PROTEOMIC ANALYSIS OF INTRALUMINAL THROMBUS HIGHLIGHTS COMPLEMENT ACTIVATION IN HUMAN ABDOMINAL AORTIC ANEURYSMS.

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ABSTRACT (241 words)

Objective: To identify proteins related to intraluminal thrombus (ILT) biological activities that could help to find novel pathological mechanisms and/or therapeutic targets for human abdominal aortic aneurysm (AAA).

Methods and results: Tissue-conditioned media from AAA patients were analyzed by a mass spectrometry (MS)-based strategy, using liquid chromatography coupled to tandem MS (LC-MS/MS). Global pathways analysis by Ingenuity software highlighted the presence of several circulating proteins, among them proteins from the complement system. Complement C3 concentration and activation were assessed in plasma from AAA patients [small AAA, AAA diameter = 3-5 cm and large AAA, AAA diameter > 5cm], showing decreased C3 levels and activation in large AAA patients. No association of a combination of single nucleotide polymorphisms in complement genes between large and small AAA patients was observed. Intense extracellular C3 inmunostaining, along with C9, was observed in AAA thrombus. Analysis of C3 in AAA tissue homogenates and tissue-conditioned media showed increased levels of C3 in AAA thrombus, as well as proteolytic fragments (C3a/C3c/C3dg), suggesting its local deposition and activation. Finally, the functional role of local complement activation in polymorphonuclear (PMN) cell activation was tested showing that C3 blockade by antiC3 antibody was able to decrease thrombus-induced neutrophil chemotaxis and ROS production.

Conclusion: A decrease of systemic C3 concentration and activity in the later stages of AAA associated to local complement retention, consumption and proteolysis in the thrombus could induce PMN chemotaxis and activation playing a detrimental role in AAA progression.

Keywords: abdominal aortic aneurysm-complement system-neutrophils-immune inflammation-thrombus

Clinical and pathophysiological evidences indicate that Intraluminal Thrombus (ILT) play a role in evolution of Abdominal Aortic Aneurysms (AAA) (1). The eccentric distribution of the ILT was associated with continuous expansion (2) and aortic ILT volume is associated with AAA growth (3-5). It has been reported that large ILT areas were significantly associated with increased AAA expansion (3). Speelman *et al* demonstrated that larger ILT in AAA was associated with a higher AAA growth rate, but also with a lower wall stress. These data suggests that weakening of the AAA wall, under the biological dynamics of ILT, might play a more imminent role in the process of AAA growth than the stress acting on the wall (4). A recent study has confirmed the association of ILT volume with AAA growth and also, with cardiovascular events (5). Finally, radiological signs of ILT lysis could precede aortic rupture (crescent sign) (1).

In parallel, accumulating data suggest that biological activities associated to leukocyte, platelet and red blood cell accumulation in ILT play an important role in AAA progression (6-11). Thus, the identification of novel proteins related to ILT biological activities could help to find novel pathogenic mechanisms, as well as therapeutic targets, of AAA. In previous studies, following a strategy based on the analysis of AAA tissue-conditioned media by gel- or array-based proteomic techniques, we identified proteins related to different pathological processes involved in AAA such as oxidation (10) and proteolysis (12). In order to explore further the pathophysiology of ILT in human AAA increasing the number of identified proteins, ILT and wall conditioned-media were analyzed in this study using liquid chromatography and tandem MS (LC-MS/MS). Global pathways analysis of identified peptides/proteins by Ingenuity software highlighted that complement system components were highly enriched in AAA tissue-conditioned media.

The complement system plays a major role in innate immunity, participating in host defense responses against microorganisms via opsonization, chemoattractation of leukocytes, cell activation and bridging innate and adaptative immunity (13-16). However, disturbances in this defense machinery contribute to the pathogenesis of various autoimmune diseases, such as systemic lupus erythematosus (SLE). SLE is characterized by decreased circulating complement components associated to their deposition and activation in host tissues. As complement proteins are mainly synthesized by the liver, we hypothesized that the high levels of complement C3 peptides/proteins identified in AAA thrombus-conditioned media could be related to its trapping from the blood and/or by increased proteolytic activation. To test this hypothesis, we first assessed C3 concentration and activity in blood of AAA patients at different stage of the disease. Second, we analyzed the presence and activation of C3 in AAA tissue and tissue-conditioned media. Finally, we studied the effect of complement activation in human AAA thrombus on neutrophil chemotaxis and oxidation, key mechanisms involved in AAA pathogenesis.

Methods

AAA patients

Spanish patients

In a first cohort, serum from 62 male patients with an asymptomatic infrarenal AAA was collected during clinical examination (aortic size = 3-5 cm, small AAA). Additionally, serum from 28 male patients with an asymptomatic infrarenal AAA was collected before surgical repair (aortic size > 5 cm, large AAA). Twenty-eight healthy male controls with non-dilated infrarenal aortas (aortic size < 3 cm, confirmed with abdominal ultrasound) and no risk factors were obtained from a screening program undertaken in our area of care. All these samples were obtained from Galdakao Ursansolo Hospital (Bilbao, Spain). In a second cohort, plasma samples were obtained from the biobank of IIS-FJD (Madrid, Spain) including 26 small AAA patients and 39 large AAA patients. Hypertension was defined as systolic blood pressure (sBP) >140 mmHg and/or diastolic pressure (dBP) ≥90 mmHg measured during the examination, after the participant had been sitting for at least 30 minutes, or the participant was already taking hypotensive medication. A patient was considered diabetic if he was under treatment (supervised diet, hypoglycaemic oral medication, insulin) or we found basal glycaemia >120 mg/dL and/or HbA1c >=6.5%. Hypercholesterolemia was defined as total basal cholesterol levels ≥200 mg/dl, LDL levels ≥100 mg/dl or the patients were receiving specific medication or a supervised diet. Cardiac disease included coronary heart disease, valvular disease, cardiomyopathy and arrhythmia. Clinical characteristics are summarized in **Table 1.** The studies were approved by Spanish center's Research and Ethics Committees, and informed consent from the patients and the controls for their inclusion in the study was obtained.

Danish patients

Blood cells were obtained from 186 patients from the randomised population based Viborg Vascular (VIVA) screening trial screening 65-74 year old men for AAA, peripheral arterial disease and unrecognised hipertension (17). Informed consent was obtained from all subjects before participation, and the study was approved by the Local Ethics Committee of the Viborg Hospital, Denmark, and performed in accordance with the Helsinki Declaration. Cases were selected according to initial size and growth rate. Clinical characteristics of the patients are included in **table 1 online.**

AAA tissue and tissue-conditioned media

Sixteen AAA thrombus and wall samples were collected from patients enrolled in the RESAA protocol (18) undergoing surgery (three for MS analysis, ten for ELISA, western-blot and immunohistochemistry and six for homogenization). One part was included in paraffin for immunohistochemistry and the rest was dissected into thrombus and wall (media and adventitia) for incubation in a RPMI protein-free medium. All patients gave their informed written consent and the protocol was approved by a French ethics committee (CPB, Cochin Hospital). Twelve control aortas (six for immunohistochemistry and six for homogenization) were sampled from

dead organ donors with the authorization of the French Biomedicine Agency (PFS 09-007). These control aortic samples were macroscopically normal, devoid of early atheromatous lesions. Different layers of AAA thrombus and wall, as well as healthy walls, were cut into small pieces (5 mm^2) and separately incubated in RPMI 1640 medium containing antibiotics and an antimycotic (Gibco) for 24 hours at 37°C (6 ml/g of wet tissue). The conditioned medium (supernatant containing proteins released by the tissue sample) was obtained after centrifugation as 3,000 g for 10 minutes at 20°C. In some cases, native C3 [purified as previously described in Alcorlo M *et al*, (19)] was incubated for 90 min at 37ºC with 1 µl of thrombus-conditioned media and then subjected to western-blot. In addition, tissues were snap-frozen in N_2 liquid and homogenates (0.2 g) were divided and resuspended for mRNA and protein analysis.

NanoLC-MS/MS analysis

Proteins from AAA–tissue conditioned media from 3 patients were precipitated using 2D clean-up kit (GE Healthcare) and re-dissolved in Triethylammonium bicarbonate (TEAB) buffer. Then, they were analysed by nLC-MS/MS, as described in **supplemental methods online.**

Quantification of C3 and C3a

Soluble concentrations of C3 in human plasma samples were automatically measured using VITROS chemistry products C3 reagents in the VITROS 5,1 FS and VITROS 5600 Integrated System analyzers, following the manufacturer´s instructions (Ortho-Clinical Diagnostics, Johnson & Johnson). Soluble concentrations of C3 in serum samples from first cohort or in plasma samples from third cohort were assayed automatically by timed nephelometry using a BNII Nephelometer (Siemens9). Both methods were standardized against the international reference preparation CRM 470 (RPPHS). C3 and C3a in conditioned media was measured with commercial kits (EC2101 Assaypro and 550499 BD, respectively) following the manufacturer's instructions.

AP50 assay

To test the hemolitic capacity of the complement system, red blood cells (RBCs) from healthy rabbits were used together with human sera as described (20) (see **supplemental methods online)**.

DNA isolation and genetic study

Genomic DNA was extracted from peripheral blood using EZ1 DNA Blood 350 µl Kit in an EZ1 Advance Robot (Qiagen) following standard procedures. DNA samples were genotyped for six single nucleotide polymorphisms (SNPs) (CFH Ile62Val, CFH c.1696+2019G>A, CFHR1 Glu175Gln, CFB Leu9His, CFB Arg32Gln/Trp) (21). The genotyping was performed using multiplex PCR and minisequencing methodology (ABI Snapshot; Applied Biosystems). Minisequecing reations were run in an automated sequencer (model 3730; ABI), and the fragments were analyzed with the appropriate software (GeneMapper Software 4.0; ABI).

Immunohistochemistry

AAA and control aorta samples were fixed in 3.7% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed using antiC3 (purified as described in 22) and anti-C9 (mAb B7, a generous gift of Prof. Paul Morgan, Cardiff University) as primary antibodies. Negative controls using the corresponding IgG were included for checking non-specific staining. The secondary antibody and ABComplex/HRP were added and sections were stained with 3,30-diaminobenzidine and mounted in DPX. For colocalization of C3 with vascular smooth muscle cells (alpha-actin), immunohistochemistry followed by immunofluorescence was performed. Colocalization of C3 with CD15/CD68?

Western blot

Equal amounts of proteins from tissue or conditioned medium (30 μg or 5 μL previously normalized to tissue weight: 1 g/6 mL, respectively) were loaded onto 12.5% polyacrylamide gels, electrophoresed and transferred to nitrocellulose membranes. Then they were blocked with 7% milk powder in TBS-T for 1 hour and incubated overnight at 4ºC with antiC3 (22). Then the membranes were washed with TBS-T and incubated with anti-rabbit antibody (1:5000) for 1 hour at RT. After 4 washes, the signal was detected using the ECL chemiluminiscence kit (GE Healthcare).

Real time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). One microgram of RNA was used to perform the reverse transcription with the high capacity cDNA archive kit (Applied Biosystems). Real-time PCR reactions were performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to the manufacturer's protocol, using the DDCt method. Human mRNA levels for C3 and 18S were done by amplification of cDNA using SYBRw Premix Ex TaqTM (Takara Biotechnology). The primer sequences are: Forward primer: AAGCGCATTCCGATTGAGGA, Reverse primer: AAGACTTCCCCACCAGGTCT. The mRNA levels of C3 were normalized to the 18S mRNA content.

Cell isolation, chemotaxis assay and ROS production.

Neutrophils were isolated from venous blood of healthy volunteers and transwell migration assays and ROS production were performed as described (12, 23) (**see supplemental methods online**)

Statistics

Normality of data was checked by probability plots. Normally distributed C3 concentrations and activity are expressed as mean±SEM. $P < 0.05$ was considered to be statistically significant. Difference among the groups in the first cohort (control, small aaa and large aaa) was analyzed by one-way ANOVA test followed by post hoc Tukey Kramer test for multiple comparisons. Differences among the groups of the second cohort and third cohort (small AAA vs large AAA) were analyzed by t-test.

Pearson correlation was used to determine correlations between two variables. Logistic or linear regression analysis adjusted by risk factors was conducted with AAA stage (small/large AAA) and aortic size as dependent variables, respectively. The Wilcoxon paired test was used to analyze differences in C3 and C3a levels between thrombus and wall supernatants of the same samples, while non-paired tests were used for pathological wall vs healthy wall supernatants comparisons. All the statistical analyses were performed by using SPSS 11.0 statistical package.

RESULTS

LC-MS/MS analysis of proteins from AAA tissue-conditioned media

Proteins obtained from AAA thrombus- and wall- conditioned media were trypsin-digested and the resulting peptides were then fractionated by 2D-LC using a strong cation exchange column followed by C18 reversed phase chromatography. Finally, the MS and MS/MS spectra were used for protein identification. **Table 2 online** lists all the proteins identified in AAA-tissue supernatants, where a 60% of them were classically secreted. To organize identified proteins, Ingenuity software was used to find the most enriched canonical pathways in our samples. A total of 257 proteins extracted from the protein lists corresponding to thrombus and wall layers supernatants were analysed in the same data set. Among others, coagulation and complement systems have been found as relatively enriched in the AAA-tissue supernatants (as compared with the human genome database) **(Figure 1A).** Interestingly, several complement-related proteins [e.g. C3, C9, clusterin, factor H] were identified, which are represented in grey colour on **Figure 1B**.

Systemic C3 concentrations and activity in AAA patients

As C3 is the central molecule in the complement cascade, we analyzed serum concentrations of C3 in a first cohort of healthy controls (n = 28) and AAA patients at follow-up [small AAA, AAA diameter = 3-5cm (n = 62)] or at surgery [large AAA, AAA diameter>5cm, (n=28)]. Increased C3 concentrations were observed in small AAA patients compared to both controls and large AAA patients (controls=148±5 vs small AAA= 177±4 vs large AAA= 124±8 mg/dl; $p<0.01$). Logistic regression analysis showed that association between increased C3 in small AAA patients vs controls remained significant when adjusted by age but was lost when adjusted for risk factors (not shown), whereas the decreased C3 in large vs small AAA patients persisted after adjustment for risk factors (**Table 3 online**). Plasma C3 concentrations correlated with lipid levels (r = 0.4 for LDL and tryglicerides and r=-0.4 for HDL, $p<0.001$ for all) and aortic size (r= -0.4, $p<0.005$). Linear regression analysis between C3 and aortic size was also independent on risk factors (Table 4 online).

In order to confirm previous data, we further analyzed in a second cohort of patients, showing that large AAA patients (n=39) have significantly decreased C3 plasma concentrations compared to small AAA patients (n =26) (122±4 vs 138±4 mg/dl, $p<0.01$, **Figure 2A**), which persisted after adjustment for risk factors (**Table 3 online**). A non-significant negative correlation was shown for C3 and aortic size (r= -0.2, p=0.1, Figure 2C). To test whether complement activity is modified in plasma of AAA patients at different stage of evolution, we performed an AP50 assay that measure the ability of the patient's plasma to lyse rabbit erythrocytes. Accordingly, large AAA patients have decreased complement activity as compared to small AAA patients [42±5 vs 75±4 % lysis; $p<0.01$, **Figure 2B**]. Logistic regression analysis showed that the significant association between AP50 in large AAA compared to small AAA patients persisted after adjustment for risk factors **(Table 3 online**). AP50 correlated with aortic size (r = -0.4, $p<0.005$, Figure 2D), which persisted after adjustment for risk factors (Table 4 online).

Genetic association study

No association of SNPs in the complement cascade in AAA patient and control studies have been recently described (24,25). To get further insight into a potential genetic association between complement and AAA evolution, we analyze whether the decrease in C3 concentrations and activity in large vs small AAA patients could be related to a particular combination of SNPs in complement genes ("complotypes"), as described for other disorders like age-related macular degeneration (reviewed in Harris *et al*, 21). However, no association was found between these complotypes and AAA in patients at different stages of the disease (large vs small AAA). In plasma samples available from these patients (n = 138), we further confirmed that C3 concentrations were decreased in large AAA patients (n = 66) compared to small AAA patients (n = 72) (194±5 vs 210±5 mg/dl, $p < 0.05$). Logistic regression analysis showed that the significant association between decreased plasma C3 in large AAA compared to small AAA patients persisted after adjustment for risk factors **(Table 3 online**). A non-significant negative correlation was shown for C3 and aortic size (r=-0.2, p=0.07).

Local complement retention and activation in AAA

We analyzed the presence of C3 in AAA tissue by immunohistochemistry, showing an intense extracellular staining in the ILT and in a lesser extent in the wall, whereas healthy wall shows weak staining (**Figure 3A**). Similarly, high C9 staining was observed in AAA thrombus compared to wall and healthy wall (**supplemental figure 1**). Interestingly, C3 and C9 deposition was observed in similar areas of the thrombus, suggesting complement activation (**Figure 3b**). Whereas C3 was mainly present in acellular areas of the thrombus, C3 in the wall was also associated to alpha-actin positive cells in the media (**supplemental figure 2**) and CD15/CD68 positive cells mainly in adventitia (**not shown?),** suggesting the possible synthesis by resident or infiltrating cells in the wall. To test this hypothesis, we performed both real time-PCR and western-blot of tissue homogenates. No detectable C3 mRNA was obtained from 3 out of 6 thrombus analyzed and very low levels were observed in the rest (0.01±0.02 a.u.). No differences were observed in C3 mRNA levels between healthy and AAA wall (0.22±0.1 vs 0.28±0.05 a.u., not shown). In contrast, C3 protein levels in tissue homogenates were higher in ILT compared to pathological wall and healthy wall, confirming the results observed by immunohistochemistry (**Figure 4A**).

We further tested C3 concentration and activation in the conditioned media of human ILT and wall of AAA, as well as in healthy media. C3 levels were increased in the AAA thrombus compared to the pathological wall and healthy wall (5.6±0.5 vs 2.4±0.2 vs 0.9±0.2 µg/ml, $p< 0.001$ for all comparisons). Moreover, C3 proteolytic fragments of 35-40 KDa [corresponding to the molecular weight of C3c/C3dg, **figure 4 A, B, C**] appear mainly in tissue and tissue-conditioned media of ILT and to a lesser extent in the wall (media and adventitia) of AAA, whereas almost no proteolytic fragments were observed in healthy wall. Since complement activation could involve proteolytic degradation of C3 by proteases such as plasmin or elastase (13) abundantly present in ILT (6,7), we assessed whether proteolysis of C3 in the thrombus of AAA could take place *ex vivo.* In this respect, when native C3 was incubated with the luminal part of the thrombus, the 35-40 KDa fragments observed in ILT conditioned media were increased **(Figure 4D).**

Role of complement activation in AAA thrombus-induced PMN chemotaxis and activation

As we did not observed the first fragment of C3 activation, C3a, by western blot probably due to its low molecular weight, we analyzed the presence of C3a in tissue-conditioned media by ELISA. We have observed that C3a levels are increased in ILT compared to wall of AAA and also to healthy wall ($p < 0.001$ for all comparisons, **Figure 5A**). Moreover, since C3a is involved in PMN chemotaxis and ROS production, we address the functional role of complement activation by proteolysis in ILT. Neutrophils were allowed to migrate through a filter into a lower chamber containing thrombus-conditioned media and the effect of C3 blocking was assessed. Neutrophils were attracted by luminal thrombus and C3 blockade by antiC3 antibody was able to decrease such effect ($p<0.001$)(**Figure 5B**). Similar effect was observed when native C3 was used as a positive control (not shown). Moreover, incubation of thrombus-conditioned media with fresh neutrophils increased ROS levels, which was prevented by antiC3 antibody (**Figure 5C**).

DISCUSSION

In the present paper, the combination of nano-Liquid Chromatography and LTQ-Orbitrap MS allowed us to identify larger lists of proteins from AAA-tissue conditioned media, as compared to array or gel-based approaches (10, 12). Several proteins previously associated to different AAA pathological mechanisms have been identified [e.g immune-inflammatory response (clusterin), thrombosis (fibrinogen)]. Interestingly, a recent proteomic study has also shown increased levels of clusterin, a complement lysis inhibitor able to block the terminal complement cascade, in AAA-thrombus conditioned media. In contrast, clusterin concentrations were decreased in AAA plasma, and the authors suggested that ILT could sequester systemic proteins (26). In agreement, the functional distribution of the identified proteins in our study has shown an enrichment in circulating proteins (e.g. coagulation and complement systems). As complement proteins are mainly synthesized by the liver, we hypothesized that the high levels of complement C3 peptides/proteins identified in AAA thrombus-conditioned media could be related to its trapping from the blood and/or by increased proteolytic activation. We first analyzed C3 concentrations in AAA patients and controls. Circulating C3 levels were increased in small AAA patients compared to controls probably suggesting an initial hepatic response to vascular injury. However, when we performed a multivariate analysis including risk factors, no significant differences were observed between small AAA patients and controls in agreement with previous data (27), discarding its potential use as a diagnostic biomarker. Regarding risk factors, we have observed a positive correlation between C3 and lipid levels. Lipids have been previously suggested as a potential mechanism leading to complement activation in experimental and human hypercholesterolemia (28). Moreover, it has been proposed that IgG is an initial mechanism leading to C3 activation in an experimental model of AAA (29) and increased IgG concentrations have been recently observed in small AAA patients vs controls. However, IgG concentrations decline in large AAA patients (30). In agreement, we have shown that C3 concentrations are decreased in large compared to small AAA patients, which was independent of different risk factors. In addition, when we performed an AP50 assay to test whether complement activity is modified during AAA evolution, we showed a negative association of complement activity with later stages of disease and with aortic size, independently on risk factors. On the whole, our data suggest that systemic C3 increase in the initial phases of AAA probably as a response to injury of the wall to increased lipid and/or IgG concentrations, while a decrease in systemic complement concentration and activation takes place in the later stages of AAA.

Complement deficiencies (inherited or acquired) could be linked to the development of autoimmunity, as shown in systemic lupus erythematous where decreased complement components are observed. To check whether genetic anomalies in complement genes could take place in AAA evolution, we performed a genetic study analyzing a particular combination of SNPs in complement genes ("complotypes"). This approach have been also described for other disorders like age-related macular degeneration [(reviewed in Harris *et al*, (21)]. No association on any of the combination of SNPs analyzed was found between small and large AAA patients, similar to previous studies where those individual SNPs were assessed in AAA patients and controls (24,25). It has been suggested that the acquired diminution in circulating complement proteins in autoimmune diseases could be associated to deposition of complement components in host tissue (13). In this respect, complement

proteins have been previously detected in human AAA wall (24,31,32). We observed that C3 protein levels were increased in tissue and tissue-conditioned media of AAA wall compared to healthy aortic wall, whereas C3 mRNA is similarly present in pathological and healthy wall in agreement with Hinterseher et al (24). But, Hinterseher *et al* did not show C3 staining in the thrombus (24). In contrast, we have shown that human AAA thrombus display an intense extracellular staining, along with increased protein levels in tissue homogenates and tissue-conditioned media of ILT compared to wall. Differences between both immunohistological studies could be related to the different antibodies used. Furthermore, C3 in thrombus from acute myocardial infarction has been also observed (33). Since the liver is the major source of complement proteins in humans and low/undetectable C3 mRNA levels were observed in thrombus homogenates, the high extracellular levels of C3 observed in AAA thrombus should be associated to its retention from serum and subsequent activation. In this regard, high C9 immunostaining was also shown in acellular areas of the thrombus and wall compared to healthy wall. In agreement, Pagano *et al* showed C5B9 in the luminal side of the wall, whereas no staining was observed in healthy wall (32). Similarly, Tulamo *et al* also observed complement activation and C5B9 formation in the less cellular part of intracranial artery aneurysm wall (34). Finally, we showed intense C9 immunostaining associated to C3 deposition, suggesting complement activation in AAA thrombus.

Complement activation involves the classical pathway (CP), the lectin pathway, the alternative pathway (AP) and the extrinsic pathway (13). The CP pathway can be activated by antibodies or by other stimulus such as CRP. The AP is part of the innate (non-antigen-specific) immune system and is important in antibody-independent defense against bacterial infection. The extrinsic pathway involves proteolytic degradation of C3 by proteases such as elastase and/or by phagocytes. Moreover, other components of the coagulation system such as plasmin could also participate in complement activation (16). In this respect, human AAA thrombus could be a privileged site for complement activation since proteases and PMNs are abundant in the ILT of AAA (6,7). When we analyzed C3 levels in AAA tissue and tissue-conditioned media by western-blot, we observed proteolytic fragments of C3 in AAA thrombus and thrombus-conditioned media. Furthermore, these products of C3 proteolysis and activation were increased when native C3 protein was coincubated with the luminal part of AAA, supporting that *in vivo* proteolysis of C3 protein could take place within the thrombus of AAA. In addition, C3c/C3dg levels were increased in AAA wall compared to healthy wall. These proteolytic products could participate in the shift from innate to adaptive immunity characteristic of the adventitial response in AAA (35). Finally, given that C3a is released in the initial step of the proteolytic processing of C3, C3a levels were assessed in AAA tissue and healthy wall. In agreement with the results obtained for C3, C3a was increased in conditioned media of ILT and wall compared to healthy aorta wall, further supporting proteolysis of C3 in AAA tissue. C3aR mRNA is upregulated in AAA tissue (24), favoring the potential interaction with C3a in AAA and its functional consequences. In this respect, it is already described that the complement acts as a critical mediator of neutrophil recruitment in AAA mice lesions (32). Interestingly, we have observed that PMN chemotaxis induced by the ILT could be modulated by incubation with an antiC3 antibody. Moreover, C3a has been involved in PMN respiratory burst (36). We have observed that NADPH-dependent ROS production is increased when fresh PMNs are incubated with ILT-conditioned media and this effect was prevented by C3 blockade.

In any case, we should take into account that these functional activities of the thrombus could be also related to the presence of other factors such as tissue factor, clotting factors (e.g. Xa) or thrombin, among others. Thrombin is involved in the activation of the complement system further confirming a coordinated action of the coagulation and complement systems (16). Interestingly, both systems have been associated with innate immunity into what *Engelmann and Massberg* recently described as immunothrombus (37). Thrombin also modulates fibrinolysis by activating the plasma carboxypeptidase, thrombin-activatable procarboxypeptidase B (pCPB). In this respect, enhanced AAA formation in pCPB−/− mice was observed associated to plasmin generation (38). On the whole, all this data suggest that complement activation by proteases present in the thrombus could contribute to AAA pathogenesis.

Several studies have recently demonstrated that genetic modification of different mediators of the complement pathway could reduce experimental AAA formation (29, 32). However, complement activation is modulated by several complement inhibitors (e.g. CD59, vitronectin). In this respect, Tulamo *et al* showed a differential distribution of complement inhibitors in different areas of the wall of intracranial artery aneurysms. These data suggest that a disturbed complement regulation is associated with an increased susceptibility to complement activation and inflammation, and maybe also cell loss (34). In our previous study, vitronectin was shown to be down-regulated in tissue of ruptured AAA compared with non-ruptured AAA (39). Finally, deficiency of CD59 accelerated, whereas transgenic overexpression of human CD59 attenuated, the progression of experimental AAA (40). At the therapeutic level, antiC5 blockade was able to reverse atherosclerosis associated to decrease complement deposition (41). Since atherosclerosis and AAA share some pathological mechanisms, it could be interesting to address whether the therapeutic modulation of complement activators or inhibitors may have a protective role in AAA progression.

On the whole, the decrease of systemic C3 concentration and activity in the later stages of AAA associated to local complement retention, consumption and proteolysis in the thrombus could induce PMN activation playing a detrimental role in AAA. Future studies targeting complement activation could be an attractive therapeutic strategy to prevent AAA progression.

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DISCLOSURES

None

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FIGURE LEGENDS

Figure 1. **Ingenuity Pathway Analysis of identified proteins by LC-MS/MS.**

A.- The bar graphic shows the canonical pathways distribution represented by gene enrichment. Ratios show the number of genes associated to each pathway found in our experiment respect the human genome database. Fisher's exact test provides p-values <0.05 for the 7 most abundant canonical pathways.

B.- Detailed inspection of the complement system cascade, where proteins identified by MS in AAA-tissue conditioned media are represented in grey colour. Protein groups or complex are yellow encircled, and receptors of different complement components are represented at the bottom of the figure.

Figure 2. **Systemic C3 concentration and activity in AAA patients.**

C3 concentration (**A**) and activity (**B**) in plasma of small AAA patients (n=26) an large AAA patients (n=39). Correlation of C3 concentration (C) and activity (D) with aortic size.

Figure 3. **Local complement retention in AAA tissue.**

Immunohistochemistry of C3 in thrombus (**A**), wall (C) and Healthy wall (D). Negative control (non-specific IgG) (B). C3 (E) and C9 (F) immunostaining is performed in serial sections of AAA thrombus. Magnification 20x. (inset: 120x, showing areas with PMNs)

Figure 4. Local complement activation in AAA tissue and tissue-conditioned media.

A.- Representative western blot of C3 in tissue homogenates of human AAA thrombus (T), pathological wall (W=media+adventitia) and healthy wall (H=media+adventitia).

B.- Representative western blot of C3 in tissue conditioned media of human AAA thrombus (T), pathological wall (media, M, and adventitia, ADV) and healthy wall (media, HM, and adventitia, HADV).

C.- Representative western blot of C3 in tissue conditioned media of human AAA pathological wall (media, M) and thrombus (T) and healthy wall (H). Fragments of C3 (C3dg, C3c, C3b, iC3b) or native C3 purified as described (20).

D.- Representative western blot of C3 in thrombus conditioned media incubated with native C3. Arrows indicated proteolytic fragments of C3.

Figure 5.-Role of complement activation in AAA thrombus-induced chemotaxis.

A.- Elisa of C3a in tissue-conditioned media of human AAA thrombus (T, n=10), pathological wall [media (M, n=10) and adventitia (ADV, n=10)] and healthy wall [media (HM, n=10) and adventitia (HADV, n=10)]. * and † $p<0.001$ for T vs M and ADV and M and ADV vs HM and HADV, respectively.

B.- Chemotaxis assay showing PMN migration towards the Thrombus (T), Thrombus preincubated with antiC3 (T+antiC3 at 1:100 or 1:10 dilution), Thrombus

preincubated with anti-IgG (T+IgG, for non-specific chemotaxis). *p<0.001 for antiC3 vs T.

C.- NADPH-dependent ROS production in PMNs stimulated during 2 min with Thrombus conditioned media, Thrombus preincubated with antiC3 (T+antiC3 at 1:100 or 1:10 dilution) or Thrombus preincubated with anti-IgG (T+IgG). *p<0.05 for antiC3 vs T.

Table 1.- Clinical characteristics of AAA patients

a) First cohort

	Small AAA (n=62)	**Large AAA (n=28)**
Sex (male/female)	62/0	28/0
Age (years±SD)	69.9±6.7	73.4±6.6
Dyslipidaemia (%)	56.5	37
Current smoking (%)	37.1	42.9
Diabetes (%)	19.4	3.6
Hypertension (%)	64.5	64.3
Heart disease (%)	32.3	21.4

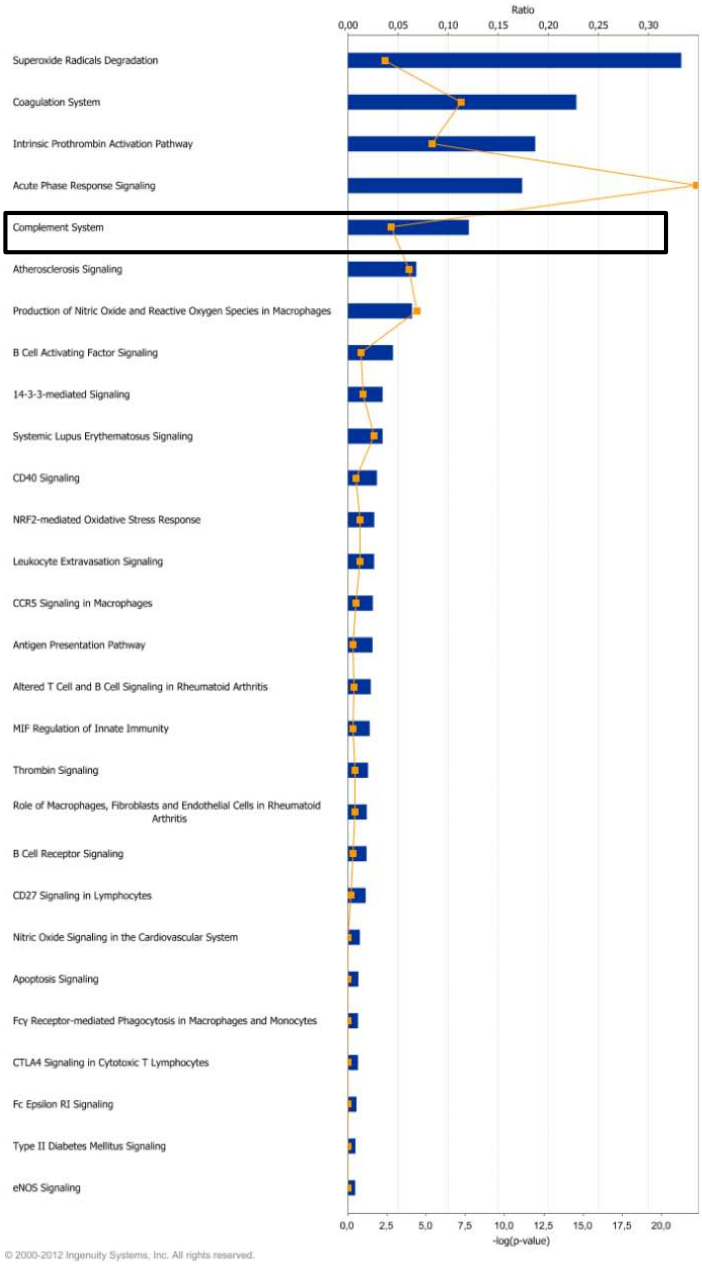
b) Second cohort

	Small AAA (n=26)	**Large AAA (n=39)**
Sex (male/female)	24/2	37/2
Age (years±SD)	79±5	70±10
Dyslipidaemia (%)	46	53
Current smoking (%)	15	30
Diabetes (%)	30	7
Hypertension (%)	73	46
Heart disease (%)	23	23

Significance

Clinical and pathophysiological evidences support that Intraluminal Thrombus (ILT) plays a role in evolution of Abdominal Aortic Aneurysms (AAA). ILT and wall conditioned-media were analyzed using a proteomic approach. Global pathways analysis of identified peptides/proteins highlighted that complement system components were highly enriched in AAA tissue-conditioned media. This could be related to its trapping from the blood (since decreased complement C3 concentration was associated to later stages of AAA in three different cohorts) and/or by increased proteolytic activation (as C3 fragments were observed in ILT). The functional consequences of complement retention and activation in AAA thrombus are related to increased PMN chemotaxis and ROS production, main mechanisms involved in AAA progression. Our data support an important role of complement activation not only in the initial phases of AAA formation (as demonstrated in experimental models of AAA) but also in human AAA progression associated to PMN recruitment and activation in ILT.

A



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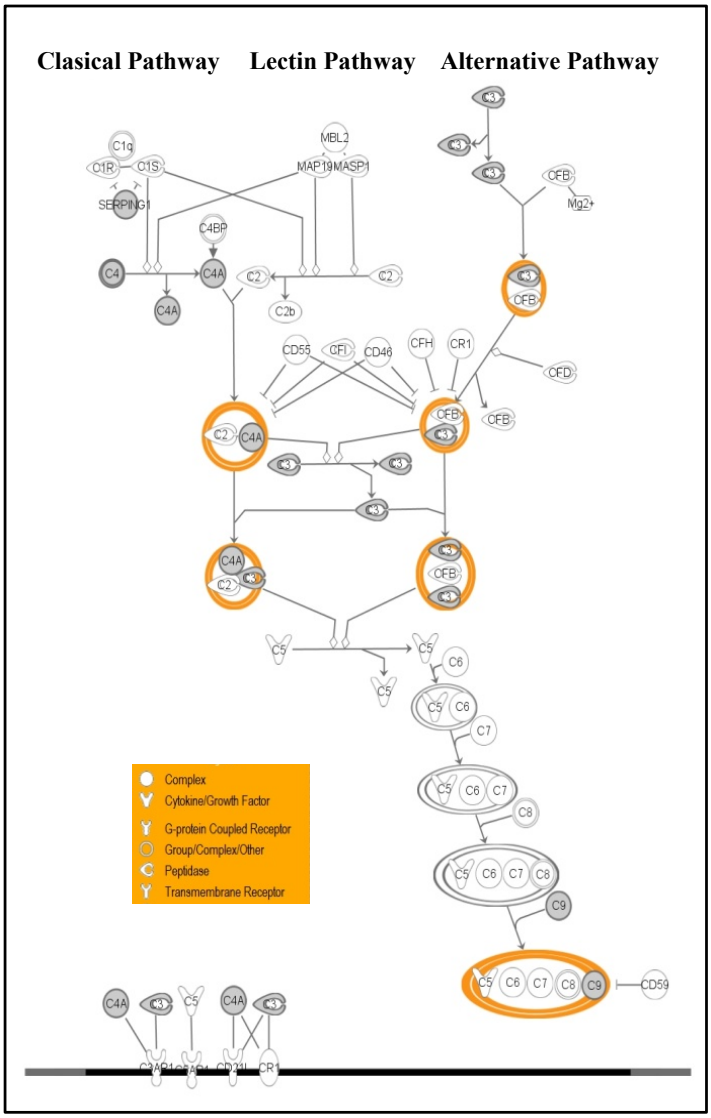

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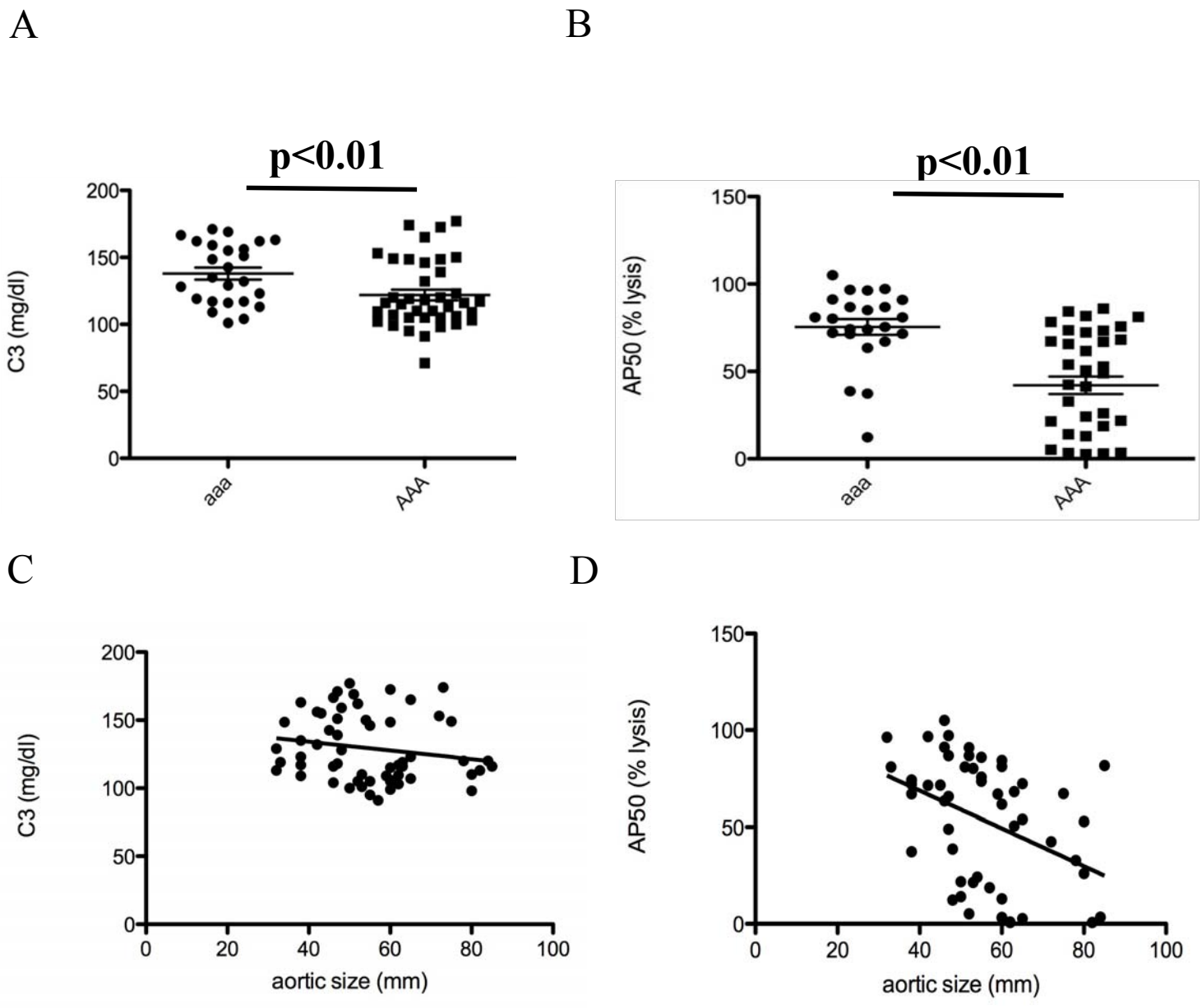


Figure 2. **Systemic C3 concentration and activity in AAA patients.**
C3 concentration **(A)** and activity (**B**) and in plasma of small AAA patients (n=26) an large AAA patients (n=39). *p<0.01 vs small AAA. Correlation between C3 **(C)** and AP50 **(D)** with aortic size

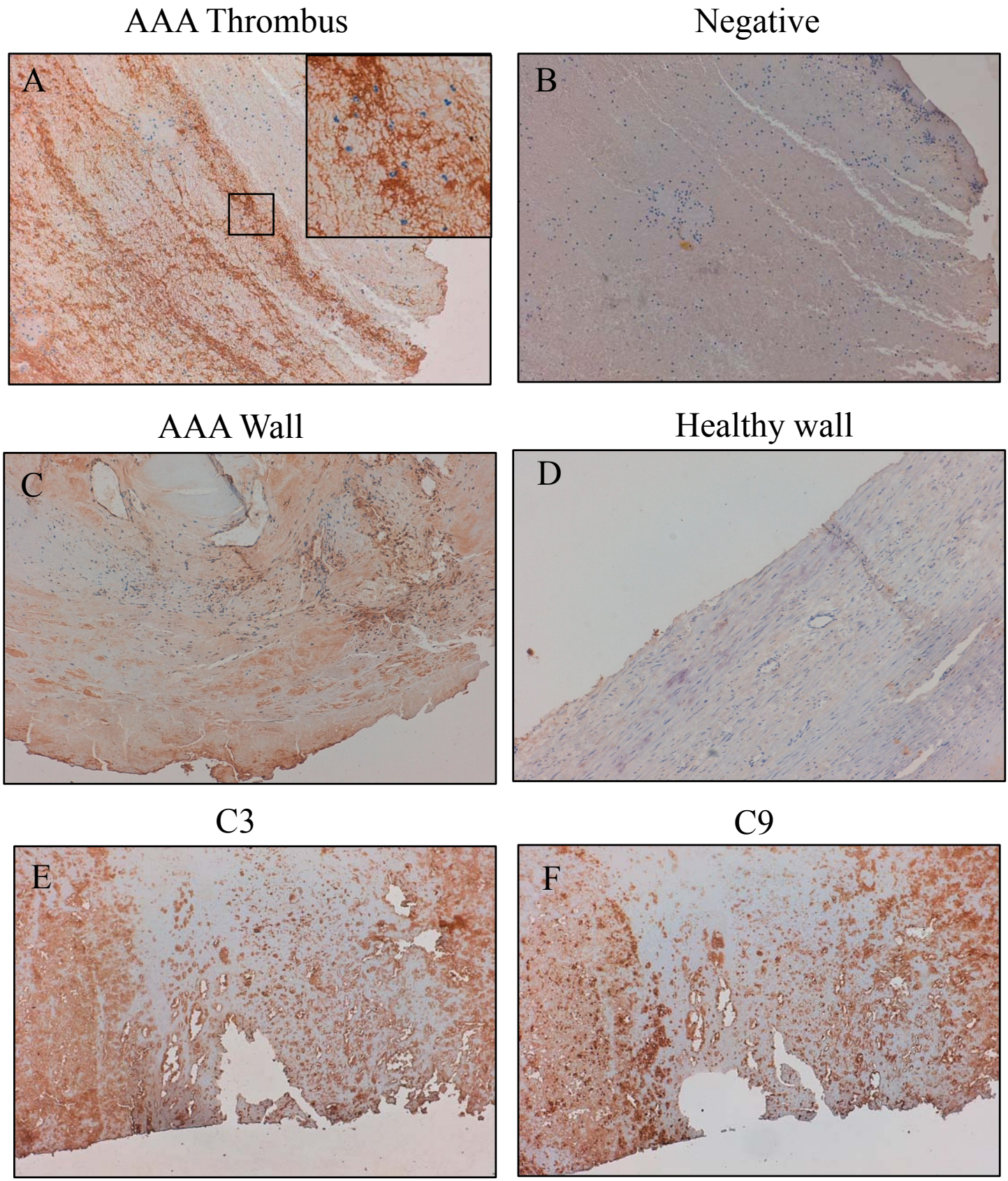
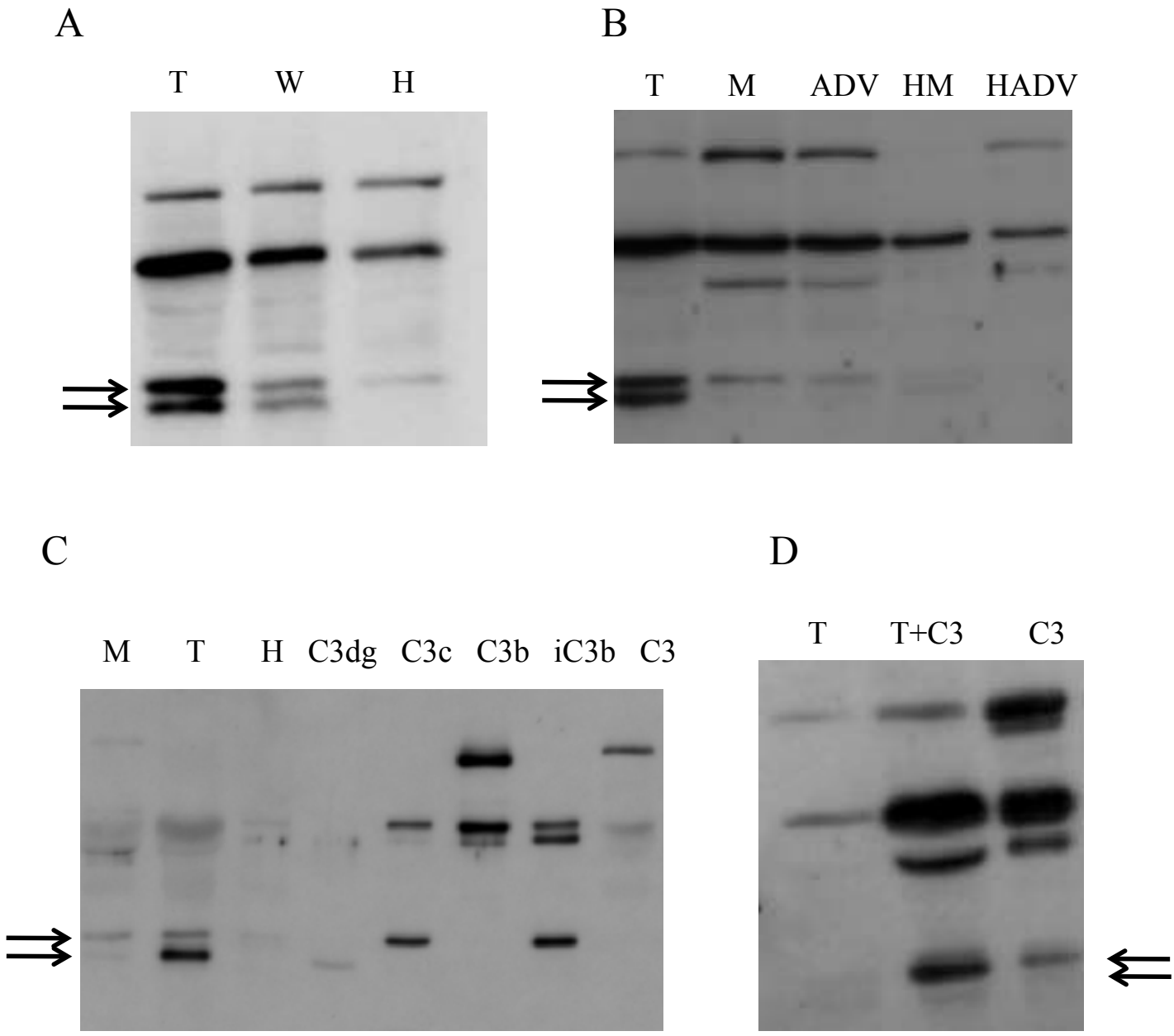


Figure 3. **Local complement retention in AAA tissue.**
Immunohistochemistry of C3 in AAA thrombus (A), AAA wall (C), healthy wall (D). (B) Negative control of C3 in AAA trhombus (IgG). Immunostaining of C3 (E) and C9 (F) in serial sections of AAA thrombus.



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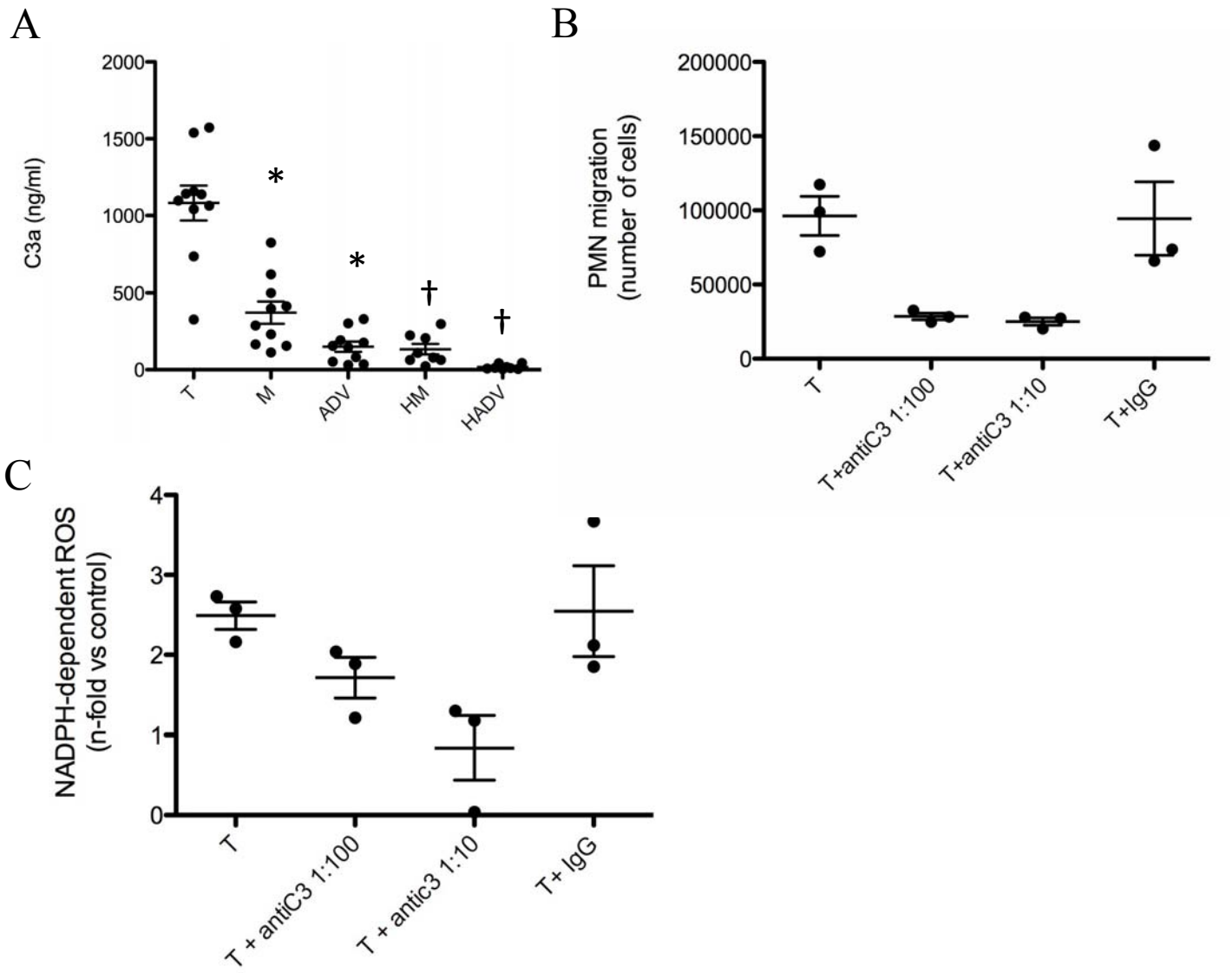
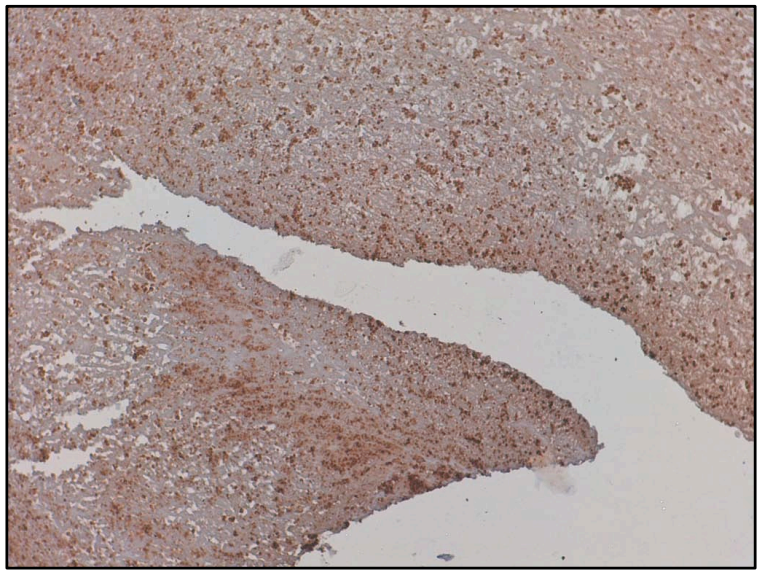


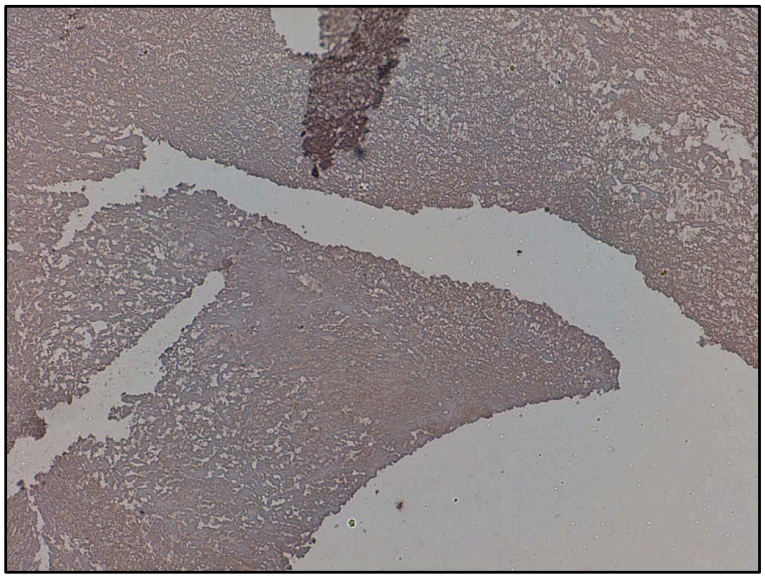
Figure 5.-Role of complement activation in AAA thrombus-induced PMN chemotaxis and activation.

A.- Elisa of C3a in tissue-conditioned media of human AAA thrombus (T, n=10), pathological wall [media (M, n=10) and adventitia (ADV, n=10)] and healthy wall [media (HM, n=10) and adventitia (HADV, n=10)]. * and † $p<0.001$ for T vs M and ADV and M and ADV vs HM and HADV, respectively. **B.-** Chemotaxis assay showing PMN migration towards the Thrombus (T), Thrombus preincubated with antiC3 (T+antiC3 at 1:100 or 1:10 dilution) or Thrombus preincubated with anti-IgG (T+IgG, for non-specific chemotaxis). *$p<0.001$ for antiC3 vs T. **C.-** NADPH-dependent ROS production in PMNs stimulated during 2 min with Thrombus conditioned media, Thrombus preincubated with antiC3 (T+antiC3 at 1:100 or 1:10 dilution) or Thrombus preincubated with anti-IgG (T+IgG). *$p<0.05$ for antiC3 vs T.

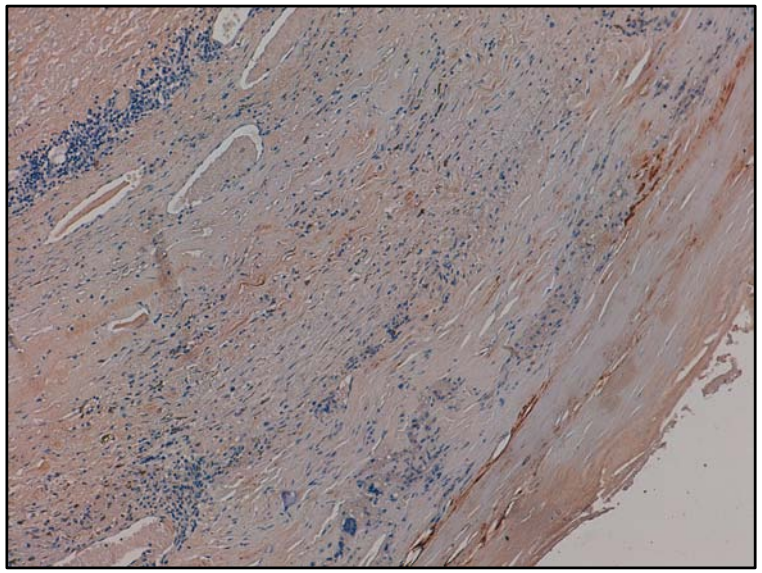
AAA thrombus



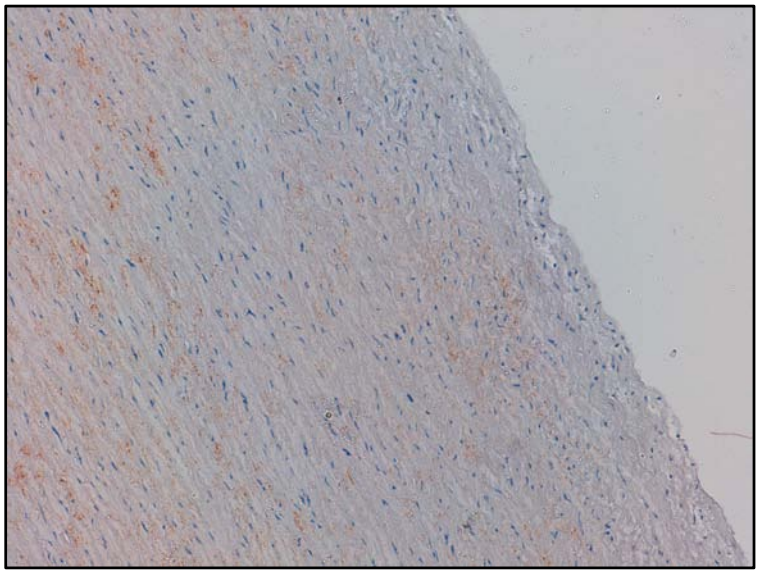
Negative



AAA wall



Healthy wall



C3

SMC

AAA wall