The pathophysiology of abdominal aortic aneurysm growth: Corresponding and discordant inflammatory and proteolytic processes in abdominal aortic and popliteal artery aneurysms

Hazem Abdul-Hussien, MD,[a] Roeland Hanemaaijer, PhD,[b] Robert Kleemann, PhD,[a,b] Ben F. J. Verhaaren, Bsc,[a] J. Hajo van Bockel, MD, PhD,[a] and **Jan H. N. Lindeman, MD, PhD,[a]** *Leiden, The Netherlands*

Objective: **There is remarkable controversy over the processes driving abdominal aneurysm growth. The inherent limitations of animal and human studies hamper elucidation of the key inflammatory and proteolytic processes. Human data are largely derived from surgical specimens that typically reflect the final stages of the disease process and thus do not allow distinction between primary and secondary processes. Clear epidemiologic and genetic associations between abdominal aortic aneurysm (AAA) and popliteal artery aneurysms (PAA) suggest that that these two pathologies share common grounds. On this basis, we reasoned that information of corresponding and discordant processes in these aneurysms might provide critical clues on the processes that are crucial for aneurysm progression.**

Methods: **Messenger RNA (semi-quantitative real-time polymerase chain reaction) and protein analysis (enzyme-linked immunosorbent assay, multiplex, Western blotting), and histology were performed on aneurysm wall samples obtained during elective PAA and AAA repair. Nonaneurysmal aorta tissue from organ donors was included as reference.**

Results: **Messenger RNA and protein analysis showed that PAA and AAA are both characterized by a marked activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) proinflammatory transcription factors, and hyperexpression of interleukin (IL)-6 and IL-8. Discordant findings were found for other inflammatory markers such as interferon-γ, interferon-inducible protein 10, tumor necrosis factor-α, monocyte chemotactic protein-1, and macrophage inflammatory protein 1α and β, which were all lower in PAA. On the cellular level, both pathologies exhibited profuse infiltration of macrophages, neutrophils, and T-helper cells. Results for B cells, plasma cells, and cytotoxic T cells were discordant, with minimal infiltration of these cell types in PAA. Evaluation of protease expression and activation showed that both conditions are dominated by increased matrix metalloproteinase 8 and 9, and cathepsin K, L and S expression and activation.**

Conclusion: **This explorative study characterizes degenerative aneurysmal disease general inflammatory conditions that are dominated by profound activation of the NF-κB and AP-1 pathways, hyperexpression of IL-6 and IL-8, and neutrophil involvement. Discordant findings for interferon γ, cytotoxic T cells, B cells, and plasma cells challenge a critical role for these factors in the process of aneurysm growth. Pharmaceutic strategies targeting the common components in AAA and PAA may prove effective for the stabilization of AAA. (J Vasc Surg 2010;51:1479-87.)**

Clinical Relevance: **An understanding of the processes driving aneurysm growth is critical for the development of new therapeutic strategies. Studies in animals and those using human material have helped to identify a variety of molecular candidates for intervention. Yet, interpretation of the data is complicated by the limited validity of animal models and because human tissue is generally obtained during surgical abdominal aortic aneurysm repair and thus represents the final stages of the disease. Strong genetic and epidemiologic associations between abdominal aortic aneurysm and popliteal aneurysms suggest that these aneurysms share a common ground. We therefore reasoned that information on parallel and incongruent pathways in these aneurysms may provide critical information on the key processes driving aneurysmal growth. Results of this study help to identify pathways that are essential to aneurysm growth.**

From the Department of Vascular Surgery, Leiden University Medical Center[a]; and TNO-Biomedical Research.[b]

Competition of interest: none.

Correspondence: Jan H. N. Lindeman, MD, PhD, Department of Vascular Surgery, K6R, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands (e-mail: Lindeman@LUMC.nl).

The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a competition of interest.

0741-5214/$36.00

doi:10.1016/j.jvs.2010.01.057

Abdominal aortic aneurysm (AAA) is a common dilating disorder of the aorta and a major cause of death due to rupture.[1-3] Despite consensus that AAA is best described as a chronic inflammatory condition with an accompanying proteolytic imbalance, the exact nature of the inflammatory cascades and the proteases driving aneurysmal growth remain unresolved. For example, although AAA was initially designated by Schonbeck et al[4] as a T-helper (Th)-2-type inflammatory disease, later studies suggest that AAA is better characterized as a Th1-dominated disease,[5] or alternatively, as a general proinflammatory disease.[6] With respect to the pro-

teases involved, attention has been primarily focused on the gelatinase matrix metalloproteinase (MMP) 9. Yet, despite ample evidence in animal models for a crucial involvement of MMP9 in AAA formation and growth of the disease, the pivotal role of MMP9 in driving human AAA growth is debated,[7] and additional proteases such as MMP8 and the cysteine proteases cathepsin K, L, and S have now also been brought forward.[8]

Interpretation of the available data is further complicated because most data are based on surgical specimens or on animal models of the disease. Surgical specimens generally represent the final stages of the disease process, making it difficult to discriminate between the primary and secondary events in the disease process.[9,10] Similarly, although animal models may provide valuable clues to the disease process, their relevance for AAA in general remains to be established.[9]

There are remarkable associations between AAA and popliteal artery aneurysms (PAA), the by far most common forms of peripheral aneurysms.[11,12] This is not only reflected by the fact that not only are about 40% to 50% of patients with a PAA also diagnosed with an AAA[13,14] but also by the aggregation of AAA and PAA in relatives of patients with these aneurysms as well as by functional analysis showing that the popliteal artery bears striking similarities to an elastic artery like the aorta.[15] These observations suggest that AAA and PAA belong to a similar family of aneurysms, the so-called degenerative aneurysms,[16] rather than aneurysms with a monogenetic origin such as aneurysms associated with Marfan syndrome or Ehlers-Danlos syndrome, which are respectively related to dysregulated transforming growth factor-β signalling[17] or defective collagen III synthesis.[18]

We thus reasoned that identification of common factors in the two conditions may provide clues on the nature of the inflammatory and proteolytic pathways causatively involved in aneurysm growth and constitutes a first important step to identify potential therapeutic targets for the pharmaceutical stabilization of AAA.

Because data from a methodic comparison of AAA and PAA are missing, we performed an explorative systematic and comprehensive analysis of inflammatory and proteolytic pathways activated in AAA and PAA tissue to identify corresponding and discordant processes in these two related pathologies. This information may identify the processes that are actually driving AAA and PAA growth as well as the bystander processes that are part of the persistent inflammatory response.

MATERIALS AND METHODS

Sample collection and handling was performed in accordance with the guidelines of the Medical and Ethical Committee in Leiden, Netherlands and the code of conduct of the Dutch Federation of Biomedical Scientific Societies (http://www.federa.org/?s=1&m=78&p=&v=4).

Tissue samples. All aneurysm samples were obtained during elective aneurysm repair. Reference samples (nonaneurysmal abdominal aortic wall samples) were obtained from the Vascular Tissue Bank at the Department of Vascular Surgery, Leiden, The Netherlands.

All samples were obtained after consent of the patients. None of the patients had a history of diabetes or chronic inflammatory disease. AAA samples (anterior lateral aneurysm wall) were obtained from 17 patients (14 men, 3 women) with an AAA >5.5 cm undergoing elective open repair (AAA group). Patients were a mean age of 72.4 ± 6.2 years (mean ± sd), and the mean AAA diameter was 6.7 ± 1.1 cm. PAA samples were from 12 patients (11 men, 1 woman) with PAA >2.0 cm[16] undergoing elective repair. These patients were aged 69.6 ± 12.1 years.

For comparison of the AAA and PAA wall with the normal aortic wall (nonaneurysmal control), we used nonaneurysmal aortic wall patches obtained during clinical organ transplantation. The grafts were derived from 11 kidney donors (7 men, 4 women) with fatal brain injury caused by a major head trauma or subarachnoidal bleeding. Only patches displaying advanced atherosclerosis with advanced atherosclerotic lesions, equalling the characteristics of grade IV to VI lesions according to the Stary classification,[19] were selected. The mean age of the control group was 55.6 ± 10.2 years, with an aortic diameter of <2.0 cm. All control samples were obtained at the level of the renal artery and during a laparotomy, that is, from comparable region and during a similar procedure as the AAA samples.

Tissue procurement. Any adhering thrombus was carefully removed and the vessel walls were halved lengthwise. One half was immediately snap frozen in liquid nitrogen and stored at −80°C for messenger RNA (mRNA) analysis by real-time polymerase chain reaction (RT-PCR) and protein analysis using Western blot and enzyme-linked immunosorbent assay (ELISA). The other half was fixed in formaldehyde for 24 hours, decalcified in Kristensen solution for 120 hours, and embedded in paraffin for histologic analysis.

Real-time PCR. Tissue samples were pulverized in liquid nitrogen, RNA was isolated, and semi-quantitative RT LightCycler PCR (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) was carried out (TaqMan method) as detailed in previous publications.[20]

Total RNA extraction was performed using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads according to the manufacturer's instructions. Copy DNA was prepared (Kit #A3500; Promega, Leiden, The Netherlands), and quantitative RT-PCR analysis was performed for human interleukin (IL)-1α, IL-1β, IL-2, IL-6, IL-8, tumor necrosis factor (TNF)α, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, macrophage inhibition factor, transforming growth factor (TGF)-β, CD4, T-Bet, GATA-3, IL-4, IL-10, IL-13, interferon γ, CD8, perforin, granzyme A, B-lymphocyte-induced maturation protein 1 (BLIMP-1), MAD4, immunoglobulin linker protein, immunoglobulin heavy chain, MMP-25, CD 337, tryptase, MMP2, MMP3, MMP9, MMP13, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, TIMP-3, cathepsin K, cathepsin L, cathepsin S, cystatin C, urokinase plasminogen activator (uPA), and plasminogen

activator inhibitor type 1 (PAI-1) on the ABI-7700 system (Applied Biosystems) using established primer/probe sets (Assays on Demand; Applied Biosystems) and Mastermix (Eurogentec, Seraing, Belgium). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a reference and for normalization.

Tissue homogenization. Snap frozen samples were crushed in liquid nitrogen and mixed with lysis buffer (10 mM Tris, pH 7.0; 0.1 mM $CaCl_2$, 0.1 M NaCl, 0.25% (v/v) Triton X-100) for protein-based assays. After centrifugation at 4°C for 15 minutes at 13,000 rpm, the supernatants were collected, and protein contents were measured (Pierce, Rockford, Ill) before storage at −80°C until use.

Multiplex assay, and IL-6, IL-8 and MCP-1 ELISAs. Concentrations of vessel wall cytokines were measured in the homogenates by the BioPlex panel (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) for IL-1α, IL-1β, IL-13, TNF-α, interferon-γ, IP-10, MIP-1α, MIP-1β, and granulocyte colony-stimulating factor (G-CSF), or by separate ELISAs (PeliKane compact kit; Sanquin, Amsterdam, The Netherlands) for IL-6 and IL-8, and the Quantikine kit (R&D Systems, Abingdon, UK) for MCP-1. We previously concluded that wall of IL-2, IL-4, IL-5, IL-7, IL-10, IL-12, IL-17A, and granulocyte macrophage colony-stimulating factor (GM-CSF) in the AAA wall are all at or below the detection threshold of the multiplex assay. Hence, these cytokines were not included in the analysis.

Western blotting. Quantifiable Western blots were performed following detailed protocols described by Kleemann et al.[21] All assays were performed on the tissue homogenates using antibodies specific for the human forms of p65-NF-κB (active form of p65-NF-κB; Chemicon, #MAB3026, Chemicon Europe Ltd, Chandlers Ford, UK), p65-NF-κB (nonactive form; sc-8008; Santa Cruz, Heerhugowaard, The Netherlands), c-Jun (sc-45), phosphor (Ser73)-c-Jun (sc-7981), CCAAAT/enhancer-binding protein (C/EBP)-α (sc-9315), C/EBP-β (sc-150), C/EBP-δ (sc-636), MMP2 (PC-158, the Bindingsite, Birmingham, UK), MMP8 (MAB3316, Chemicon, Chemicon Europe, Ltd, Chandlers Ford, UK), MMP9 (TNO-BEA-21), cathepsin K (IM55L, Calbiochem, Breda, The Netherlands), cathepsin L (AF952, R&D systems, Abingdon, UK), cathepsin S (sc-6505, Santa Cruz), and α-actin (sc-1615, Santa Cruz) for normalization. All protease antibodies were specifically chosen to effectively detect both the pro and activated forms of the proteases.[8]

All secondary antibodies were obtained from Santa Cruz Biotechnology. Immunoblots were visualized using Super Signal West Dura Extended Duration Substrate (Perbio Science, Etten-Leur, The Netherlands) and a luminescent image workstation (UVP, Cambridge, UK). Immunoblots were quantified using LabWorks 4.6 software (Bio-Rad, Hercules, Calif).

Immunohistochemistry. Immunohistochemistry was performed using deparaffinized, ethanol rehydrated tissue 4-γm-thick cross-sections as reported previously.[22] Sections were incubated overnight with polyclonal antibodies specifically for human myeloperoxidase (DAKO, Heverlee, Belgium), CD4 (clone 1F6, DAKO), CD8 (clone 4B11, Novocastra, Valkenswaard, the Netherlands), CD20 (clone L26, DAKO), CD68 (clone KP6, DAKO), and CD138 (clone B-B4, Serotec, Oxford, UK). Conjugated biotinylated antigoat or rabbit anti-immunoglobin (Ig) G were used as secondary antibodies. Sections were developed with Nova Red (Vector Laboratories, Burlingame, Calif) and counterstained with Mayer hematoxylin, allowing morphologic analysis. Specificity of the antibody staining was confirmed by omitting the primary antibody. Specimens were quantified by counting the number of cells per unit area for at least 20 fields at original magnification ×400.

Table I. Patient characteristics

Variable	*AAA*	*PAA*	*Control aorta*
Evaluable patients, No.	15	12	11
Age, mean ± SD, y	74.8 ± 6.2	69.6 ± 12.1	55.6 ± 10.2
Diameter, mean ± SD, cm	6.7 ± 1.1	3.35 ± 1.36	<2.0
Female sex, No.	1	1	4
Aneurysm elsewhere, No.	1	4	0
Current smoker, No.	6	4	4
Statin use, No.	1	3	0
Antihypertensives, No.	8	5	0
Antiplatelet therapy, No.	10	9	0

AAA, Abdominal aortic aneurysm; *PAA,* popliteal artery aneurysm; *SD,* standard deviation.

Statistical analysis. Messenger RNA and protein expressions, results of the Western blots and immunohistochemistry were analyzed by Wilcoxon-Mann-Whitney *U* test to compare the different groups. Statistical significance was accepted at $P < .05$. Most of the data in this study reflect coherent data that fits in the theoretic inflammatory frame works. As such, noncorrected data are provided,[23] yet a Bonferroni correction should be considered when interpreting noncoherent data. All analyses were performed using SPSS 16.0 software (SPSS Inc, Chicago, Ill).

RESULTS

Patient characteristics are reported in Table I.

Cellular composition and cellular markers in AAA and PAA walls. We first performed a histologic evaluation of infiltrating leucocytes in AAA, PAA, and nonaneurysmal control aorta. Results of these analyses are shown in Fig 1. AAA and PAA tissues were characterized by profuse and diffuse infiltration of monocytes (CD68+), neutrophils (MPO+), and T-helper cells (CD4+). Findings for B-cells (CD20+), plasma cells (CD138+), and cytotoxic T-cells (CD8+) content, on the other hand, were clearly discordant between PAA and AAA, with CD20+, CD138+ and CD8+ abundance in AAA but with very limited presence in PAA.

The findings for the reduced B-cell, plasma cell, and cytotoxic T-cell content in PAA were in accordance with a

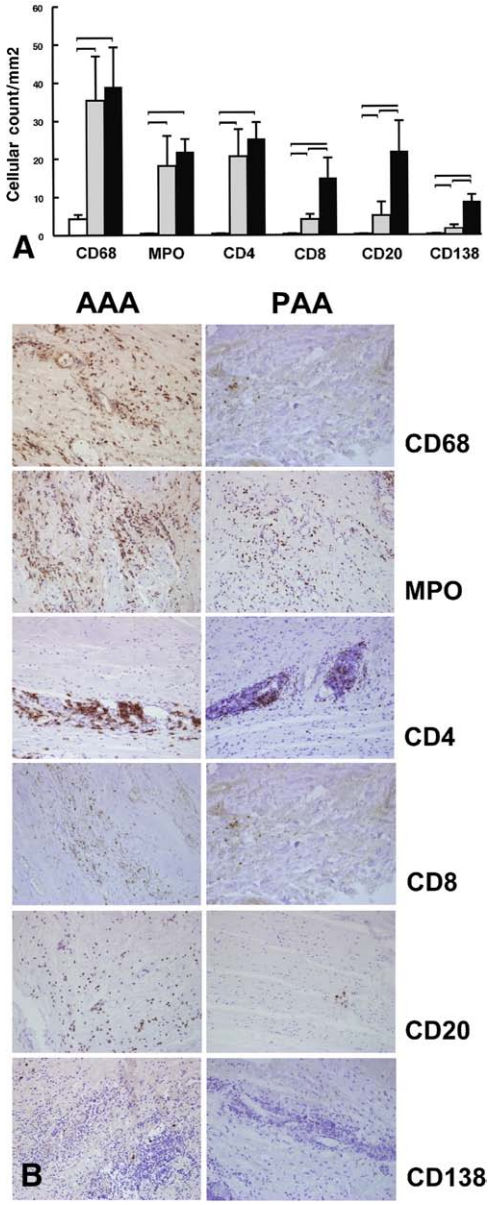

Fig 1. A, The cellular composition of abdominal aortic aneurysm *(AAA; black)*, popliteal artery aneurysm *(PAA; gray)*, and control aorta (*white*) tissue by immunohistochemical analysis is shown in graph form. The amount of cells/mm^2 in AAA and PAA tissues, and control aorta is shown after staining with antibodies specifically detecting monocytes and macrophages (*CD68*), neutrophils (myeloperoxidase *[MPO]*), T helper cells (*CD4*), cytotoxic T cells (*CD8*), B-lymphocytes (*CD20*), and plasma cells (*CD138*). There was no significant difference between AAA and PAA with respect to CD68 (P = .672), MPO (P = .075), and CD4 (P = .181). Significant differences were found in the CD8 (P = .026), CD20 (P = .026), and CD138 (P = .001) content. A statistical difference (P < .05) between two groups is indicated by a *horizontal bar* above the bars. Representative examples of the staining are shown in section **B**.

reduced mRNA expression of B-cell, plasma cell, and cytotoxic T-cell markers in PAA (Table II). In line with the cellular observations, no difference was found for the expression of monocyte/macrophage, neutrophil and the T-helper cell markers in AAA and PAA (Table II).

Analysis of inflammatory molecular factors in AAA and PAA walls. Comparison of the AAA and PAA inflammatory fingerprint on the mRNA level (semi-quantitative RT-PCR) and protein level shows that PAA and AAA are characterized by particularly prominent IL-6 and IL-8 expression (Table II, Fig 2), although the increase in IL-6 levels was less prominent in PAA than in AAA (P = .038; Fig 2). Findings for the other proinflammatory factors such as TNF-α, interferon γ, and IP-10, as well as MCP-1, MIPα, and MIP-β, were clearly discordant between AAA and PAA (P < .008, Table III).

The congruent IL-8 hyperexpression in AAA and PAA prompted us to quantify the baseline levels of the general proinflammatory transcription factors NF-κB, AP-1, and C/EBP. Similar baseline but clearly increased NF-κB and AP-1 activation (active confirmation [NF-κB] and phosphor-c-jun [AP-1]) show that AAA and PAA are both associated with a hyperactivation of these systems[6,24] (Fig 3). Abundance of the C/EBP isoforms α, β, and δ was less outspoken in PAA (P < .021; Fig 3).

Protease profiles in AAA and PAA. MMP2, 8, and 9, and cathepsin K, L, and S have been implicated as the primary proteolytic culprits in AAA.[8] Evaluation of these proteases on the messenger and protein level showed a similar profile in PAA (Table II), although a trend towards lower expression of the cysteine proteases was observed in PAA. Activation of these proteases was assessed by quantification of the activated forms in Western blot analysis.[8] With the sole exception of reduced cathepsin K and increased MMP2 activation in PAA (P = .005), similar activation profiles were found in AAA and PAA (Fig 4).

DISCUSSION

The primary processes driving progression of AAA and PAA, the so-called degenerative aneurysms, are still unresolved. To obtain clues on the factors that are generic for aneurysm growth, we performed a systematic comparison of inflammatory and proteolytic processes in AAA and PAA. Our findings demonstrate that both pathologies share a general proinflammatory profile that is dominated by IL-8 and IL-6 hyperexpression. Abundance of neutrophils, macrophages, and T-helper cell infiltration, as well as ample expression of MMP and cysteine proteases, are clear features of the diseases. Distinct differences were found for the presence of cytotoxic T cells, B cells, and plasma cells, as well as for their associated markers, suggesting that the abundance of these cells in AAA is not generic to the progression of degenerative aneurysms.

The pathology of AAA is complex and poorly understood,[1] and very limited information is available for PAA. Remarkable controversy exists on the inflammatory cascades driving AAA aneurysm growth.[25] In a first evaluation, Schonbeck et al[4] reported dominance of the T-helper (Th)

Table II. Log relative expression of markers of inflammatory responses in abdominal aortic aneurysm, popliteal artery aneurysm, and nonaneurysmal aortic control[a]

Variable	*PAA* *Median (IQR)*	*AAA* *Median (IQR)*	P *value* *(PAA vs AAA)*	*Aortic control* *Median (IQR)*	P *value* *(PAA vs control)*
Cytokines					
IL-1α	−3.12 (−3.32 to −2.81)	2.49 (−3.02 to −1.80)	.065	ND	.071
IL-1β	−1.40 (−2.03 to −1.31)	−0.80 (−1.17 to −0.53)	.007[b]	−1.91 (−2.74 to −1.35)	.270
IL-2	−3.15 (−4.83 to −2.91)	−2.92 (−3.25 to −2.34)	.135	−4.86 (ND to −3.60)	.091
IL-6	−0.76 (−0.84 to −0.73)	−0.77 (−1.07 to −0.45)	.604	−2.19 (−2.81 to −1.84)	.00013
IL-8	−0.56 (−1.28 to −0.14)	−0.24 (−0.84 to 0.02)	.348	−1.53 (−2.61 to −0.86)	.085
TNF-α	−1.95 (−2.72 to −1.74)	−2.43 (−2.65 to −1.92)	.452	−2.81 (−3.80 to −1.99)	.188
MCP-1	0.11 (ND to 0.64)	0.54 (0.10 to 0.92)	.26	0.11 (−0.41 to 0.47)	.758
MIP-1β	−0.71 (−0.84 to −0.72)	−0.40 (ND to −0.11)	.008[b]	−1.05 (−1.58 to −0.44)	.230
MIF	0.49 (0.32 to 0.59)	0.56 (0.41 to 0.83)	.222	0.28 (0.20 to 0.37)	.246
TGF-β	−0.18 (−0.49 to 0.12)	0.30 (0.04 to 0.48)	.001[b]	0.14 (−0.05 to 0.34)	.069
T-helper cells					
CD4	−2.75 (−2.89 to −2.12)	−2.38 (−2.74 to −2.26)	>.99	−3.46 (−4.22 to −2.12)	.328
T-Bet	−2.67 (−3.44 to −2.29)	−2.64 (−4.96 to −1.81)	.72	−4.01 (ND to −3.25)	.069
GATA-3	−3.04 (−3.38 to −2.92)	−2.04 (−2.64 to −1.46)	.001[b]	−3.13 (ND to −2.35)	.930
IL-4	ND	−3.59 (−4.11 to −3.38)	.010[b]	−3.83 (−4.62 to −3.46)	.126
IL-10	−2.09 (−2.50 to −1.92)	−1.74 (−2.22 to −1.35)	.106	−1.89 (−2.28 to −1.35)	.525
IL-13	ND	−3.29 (−4.88 to −2.47)	.166	ND	.375
Interferon-γ	−4.97 (ND to −2.92)	−2.71 (−3.05 to −2.23)	.008[b]	−5.52 (ND to −3.04)	.536
Cytotoxic T cell					
CD8	−2.81 (−3.12 to −2.10)	−1.49 (−1.75 to −1.06)	.001[b]	−2.91 (−4.30 to −1.89)	.596
Perforin	−2.85 (−3.31 to −2.72)	−2.25 (−2.76 to −1.53)	.034[b]	−3.38 (−4.07 to −2.88)	.230
Granzyme A	−1.67 (−1.92 to −1.41)	−0.83 (−1.13 to −0.50)	.003[b]	−1.96 (−2.41 to −1.04)	.179
B/Plasma cell					
BLIMP-1	−2.25 (−2.82 to −2.04)	−1.39 (−2.05 to −1.25)	.023[b]	−2.92 (−3.47 to −2.48)	.081
MAD4	−1.45 (−1.63 to −1.31)	−1.05 (−1.49 to −0.83)	.044[b]	−1.41 (−1.56 to −1.05)	.808
Ig linker protein	−0.40 (−0.92 to −0.44)	0.26 (−0.02 to −0.63)	.001[b]	−1.21 (−1.70 to −0.73)	.122
Ig heavy chain	0.17 (−0.44 to −0.74)	1.76 (1.29 to 2.10)	.001[b]	0.18 (−1.18 to 0.56)	.536
Neutrophil					
MMP25	−1.59 (−1.91 to −0.92)	−1.80 (−2.01 to −1.12)	.757	−2.94 (−3.52 to −2.73)	.027
NK cell					
CD337	−3.35 (−3.79 to −2.77)	−2.22 (−2.75 to −1.39)	.006[b]	−4.16 (−4.99 to −3.12)	.180
Proteases					
MMP2	−1.02 (−1.35 to −0.96)	−2.64 (−3.31 to −2.13)	.001[b]	−2.55 (−3.09 to −1.60)	.015
MMP3	−2.21 (−2.87 to −1.35)	−2.42 (−3.17 to −2.05)	.349	−3.38 (−4.53 to −2.61)	.027
MMP9	−1.06 (−1.26 to −0.82)	−1.27 (−1.56 to −0.87)	.288	−2.54 (−3.34 to −1.24)	.011
MMP13	−2.11 (−2.36 to −1.25)	−3.15 (−3.51 to −2.74)	.018[b]	−3.46 (−3.66 to −2.66)	.043
Cathepsin K	−1.79 (−2.71 to −1.69)	−1.83 (−2.20 to −1.37)	.376	−2.30 (−2.95 to −1.87)	.375
Cathepsin L	0.67 (0.22 to 0.81)	0.18 (−0.16 to 1.05)	.619	−0.14 (−0.62 to 0.55)	.020
Cathepsin S	−0.52 (−0.87 to −0.48)	−0.71 (−0.84 to −0.40)	.619	−1.19 (−2.13 to −0.32)	.211
TIMP-1	0.74 (0.01 to 0.87)	0.63 (0.22 to 1.26)	.418	0.25 (0.03 to 0.85)	.536
TIMP-2	−1.50 (−1.72 to −1.36)	−1.37 (−1.54 to −0.93)	.178	−1.78 (−2.03 to −1.33)	.216
TIMP-3	−0.54 (−0.81 to −0.35)	−0.54 (−0.90 to 0.17)	.973	−0.45 (−0.62 to −0.17)	.301
Cystatin C	0.72 (0.32 to 0.91)	0.93 (0.69 to 1.20)	.087	0.87 (0.75 to 1.01)	.270

BLIMP-1, B-lymphocyte-induced maturation protein 1; *Ig*, immunoglobulin; *IL*, interleukin; *IQR*, interquartile range; *MCP-1*, monocyte chemotactic protein; *MIF*, macrophage inhibition factor; *MIP*, macrophage inflammatory protein; *MMP*, matrix metalloproteinase; *NK*, natural killer; *ND*, not detectable; *TGF*, transforming growth factor; *TIMP*, tissue inhibitor of metalloproteinase; *TNF*, tumor necrosis factor.

[a]Expression normalized on basis of glyceraldehyde-3-phosphate dehydrogenase; *P* value is for the comparison of PAA vs AAA (middle column) or PAA vs control (final column) responses.

[b]Significant difference between PAA and AAA.

2-associated cytokines IL-4, IL-5, and IL-10, along with minimal expression of the Th1-associated cytokine interferon-γ in AAA.[4] On this basis, the authors concluded that AAA is best described as a Th2-driven disease. Contrary to these findings, Xiong et al[26] (mouse model) and Galle et al[5] (human AAA) reported Th1 polarization in AAA. Because of these apparent contrasting findings, we performed a comprehensive evaluation of the inflammatory fingerprint of AAA and concluded that AAA is best described as a general proinflammatory condition with IL-6 and IL-8 hyperexpression and dominance of IL-6– and IL-8–related responses.[6]

The persistent inflammatory state in AAA is held responsible for a proteolytic imbalance that underlies the excess matrix degradation in the disease. With respect to the proteases involved, attention has been primarily focussed on the gelatinases MMP2 and MMP9.[27,28] Yet, it has been pointed out that these proteases cannot degrade

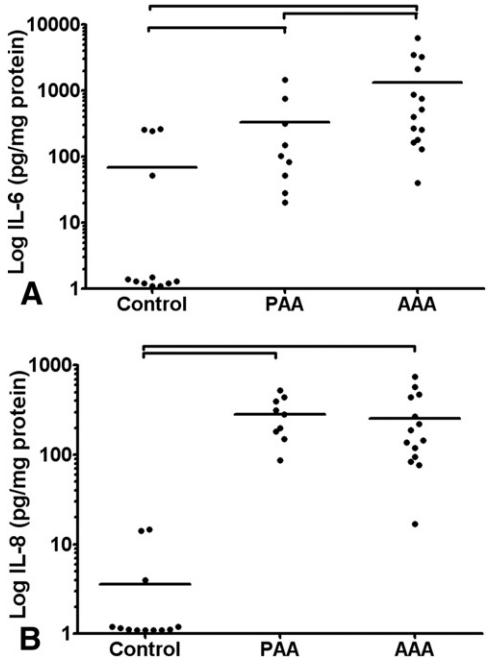

Fig 2. Concentrations of **(A)** interleukin *(IL)*-6 and **(B)** IL-8 protein are characteristic for abdominal aortic aneurysm *(AAA)* and popliteal artery aneurysm *(PAA)* wall. Aortic wall protein expression levels of IL-6 and IL-8 were determined by specific enzyme-linked immunosorbent assays in PAA *(gray)* and AAA *(black)* wall and control aorta *(white)* samples. There was a strong significant difference between the control aorta and the aneurysm group ($P < .0001$) with respect to IL-6 and IL-8. Focussing on AAA and PAA, IL-8 hyperexpression was found in the two conditions ($P = .378$) but IL-6 hyperexpression was less obvious in PAA ($P = .038$). A statistical difference ($P < .05$) between two groups is indicated by the *horizontal bar* above the bars.

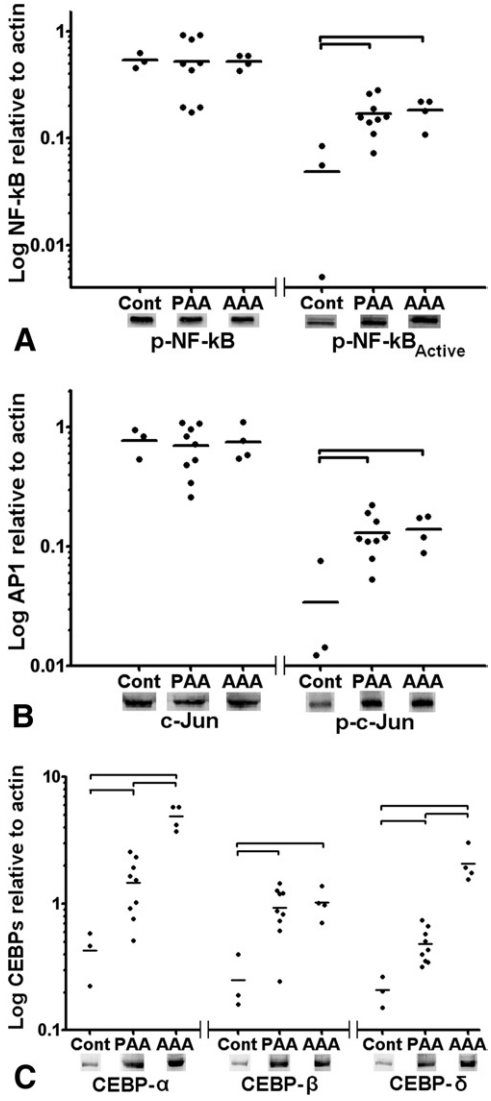

Fig 3. Concentrations of inflammatory transcription factors are characteristic for abdominal aortic aneurysm (*AAA*) and popliteal artery aneurysm (*PAA*) wall. Relative (normalized on basis of β-actin levels) basal levels of **(A)** p65-NF-κB, **(B)** c-jun protein, and their activated forms (NF-κB active and p-c-jun) and **(C)** CCAAAT/enhancer-binding protein (C/EBP)-α, C/EBP-β, and C/EBP-δ in homogenates of AAA *(black)*, PAA (gray), and control aorta *(white)*. NF-κB and activator protein-1 *(AP-1)* baseline and activation levels were comparable in AAA and PAA (pNFκB, $P = .537$; pNF-κB active, $P = .643$; c Jun, $P = .877$; p-c-Jun, $P = .643$). The three isoforms C/EBP-α, C/EBP-β, and C/EBP-δ were significantly lower in PAA (C/EBP-α and C/EBP-β; $P = .005$; C/EBP-δ, $P = .009$). A statistical difference ($P < .05$) between two groups is indicated by a *horizontal bar* above the bars.

Table III. Aortic wall cytokine protein levels normalized on basis of protein levels

Variable	*PAA, pg/mg Median (IQR)*	*AAA, pg/mg Median (IQR)*	P *value*[a]
IL-1α	ND (ND-0.14)	0.14 (ND-0.34)	.131
IL-1β	1.50 (0.87-2.46)	2.81 (2.00-7.54)	.04
IL-13	ND (ND-0.06)	0.18 (0.09-0.28)	.001
TNF-α	ND (ND-0.01)	0.04 (0.01-0.11)	.007
Interferon-γ	ND (ND-ND)	1.32 (ND-3.62)	.008
IP-10	5.7 (1.3-38.8)	141.2 (63.0-237.0)	.0001
MCP-1	67.3 (14.2-127.3)	197.2 (102.1-212.7)	.006
MIP-1α	1.29 (0.38-1.83)	4.65 (3.52-9.81)	.0003
MIP-1β	2.77 (0.55-5.71)	20.0 (10.1-26.6)	.001
G-CSF	ND (ND-0.13)	0.73 (0.40-1.77)	.001

AAA, Abdominal aortic aneurysm; *G-CSF,* granulocyte colony-stimulating factor; *IL,* interleukin; *IP-10,* interferon-γ-induced protein; *IQR,* interquartile range; *MCP-1,* monocyte chemotactic protein; *MIP-1,* macrophage inflammatory protein-1; *ND,* not detectable; *PAA,* popliteal artery aneurysm; *TNF,* tumor necrosis factor.

[a] *P* value is for PAA vs AAA.

the load-bearing fibrillar collagens in the aortic wall[7,8] and that it is thus unlikely that these proteases are directly responsible for the weakening of the aortic wall. As such, it is very likely that other proteases contribute to the weakening of the aortic wall as well.

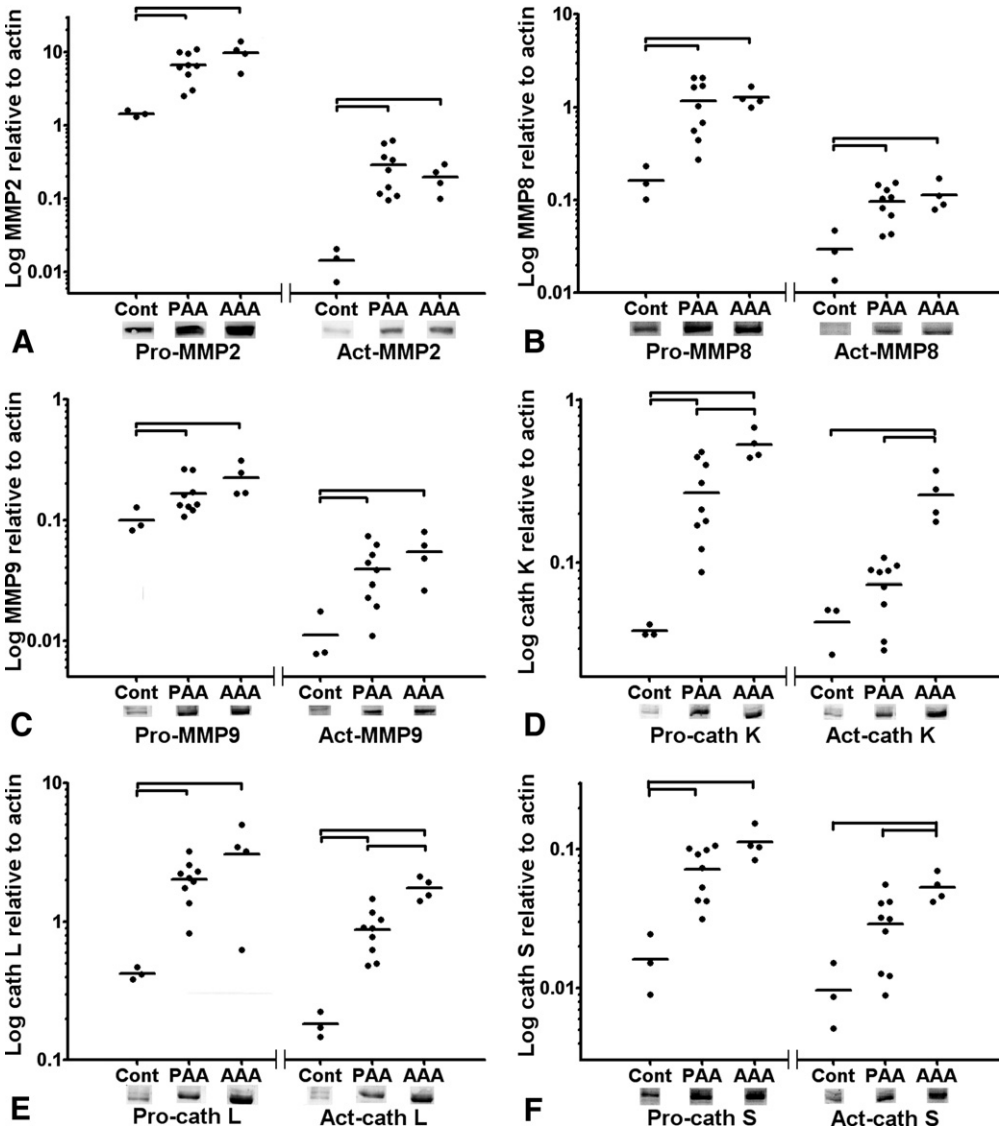

Fig 4. Pro and active forms of matrix metalloproteinase *(MMP)* and cathepsin proteases are characterized for abdominal aortic aneurysm *(AAA)* and popliteal artery aneurysm *(PAA)*. Western blot analysis of pro and activated forms of **(A)** MMP2, **(B)** MMP8, **(C)** MMP9, and **(D)** cathepsin K, **(E)** L and **(F)** S in AAA *(black)*, PAA *(gray)*, and aorta control *(white)* tissues. Equal dominance of MMP2, MMP8, and MMP9 were found in AAA and PAA both for the pro and active (act) form (pro MMP2, $P = .217$; act MMP2, $P = .537$; pro MMP8, $P = .758$; act MMP8, $P = .537$; pro MMP9, $P = .123$; act MMP9, $P = .280$). Results for the cathepsins were more variable and less outspoken in PAA than in AAA (pro cathepsin K, $P = .021$; act cathepsin K, $P = .005$; pro cathepsin L, $P = .165$; act cathepsin L, $P = .009$; pro cathepsin S, $P = .065$; act cathepsin S, $P = .021$). A statistical difference ($P < .05$) between two groups is indicated by a *horizontal* bar above the bars.

As for the large number of remaining open questions, the complexity of the inflammatory and proteolytic cascades in AAA, and the scarcity of data on PAA, we reasoned that a systematic exploration of common and discordant inflammatory and proteolytic processes in AAA and PAA might provide critical clues on the processes that drive progression of degenerative aneurysms.

An evaluation of the inflammatory and proteolytic fingerprints in AAA and PAA showed remarkable similarities and dissimilarities. Both conditions are characterized by a strong activation of the general inflammatory transcription factors NF-κB and AP-1 (c-jun), profuse IL-8 and IL-6 expression, a high neutrophil and macrophage content, and abundant expression and activation of the MMP proteases neutrophil collagenase (MMP8) and gelatinase (MMP9) and the cysteine proteases cathepsin K, L, and S. Unlike the congruent findings for IL-6 and IL-8, much lower levels were found for soluble factors such as interferon-γ, the

interferon-γ–induced protein (IP-10, CXCL-10), TNF-α, MCP-1, MIP-1α and MIP-1β in PAA. These latter observations may indicate that these factors are less critical to aneurysm growth and particularly challenge a role for interferon-γ in the progression of AAA.[29]

Dominance of neutrophil-derived proteases and neutrophil abundance in AAA and PAA biopsy specimens suggests that these cells are generic for these forms of aneurysmal disease. Neutrophils have long been considered part of an acute inflammatory response, and their role in chronic conditions has long been ignored.[30] Recent studies, however, show that neutrophils do participate in chronic inflammatory processes such as chronic obstructive pulmonary disease and rosacea.[31,32] We previously identified the neutrophil-derived collagenase MMP8 as the most prominent MMP protease in AAA and as an important target for doxycycline therapy in AAA,[8,33] suggesting that neutrophils may also be actively involved in AAA growth. This notion is supported by animal studies by Eliason et al[34] and Pagano et al,[35] both of which showed that abrogated neutrophil influx in an animal models of AAA inhibited AAA development. Neutrophil abundance in AAA and PAA can well be explained by the local IL-8 hyperexpression that is found in the two pathologies. This notion is supported by observations from a clinical trial that showed a strong association ($r = 0.84$) between aortic wall neutrophil content and IL-8 levels.[8]

Remarkable differences were found with respect to the cytotoxic T cell, B cell, and plasma cell content between AAA (abundant) and PAA (minimal). These differences on the histologic levels were confirmed by a clear reduction of cell-type specific markers on the mRNA level. A clear-cut explanation for this finding is missing, but possible explanations are the reduced expression of the C/EBP family of transcription factors in PAA[36,37] or, alternatively, that the cytotoxic T-cell, plasma-cell, and B-cell abundance in AAA reflects presence of the vascular associated lymphoid tissue[38] in this section of infrarenal aorta.

It is possible that the reduced cytotoxic T-cell content reflects skewing of the Th1/Th2 balance towards a more Th1-dominated response, as indicated by similar T-bet but lower GATA-3 levels in PAA. Yet, the reduced interferon-γ and IP-10 levels in the disease do not support such a scenario. Another possibility is that the differences in the T-helper cell and cytotoxic T-cell content relates to diverging roles of specific signaling pathways in CD8+ T-cell and CD4+ T-cell biology.[39,40] Minimal B cell and plasma cell infiltration in PAA challenges a critical role for these cells—and the concept of AAA as an autoimmune phenomenon—in aneurysm growth. Yet, little is known about the pathophysiologic role of these cells in the context of AAA, and further mechanistic studies are necessary to investigate the contribution of these cells and the relevance of a possible autoimmune phenomenon[41,42] to AAA formation and progression.

The proteolytic imbalance in AAA with prominent expression of MMP2[8,9] and the cysteine proteases cathepsin K, L, and S is a key factor of AAA. With the notable exception of reduced cathepsin K activation and increased MMP2 in PAA, the inflammatory differences between AAA and PAA were not followed by a change in MMP or cysteine protease levels or activation. We have no clear explanation for the reduced cathepsin K and increased MMP2 activation in PAA. One possible explanation for the reduced cathepsin K activation in PAA is that it reflects reduced expression and/or activity of the osteoclastic proton pump V-H-adenosine triphosphatase[8] that is required for maintenance of an acidic pericellular microenvironment that is required for cathepsin K activation and stability.

A possible limitation of this study is that it is based on assumed parallels in the pathophysiology. Despite the clear epidemiologic links between AAA and PAA, it is important to realize that the biologic profiles of the two vessels may differ; for example, elastic vs muscular artery, different in flow profiles, complications through rupture (AAA) vs thrombosis (PAA). Yet, a functional analysis of the popliteal artery showed that the wall function of the popliteal artery clearly differs from other peripheral arteries, and instead shows striking similarities to the abdominal aorta.[15] Similarly, although AAA and PAA may differ in their flow profile, previous work has shown that the flow velocities in larger vessels prevent adhesion and tissue migration of T cells and monocytes into the wall and that inflammatory cells require the vasa vasora in the adventitial rather than the intimal layer to enter the vessel.[43]

CONCLUSION

This explorative study demonstrates a remarkable overlap between AAA and PAA and characterizes the two pathologies as general inflammatory conditions dominated by activation of the NF-κB and AP-1 pathways, IL8 hyperexpression and neutrophil involvement, as well as ample activation of selected MMP and cysteine proteases. Remarkable and consistent differences were found with respect to B cells, plasma cells and cytotoxic T cells and their markers, suggesting that these cell types are specific for AAA and are not critical to the process of aneurysmal growth. Pharmaceutic strategies targeting the generic components in AAA and PAA may prove effective for the stabilization of AAA.[10]

AUTHOR CONTRIBUTIONS

Conception and design: JL
Analysis and interpretation: HA-H, JL, RK, BV
Data collection: HA-H
Writing the article: JL, RK, H-AH
Critical revision of the article: JvB
Final approval of the article: JL, RK, HA-H
Statistical analysis: JL, HA-H
Obtained funding: JL
Overall responsibility: JL

REFERENCES

1. Sakalihasan N, Limet R, Defawe OD. Abdominal aortic aneurysm. Lancet. 2005;365:1577-89.

2. Lindholt JS, Shi GP. Chronic inflammation, immune response, and infection in abdominal aortic aneurysms. Eur J Endovasc Surg 2006;31: 453-63.
3. Knox JB, Sukhova GK, Whittemore AD, Libby P. Evidence for altered balance between matrix metalloproteinases and their inhibitors in human aortic diseases. Circulation 1997;95:205-12.
4. Schonbeck U, Sukhova GK, Gerdes N, Libby P. T(H)2 predominant immune responses prevail in human abdominal aortic aneurysm. Am J Pathol 2002;161:499-506.
5. Galle C, Schandene L, Stordeur P, Peignois Y, Ferreira J, Wautrecht JC, et al. Predominance of type 1 CD4+ T cells in human abdominal aortic aneurysm. Clin Exp ImmunoI 2005;142:519-27.
6. Lindeman JH, Abdul-Hussien H, Schaapherder AF, Van Bockel JH, Von der Thüsen JH, Roelen DL, et al. Enhanced expression and activation of pro-inflammatory transcription factors distinguish aneurysmal from atherosclerotic aorta: IL-6- and IL-8-dominated inflammatory responses prevail in the human aneurysm. Clin Sci (Lond) 2008; 114:687-97.
7. Carmeliet P. Proteinases in cardiovascular aneurysms and rupture: targets for therapy? J Clin Invest 2000;105:1519-20.
8. Abdul-Hussien H, Soekhoe RG, Weber E, von der Thüsen JH, Kleemann R, Mulder A, et al. Collagen degradation in the abdominal aneurysm: a conspiracy of matrix metalloproteinase and cysteine collagenases. Am J Pathol 2007;170:809-17.
9. Daugherty A, Cassis LA. Mouse models of abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol 2004;24:429-34.
10. Thompson RW, Curci JA, Ennis TL, Mao D, Pagano MB, Pham CT. Pathophysiology of abdominal aortic aneurysms: insights from the elastase-induced model in mice with different genetic backgrounds. Ann N Y Acad Sci 2006;1085:59-73.
11. Wychulis AR, Spittell JA Jr, Wallace RB. Popliteal aneurysms. Surgery 1970;68:942-52.
12. Jacob T, Hingorani A, Ascher E. Examination of the apoptotic pathway and proteolysis in the pathogenesis of popliteal artery aneurysms. Eur J Vasc Endovasc Surg 2001;22:77-85.
13. Ravn H, Wanhainen A, Björck M. Risk of new aneurysms after surgery for popliteal artery aneurysm. Br J Surg 2008;95:571-5.
14. Diwan A, Sarkar R, Stanley JC, Zelenock GB, Wakefield TW. Incidence of femoral and popliteal artery aneurysms in patients with abdominal aortic aneurysms. J Vasc Surg 2000;31:863-9.
15. Debasso R, Astrand H, Bjarnegård N, Rydén Ahlgren A, Sandgren T, Länne T. The popliteal artery, an unusual muscular artery with wall properties similar to the aorta: implications for susceptibility to aneurysm formation? J Vasc Surg 2004;39:836-42.
16. Van Bockel JH, Hamming JF. Lower extremity aneurysms. In: Rutherford RB, editor. Vascular surgery. Philadelphia: Elsevier Saunders; 2005;1534-51.
17. Mizuguchi T, Matsumoto N. Recent progress in genetics of Marfan syndrome and Marfan-associated disorders. J Hum Genet 2007;52: 1-12.
18. Milewicz DM. Molecular genetics of Marfan syndrome and Ehlers-Danlos type IV. Curr Opin Cardiol 1998;13:198-204.
19. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, Rosenfeld ME, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Arterioscler Thromb Vasc Biol 1995;15: 1512-31.
20. Lindeman JH, Hanemaaijer R, Mulder A, Dijkstra PD, Szuhai K, Bromme D, et al. Cathepsin K is the principal protease in giant cell tumor of bone. Am J Pathol 2004;165:593-600.
21. Kleemann R, Gervois PP, Verschuren L, Staels B, Princen HM, Kooistra T. Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing nuclear p50-NFkappa B-C/EBP-beta complex formation. Blood 2003;101:545-51.
22. Verschuren L, Lindeman JH, van Bockel JH, Abdul-Hussien H, Kooistra T, Kleemann R. Up regulation and coexpression of MIF and matrix metalloproteinases in human abdominal aortic aneurysms. Antioxid Redox Signal 2005;7:1195-202.
23. Perneger TV. What's wrong with Bonferroni adjustments. BMJ 1998; 316:1236-8.
24. Yoshimura K, Aoki H, Ikeda Y, Fujii K, Akiyama N, Furutani A, et al. Regression of abdominal aortic aneurysm by inhibition of c-Jun N-terminal kinase. Nat Med 2005;11:1330-8.
25. Shimizu K, Mitchell RN, Libby P 2006. Inflammation and cellular immune responses in abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol 2006;26:987-94.
26. Xiong W, Zhao Y, Prall A, Greiner TC, Baxter BT. Key roles of CD4+ T cells and IFN-gamma in the development of abdominal aortic aneurysms in a murine model. J Immunol 2004;172:2607-12.
27. Freestone T, Turner RJ, Coady A, Higman DJ, Greenhalgh RM, Powell JT. Inflammation and matrix metalloproteinases in the enlarging abdominal aortic aneurysm. Arterioscler Thromb Vasc Biol 1995;15: 1145-51.
28. Thompson RW, Geraghty PJ, Lee JK. Abdominal aortic aneurysms: basic mechanisms and clinical implications. Curr Probl Surg 2002;39: 110-230.
29. King VL, Lin AY, Kristo F, Anderson TJ, Ahluwalia N, Hardy GJ, et al. Interferon-gamma and the interferon-inducible chemokine CXCL10 protect against aneurysm formation and rupture. Circulation 2009;119: 426-35.
30. Cowburn AS, Condliffe AM, Farahi N, Summers C, Chilvers ER. Advances in neutrophil biology: clinical implications. Chest 2008;134: 606-12.
31. Barnes PJ. New molecular targets for the treatment of neutrophilic diseases. J Allergy Clin Immunol 2007;119:1055-62.
32. Millikan L. The proposed inflammatory pathophysiology of rosacea: implications for treatment. Skinmed 2003;2:43-7.
33. Lindeman JH, Abdul-Hussien H, van Bockel JH, Wolterbeek R, Kleemann R. Clinical trial of doxycycline for matrix metalloproteinase-9 inhibition in patients with an abdominal aneurysm: doxycycline selectively depletes aortic wall neutrophils and cytotoxic T cells. Circulation 2009;119:2209-16.
34. Eliason JL, Hannawa KK, Ailawadi G, Sinha I, Ford JW, Deogracias MP, et al. Neutrophil depletion inhibits experimental abdominal aortic aneurysm formation. Circulation 2005;112:232-40.
35. Pagano MB, Bartoli MA, Ennis TL, Mao D, Simmons PM, Thompson RW, et al. Critical role of dipeptidyl peptidase I in neutrophil recruitment during the development of experimental abdominal aortic aneurysms. Proc Natl Acad Sci U S A 2007;104:2855-60.
36. Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 2002;365:561-75.
37. Takata Y, Kitami Y, Yang ZH, Nakamura M, Okura T, Hiwada K. Vascular inflammation is negatively autoregulated by interaction between CCAAT/enhancer-binding protein-delta and peroxisome proliferator-activated receptor-gamma. Circ Res 2002;91:427-33.
38. Bobryshev YV, Lord RS. Vascular-associated lymphoid tissue (VALT) involvement in aortic aneurysm. Atherosclerosis 2001;154:15-21.
39. Rincón M, Pedraza-Alva G. JNK and p38 MAP kinases in CD4+ and CD8+ T cells. Immunol Rev 2003;192:131-42.
40. Schade AE, Wlodarski MW, Maciejewski JP. Pathophysiology defined by altered signal transduction pathways: the role of JAK-STAT and PI3K signaling in leukemic large granular lymphocytes. Cell Cycle 2006;5:2571-4.
41. Kuivaniemi H, Platsoucas CD, Tilson MD 3rd. Aortic aneurysms: an immune disease with a strong genetic component. Circulation 2008; 117:242-52.
42. Jagadesham VP, Scott DJ, Carding SR. Abdominal aortic aneurysms: an autoimmune disease? Trends Mol Med 2008;14:522-9.
43. Pryshchep O, Ma-Krupa W, Younge BR, Goronzy JJ, Weyand CM. Vessel-specific Toll-like receptor profiles in human medium and large arteries. Circulation 2008;118:1276-84.

Submitted Jun 26, 2009; accepted Jan 19, 2010.