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End of Term Final Report

Student Name: Lauren Bath **Date:** 05 August 2018

Project Title: The relationship between active proteolytic enzymes and intraluminal thrombus in human abdominal aortic aneurysms

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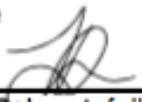
Summary (250 words max single spaced):
We have previously shown that the region of abdominal aortic aneurysms (AAA) containing intraluminal thrombus may be at higher risk of rupture. The purpose of this study is to evaluate differential expression of macrophage derived protease and cytokines across the aneurysm, to evaluate the regional differences in AAA that may contribute to rupture.

Full thickness tissue samples were collected from twenty-one participants using a systematic map, including specimens from thrombus adjacent and thrombus free wall. Protein array was performed for matrix metalloproteinase (MMP)-12, interleukin (IL)-6, IL-10, and macrophage chemoattractant protein (MCP)-1. Eight participants undergoing aortobifemoral bypass for aortoiliac occlusive disease were included as control.

We demonstrated inflammation, specifically, CD68+ macrophage are elevated in thrombus adjacent AAA compared to control. This supports the body of literature identifying AAA as an inflammatory process. Contrary to our hypothesis, there was no difference in inflammation between ILT and non ILT containing regions of the AAA. There was additionally no difference in MMP-12, IL-6, IL-10 and MCP-1 between regions of high ILT, no ILT, and control. This suggests that in addition to presence, macrophage function may not significantly differ between regions of the AAA. These results propose that while inflammation is a hallmark of AAA, macrophage may play a limited role in AAA rupture.

Student Signature 

Primary Supervisor Signature **April J. Boyd** Digitally signed by April J. Boyd DN: cn=April J. Boyd, o=Health Sciences Centre, ou=Vascular Surgery, email=abboyd2@hsc.mb.ca, c=CA Date: 2018.07.31 18:27:15 -05'00'

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Introduction

Abdominal aortic aneurysms (AAA) are present in up to 8% of men over 65, with rupture associated with significant mortality.[1] Currently, the decision to repair AAA is based on a diameter of ≥5.5 cm in males and ≥5.0 cm in females, symptomatology, or rupture.[2] However, size as a single criterion for rupture fails to explain AAA that rupture at much smaller sizes, and AAA that reach extreme sizes without rupture.[3,4] Investigation into pathogenesis and pathophysiology of AAA may elucidate reason for rupture, and provide novel mechanisms for ascertaining which patients need repair.

Contributing factors to the development of AAA may include genetics, inflammation, proteolysis, and hemodynamics.[5] The presence of proteolytic factors in the aneurysmal tissue has been well described, although their role in AAA expansion and rupture is inconclusive.[6] Matrix metalloproteases (MMPs) have been implicated in AAA as they play a role in inflammation, apoptosis, and ECM remodelling.[7]

Macrophage metalloelastase (MMP-12) is involved in macrophage mediated extracellular proteolysis, and is an understudied protease in the literature surrounding AAA pathogenesis. Incubation of aortic tissue with MMP-12 in vitro has demonstrated degradation of healthy tissue, including extensive collagen fragmentation.[8] In mice, MMP-12 gene knockout results in attenuated aneurysm growth, supporting the role of this protease in AAA expansion.[9] In the human AAA, MMP-12 is histologically associated with macrophage infiltration and elastin degradation.[6] Measurement of MMP-12 in AAA has demonstrated significant elevations in comparison to control, furthering supporting its role in AAA pathogenesis.[6] However, there is limited investigation into the differential expression of MMP-12 across the aneurysm sac. In vitro studies have shown intraluminal thrombus (ILT) actively secretes proteolytic factors, including MMPs.[10] Presence and size of ILT has even been offered as an independent risk factor for AAA expansion.[11] Our group has previously described that rupture of AAA occurs in regions of low wall shear stress, where flow recirculation and ILT predominate.[12] Therefore, it may be hypothesized that MMP-12 is higher in aneurysmal wall adjacent to thrombus, contributing to AAA degeneration and rupture.

Supporting the role of MMP-12 in AAA, is the considerable presence of macrophage. Inflammatory cell infiltration is a histological hallmark of AAA, with macrophage being a predominant intramural infiltrate.[13] Although present throughout the aneurysm, there may be higher percentages of macrophage in regions of high ILT.[14] To establish their presence, monocyte chemoattractant protein-1 (MCP-1) is secreted by macrophage, and functions in recruitment and infiltration of macrophage to site of inflammation.[15,16] Indeed, MCP-1 elevation has been found in AAA compared to control.[17] However, the function of MCP-1 in aneurysm pathogenesis and rupture remains to be understood. As macrophage are hypothesized to play a role in AAA rupture, it is expected MCP-1 and macrophage will be elevated in ILT containing regions of the aneurysm that have greater propensity for rupture.

In addition to MCP-1, many macrophage associated cytokines are elevated in AAA. Interleukin (IL)-6 being one of most abundantly expressed and well-studied in aneurysmal tissue.[13] IL-6 function may include stimulation of MMP expression, angiogenesis, cell activation, differentiation, recruitment, and chronic inflammation.[18] Along with strong association to AAA presence, it has previously been demonstrated that IL-6 may even correlate with increasing aneurysm diameter.[19] Along with IL-6, IL-10 is an additional macrophage associated cytokine implicated in AAA. IL-10 functions to ameliorate excessive inflammatory response, blunting activity and thus preventing tissue damage.[20] In AAA, researchers have documented decreased

circulating IL-10 compared to control.[21] True to its proposed function, there has also been negative correlation demonstrated in AAA tissue between IL-10 and IL-6.[22] Despite these correlations, the role the described chemokines play in pathogenesis and rupture remain unclear. There is also lack of literature describing differential expression of IL-6 and -10 in relation to the ILT and thus region of AAA rupture. It can be hypothesized pro-inflammatory IL-6 will be elevated alongside macrophage presence in ILT adjacent tissue, while IL-10 levels will be decreased, contributing to tissue degradation and ultimately rupture.

In the present study, we analyzed differences in macrophage mediated inflammation on thrombus and non-thrombus adjacent AAA tissue in comparison with control. We hypothesize cumulative inflammation and CD68+ macrophage presence will be higher in AAA compared to control. Further, we hypothesize this inflammation and subsequent macrophage mediated proteolytic activity will be localized to ILT containing regions of the AAA. Therefore, we expect elevated MMP-12, IL-6, MCP-1, and decreased IL-10 on thrombus containing regions of the aneurysm. Regional differences in areas of the AAA that tend to rupture may elucidate important factors that may contribute to aneurysm rupture, and lead to novel mechanisms to identify which AAA may need intervention.

Methods

Patient selection

Participants were selected among patients presenting to the authoring vascular surgeon at one Winnipeg centre. Patients with infra-renal AAA necessitating open repair were eligible. As control, patients undergoing aortobifemoral bypass (ABF) for aortoiliac occlusive disease were also eligible. Research nurse obtained Informed consent prior to surgery under REB B2013:130. Participant demographics were obtained from pre-surgical clinic records.

Sample Acquisition

The inclusion of venous blood samples was added partway through the study. Venous blood samples (5ml) were obtained from participants in the operating room prior to surgery. Samples were transported on ice and centrifuged 10 minutes at 2000 rcf and 4 degrees. Plasma was extracted and placed in a cryogenic tube (Fisher) in a -80 freezer.

During AAA repair, roughly 1 cm^2 of full thickness aneurysmal aortic tissue was excised from six systematic locations (figure 1). During ABF, roughly 1 cm^2 of full thickness healthy appearing aortic tissue was sampled at the discretion of the surgeon from one location on the anterior wall just below the renal arteries. All tissue samples were halved. One half was placed in a cryogenic tube and into a dewar containing liquid nitrogen for transport to a -80 freezer. The second half was placed into a tube containing formalin, for histological analysis.

Thrombus thickness at each of the six systematic locations in AAA participants was measured utilizing CT angiography (CTA) done prior to surgery. Measurements were taken by two investigators and compared, a third investigator was consulted if discrepancy existed.

Protein Extraction

Aortic tissue samples were removed from a -80 freezer and separately homogenized utilizing a pestle and mortar. Drops of liquid nitrogen were added as needed to facilitate grinding to powder. Ground samples were mixed with 1200μl of of 0.1% Triton-X-100 (Sigma) 50mM Tris-

HCl (Baker) lysis buffer and the suspensions were permitted to lyse for 15 minutes. Samples were then centrifuged 20 minutes at 16000 rcf and 4 degrees. Supernatant (protein extract) was extracted, and aliquots were stored in a -80 freezer.

Aliquots corresponding to the six locations harvested from each AAA participant underwent MMP-12 immunoassay. Aliquots corresponding to the AAA wall region of greatest adjacent thrombus diameter and absent adjacent thrombus from patients with eccentric thrombus were sent for IL-6, IL-10, and MCP-1 analysis. Control tissue, control plasma, and corresponding AAA plasma was additionally assessed for IL-6, IL-10, and MCP-1 analysis. Coded samples were shipped on dry ice, and analysis was performed in triplicates by commercial company Eve Technologies (Calgary) utilizing a Bio-plex 200^{TM}.

The second aliquot of protein extract from each AAA and control location was used to perform protein assay. Samples were labelled with patient number and location, key connecting location to thrombus thickness was not available during analysis. A microtiter plate was prepared according to recommended protocol and analyzed by BioTek^{TM} microplate reader to determine protein concentration in mg/ml. Results were accepted at R^2 of standard curve >0.90. Plasma protein was estimated at 80g/L.[23]

Histological Analysis

Aortic tissue placed in formalin was allowed to fix overnight. Tissue was trimmed to 10x10x3mm and placed in a cassette. Cassettes were stored in 70% ethanol to prevent drying of tissue. Tissue was processed utilizing a Shandon Citadel® Tissue processor. Resultant paraffin embedded tissue blocks were shaved to achieve uniform size. A microtome was then used on each paraffin block to create sections at 5μm thickness. Sections were placed on (3-Aminopropyl)triethoxysilane coated slides and dried overnight. Slides were labelled with patient number and location number, key corresponding location number to thrombus thickness was not available during histological analysis.

Hematoxylin and eosin (H&E) stain was performed on 2-3 AAA sections from locations 1-4. H&E stain was additionally performed on 2-3 sections from the 1 location taken from control participants. De-paraffinization of formalin-fixed paraffin-embedded tissue was performed. Paraffin sections were cleared in xylene (Merck) and rehydrated in decreasing concentrations of ethanol (Fisher)(100-70%, 5 minutes for each step). Slides were then washed in distilled water (dH2O). Primary staining was performed by placing slides in a hematoxylin bath (Sigma) for 7 minutes. Staining was followed with dH2O wash, and 1% acid ethanol (Fisher) was used to de-stain slides. DH2O wash was repeated, and slides were subjected to eosin staining solution (Sigma) for 1 minute. Dehydration of slides was performed through sequential ethanol washes (95-100%). Slides were cleared with xylene and coverslips were mounted.

H&E stained tissue was analyzed at 100x and 200x magnification. Multiple images were taken to represent each sample. Images were taken by jumping in a zigzagging motion across the tissue, capturing both adventitia and media. The number of images taken was dependant on the size of the tissue and number of sections mounted. Utilizing Zeiss Zen pro image analyzer, nucleated cells were labelled based on wavelength and percentage area occupied was calculated. Each image was reviewed to ensure automatic cell selection was correct. Nucleated cells were assumed to be neutrophils, macrophage, lymphocytes, and fibroblasts.

Immunohistochemical Analysis

AAA containing eccentric thrombus were analyzed by CTA to identify tissue locations corresponding to absent adjacent thrombus and thick adjacent thrombus. Tissue samples from the respective locations, along with control tissue samples, were mounted in 2-3 sections and immunohistochemical analysis was performed. A section of human spleen was used as positive control. Following de-paraffinization and rehydration, slides were washed with dH2O and a barrier was drawn around tissue samples with hydrophobic barrier pen. Antigens were unmasked by boiling slides in 10mM sodium citrate buffer (pH 6.0, Sigma) for 20 minutes. Slides were cooled and rinsed with dH20. Endogenous peroxidase activity was blocked by incubating sections in 3% hydrogen peroxide (Merck) and 10% methanol (Merck) for 10 minutes. After TBST (Merck) wash (3x2 minutes), sections were incubated with avidin/biotin blocking system (Biolegend). Following TBST wash, sections were incubated with goat serum blocking solution (Jackson)(1:20, 1 hour). Blocking solution was cleared with TBST wash, and sections were incubated with primary antibody CD68/SR-D1 (Novus)(1:50) overnight at 4 degrees. One section from each sample was not inoculated with CD68 to act as negative control. TBST wash was performed and sections were incubated with secondary antibody biotinylated goat anti-mouse (Novus) for 1 hour. Incubation was followed by TBST wash. Streptavidin-HRP (Jackson)(1:500) was added for 30 minutes, followed by TBST wash. DAB (Fisher) was added to each section for 5 minutes, followed by rinse in dH2O. Counter stain was performed by adding hematoxylin for 40 seconds, followed by rinse in dH2O. Sections were then incubated in lithium carbonate for 1 minute, before running under H2O for 2 minutes. Sections were dehydrated and mounted with coverslips.

Slides were analyzed at 200x and 400x magnification, images were taken in the same manner as performed on H&E slides. Utilizing Zeiss Zen pro image analyzer, CD68+ cells and hematoxylin cells were labelled based on wavelength and percentage area was calculated for each. Each image was reviewed to ensure automatic cell selection was correct. Hematoxylin labelled cells were assumed to be neutrophils, lymphocytes, and fibroblasts. CD68+ cells were assumed to be macrophage.

Statistical Analysis

Categorical data are expressed as number (percentage), and were analysed using Fisher's exact test. Continuous variables are expressed as mean +/- standard error, and were analyzed using independent two-tailed t-test. MMP-12, IL-6, IL-10, MCP-1 triplicate results were averaged prior to analysis to reduce influence of technical variance. Otherwise, raw data was used as this was assumed to represent biological variance. Univariate analysis was performed to determine correlation between variables using Pearson correlation.

Results

Patient Demographics

A total of 32 participants were enrolled in this study, 24 of which had AAA and 8 of which were control (aortoiliac occlusive disease). The first AAA participant was used to assess safety and feasibility of tissue harvest, and data were not analyzed. One AAA participant was excluded due to lack of available liquid nitrogen at time of harvest. An additional AAA participant was excluded due to lack of available pre-operative imaging. Of the 21 remaining AAA participants, 10 had eccentric thrombus, 8 had concentric thrombus, and 3 had no thrombus as captured by the systematic sampling procedure. When using thickest measurement of thrombus from each participant mean was 0.77 ± 0.17 cm. Mean of all thrombus measurements for all participants was 1.32 ± 0.11 cm. Venous blood was collected from 4 AAA participants. One control

participant provided only venous blood, as tissue sample was unable to be harvested. A total of 7 control tissue samples and 4 control venous blood samples were collected. Clinical factors in AAA and control are summarized in table 1. Mean AAA diameter was 6.21 ± 0.24 cm. There were no significant differences between groups, aside from higher rates peripheral vascular disease (PVD) and younger age in the control group (p=0.001, p=0.005).

MMP-12

Aliquots of 6 homogenized tissue samples from each of the 21 AAA participants underwent MMP-12 protein analysis. Reported MMP-12 concentration was divided by protein concentration, as determined by protein assay, to determine protease concentration in pg/mg protein. Three samples with immeasurable total protein concentration with measurable protease levels was assumed to represent variance in sensitivity between devices and was excluded. There was no difference found in pg MMP-12/mg protein between thrombus and non-thrombus adjacent regions of the aneurysmal tissue (540 ± 62, 634 ± 128, p=0.46). There was additionally no correlation between thrombus thickness and MMP-12 levels (R=0.061. p=0.80).

IL-6, IL-10, MCP-1

Tissue samples from 2 locations in each of the participants with eccentric thrombus (n=10 underwent IL-6, IL-10, and MCP-1 analysis. Tissue samples corresponded to area adjacent to greatest thrombus deposition, and area of absent adjacent thrombus. Additionally, tissue from control (n=7), plasma from control (n=4), and plasma from eccentric AAA participants (n=4) underwent analysis. Reported cytokine concentration was divided by protein concentration, as determined by protein assay, to determine cytokine concentration in pg/mg protein. Results are reported in pg cytokine/mg protein, and are summarized in figure 2. There was no difference in IL-6 in AAA wall adjacent to thrombus compared to no thrombus or control (453 ± 226, 215 ± 82, p=0.34, 66 ± 31, p=0.21). Additionally, there was no difference between plasma or control IL-6 (0.079 ± 0.049, 0.035 ± 0.017, p=0.50). IL-10 analysis yielded no difference in AAA wall adjacent to thrombus compared no thrombus or control (1.11 ± 0.33, 0.64 ± 0.11, p=0.19, 0.36 ± 0.19, p=0.12). There was additionally no difference in plasma IL-10 between AAA and control (0.040 ± 0.0020, 0.029 ± 0.0086, p=0.26). MCP-1 analysis showed no difference in AAA wall adjacent to thrombus compared to no thrombus or control (1700 ± 329, 1064 ± 211, p=0.12, 1023 ± 394, p=0.22). Additionally, there was no difference in plasma MCP-1 between AAA and control (3.03 ± 0.75, 5.05 ± 0.51, p=0.96).

Correlation analysis yielded no correlation between thrombus thickness and IL-6 or IL-10 (R=0.12, p=0.62, R=0.0053, p=0.98). There was a weak positive correlation between thrombus thickness and MCP-1, although significance is lost when Bonferroni correction is applied (R=0.48, p=0.031, Bonferroni corrected significance at p<0.017).

Histological Analysis

Tissue samples from AAA and control participants were analyzed using H&E (n=21, n=7). A mean of 19 images for each AAA participant, and 5 images for each control participant were taken. Utilizing Zeiss Zen Pro image analyzer, nucleated cells were quantified as percentage area of each image taken. Select histology images are depicted in figure 3. It was found there was significantly more nucleated cells in AAA patients compared to control (3.11 ± 0.19%, 1.21 ± 0.16%, p=0.0011).

Immunohistochemical Analysis

Immunohistochemical analysis was performed on samples from AAA participants with eccentric thrombus (n=10). A mean of 8 images were analyzed from each area of greatest and absent ILT. A mean of 8 images from each control participant was additionally included in analysis. Percentage area occupied by CD68+ cells and hematoxylin staining nuclei were quantified utilizing Zeiss Zen Pro image analyzer. Select histology images are depicted in figure 3, results are summarized in figure 4. CD68+ cells and hematoxylin staining cells were added to represent total inflammation. Following analysis, significantly greater inflammation was found in AAA tissue compared to control (4.70 ± 0.35%, 1.11 ± 0.29%, $p<0.00001$). There was no difference in area occupied by CD68+ cells between thrombus and non-thrombus containing regions (1.54 ± 0.19%, 1.81 ± 0.21%, $p=0.33$). There was a significantly greater area occupied by CD68+ cells in thrombus containing regions compared to control (1.54 ± 0.19%, 0.24 ± 0.075%, $p<0.0001$). CD68+ cell percentage area was divided by total inflammatory percentage area to represent percentage of inflammatory cells positive for CD68. There was no difference found between thrombus adjacent regions compared to non-thrombus or control tissue (36 ± 2.6%, 44 ± 3.0%, $p=0.052$, 39 ± 4.8%, $p=0.56$).

Relationship between CD68+ area percentage and dependent variables was investigated. There was no relationship between CD68+ area percentage and MMP-12 pg/mg protein for a given harvest location ($R=0.0088$, $p=0.97$). There was additionally no relationship between CD68+ area percentage and IL-6, IL-10, or MCP-1 ($R=-0.048$, $p=0.84$, $R=0.27$, $p=0.25$, $R=-0.16$, $p=0.50$).

Discussion

Our group has previously described that interestingly, rupture of AAA occurs not in regions of high wall shear stress, but in regions of ILT deposition and flow recirculation.[12] There is an accompanying body of literature that supports the role of the ILT in AAA progression and rupture.[11,24] As a result, there is increasing interest as to the regional differences throughout the aneurysm. Differences in the ILT containing regions of the aneurysm may suggest factors that are responsible for AAA rupture, and provide novel mechanisms to determine AAA in need of repair. This study utilized immunohistochemistry and protein array to compare CD68+ macrophage presence, IL-6, IL-10, MCP-1, and MMP-12 in human AAA tissue samples from stereotyped locations throughout the aneurysm.

This study included both participants with AAA and participants undergoing repair for aortoiliac occlusive disease. It was noted that participants in the ABF group were significantly younger than the AAA group. This may be expected, as mean age of ABF for occlusive disease in one large retrospective study was 60 years, while the mean age for AAA open repair trends towards 72 years old.[25,26] Control participants also held higher rates of PVD, which may be expected as by definition 100% of patients with aortoiliac occlusive disease have PVD.

H&E was utilized to quantify inflammation in tissue samples from 21 participants with AAA and 7 controls with aortoiliac occlusive disease. Nucleated cells occupied a greater area in AAA compared to control. Nucleated cells identified by H&E are assumed to represent lymphocytes, macrophage, neutrophils, and fibroblasts. The greater presence of nucleated cells in AAA thus represents inflammation, which has been well described in aneurysmal tissue.[13] This finding adds support to the body of literature highlighting inflammation in the pathophysiology of AAA.

Immunohistochemical analysis was then performed to evaluate variance in inflammation across the ILT and non-ILT containing regions of the aneurysm. Of the intramural infiltrate, macrophage

are a predominate shareholder.[13] However, little is known about the distribution of macrophage in relation to the intraluminal thrombus. CD68+ was used to identify macrophage, and hematoxylin identified remaining neutrophils, lymphocytes, and fibroblasts. Consistent with H&E measurements, there was greater inflammation in AAA than control. Additionally, we were able to demonstrate greater inflammation in ILT containing regions compared to control tissue. Contrary to our hypothesis, there was no difference noted in inflammatory cell area between ILT and non-ILT adjacent AAA tissue. This finding contrasts a previous smaller study that, using H&E, found regions of thick ILT had greater inflammatory cell area than regions of no ILT.[14] However, Vorp *et al.* included regions containing ILT in the non-ILT group, and thus their findings do not completely represent thrombus free tissue.[14] Additionally, our study analyzed media and adventitia where inflammation in AAA is greatest, while it is unclear what areas of the AAA were analyzed by Vorp *et al*.[14] We used an automated cell counting procedure to reduce bias that included fibroblasts in total nucleated cell count, whereas Vorp *et al*. used a semi-automated method, where automatic count was manually altered to remove areas occupied by fibroblasts.[14] Our finding of no difference in gross nucleated cells between areas of no ILT and areas of high ILT that tend to rupture, corroborate that more finite changes are involved in AAA pathophysiology.

When CD68+ macrophage were isolated, we found a greater amounts in AAA compared to control. Specifically, we were able to identify greater CD68+ presence in regions of ILT deposition compared to control. This finding aligns with a previous study evaluating elastin degradation that also noted greater CD68+ macrophage in AAA compared to patients with aortoiliac occlusive disease.[27] Another study found no difference between AAA CD68 levels with control.[28] However, Kasashima *et al.* used control tissue from deceased patients, and only adventitia was analyzed.[28] The greater presence macrophage in AAA compared control tissue advocates their involvement in aneurysm pathophysiology. Differing from our initial hypothesis, there was no difference in macrophage between the ILT and non-ILT containing regions of the aneurysm. This finding builds on previous work that found no difference in estimated amount of CD68+ macrophage between thrombus and non-thrombus containing regions of the aneurysm.[29] Our study was able to add quantitative counts of CD68+ cells, and analysis of full thickness tissue. A more recent study from the same group measured CD68+ cells using mRNA, finding greater amounts of macrophage in the thrombus free wall.[30] However, this study did not use a stereotyped manner or harvest and used a smaller sample size.[30]

While we did not find greater macrophage content in areas of AAA containing ILT compared to non-ILT, macrophage content may not necessarily represent activity. MMP-12 is expressed by macrophage, and has previously been demonstrated to be elevated in AAA compared to control.[7,8,31] MMP-12 has further been co-localized with macrophage in AAA using mRNA.[32] Employing protein assay, we found no difference in MMP-12 levels between ILT and non-ILT containing regions of the AAA. A similar study found MMP-12 elevated in thrombus free wall, however this relationship only existed between pooled samples and in a smaller sample size.[30] Lack of differential MMP-12 expression suggests it may not be involved in AAA rupture. We also found no relationship found between CD68 cell amount and MMP-12 levels for a given sample. This may suggest MMP-12 is not expressed equally by all macrophage in AAA.

To further investigate the role macrophage may play in AAA, differential expression of macrophage released cytokines was investigated. Protein array for IL-6, IL10, and MCP-1 showed no difference between ILT adjacent tissue and non-ILT tissue or control. Previous studies have found elevations between IL-6 in aneurysmal tissue and control, although this may be limited to large aneurysms.[33,34] Timing may also play an important role in cytokine expression, as demonstrated by elevation with AAA rupture, compared to intact AAA.[35] IL-6 and

IL-10 have been associated with macrophage in explanted media from AAA.[22] However, we detected no association between CD68+ macrophage amount and IL-6, IL-10, or MCP-1 levels, suggesting elements of the in-situ environment such as thrombus and blood flow may induce heterogeneity of macrophage activity within the aneurysm. Further, tissue cytokines may not even play a large role in AAA expansion or rupture, contrary to previous suspicion.

Finally, we analyzed plasma cytokine levels to see if circulating cytokine load differed between AAA and control, as there remains significant interest into identifying novel biomarkers of AAA. We were unable to detect a difference in IL-6, IL-10, and MCP-1 between AAA and control. AAA have been previously shown to secrete IL-6 into circulation, by measuring plasma IL-6 content in regions surrounding the aneurysm.[36] A recent meta-analysis showed venous IL-6 was higher in AAA compared to controls, however controls in this study were healthy subjects.[37] Our control participants had aortoiliac occlusive disease, which a condition that is also marked by inflammation.[38] IL-10 and MCP-1 have previously been shown not to be elevated compared to healthy controls.[22,33] Our finding of no significant difference in plasma cytokine levels between AAA and controls with aortoiliac occlusive disease highlights the limitations of using circulating biomarkers to identify AAA in patients with comorbidities.

It is important to address the limitations of this study. Like many groups harvesting AAA tissue, large samples size is difficult to obtain. However, our samples size of 21 does outpace several similar studies. The limited sample size combined with a large standard deviation resulted in this study being sufficiently underpowered to detect type 2 error (data not shown). Another weakness of this study is in part to the nature of the tissue studied. Several AAA specimens displayed heavy calcification, potentially limiting qualitative histological analysis. Unlike other studies, control tissue was included in analysis. However, it should be noted that control tissue was from patients undergoing ABF, and thus is likely to incompletely represent healthy tissue.

Using stereotyped harvest locations from AAA and control participants, we were able to demonstrate inflammation, and specifically CD68+ macrophage are elevated in thrombus adjacent AAA compared to control. Contrary to our hypothesis, there was no difference in ILT and non-ILT of the aneurysm with regards to inflammation, macrophage, or macrophage related proteases and cytokines. As there is a difference in rupture risk between the ILT and non-ILT regions of the AAA, our findings suggest macrophage may have a limited role in AAA rupture. Future investigation is needed to examine regional differences in the AAA inflammatory infiltrate, to characterise factors that may be important in AAA rupture.

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Tables and Figures

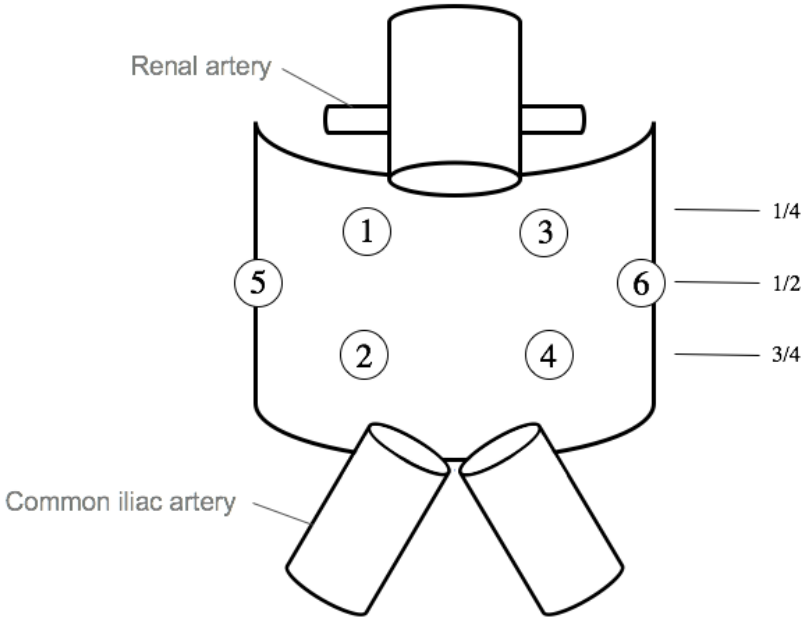

Figure 1. Location of AAA wall sample harvest. Length of the aorta from renal arteries to iliac bifurcation was divided into quarters. Samples 1 and 3 were obtained ¼ down samples 2 and 4 ¾ down, left and right posteriorly. Samples 5 and 6 were obtained ½ down, left and right anteriorly.

Variables	AAA (n=21)	Control (n=8)	p value
AAA size (cm)	6.21 (± 0.24)		
Age (yr)	71 (±7)	63 (±6)	<0.005*
Male	14 (67)	3 (38)	0.22
Ever smoked	19 (90)	7 (88)	>0.99
Current smoker	9 (43)	3 (38)	>0.99
Previous smoker	10 (48)	4 (50)	>0.99
Hypertension	13 (62)	4 (50)	0.68
Diabetes mellitus	9 (43)	2 (25)	0.67
Statin use	16 (76)	4 (50)	0.21
Peripheral vascular disease	3 (14)	8 (100)	<0.001*
Chronic obstructive pulmonary disease	4 (19)	1 (13)	>0.99
Cerebrovascular accident	2 (10)	1 (13)	>0.99
Coronary artery disease	9 (43)	1 (13)	0.20
Chronic kidney disease	1 (5)	0 (0)	0.48

Table 1. Participant baseline characteristics. * denotes p <0.05.

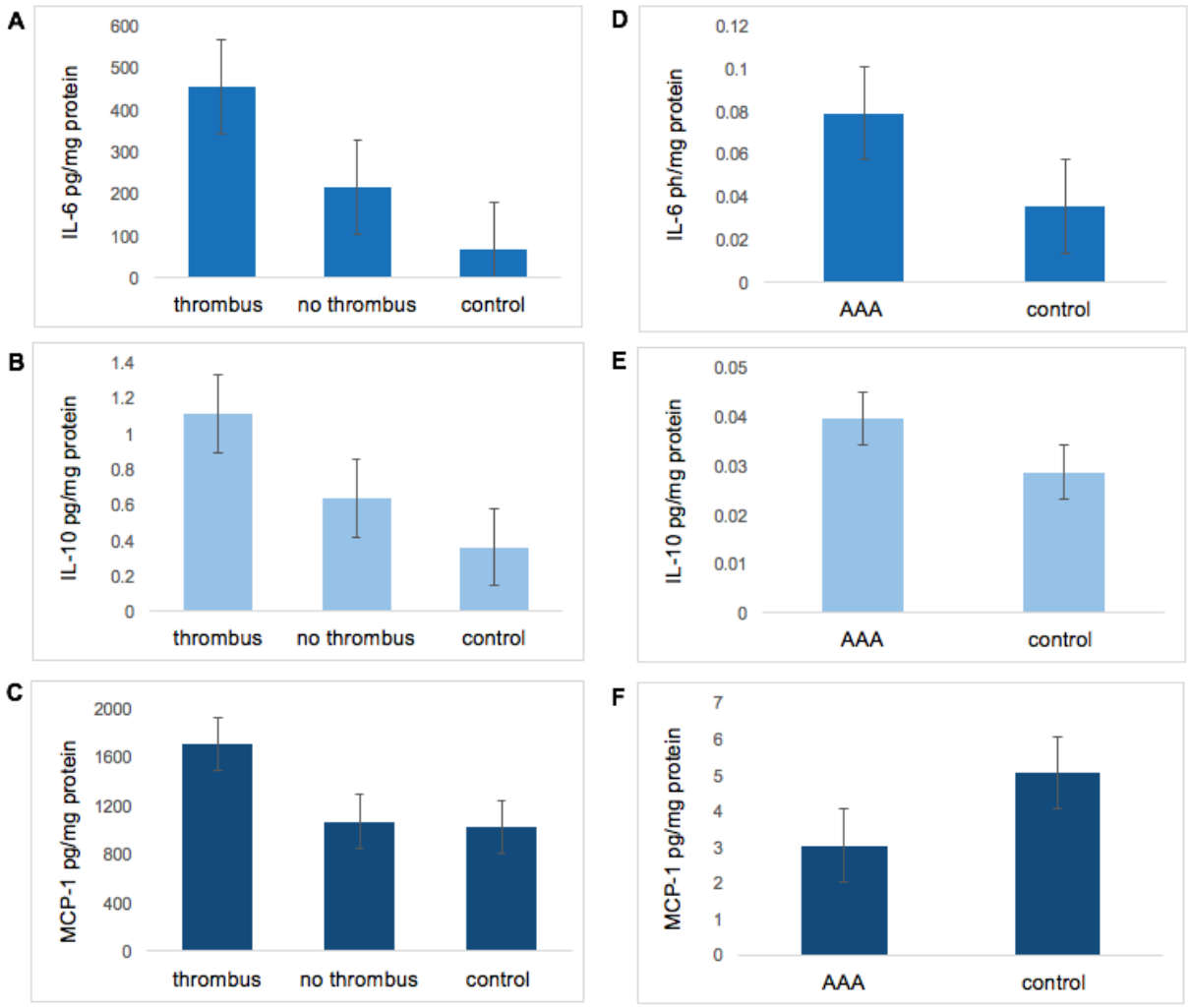

Figure 2. Mean aortic wall IL-6 (A), IL-10 (B), and MCP-1 (C) ± standard error. Measurements in participants with eccentric thrombus in areas adjacent to high thrombus, no thrombus, and in control participants. Mean plasma IL-6 (D), IL-10 (E), MCP-1 (F) ± standard error. Measurements in participants with eccentric thrombus and in control participants.

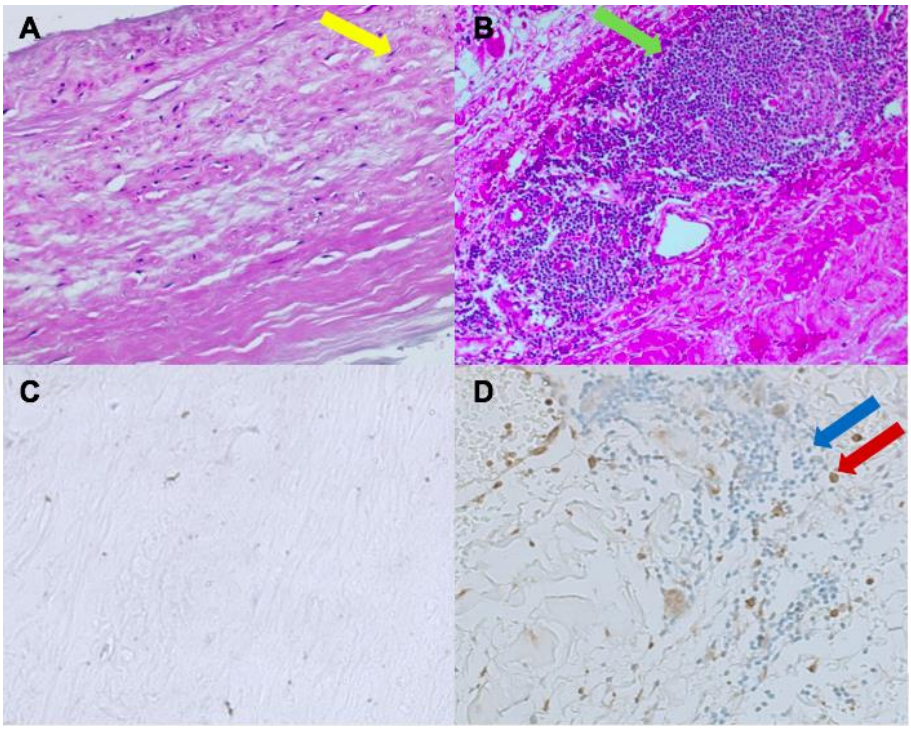

Figure 3. Select histology images. Control aortic (A) and AAA tissue (B) stained with H&E. Dark blue staining nuclei represent both fibroblasts (yellow arrow) and inflammatory cells (green arrow). Control aortic (C) and AAA tissue (D) stained with CD68 and hematoxylin. Brown stain indicates CD68+ cells (red arrow), blue stain indicates inflammatory cells (blue arrow) and fibroblasts.

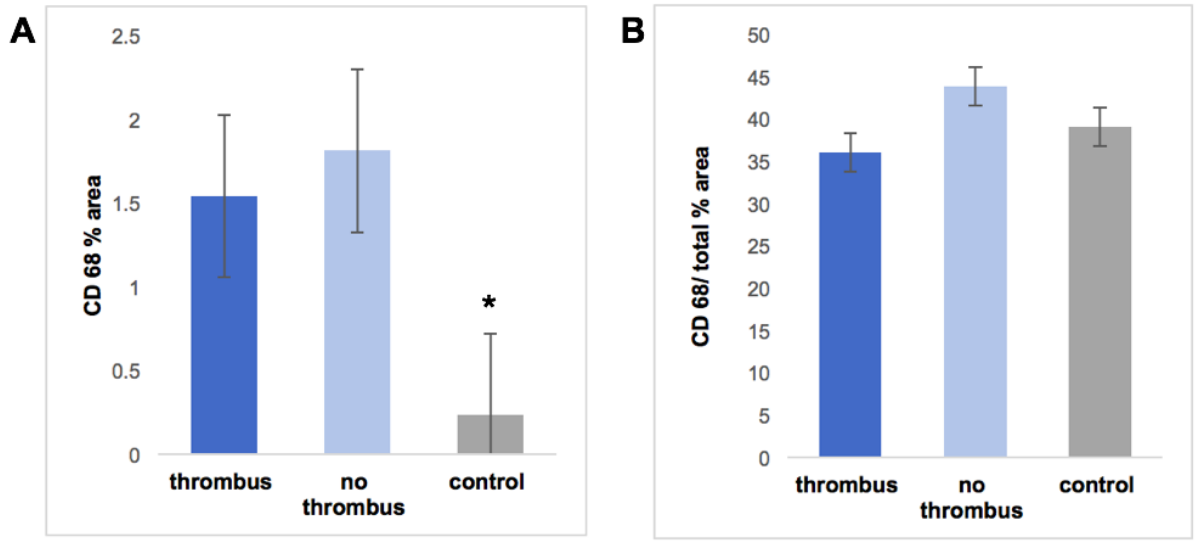

Figure 4. Percentage area occupied by CD68+ cells in thrombus adjacent regions of AAA compared to non-thrombus adjacent AAA and control (A). Percentage of inflammatory cells CD68+ in thrombus adjacent regions compared to non-thrombus containing regions and control (B). * denotes $p<0.05$.

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