Laboratories, Inc.). The labeled cDNA samples were purified by NucleoSpin Extraction Spin Column chromatography to remove unincorporated isotope before scintillation counting. The labeled probes were added to 10 $\times$  denaturing solution (1 M NaOH, 10 mM EDTA) and incubated at  $68^{\circ}\text{C}$  for 20 min. Next, 2 $\times$  neutralizing solution (1 M  $\text{NaH}_2\text{PO}_4$ , pH 7.0) and Cot-1 DNA (as a blocking agent) were added, and the solution was incubated at  $68^{\circ}\text{C}$  for 10 min. At the same time, the nylon array membranes were prehybridized with Express Hyb solution (Clontech Laboratories, Inc.) and heat-denatured salmon sperm DNA in a glass tube, with continuous agitation (rolling) at  $68^{\circ}\text{C}$  for 30 min. The treated probes were placed into a glass tube and agitated continuously at  $68^{\circ}\text{C}$  overnight. The arrays were washed four times with prewarmed 2 $\times$  saline-sodium citrate (SSC) and 1% sodium dodecyl sulfate, and twice with 0.1 $\times$  SSC and 0.5% sodium dodecyl sulfate at  $68^{\circ}\text{C}$ , and washed with 2 $\times$ SSC (200 ml) at room temperature.

*Microarray data acquisition and processing.* The array membrane was sealed in a plastic wrap and was exposed to either X-ray film or phosphorimager screen using a Cyclone phosphorimager (PerkinElmer, Waltham, MA, USA) for 16 to 48 h. The autoradiographs were scanned, readings for each gene from different samples were obtained and analyzed using AtlasImage 1.01a software (Clontech Laboratories, Inc.) and were normalized based on the sum of signal data from all measured positions on the membrane minus background signals, as described (53, 54). Two additional normalization methods were used and are based on: (i) the sum of all nine housekeeping genes included in the array; (ii) glyceraldehyde 3-phosphate dehydrogenase or ribosome protein S9 housekeeping gene expression levels alone. Similar results were obtained from all three different applied

normalization algorithms, the global method, the sum of the housekeeping genes, and the individual housekeeping genes. Individual genes were considered upregulated or downregulated when the  $\log_2$  ratio of the differences of the average expression values between AAA and the normal aorta controls was  $\geq 1$  or  $\leq -1$ , respectively, as described in previous microarray analysis studies (37, 41), and the difference of the average expression values between aneurysmal and normal aortic tissues was greater than background level.

**Quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR).** SYBR green-mediated qRT-PCR was employed to study quantitative differences of certain genes in AAA versus normal aorta. The primers used for qRT-PCR are listed in Table II. Briefly, reverse transcription was performed with 10  $\mu\text{g}$  of total RNA from AAA or normal aorta specimens. The total RNA was primed by oligo-(dT)<sub>15</sub>-NotI (Promega, Madison, WI, USA) and a SuperScript II (GibcoBRL, Indianapolis, IN, USA) cDNA synthesis kit was used following the manufacturer's specifications. Prior to use, qRT-PCR primers (Table II) were tested by traditional PCR followed by agarose gel electrophoresis to assure that a single amplification product of appropriate size was produced. Triplicate reactions containing 2 ng of cDNA from each sample and 10 pmol of each set of PCR primers were amplified in SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) in 20  $\mu\text{l}$ . An Mx 3000P Real-Time PCR system (Stratagene) was used to carry out qRT-PCR reactions, as recommended by the manufacturer. The reaction mixture was preheated at 95°C for 10 min, followed by 45 cycles of heating at 95°C for 15 s and at 60°C for 1 min. The qRT-PCR results were normalized to the mean concentration of ribosomal protein S9 (a housekeeping gene) mRNA. Fold-enrichment values for each amplified DNA sequence were determined on the basis of the cycle threshold (Ct) for each AAA and control aorta specimens. All data shown represent the mean of triplicate determinations under the same experimental conditions. Experiments were repeated at least three times.

**DNA-based HLA-typing.** DNA was isolated from AAA specimens and DNA-based HLA-typing for *DRB1*, *DQA1* and *DQB1* loci was carried out as described elsewhere (14, 15).

**Statistical analysis of microarray and qRT-PCR results.** After global normalization of the data from each analysis (37, 41, 53, 54), the results from normal control aortas and from AAA specimens were pooled, separately. The analysis of the AAA gene array was a pilot study to determine possible targets of research into the disease. The number of subjects in this study was comparable to those used in several other microarray studies (30, 36-38, 44, 48-50) (see the Discussion). A nominal level of 0.05 was used to determine statistical significance between AAA and controls using Student's *t*-test. Genes were ranked by the calculated *t*-value and *p*-value. The Benjamini Hochberg false-discovery rate was calculated based on the results of the *t*-tests. Of 25 statistically significantly differentially expressed genes, 5% were considered to be false-positives (since a level of significance of  $p < 0.05$  was selected), leading to a false-discovery rate of 5% (determined as follows:  $0.05 \times 25$  genes = 1.25 as the number of false-positive genes;  $1.25/25$  genes with statistically different expression = 5%). Following the ranking, candidate genes were further considered using the  $\log_2$  ratio fold change (increase or decrease). The  $\log_2$  ratio was used to judge biological/clinical importance of the expression of each gene. Those with the largest (or smallest)  $\log_2$  ratio were validated using qRT-PCR.

## Results

**Differential expression of transcripts in AAA lesions versus normal aorta.** The expressions of 588 genes were studied in AAA lesions versus normal aorta. These genes were involved primarily in cardiovascular processes. Nine common house-keeping genes were also included in the microarray for normalization and reference purposes. Hybridization signals of a total of 232 genes were detected on microarrays from at least one AAA specimen, and a total of 208 genes were detected on microarrays from at least one specimen of a normal aorta. Of the expressed

Table II. Primers used for quantitative real time reverse transcriptase-polymerase chain reaction amplifications.

Gene symbol	Gene name	5' End primer	3' End primer
<b>Collagens</b>			
<i>COL1A1</i>	Collagen type I alpha 1 chain	GCTTCACCTACAGCGTCACTGTGCG	GAGGGAGTTTACAGGAAGCAGACAG
<i>COL1A2</i>	Collagen type I alpha 2 chain	AGGTGTAAGCGGTGGTGTATGAC	CCGGATACAGGTTTCGCCAGTAGAG
<i>COL2A1</i>	Collagen type II alpha 1 chain	TCAACAACCAGATTGAGAGCATCCGC	GATTGGGGTAGACGCAAGTCTCGCC
<i>COL3A1</i>	Collagen type III alpha 1 chain	AGCCTCATTAGTCTGTATGGTTCTCG	CTTCTCAGCACTAGAATCTGTCCACC
<i>COL4A1</i>	Collagen type IV alpha 1 chain	CCGATGGGTGGCCAGGATCCATG	GAAGGGCATTGTGCTGAACTTGCG
<i>COL4A4</i>	Collagen type IV alpha 4 chain	ATTGCAGTGAACCTCCAGACAGACTG	GTGTGGAGCAAAGTGTCCGTTGTG
<i>COL4A3</i>	Collagen type IV alpha 3 chain	CCAATGAACATGGCTCCCATTACTGG	CTGTAGGAATTTGAATAGTAGTTGCACG
<i>COL4A6</i>	Collagen type IV alpha 6 chain	CAGTCACCTCACAACTGAATGG	AGAGCTTCCGATCAAGACGGTAAGG
<i>COL5A1</i>	Collagen type V alpha 1 chain	CATCTGTCTCAGAGCTCCACTCAG	GCCTGTATCCTTCGATTGGCACC
<i>COL5A2</i>	Collagen type I alpha 2 chain	TCAGCAAACCCATCCAGTGTACCAC	TGAGGTTCTTAGCTTGATCGTCCATG
<i>COL6A1</i>	Collagen type VI alpha 1 chain	GGCCTGTGCCGCACTAGCCTC	CGAGTCGCCGAGATTTATGTCTGC
<i>COL6A2</i>	Collagen type VI alpha 2 chain	GTGTTTGGCGGTGGTCATCACGGAC	TCAGCGACCAGTCCGGAGAACAG
<i>COL6A3</i>	Collagen type VI alpha 3 chain	CTGGGCTAAATGGAACAACAGGACCC	ATACTCAAGACCACATCTCGCATCCG
<i>COL8A1</i>	Collagen type VIII alpha 1 chain	TGCATTTACCGCCGAGCTAACCGC	GCAGCCTGTTCTGAGGGCATCTGG
<i>COL8A2</i>	Collagen type VIII alpha 2 chain	GGGCCTTCGATGAGACTGGCATC	GTAGCCGCTGTGGCCATTGTAGAG
<i>COL9A1</i>	Collagen type IX alpha 1 chain	GGAGCAAGTTGGCATAAAGATTAATGGC	CAGCAAAGCCATCAATGTCAATTGGGC
<i>COL9A2</i>	Collagen type IX alpha 2 chain	CTATGGCTCTGCCCGCCTTACAG	CTFCGACTCCATCGACACCACCTTG
<i>COL9A3</i>	Collagen type IX alpha 3 chain	CCGAAGCACAGTGGACGGTCAATG	TGGGCTTACAGGTTTATTAGCTAGGG
<i>COL11A1</i>	Collagen type XI alpha 1 chain	GCCAAAGGGCACTTCAGGTGGCG	CACCGGTTGGTCCCTGTGGTCC
<i>COL11A2</i>	Collagen type XI alpha 2 chain	CCACCCAGAGCTTCCGATGGAG	GGCTCAGCTCATCCTCATTGGCCC
<i>COL15A1</i>	Collagen type XV alpha 1 chain	GCCACGGAGGTCAAGTCAATATGC	TAGGACAATTAGCCGATTAGCACAGC
<i>COL16A1</i>	Collagen type XVI alpha 1 chain	AGATGGGTGCAACAGGACCAATGGCC	CCAAAGGCAGGTGGGAATTTACAGCC
<b>MMPs and TIMPs</b>			
<i>MMP1</i>	Matrix metalloproteinase 1	TCAGTCTAGCTCAGGATGACATTGATG	TCAAGCCCATTTTGGCAGTTGTGGC
<i>MMP2</i>	Matrix metalloproteinase 2	GAATACCATCGAGACCATGCGGAAG	GGGTACATCGCTCCAGACTTGG
<i>MMP3</i>	Matrix metalloproteinase 3	AGAGGTGACTCCACTCACATTCTCC	ATTGGTCCCTGTTGTATCCTTTGTCC
<i>MMP7</i>	Matrix metalloproteinase 7	AGATGTGGAGTGCCAGATGTTGCAG	AAGCCAATCATGATGTCAGCAGTTCC
<i>MMP8</i>	Matrix metalloproteinase 8	CCCAAGTGGGAACGCACTAAGTTG	GCATGAGCAAGGATTCATTTGGGCT
<i>MMP9</i>	Matrix metalloproteinase 9	GCGCTACCACCTCGAECTTTGACA	CCTCAGTGAAGCGGTACATAGGGT
<i>MMP11</i>	Matrix metalloproteinase 11	GCCCTAAAGGTATGGAGCGATGTG	TCTCATCATAGTCCGAAGTGGACATCC
<i>MMP12</i>	Matrix metalloproteinase 12	ATTCTAGTGATCCAAAGGCTGTAATGTT	AAAGATCTTATTTCCACCGTAGTGAC
<i>MMP13</i>	Matrix metalloproteinase 13	TGAGAGGCTCCGAGAAATGCAGTC	CAGCAATGCCATCGTGAAGTCTGG
<i>MMP14</i>	Matrix metalloproteinase 14	GGAACCTGTAGCTTTGTGTCTGTG	TCTCTACCTCAACAAGATTAGATTCC
<i>MMP15</i>	Matrix metalloproteinase 15	GCGTGGTCTGCCCGGTGACATC	CGTTGAAGCGCCAGTACCTGTCC
<i>MMP16</i>	Matrix metalloproteinase 16	ACTGGAAGACGGTTGGATTTCTGTGC	ATGTTAATGCCATAGAAGTCTGCATG
<i>MMP17</i>	Matrix metalloproteinase 17	TACGATGACCACAGGACCATG	CATCGGCTGTGAGTCTCCACAC
<i>MMP19</i>	Matrix metalloproteinase 19	CAGCCCTCGGCTGTATGAGTTGG	CCAAGATGTGTCTGTGGGTGAGCA
<i>TIMP1</i>	TIMP metalloproteinase inhibitor 1	CCGCTGACATCCGGTTCTGTCTAC	CCAACAGTGTAGGCTTGTGGTGAAGC
<i>TIMP2</i>	TIMP metalloproteinase inhibitor 2	AGAGGATCCAGTATGAGATCAAGCAG	TGGTACCTGTGGTTACAGGCTCTTC
<i>TIMP3</i>	TIMP metalloproteinase inhibitor 3	TTCCCATTTGGTGCACGCCATTTGG	GGGAACATGTGTAGGTTGTCAGG
<i>TIMP4</i>	TIMP metalloproteinase inhibitor 4	ATTAGTCTAGCCTTTGTAGCTGTTACTG	CTGTCAAACCACTTCTGATTCTGTAC
<b>Housekeeping genes</b>			
<i>ACTB</i>	Actin beta	CTACGTGCGCCTGGACTTTCGAGC	GATGGAGCCCGGATCCACACGG
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	GGCTCTCCAGAACATCATCCCTGC	GGGTGTCTGCTGTTGAAGTCAGAGG
<i>HLAC</i>	Major histocompatibility complex, class I, C	GCAAGGATTACATCGCCCTGAACGAG	CATCATAGCCGTGACCACAGTCCAA
<i>HPRT1</i>	Hypoxanthine phosphoribosyltransferase 1	TTCTTTGCTGACCTGCTGGATTACAT	GACCATCTTTGGATTATACTGCCTGA
<i>RPL13A</i>	Ribosomal protein L13a	TAAACAGGTAAGTCTGGGCCGGAAGGTG	CACGTTCTTCTCGGCTGTTTCCGTAGC
<i>RPS9</i>	Ribosomal protein S9	GATGAGAAGGACCCACGGCTCTGTTCG	GAGACAATCCAGCAGCCAGGAGGGAC
<i>TUBA4A</i>	Tubulin alpha 4a	CACCCGTCTTCAGGGCTTCTTGGTTT	CATTTACCATCTGGTTGGCTGGCTC
<i>UBC</i>	Ubiquitin C	AGTCTACCTGCACCTGGTGTCTCCG	CCTCAAGCGCAGGACCAAGTGCAGAG
<i>YWHA1</i>	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein eta	GAGAAAATTGAGACGGAGCTAAGAGA	GCCACCTCAAGATGAAAACAGATAAC

Table II. Continued

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Gene symbol	Gene name	5' End primer	3' End primer
Other genes of interests			
<i>FLNA</i>	Filamin A	CCTCACTGTTTCTAGCCTTCAGGA	GCGCACAGCATACTTATCTTGCTC
<i>ACTN1</i>	Actinin alpha 1	GCCATGCTGCGACAGAAGGACTAT	CTTCTTGAGCAGGGCCTTGATCTC
<i>NPR1</i>	Natriuretic peptide receptor 1	GTGAACACCATCCCAGCATCCTTC	AGTGACGGAGACTCTGGCACATGG
<i>ANXA1</i>	Annexin A1	GCCAAAGACATAACCTCAGACACATC	TGGTGGTAAGGATGGTATTGAACACC
<i>ANXA6</i>	Annexin A6	AGTGAGCGAGGACCTGGTACAACAG	TCACTACGGCCAGCATTAGCTTCTC
<i>APOD</i>	Apolipoprotein D	TGGCCACCGACTATGAGAACTATGC	CCTGTAGAACCTGGTTACGAGAGC
<i>APOE</i>	Apolipoprotein E	CAATCACAGGCGAGGAAGATG	CTCTGTCTCCACCGCTTGCT
<i>BGN</i>	Biglycan	AACTGGAGGACCTGCTTCGCTACTC	GCTGATGCCGTTGTAGTAGGCCCG
<i>CAV3</i>	Caveolin 3	GGATTCTATTGTTTGTGGAAACCGTAC	CCTCATGTCAAATGGCAGTACTGGG
<i>CREBBP</i>	CREB binding lysine acetyltransferase	TGCAGTCCATCAACGAGTGGGCCG	TCGTGCTGCTAGTAGTGTAGAGGC
<i>CDH11</i>	Cadherin 11	GAGAGTCCAACCACACTTACGTCA	AGGATTCTGGAGGGTGGCAATATC
<i>CIB1</i>	Calcium and integrin binding 1	GAGTCGTCACCTTCGGGCACAAAGTG	GATGCGGAAGGCATAATGGGACTTC
<i>DCN</i>	Decorin	TCTCTACATCCGCATTGCTGATAACC	CTTGTGTTGTCCAAGTGAAGCTCCC
<i>ENG</i>	Endoglin	CGGAGCTGCTCATGCTTGTGATCC	GATATTGACCACCGCTCATTGCTG
<i>FABP4</i>	Fatty acid binding protein 4	AAAGTCAAGAGCACCATAACCTTAGATG	AGAGTTCAATCGAACTTCAGTCCAG
<i>LGALS1</i>	Galectin1	GTGGCCCTGCCCGGGAACATC	CGCCGTGGGCGTTGAAGCGAG
<i>GJA1</i>	Gap junction protein alpha 1	TTCATGCTGGTGGTGTCTTGGTG	GCTCACTTGCTTGCTTGTGTAATTGC
<i>ITGB3</i>	Integrin subunit beta 3	ACACTGGCAAGGATGCAAGTGAATTGTAC	CGTGATATTGGTGAAGGTAGACGTGGC
<i>H19</i>	H19 imprinted maternally expressed transcript	TCCACGGAGTCGGCACACTATGG	GGGCTGATGAGGTCTGGTTCCTC
<i>ICAM2</i>	Intercellular adhesion molecule 2	AAACATCTCCATGACACGGTCCCTC	CAGAGTCTCATTGCCACGGAACAGG
<i>ITGA5</i>	Integrin subunit alpha 5	GCTGAGCTTCGGGTCACCGCC	GGAAACCACGTCGCTTTCGAGTTGT
<i>ITGAL</i>	Integrin subunit alpha L	GTCAGGGCGTGGGCATCTAGTAGG	TGGAGTGAATGGCGCAATCTTGGCT
<i>LDLR</i>	Low density lipoprotein receptor	CCTTCAGTGGCCACGAACATTTTGG	CCTCATATGATTTGGCCCAATTAATCC
<i>LRP1</i>	LDL receptor related protein 1	GACACTGGTGCAGGACAACATTCA	CAGCCGCTCATTGTGATAATCCAC
<i>CCL2</i>	C-C motif chemokine ligand 2	TTCTCAAAGTGAAGCTCGACTCTCGCC	TGTGGAGTGAGTGTCAAGTCTTCGGAG
<i>MCAM</i>	Melanoma cell adhesion molecule	GAAGGGCAAGGCTGCCGTGCAGG	TGGGATGAGCTTCACTCAACGTGGAG
<i>CYP1A1</i>	Cytochrome P450 family 1	CCATGTCCGGCCAGGAGTTTCTTC	CCCCATACTGCTGGCTCATCCTTG
<i>PDGFA</i>	Platelet derived growth factor subunit A	GCATCCGGGACCTCCAGCGACTCCT	AGGCTTGTGGTCCGCGAGGCGCACT
<i>PDGFRB</i>	Platelet derived growth factor receptor beta	ACGCAGTGCAGACTGTGGTCCGCCA	CGTAGCCGCTCAACCACGGTATGT
<i>PLA2G1B</i>	Phospholipase A2 group IB	AACTACGGCTGCTACTGTGGCTTGG	AAAGCAGATGGCAGCGTTGCGGTC
<i>PLA2G2A</i>	Phospholipase A2 group IIA	TGCATTTGTCAACCAAGAACTTACC	CCCGAGTTGCTAAACTTGTAGCTCAG
<i>PLTP</i>	Phospholipid transfer protein	CACGTTTCATACCTCAGGGA	CAGGAGGGAGTTGAGCAGGA
<i>SOD3</i>	Superoxide dismutase 3	GAGCACTCAGAGCGCAAGAA	TGCCTTTGAGCTTCTCTCT
<i>TNC</i>	Tenascin C	ATGCCAAGACTCGCTACAAGCTGA	GCACAGTTGGTGTGGCTGAATCT
<i>PLAU</i>	Urokinase-type plasminogen activator	TAGCCAATGTGGGAGCAGCGTTTGG	ATAAGTACATTTCCAGGCACCTGTCACGT
<i>VWF</i>	von Willebrand factor	ACAGATCATGACTGAAGCGTGATG	TGGCAGTGATGCTGTTGCACTCAG

Primers were provided by Clontech Laboratories, Inc. Certain primers were reported in the literature, or designed by the Authors.

genes, 35 were differentially expressed, on the basis of the criteria mentioned elsewhere (37, 41, 53, 54), with either  $\log_2$  ratio of AAAs/controls  $\geq 1$ , *i.e.* upregulated, or  $\leq -1$  *i.e.* downregulated. In 25 out of these 35 genes (71%), the differences were statistically significant ( $p \leq 0.05$ ) (Table III and Figure 1). Results from all AAA specimens and all normal aorta controls are shown in Table III.

Twenty of these 35 genes (57%) were upregulated (Table III and Figure 2) in AAA lesions compared with normal aorta. These results were statistically significant

( $p \leq 0.05$ ) for 16 out of these 20 genes (80%). The expression of the remaining 15 genes (43%) was downregulated (Table III and Figure 3) in AAA lesions *versus* normal aorta. These results were statistically significant ( $p \leq 0.05$ ) for nine out of these 15 genes (60%). Our studies are focused on identifying differential expressions, as designated by the  $\log_2$  ratio of AAAs/controls of cardiovascular transcripts, and not on the strong or weak expression of transcripts in both AAA and normal aorta controls.

Table III. Microarray results of differentially expressed genes in abdominal aortic aneurysms (AAAs) versus normal aortas.

Class and gene name	Gene symbol	Ratio	Log <sub>2</sub> ratio	p-Value	Class and gene name	Gene symbol	Ratio	Log <sub>2</sub> ratio	p-Value
Matrix metalloproteinases and TIMP metalloproteinase inhibitors					Apolipoprotein D	APOD	2.67	1.42	0.018
Matrix metalloproteinase 9	MMP9	11.50	3.52	0.025	Phospholipid transfer protein	PLTP	2.84	1.51	0.010
TIMP Metalloproteinase inhibitor 3	TIMP3	3.07	1.62	0.0014	Phospholipase A2 group IIA	PLA2G2A	4.42	2.14	0.050
Collagens					Galectin-1	LGALS1	0.11	-3.18	0.037
Collagen type I alpha 1 chain	COL1A1	2.22	1.15	0.017	Low-density lipoprotein receptor-related protein 1	LRP1	0.17	-2.56	0.073
Collagen type IV alpha 4 chain	COL4A4	0.15	-2.74	<0.001	Acetyl-CoA acyltransferase	ACAA	0.44	-1.18	0.075
Collagen type VI alpha 3 chain	COL6A3	6.09	2.61	0.032	Coagulation and fibrinolysis				
Collagen type XI alpha 2 chain	COL11A2	0.14	-2.84	0.109	Von Willebrand factor	VWF	3.53	1.82	0.159
Collagen type XIV alpha 1 chain	COL15A1	4.53	2.18	0.039	Calcium and integrin binding 1	CIB1	2.30	1.20	0.018
Extracellular matrix proteins, adhesion molecules and cell surface antigens					Serpin family B member 6	SERPINB6	4.07	2.03	0.026
Intercellular adhesion molecule 2	ICAM2	4.22	2.08	0.024	Urokinase-type plasminogen activator	PLAU	5.43	2.44	0.103
Intercellular adhesion molecule 1	ICAM1	2.47	1.30	0.109	Serpin family E member 1	SERPINE1	0.27	-1.87	0.16
Decorin	DCN	4.80	2.26	0.0046	Other				
Endoglin	ENG	2.04	1.03	0.086	H19 imprinted maternally expressed transcript	H19	4.42	2.14	0.006
Gap junction protein alpha 1	GJA1	0.32	-1.64	0.001	C-C motif chemokine ligand 2	CCL2	9.05	3.18	0.012
Biglycan	BGN	0.48	-1.06	0.139	Platelet derived growth factor receptor beta	PDGFRB	2.05	1.04	0.039
Integrin subunit alpha 8	ITGA8	0.23	-2.12	0.057	Melanoma cell adhesion molecule	MCAM	0.14	-2.84	0.021
Fatty acid metabolism					Sodium channel epithelial 1 subunit beta	SCNN1B	0.35	-1.51	0.0016
Apolipoprotein E	APOE	2.21	1.14	0.044	Natriuretic peptide receptor 1	NPR1	0.19	-2.40	0.039
					Superoxide dismutase 3	SOD3	0.36	-1.47	0.050
					Actinin alpha 1	ACTN1	0.16	-2.64	0.028
					Phospholamban	PLN	0.23	-2.12	0.012

Results were calculated and statistically analyzed as described in the Materials and Methods. Ratios were calculated for all AAAs/all normal aortas from the scanning results of the autoradiographs, which were analyzed using AtlasImage 1.01a software, as described in the Materials and Methods. Statistical significance was determined using Student's *t*-test at  $p \leq 0.05$ . Positive ratios represent upregulation of genes and negative values represent downregulation.

**Tissue remodeling.** The expression of *MMP9* was upregulated in AAA lesions versus normal aorta controls and these results were statistically significant ( $\log_2$  ratio=3.52,  $p=0.025$ ) (Table III and Figure 2). Increased expression of *MMP14* in AAA lesions was also observed, although it was not statistically significant (data not shown). *TIMP1*, *TIMP2*, *TIMP3* and *TIMP4* expression was investigated. The first three types of TIMPs were expressed robustly in both AAA lesions and normal aorta, but only *TIMP3* was statistically significantly upregulated in AAA lesions ( $\log_2$  ratio=1.62,  $p=0.0014$ ) (Table III). Results obtained with *TIMP1*, *TIMP2*, and *TIMP4* are not shown.

**Collagens.** The expression of 3 out of 25 genes encoding different collagen subunits examined in the microarrays were increased, in a statistically significant manner, in AAA lesions versus normal aorta. These were collagen type I  $\alpha$  1 chain (*COL1A1*) ( $\log_2$  ratio=1.15,  $p=0.017$ ), *COL6A3* ( $\log_2$  ratio=2.61,  $p=0.032$ ), and *COL15A1* ( $\log_2$  ratio=2.18,  $p=0.039$ ) (Table III and Figure 2). Two collagen subunits examined in the microarrays were reduced in AAA lesions, however, the decrease was statistically significant only for *COL4A4* ( $\log_2$  ratio=-2.74,  $p<0.001$ ), but not *COL11A2* ( $\log_2$  ratio=-2.84,  $p=0.109$ ) (Table III and Figure 3). *COL1A1* was the collagen transcript with the highest expression in both AAA lesions and normal aortas (data not shown).

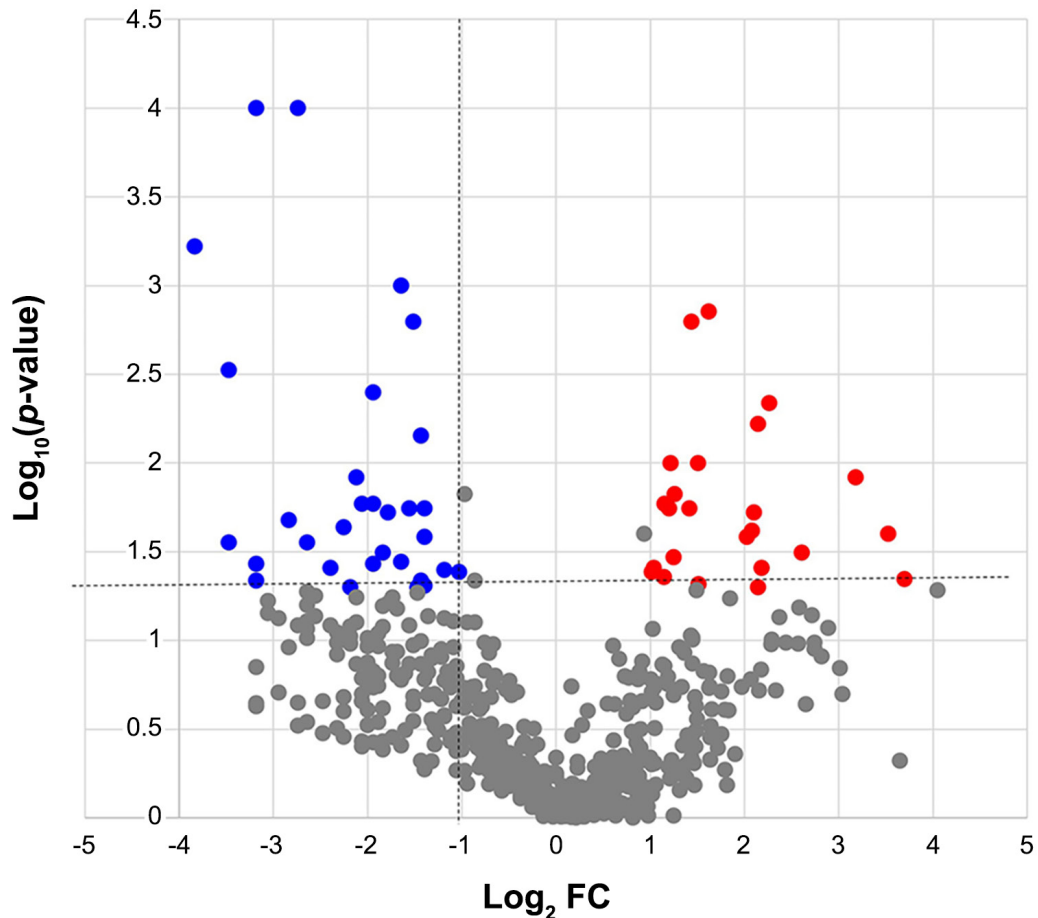


Figure 1. Volcano plot of gene expression by microarray analysis. Red colored dots represent genes in abdominal aortic aneurysm that were statistically significantly upregulated compared with normal aortas. Blue colored dots represent genes statistically significantly downregulated. Grey colored dots represent genes with a fold-change (FC) of less than 2 ( $\log_2 < 1$ ), or genes with no statistically significant change.

Extracellular matrix (ECM) proteins, adhesion molecules and cell-surface antigens. ECM proteins and cell-adhesion molecules interact with each other forming a complex network important in maintaining the normal structure and function of the aorta. The transcripts of ECM or adhesion molecules upregulated in AAA lesions versus normal aorta were the following: intercellular adhesion molecule 2 (*ICAM2*) ( $\log_2$  ratio=2.08,  $p=0.024$ ), *ICAM1* ( $\log_2$  ratio=1.30,  $p=0.109$ ), decorin (*DCN*) ( $\log_2$  ratio=2.26,  $p=0.0046$ ), and endoglin (*ENG*) ( $\log_2$  ratio=1.03,  $p=0.086$ ) (Table III and Figure 2). Of the transcripts of ECM or adhesion molecules downregulated in AAA lesions, a

statistically significant decrease was observed for gap junction protein  $\alpha$  1 (*GJA1*) ( $\log_2$  ratio=-1.64,  $p=0.001$ ), while non statistically significant downregulation changes were observed for biglycan (*BGN*) ( $\log_2$  ratio=-1.06,  $p=0.139$ ), and integrin subunit  $\alpha$  8 (*ITGA8*) ( $\log_2$  ratio=-2.12,  $p=0.057$ ) transcripts (Table III and Figure 3).

**Fatty acid metabolism.** Transcription of the following genes of fatty acid metabolism was upregulated in AAA lesions vs. normal aortas: apolipoprotein E (*APOE*) ( $\log_2$  ratio=1.14,  $p=0.044$ ), *APOD* ( $\log_2$  ratio=1.42,  $p=0.018$ ), phospholipid transfer protein (*PLTP*) ( $\log_2$  ratio=1.51,  $p=0.01$ ), and

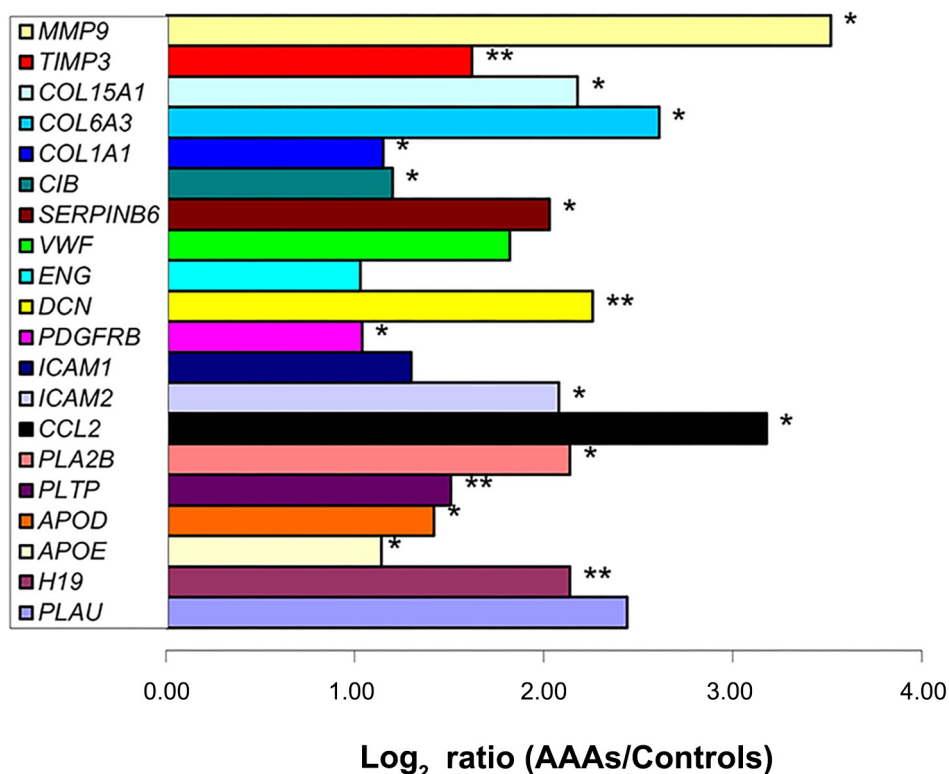


Figure 2. Genes upregulated in abdominal aortic aneurysms (AAA) as compared with normal aortas. Transcript levels were determined and the results were analyzed as described in the Materials and Methods. Statistical significance was determined using Student's t-test: \* $p \leq 0.05$  and \*\* $p \leq 0.01$ . APOD: Apolipoprotein D; APOE: apolipoprotein E; CCL2: C-C motif chemokine ligand 2; CIB1: calcium and integrin binding 1; COL15A1: collagen type XV  $\alpha$  1 chain; COL1A1: collagen type I  $\alpha$  1 chain; COL6A3: collagen type VI  $\alpha$  3 chain; DCN: decorin; ENG: endoglin; H19: H19 imprinted maternally expressed transcript; ICAM1: intercellular adhesion molecule 1; ICAM2: intercellular adhesion molecule 2; MMP9: matrix metalloproteinase 9; PDGFRB: platelet-derived growth factor receptor beta; PLA2G2A: phospholipase A2 group IIA; PLAU: urokinase-type plasminogen activator; PLTP: phospholipid transfer protein; SERPINB6: serpin family B member 6; TIMP3: TIMP metalloproteinase inhibitor 3; VWF: von Willebrand factor.

phospholipase A2 group IIA (PLA2G2A) ( $\log_2$  ratio=2.14,  $p=0.05$ ) (Table III and Figure 2). Transcription of galectin-1 (LGALS1) ( $\log_2$  ratio=-3.18,  $p=0.037$ ), low-density lipoprotein receptor-related protein-1 (LRP1) ( $\log_2$  ratio=-2.56,  $p=0.073$ ), and acetyl-CoA acyltransferase (ACAA) ( $\log_2$  ratio=-1.18,  $p=0.075$ ) was downregulated in AAA versus normal aorta (Table III and Figure 3).

**Blood coagulation and fibrinolysis.** Several genes related to blood coagulation and fibrinolysis exhibit different levels of expression in AAA lesions compared with normal aortas. The expression of the following genes was upregulated in AAA lesions: (i) calcium and integrin binding 1 (CIB1) ( $\log_2$  ratio=1.20,  $p=0.018$ ); serpin family B member 6 (SERPINB6)

( $\log_2$  ratio=2.03,  $p=0.026$ ), and both these increases were statistically significant; and (ii) von Willebrand factor (VWF) ( $\log_2$  ratio=1.82,  $p=0.159$ ), urokinase-type plasminogen activator (PLAU) ( $\log_2$  ratio=2.44,  $p=0.103$ ), however, both these increases were not statistically significant (Table III and Figure 2). In contrast, the expression of serpin family E member 1 (SERPINE1) was downregulated ( $\log_2$  ratio=-1.87,  $p=0.16$ ) in AAA lesions versus normal aorta but this decrease was not statistically significant (Table III and Figure 3).

**Other genes assayed.** Our studies focused on the study of the expression of genes related to the cardiovascular system in AAA lesions and in normal aortas and determinations to

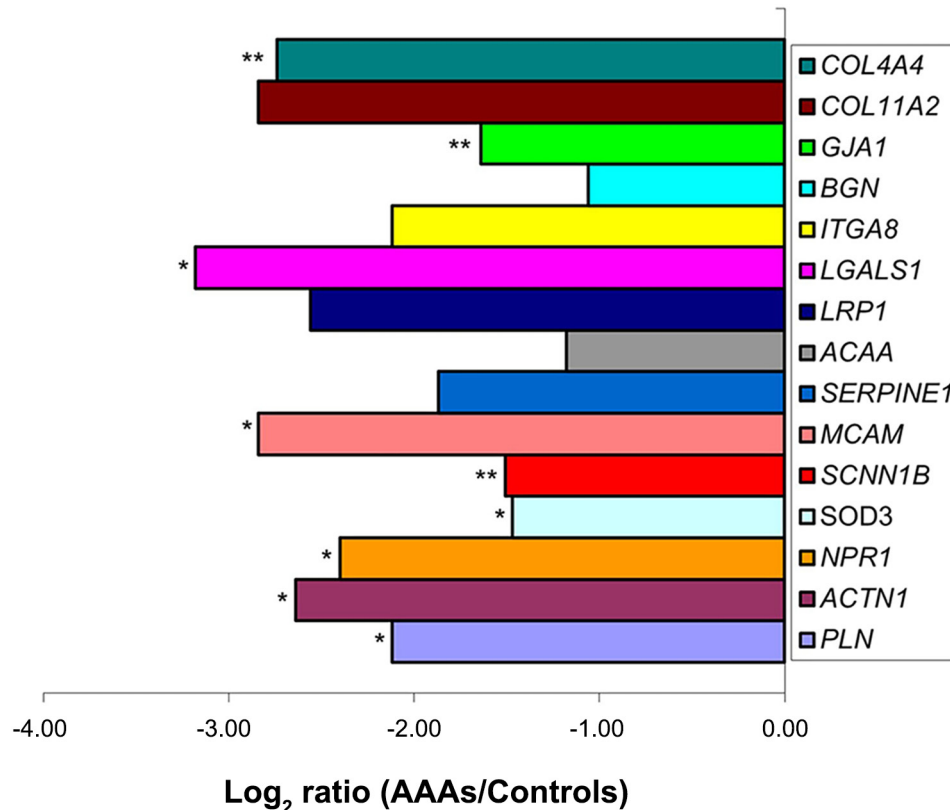


Figure 3. Genes downregulated in abdominal aortic aneurysms (AAA) as compared with normal aortas. Transcript levels were determined, and the results were analyzed as described in the Materials and Methods. Statistical significance was determined using Student's *t*-test: \* $p \leq 0.05$  and \*\* $p \leq 0.01$ . ACAA: Acetyl-CoA acyltransferase; ACTN1: actinin  $\alpha$  1; BGN: biglycan; COL11A2: collagen type XI  $\alpha$  2 chain; COL4A4: collagen type IV  $\alpha$  4 chain; GJA1: gap junction protein  $\alpha$  1; ITGA8: integrin subunit  $\alpha$  8; LGALS1: galectin-1; LRP1: low-density lipoprotein receptor-related protein 1; MCAM: melanoma cell adhesion molecule; NPR1: natriuretic peptide receptor 1; PLN: phospholamban; SCNN1B: sodium channel epithelial 1 subunit beta; SERPINE1: serpin family E member 1; SOD3: superoxide dismutase 3.

examine the expression of genes of cytokines, chemokines and their receptors, and of growth factors were limited. The expression of the H19 imprinted maternally expressed transcript (*H19*) gene was upregulated in AAA lesions compared with normal aorta, and this increase was statistically significant ( $\log_2$  ratio=2.14,  $p=0.006$ ) (Table III and Figure 2). C-C motif chemokine ligand 2 (*CCL2*) ( $\log_2$  ratio=3.18,  $p=0.012$ ) and platelet-derived growth factor receptor beta (*PDGFRB*) ( $\log_2$  ratio=1.04,  $p=0.039$ ) were upregulated in AAA lesions.

Genes downregulated in AAA lesions included melanoma cell adhesion molecule (*MCAM*) ( $\log_2$  ratio=-2.84,  $p=0.021$ ), sodium channel epithelial 1 subunit

beta (*SCNN1B*) ( $\log_2$  ratio=-1.51,  $p=0.0016$ ), natriuretic peptide receptor 1 (*NPR1*) ( $\log_2$  ratio=-2.40,  $p=0.039$ ), superoxide dismutase 3 (*SOD3*) ( $\log_2$  ratio=-1.47,  $p=0.05$ ), actinin  $\alpha$  1 (*ACTN1*) ( $\log_2$  ratio=-2.64,  $p=0.028$ ), and cardiac phospholamban (*PLN*) ( $\log_2$  ratio=-2.12,  $p=0.012$ ) (Table III and Figure 3). These results were statistically significant.

*qRT-PCR confirmation of microarray results.* To confirm the microarray results of the expression of certain genes that were upregulated or downregulated in AAA lesions, we employed qRT-PCR using AAA tissue from a patient (AAA10) and control normal aortic tissue (aorta-1). Representative qRT-PCR results are summarized and

Table IV. Comparison of microarray and quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR) results of gene-expression profiles in abdominal aortic aneurysm (AAA) lesions from patient AAA10 and normal control (aorta-1).

Gene	Gene symbol	Microarray		qRT-PCR	
		Ratio*	Log <sub>2</sub> ratio*	Ratio*	Log <sub>2</sub> ratio*
<b>Upregulated genes</b>					
Matrix metalloproteinase 9	MMP9	5.64	2.50	26.23	4.71
TIMP metalloproteinase inhibitor 3	TIMP3	3.68	1.88	3.55	1.83
Collagen type XV alpha 1 chain	COL15A1	6.43	2.69	4.74	2.25
Collagen type VI alpha 3 chain	COL6A3	14.75	3.88	1.34	0.42
Collagen type I alpha 1 chain	COL1A1	1.72	0.78	2.23	1.16
von Willebrand factor	VWF	11.41	3.51	9.93	3.31
Decorin	DCN	12.18	3.61	19.05	4.25
Intercellular adhesion molecule 2	ICAM2	1.87	0.90	5.37	2.42
C-C motif chemokine ligand 2	CCL2	6.22	2.64	10.77	3.43
Phospholipid transfer protein	PLTP	4.33	2.12	4.09	2.03
Apolipoprotein D	APOD	5.43	2.44	3.05	1.61
Apolipoprotein E	APOE	4.32	2.11	2.97	1.57
H19 imprinted maternally expressed transcript	H19	8.00	2.96	9.38	3.23
Urokinase-type plasminogen activator	PLAU	2.73	1.45	6.95	2.80
Phospholipase A2 group IIA	PLA2G2A	9.91	3.31	2.83	1.5
<b>Downregulated genes</b>					
Natriuretic peptide receptor 1	NPR1	0.11	-3.22	0.02	-5.45
Superoxide dismutase 3	SOD3	0.20	-2.31	0.36	-1.47
Low-density lipoprotein receptor-related protein 1	LRP1	0.11	-3.13	0.06	-4.09
Galectin-1	LGALS1	0.06	-4.17	0.46	-1.12
Melanoma cell adhesion molecule	MCAM	0.01	-6.11	0.07	-3.76
Biglycan	BGN	0.21	-2.23	0.05	-4.33
Actinin alpha 1	ACTN1	0.08	-3.61	0.13	-2.99
Gap junction protein alpha 1	GJA1	0.65	-0.63	0.55	-0.86
Ribosomal protein S9 (house-keeping gene)	RPS9	1.00	0	1.00	0

AAA10 data were adjusted by house-keeping gene S9. \*Ratio of AAA10/aorta-1.

compared to the microarray results in Table IV, Figure 4 and Figure 5. The qRT-PCR results were in good agreement in general with those obtained from the microarray analysis,

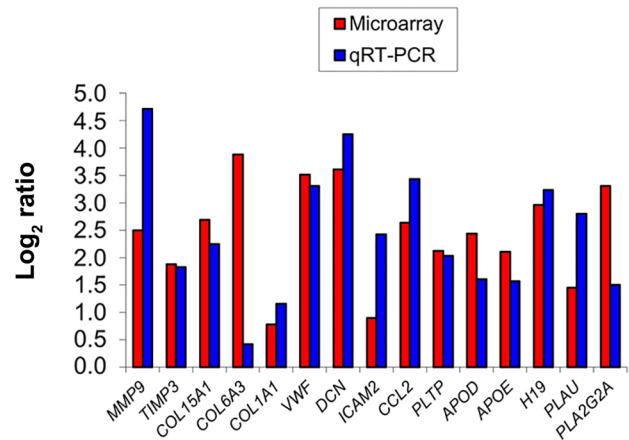


Figure 4. Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) confirmation of genes found by microarray analysis to be upregulated. Log<sub>2</sub> ratios of gene expression in a patient with abdominal aortic aneurysm (AAA10) to those in control aorta (aorta 1) by microarray analysis and qRT-PCR analysis. APOD: Apolipoprotein D; APOE: apolipoprotein E; CCL2: C-C motif chemokine ligand 2; COL15A1: collagen type XV  $\alpha$  1 chain; COL1A1: collagen type I  $\alpha$  1 chain; COL6A3: collagen type VI  $\alpha$  3 chain; DCN: decorin; H19: H19 imprinted maternally expressed transcript; ICAM2: intercellular adhesion molecule 2; MMP9: matrix metalloproteinase 9; PLA2G2A: phospholipase A2 group IIA; PLAU: urokinase-type plasminogen activator; PLTP: phospholipid transfer protein; TIMP3: TIMP metalloproteinase inhibitor 3; VWF: von Willebrand factor.

with relatively few exceptions. The AAA10/aorta-1 ratios were calculated based on triplicate determinations under the same conditions. Analyses were repeated three times; representative data are shown. Additional analyses were carried out on other genes not listed in Table IV (data not shown). qRT-PCR is substantially more sensitive than cDNA microarrays and requires 1,000-fold less RNA than DNA microarrays (55). We used 2 ng cDNA for each qRT-PCR and 5  $\mu$ g RNA for each microarray. Furthermore, certain differences between the log<sub>2</sub> ratio of AAAs/normal aortas obtained using microarray compared with qRT-PCR (Table IV, Figure 4 and Figure 5) may be, at least in part, due to the different hybridization kinetics of the probe sets for each gene (56).

The qRT-PCR results of the expression of H19 gene and housekeeping gene S9 in AAA lesions from patient AAA10 versus normal tissue from aorta-1 are shown in Figure 6. The Ct values of S9 were very similar (27.15 and 27.20, respectively), indicating that the original

template transcripts for qRT-PCR in both tissues were quantitatively similar (Figure 6). However, the Ct values of *H19* were different by 3.7 cycles, indicating that the original template transcripts of *H19* in AAA10 lesions were at an approximately 12.9-fold higher level than in normal aorta-1, along the lines, in general, with the results from microarray analysis (7.8-fold) (Figure 6). Differences in hybridization kinetics would explain the differences observed between the  $\log_2$  ratio of AAAs/normal aortas obtained using microarrays versus those with qRT-PCR in Figure 6, as was discussed above.

**DNA-based HLA-typing for HLA-DRB1, -DQA1 and -DQB1.** Patients AAA02, AAA09 and AAA10 with AAA were HLA-typed by DNA-based approaches for HLA-DRB1, HLA-DQA1 and HLA-DQB1 (Table V). All these three patients expressed *DRB1* alleles with the DR $\beta$ Gln70 amino acid residue that has been associated with AAA (19). These results have been reported previously (14) as part of a larger series and are included here to present a more complete picture.

## Discussion

Cardiovascular microarray was utilized to study the gene-expression profiles in AAA lesions from patients with AAA and compare them to normal aorta. We selected to focus our studies on the expression primarily of cardiovascular genes because the expression of genes associated with immune-related pathways, leukocyte migration, adhesion molecules, cytokines, chemokines and their receptors, ECM degradation, and others, have already been investigated to a substantially greater extent (30-44). The focus of the study on the expression of primarily cardiovascular genes limited the number of genes studied to 588. We identified for the first time in this study 14 genes which were upregulated or downregulated in AAA lesions compared with normal aortas, which were not reported previously. These genes were: *COL6A3*, *COL15A1*, *ICAM2*, *APOD*, *CIB1*, *SERPINB6*, *COL11A2*, *GJA1*, *ITGA8*, *ACAA*, *SCNN1B*, *NPR1*, *ACTN1* and *PLN*. The first six of these genes were

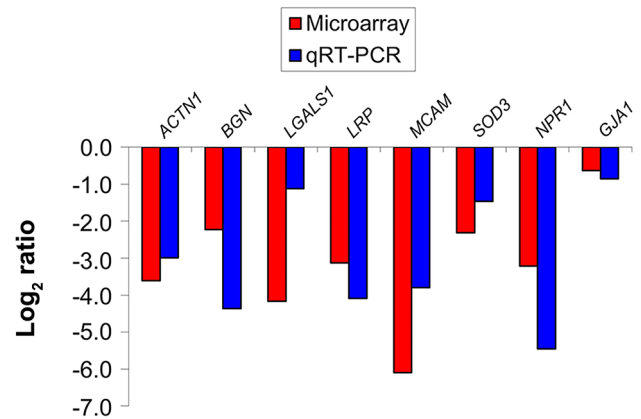


Figure 5. Quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR) confirmation of genes found by microarray analysis to be downregulated.  $\log_2$  ratios of gene expression in a patient with abdominal aortic aneurysm (AAA10) to those in control aorta (aorta 1) by microarray analysis and qRT-PCR analysis. ACTN1: Actinin  $\alpha$  1; BGN: biglycan; GJA1: gap junction protein  $\alpha$  1; LGALS1: galectin-1; LRP1: low-density lipoprotein receptor-related protein 1; MCAM: melanoma cell adhesion molecule; NPR1: natriuretic peptide receptor 1; SOD3: superoxide dismutase 3.

upregulated and the remaining eight were downregulated in AAA compared with normal aorta (Table III). The differential expression (upregulation or downregulation) in AAA lesions was statistically significant in 11 out of these 14 genes (Table III). Studies are needed to identify further the role of these genes in AAA.

Twenty-one genes that were differentially expressed (either upregulated or downregulated) in AAA lesions were also identified; these were previously reported in the literature and confirmed in this study. These genes were the following (Table III): *MMP9*, *TIMP3*, *COL1A1*, *COL4A4*, *ICAM1*, *DCN*, *ENG*, *BGN*, *APOE*, *PLTP*, *PLA2G2A*, *LGALS1*, *LRP1*, *VWF*, *PLAU*, *SERPINE1*, *H19*, *CCL2*, *PDGFRB*, *MCAM* and *SOD3*. These microarray results were confirmed by qRT-PCR.

As was mentioned previously, the number of subjects in this study was small ( $n=5$ , see Table I) but comparable to the number used in several earlier microarray studies (30, 36-38, 44), including those using microarray datasets of gene-expression profiles to identify hub genes (48), to carry out weighted gene co-expression network analysis (49), and to perform GeneSet enrichment analysis with the objective of identifying potential key genes and pathways

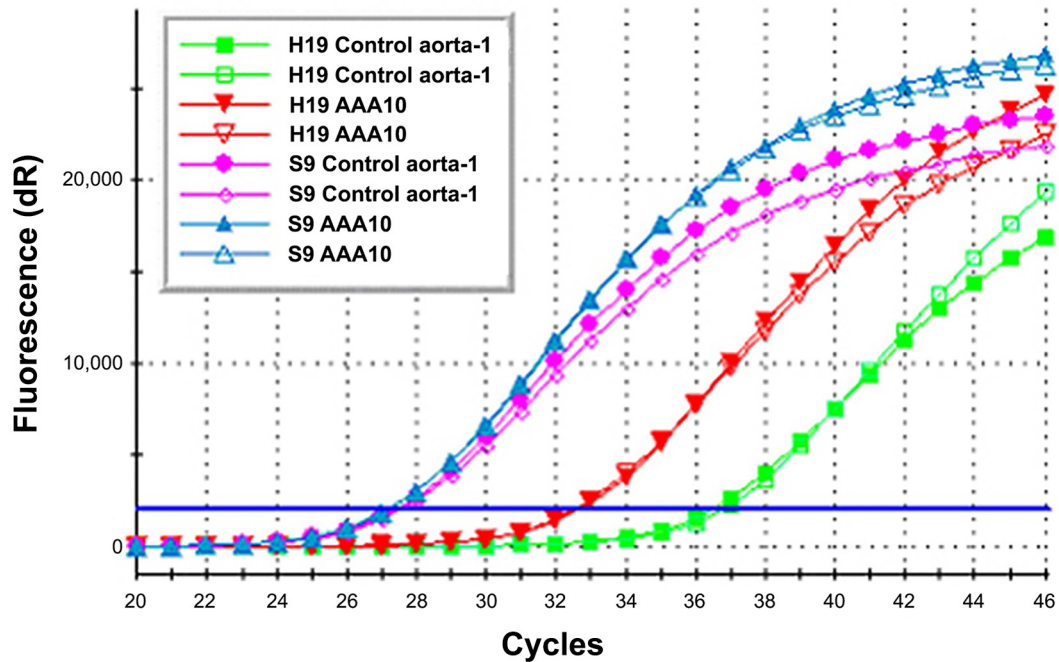


Figure 6. Quantitative real time reverse transcriptase-polymerase chain reaction results of *H19* imprinted maternally expressed transcript (*H19*) and ribosomal protein *S9* genes in a patient with abdominal aortic aneurysm (AAA10) and in control aorta (aorta 1). Note the blue horizontal line is the detection threshold. The crossing point of the blue line and amplification curve is the *Ct* value.

in AAA (49, 50). Moreover, as reported in the previous paragraph, we confirmed the results of others with 21 genes that were either upregulated or downregulated. The agreement of our results with these 21 genes reported in previously published studies, validates our approach regarding the upregulation or downregulation of the 14 additional genes that we report here.

The structural components of aorta (collagens, elastin, smooth muscle, others), and proteolytic enzymes (MMPs) and their tissue inhibitors are actively involved in tissue remodeling in AAA, and have been studied previously (30-44). Their expression was altered in AAA, providing additional evidence for their role in the pathogenesis of AAA. Other participants include molecules involved in fatty acid metabolism, blood coagulation and others. We would like to discuss further certain of these molecules.

Adhesion molecules play a critical role in the infiltration of mononuclear cells in the aortic wall in AAA (57). *ICAM1*, -2 (Table III), and -3 (data not shown) were

upregulated in this study, with *ICAM2* reaching statistical significance ( $\log_2$  ratio=2.08,  $p=0.024$ ). *CD18*, the subunit of specific ligand for *ICAM1*, was also upregulated (data not shown). The role of *ICAM2* in the pathogenesis of AAA has not been previously reported. However, *ICAM1* and *CD18* expression was elevated in a previous study (37). ICAMs are consistently associated with both acute and chronic inflammation in many diseases and in AAA (44, 58) and participate in T-cell activation and proliferation, in addition to mediating the adhesive interaction between leukocytes and endothelial cells, a vital step for leukocyte extravasation through the endothelial layer to sites of inflammation (59). Once adhesion of leukocytes to the endothelium is complete, the next step is diapedesis into the *intima*. This is regulated by chemokines and adhesion molecules (60). *CCL2*, also known as monocyte chemoattractant protein 1, is an active player in this process (61), and its expression has been correlated with macrophage densities in the media and adventitia, in both

Table V. DNA-based typing for human leukocyte antigen (HLA)-DRB1, HLA-DQA1, and HLA-DQB1 loci of patients with abdominal aortic aneurysm (AAA)\*.

Patient ID	DRB1-1	DRB1-2	DQA1-1	DQA1-2	DQB1-1	DQB1-2	DRβQ70
AAA02	0301	1104	0501	0505	0201	0301	+
AAA09	0101	0701	0101	0201	0202	0501	+
AAA10	0301	1501	0102	0501	0201	0602	+

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atherosclerosis and elastase-induced AAA (62). *CCL2* was significantly upregulated ( $\log_2$  ratio=3.18,  $p=0.012$ ) in AAA tissue versus normal aorta controls.

Long non-coding RNA *H19*, an imprinted maternally expressed untranslated mRNA, is abundant in extraembryonic and fetal tissues, but it drops significantly after birth. Recent studies show *H19* might be involved in tumorigenesis, cardiovascular disease, chronic inflammation, metabolic disorders, among others (reviewed in 63-66). *H19* is one of the most highly upregulated transcripts in two mouse aneurysm models (67), is increased in human and mouse AAA, and the *H19*/LET-7a/interleukin-6 (IL6) pathway may be potentially pathogenic in AAA (68). *ACTN1*, an actin-binding protein, was significantly downregulated in AAA (Table III), and in saccular intracranial aneurysms (69). *LGALS1* exhibits anti-inflammatory activity by inhibiting chemotaxis and transendothelial leukocyte migration (70). The expression of *LGALS1* was downregulated in AAA lesions (Table III). However, in another study, *LGALS1* was upregulated in AAA and contributed to the progression of AAA (71). *MCAM* is mostly located in the epithelial cells of the *intima*. It is involved in angiogenesis and metastasis of certain tumors. *MCAM* mRNA and protein expression were found to be significantly downregulated in AAA in comparison to normal arteries (72). Our findings are in agreement with these.

Evidence has accumulated suggesting that AAA is an autoimmune (1-6, 10) trimolecular complex (TCR/peptide/major histocompatibility complex) disease (10-20, 26, 73-83). We have suggested that AAA is a specific antigen-driven T-cell disease (10). Molecular mimicry [reviewed in (84, 85)] may be involved (26). Chronic

inflammatory mechanisms in AAA are typical to those in autoimmune disease (86) and the antitumor response (87). The autoimmune hypothesis is becoming more popular for the etiology of AAA versus the atherogenic theory (6, 12, 23, 33, 39, 77, 79, 80).

We used fresh (cryopreserved for up to 2 weeks) AAA specimens and normal aortas, and not cells expanded in culture, because this may result in selection of particular cell populations. For example, when compared with fresh T-cells from the same donor, different T-cells expanded in culture with recombinant IL2, produced different cytokines and exhibited different properties (88). This may be the case with other cell types. T-cell clones with different properties exhibit different growth rates in culture with recombinant IL2 (89). T-cells exhibit cytotoxic and helper functions and produce directly or indirectly – by affecting other cells – a large number of molecules responsible for the destruction of the aortic wall. Studies to characterize genes expressed in AAA lesions have been carried out in general using total (unseparated) populations of cells (30, 33-44, 48, 49). Limited studies (12, 50, 77) investigating the differential expression of genes in AAA at single cell level have been reported, and they are focused primarily on immune cells, more specifically on T-cells. Additional single cell studies are needed. Microarrays have been used to study gene expression in AAA, including studies on differential gene expression in AAA tissues and normal aortas (35, 37), ruptured AAA and anterior sac as control (38), and in comparison with aortic occlusive disease (44), small AAA ( $\leq 55$  mm), large AAA ( $> 55$  mm), aortic occlusive disease versus normal aortas (41), or from patients with thoracic aortic aneurysm (42) compared with normal aortas.

A large number of loci are associated in genome-wide association study (GWAS) with AAA (90-92). A total of 33 single nucleotide polymorphisms have been identified in AAA (92). We searched for differentially expressed genes in AAA *versus* control aorta tissues in this and other (35-44) microarray or individual gene studies, which were also associated with AAA by GWAS. A limited number of such genes were found. These were *LRP*, *APOE* and *PLTP*.

LRP is a member of the low-density lipoprotein receptor family and can bind to many ligands including APOE, PDGF and MMP. LRP1 is associated in GWAS with AAA (91-93), but this association was not observed by others (90). The lead SNP is associated with downregulation of *LRP1* expression in aortic adventitia (93). LRP1-knockout mice displayed distended and dilated aortas and eventually developed AAA (94). Reduced LRP protein, but not mRNA, was found in human AAA (95). In our study *LRP1* transcripts were downregulated in AAA ( $\log_2$  ratio=-2.56), although the results were not statistically significant ( $p=0.073$ ) (Table III).

APOE regulates cholesterol and lipid homeostasis. It is associated with AAA by GWAS (91). APOE-knockout mice have long been used as an animal model to study AAA pathogenesis and therapeutic intervention (96). *APOE* expression was increased by 13-fold in AAA tissue by cDNA expression microarray analysis (37). Our results are in agreement with increased *APOE* expression in AAA.

PLTP is a cardiovascular risk factor and plays an important role in lipoprotein and high-density lipoprotein remodeling. It is associated with AAA by GWAS (90). Increased *PLTP* expression, similar to the results in our study, has been reported in AAA lesions *versus* control aorta (90). The development of AAA is reduced in mice with PLTP deficiency and is associated with reduced elastin degradation, infiltration by macrophages and CD4<sup>+</sup> T-cells, vascular smooth muscle cell depletion, and MMP expression and activity (97).

Differentially expressed genes are perhaps associated with propagation of AAA and are more involved in end-stage disease. Single nucleotide polymorphisms identified by GWAS may be involved in the initiation of AAA.

## Conclusion

We studied the expression of 588 genes related to the cardiovascular system in AAA lesions and in normal aorta controls by microarray, and validated the altered expression of certain genes by qRT-PCR. Many genes are involved in the pathogenesis of AAA, including genes associated with tissue remodeling, structural proteins, inflammatory and immune-related pathways, intracellular signaling, ECM degradation, leukocyte migration, lipid metabolism, adhesion molecules, cytokines, chemokines and their receptors, fatty acid metabolism, blood coagulation, and others (24-39). We have identified certain genes with differential expression in AAA that were not previously reported. Additional studies are needed to elucidate further the role of these genes in the development of AAA.

## Supplementary Material

All genes are listed in the Supplementary Table, available at: [https://drive.google.com/file/d/1rBZSRJwm7Rlx7A4yi6DPvTuL\\_kuhm0T1/view?usp=sharing](https://drive.google.com/file/d/1rBZSRJwm7Rlx7A4yi6DPvTuL_kuhm0T1/view?usp=sharing)

## Conflicts of Interest

The Authors have no conflicts of interest to declare.

## Authors' Contributions

All Authors materially participated in the research/article preparation and have reviewed and approved the final article. Song Lu, Chris D. Platsoucas, and Emilia L. Oleszak wrote the manuscript. Song Lu, Chris D. Platsoucas and Emilia L. Oleszak designed the study. Song Lu, Li Ping Li, Xiaoying Zhang, Ifeyinwa Nwaneshiudu, Adaobi Nwaneshiudu, Nectaria Ntaoula, Dimitri S. Monos, Wan-Lu Lin, and Charalambos C. Solomides performed the experiments and analyzed and evaluated the results. Song Lu, John Gaughan, John V. White, Emilia L. Oleszak, and Chris D. Platsoucas analyzed and evaluated the results. John V. White recruited the patients.

## Funding

This work was supported in part by grant RO1 HL64340 from the National Institutes of Health, Bethesda, MD, USA.

## Artificial Intelligence (AI) Disclosure

No artificial intelligence (AI) tools, including large language models or machine-learning software, were used in the preparation, analysis, or presentation of this manuscript.

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