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Dietary Supplementation with Omega-3 Polyunsaturated Fatty Acids Modulate Matrix Metalloproteinase Immunoreactivity in a Mouse Model of Pre-abdominal Aortic Aneurysm

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Abstract

Background
Two-day infusion of angiotensin II to apolipoprotein E-deficient (ApoE-/-) mice provides a model of pre-abdominal aortic aneurysm. Long chain omega-3 polyunsaturated fatty acids (n-3 PUFAs) have anti-inflammatory effects. This study examined the effect of an eight-week low or high n-3 PUFA diet in ApoE-/- mice on matrix metalloproteinase (MMP) expression and elastin degradation.

Methods
ApoE-/- mice were fed a low or high n-3 PUFA diet for eight weeks prior to two-day infusion with angiotensin II. The omega-3 index, MMP-2, MMP-9, TIMP-1, and TGF-β1 immunoreactivity, and elastin fragmentation were measured.

Results
The omega-3 index with the low and high n-3 PUFA diet was 3.78% and 13.03%, respectively. MMP-9 immunoreactive stain intensity was lower in mice fed the high, compared to the low n-3 PUFA diet in endothelial cells (suprarenal aorta), and inflammatory cells (suprarenal and infrarenal aorta). Inflammatory cells had higher TIMP-1 and TGF-β1 stain intensity in mice fed the high, compared to the low n-3 PUFA diet (suprarenal aorta). MMP-2 immunoreactivity was unaffected by diet. A non-significant trend for reduced elastin fragmentation was observed in mice fed the high n-3 PUFA diet.

Conclusion
Dietary supplementation with n-3 PUFAs may have protective anti-inflammatory effects mediated through modulation of MMPs and TIMPs.

Key words: Abdominal aortic aneurysm; Matrix metalloproteinases; Long chain omega-3 polyunsaturated fatty acids; Transforming growth factor-β1
Introduction

Abdominal aortic aneurysm (AAA) is a cardiovascular disease (CVD) of increasing prevalence and incidence [1]. AAA is characterised by progressive dilation of the aorta as a result of inflammatory, apoptotic and proteolytic processes damaging the aortic wall [1, 2]. AAA is defined as a permanent dilation of the infrarenal aorta to at least 30 mm, or 1.5 times the expected infrarenal aortic diameter [1, 2]. When peak wall stress exceeds the local strength of the aortic tissue, rupture ensues [3]. Ruptured AAA is associated with a mortality rate up to 90% [2], and is responsible for an estimated 7,000 deaths each year in the United States alone [1]. Open surgery and endovascular repair are currently the only validated therapeutic options recommended for AAA [1]. However, surgical repair costs in excess of US$20,000 per patient and carries an operative mortality rate up to 5% [4-6], highlighting the need to identify non-surgical treatments capable of slowing AAA expansion and minimising AAA associated complications.

Collagen and elastin provide tensile strength and elasticity to the aortic wall. Matrix metalloproteinases (MMPs) have a central role in tissue remodelling through the degradation of these matrix proteins [7]. A dynamic equilibrium between MMPs and their tissue inhibitors (TIMPs) is crucial for tissue destruction and repair homeostasis, with TIMPs binding to the catalytic site of the MMPs to reduce their activity [7]. A 1:1 stoichiometric balance normally exists between MMPs and TIMPs, with an imbalance in this ratio resulting in a loss of tissue destruction and repair homeostasis [7]. In particular, excess MMP-2 and MMP-9, and absence of TIMP-1 potentiate aneurysm formation and rupture [8-10]. Cytokines, including monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), and transforming growth factor-β1 (TGF-β1) are implicated in the pathogenesis, or in some instances in the protection against the development of abdominal aortic aneurysm [11-13]. MCP-1 is highly expressed in AAA, and exposure of human aortic smooth muscle cells to MCP-1 increased MMP-9 expression and activity [11]. Neutralisation of TGF-β1 lead to an increase in MMP-9
gelatinolytic activity in mouse abdominal aortic segments, indicative of a possible protective effect of TGF-β1 against extracellular matrix destruction [12].

The long chain omega-3 polyunsaturated fatty acids (n-3 PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have anti-inflammatory activities following their incorporation into cell membrane phospholipids [14]. Recently, it has been shown that supplementation of BALB/cA mice with a diet fortified in EPA (10% wt/wt) for four days protected mice against aneurysm development following peri-aortic application of CaCl$_2$ [15]. In that study, EPA reduced smooth muscle MMP-2 and macrophage MMP-9 expression, without effect on TIMP-1 or TIMP-2 gene expression. Whether similar findings can also be seen in other models of AAA, or with other n-3 PUFAs such as DHA, has not yet been investigated.

Subcutaneous infusion of angiotensin-II (AngII) promotes AAA formation in C57BL/6 mice [4]. Frequency and size of aneurysms greatly increases when AngII is infused into hyperlipidaemic mice, such as those deficient in the gene encoding apolipoprotein-E (ApoE$^{-/-}$) [4]. Medial degradation, luminal dilation and intraluminal thrombus formation are characteristic of AAA development in the ApoE$^{-/-}$ AngII-infused mouse model, and are all hallmarks of human AAAs [4]. This model of AAA is also similar to humans in that there is greater proclivity for aneurysm formation in males [4]. This model is not without limitations. Human AAA normally develops in the infrarenal aorta, and does not usually dissect [4]. In contrast, aneurysms in the ApoE$^{-/-}$ AngII-infused mouse model form in the suprarenal aorta, with dissection a common feature [4].

Studies using the ApoE$^{-/-}$ AngII-infused mouse as a model for AAA typically deliver AngII over 28 days [16]. However, initial changes to the aorta, including medial infiltration of macrophages and elastin degradation, are observed within the first four days of initiating AngII infusion [17]. Transmedial dissection and a resultant expanded lumen are evident within seven days of AngII infusion [16]. Haematoma development occurs between 4-10 days [17], during which time up to 10% of mice die due to ruptured AAA [17]. This suggests that two-day infusion of AngII into male ApoE$^{-/-}$ mice will be ideally suited to examine the effects of n-3 PUFAs on the early inflammatory response, prior to aneurysm development.
The aims of this study were to characterise MMP-2, MMP-9 and TIMP-1 immunoreactivity in endothelial cells lining the vessel lumen, smooth muscle cells of the media and adventitial inflammatory infiltrates of the abdominal aorta in ApoE<sup>−/−</sup> AngII-infused mice fed a diet with low (DHA, 0%; EPA, 0%; total n-3 PUFA, 0.14%) or high n-3 PUFA content (DHA, 0.3%; EPA, 0.07%; total n-3 PUFAs, 0.7%).

**Materials and Methods**

**Dietary Supplementation of Animals**

Three week old male ApoE<sup>−/−</sup> mice on a C57BL/6 background (n = 20) were housed in groups of five in a 12 h light/dark cycle, with ad libitum access to sterile water and feed. Mice were randomised to receive a meat-free rat and mouse chow diet containing low or high n-3 PUFA content (Table 1). Animal feed was autoclaved (121°C, 25 min) to minimise risk of infection to the mice. Fatty-acid content of pre- and post-autoclaved feed has previously been analysed in this laboratory, with gas chromatography-mass spectrometry (GC-MS) analysis finding only a small diminution in n-3 PUFA content post-autoclaving. All animals received humane care in accordance with the 'Statement on Animal Experimentation' by the National Health and Medical Research Council of Australia. The protocol was approved by the Animal Ethics Committee of the University of the Sunshine Coast (Approval Number: AN/A/13/70).

Mice were fed the low or high n-3 PUFA diet for eight weeks, anaesthetised using sodium pentobarbital (32.5 µg/g body weight in 0.9% saline) prior to subcutaneous insertion of osmotic minipumps, which were primed to infuse AngII (1000 ng/kg/min in 0.9% saline) for two days. Animals received analgesic (buprenorphine, 330 ng/g body weight), and were maintained on the same low or high n-3 PUFA diet post-surgery. On day 2 post-surgery, mice were anaesthetised with pentobarbital (325 µg/g body weight in 0.9% saline). Whole blood was collected by cardiac puncture and placed in 2 mL EDTA tubes. Samples were centrifuged at 1500 x g for 10 min at 22°C, and erythrocytes and plasma were placed in separate eppendorf tubes, frozen on dry ice, and stored in a -
80°C freezer until use. The aorta was harvested and fixed in 10% neutral buffered formalin for 24-48 h at 22°C to maintain morphology, and embedded in paraffin.

**Determination of the Omega-3 Index**

GC-MS was used to determine the ratio of DHA or EPA to all other fatty acids in erythrocyte membrane phospholipids. Briefly, erythrocytes (~300 μL) were placed in a glass tube with 600 μL methanol containing 0.02% BHT, and membranes were disrupted by mechanical homogenisation using a glass rod for 1 min. Samples were covered with nitrogen, sealed and placed on ice for 30 min. Chloroform (600 μL) was added, cells were homogenised as before, and samples were stored on ice with nitrogen for 30 min. Tubes were centrifuged at 3000 x g for 5 min at 4°C. The homogenisation procedure was repeated twice, but using 300 μL volumes of methanol/BHT and chloroform, and 10 min incubations of tubes on ice. The lipid supernatant was collected after each centrifugation step, and placed on ice. The pooled lipid layer (1 mL) was combined with 800 μL chloroform and 460 μL of 0.05M KCl, vortexed in the presence of nitrogen, and centrifuged at 3000 x g for 10 min at 4°C. The bottom lipid layer was transferred to a 2 mL GC-MS vial and dried under a stream of nitrogen. The sample was hydrolysed with a 500 μL solution containing 9 M HCl, milliQ water, and acetonitrile in a 1:1:18 ratio, and 0.05% BHT. Samples were covered with nitrogen, sealed, and incubated overnight at 65°C. The sample was dried under a stream of nitrogen, freeze dried for 20 min, and then combined with hexane (250 μL) containing derivatising agent, N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide, with 1% TBDMSI, 97% (15 μL). Samples were covered with nitrogen, incubated for 2h at 37°C, and then transferred to 2 mL GC-MS vials. GC-MS was carried out as described above, with the following modification: the split ratio was 10:1 on injection, opening to 30:1 after 1.0 min. The injector was at 300°C with a temperature program of 170°C initial, ramping at 6.0°C/min until 310°C, and then holding for 5.0 min. The mass spectrometer scanned over 45 to 450 m/z+ for 4.0-28.2 min.

**Detection of MMP-2, MMP-9, TIMP-1, TGF-β1, MCP-1, and IL-1β Immunoreactivity**
Paraffin embedded tissues were cut using a Computer Microtome (YIDI, model YD-335A). Serial 4 μm sections were collected on gelatin/chrome alum-coated microscope slides. Sections were deparaffinised by consecutive changes in xylene (1 x 10 min, 2 x 2 min) and absolute ethanol (3 x 2 min), and rinsed in dH₂O. Immunohistochemistry (IHC) was used for the quantitation of matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteinase 1 (TIMP-1) in the endothelial cells of the tunica intima, smooth muscle cells of the tunica media, and inflammatory cells of the tunica adventitia. Inflammatory cells were also investigated for expression of monocyte of chemoattractant protein-1 (MCP-1), transforming growth factor-1 (TGF-β1) and interleukin-1β (IL-1β). Optimal conditions for immunohistochemistry were determined by varying the concentration of primary antibody and incubation times with SIGMAFAST™ metal enhanced 3,3’-diaminobenzidine tetrahydrochloride (DAB).

Antigen-retrieval, involving incubation of sections for 20 min in sodium citrate buffer (trisodium citrate, 11.4 mM, containing 0.05% Tween-20, pH 6.0) at 95°C, then cooling in citrate buffer for a further 40 min, was carried out for detection of MMP-9 and TIMP-1. Endogenous peroxidase activity was blocked using 0.3% H₂O₂ (30 min), followed by washing in PBS (NaCl, 138, KCl, 2.7, Na₂HPO₄, 10, KH₂PO₄, 1.8 mM, pH 7.5) for detection of MMP-2, or PBS containing 0.05% Tween-20 for detection of MMP-9 and TIMP-1. Sections were incubated with rabbit serum (1.5%, diluted in PBS), goat serum (5%, diluted in PBS) or horse serum (5%, diluted in PBS), for 20 min. Excess serum was blotted, and sections incubated with goat polyclonal antibodies that were raised against mouse MMP-2 (10 μg/mL in PBS, containing 0.1% BSA; R&D Systems, Catalogue # AF1488), MMP-9 (5 μg/mL in PBS, containing 0.1% BSA, R&D Systems, Catalogue # AF909), TIMP-1 (10 μg/mL in PBS, containing 0.1% BSA, R&D Systems, Catalogue # AF980), TGF-β1 (5 μg/mL in PBS, Promega, Catalogue # G1221), MCP-1 (13.3 μg/mL in PBS, Abcam, Catalogue # ab7202), IL-1β (10 μg/ML in PBS, Abcam, Catalogue # ab9722), or antibody diluent alone, for 30 min. Slides were rinsed in PBS, incubated with rabbit α-goat or goat α-rabbit IgG biotinylated secondary antibody (0.5% in PBS, containing 1.5% rabbit serum or 1.5% goat serum) for 30 min, washed in PBS (5 min), and then incubated with Vectastain Elite ABC reagent for 30 min. Slides were washed for 5 min in PBS, followed by incubation with DAB, using a microscope to determine optimal development time.
The measurement of stain intensity for the negative control was subtracted from the measurement of stain intensity determined using the primary antibodies. To determine whether stain intensity deteriorated over the duration in which photography was undertaken for sections stained for MMP-2, MMP-9 and TIMP-1, photographs of the same region were taken at various time points over a 4 h period. Stain intensity was consistently intense over the 4 h duration ($P = 0.760$, data not shown).

**Zymography**

Plasma samples were diluted in a 2:5:3 ratio with 2x Tris-glycine sample buffer and distilled water, and loaded on to 10% zymogram gelatin gels (Novex, Life Technologies, CA, USA). Molecular weight was determined by loading a prestained protein molecular weight marker (Thermo Fisher Scientific, Vic, Australia). Gels were placed in running buffer (Tris base, 25 mM; glycine, 246 mM; sodium dodecyl sulfate, 0.1%; pH 8.3) and proteins were separated by electrophoresis (150 V, 90 min, 22°C). Following separation, gels were denatured in 2.5% Triton X in distilled water (2x30 min, 22°C) with gentle rocking, washed in distilled water, and then placed in zymogram developing buffer (Novex Life Technologies) for 18 h at 37°C, with gentle shaking. The gels were washed and stained with Simply Blue Safe Stain (Invitrogen Life Technologies, Vic, Australia) for 1 h at 22°C with gentle rocking. Bands were photographed and images were quantitated using Image J software (1.45s, NIH).

**Quatitation of Elastin Fragmentation**

Verhoeff-van Gieson stain was used to examine the integrity of elastin within the vessel wall. Sections were covered with Verhoeff’s haematoxylin (5% alcoholic haematoxylin: 10% ferric chloride: Lugol’s iodine, 2.5:1:1) for 30 min before rinsing with tap water. Ferric chloride (2%) was applied, with the time for staining determined empirically under a brightfield microscope. Sections were rinsed in dH$_2$O when black elastin fibres were easily distinguished from a light grey background (typically 60 - 90 s), covered in sodium thiosulphate (5%) for 1 min and counterstained with van Gieson’s solution (1% acid fuschin: saturated picric acid, 1:45) for 5 min, rinsing in dH$_2$O between steps. Elastin fragmentation was assessed by two independent assessors, both of whom were blinded.
to the treatment group of each vessel. Elastin fragmentation was expressed as the number of breaks in elastin fibres/mm² media.

Sections were mounted with glycerol and coverslips, and photographed using a Nikon DS-Fi2 camera attached to a Nikon Eclipse Ti microscope. A stitched image of each vessel was taken using a 10x objective lens, and individual images of the top, bottom, left and right of the aortas were obtained using a 20x objective lens.

Statistical Analysis

Statistical analyses were performed using SPSS software (Version 19). Data are presented as mean±standard error of the mean (SEM). Significance was defined as $P<0.05$. ImageJ (Version 1.48d) software was used to determine the intensity of positive staining for MMP-2, MMP-9 and TIMP-1 in endothelial cells, smooth muscle cells and inflammatory cells. Shapiro–Wilk test was used to determine whether data was normally distributed. Difference in stain intensity between mice fed the low or high n-3 PFA diet was determined by independent samples t-test for normally distributed data, and Mann-Whitney U test for data that was not normally distributed.

Results

Mice fed the low and high n-3 PUFA diet consumed similar amounts of feed per day, and had a similar increase in body weight (data not shown). The omega-3 index for mice fed the low and high n-3 PUFA diets was 3.78±0.26% and 13.03±0.22% ($P<0.001$). Four of the 10 mice fed the low n-3 PUFA diet, and none of the 10 mice fed the high n-3 PUFA diet, had a dissected aorta.

Positive MMP-2, MMP-9 and TIMP-1 immunoreactive staining was detected in endothelial cells, smooth muscle cells, and inflammatory cells infiltrating the adventitia (Fig. 1). Immunoreactivity of MMP-2 in endothelial cells, smooth muscle cells and inflammatory cells of the suprarenal and infrarenal aortas was similar in mice fed the low or high n-3 PUFA diet (Fig. 2). Endothelial cells in the suprarenal aorta, and inflammatory cells in the suprarenal and infrarenal aorta
of mice fed the high n-3 PUFA diet had significantly lower MMP-9 immunoreactive stain intensity per cell, compared to mice fed the low n-3 PUFA diet (Fig. 2). There was no difference in MMP-9 immunoreactivity in smooth muscle cells in the suprarenal or infrarenal aortas of mice fed the low or high n-3 PUFA diet (Fig. 2). Stain intensity for TIMP-1 immunoreactivity in endothelial cells and smooth muscle cells in the suprarenal and infrarenal aortas did not differ between mice fed the low or high n-3 PUFA diet. Inflammatory cells infiltrating the adventitial region of the suprarenal aorta had higher TIMP-1 immunoreactive stain intensity in mice fed the high, compared to the low n-3 PUFA diet (Fig. 2).

Zymography was used to examine systemic, latent MMP-9 and active MMP-2 activity. Latent MMP-9 activity was greater in ApoE<sup>-/-</sup> mice that were fed the low, compared to the high n-3 PUFA diet (Fig. 3). There was no difference in systemic, active MMP-2 activity in mice that were fed the low or high n-3 PUFA diet. The band for active MMP-9 co-located with the band for latent MMP-2, thus precluding their quantitation.

Fragmentation of elastin was detected in the aortic media of mice on the low and high n-3 PUFA diets (Fig. 4). Large fibrotic regions of media were observed in some aortas of mice that were fed the low n-3 PUFA diet. A non-significant trend for reduced elastin fragmentation was observed in mice that received the high, compared to the low n-3 PUFA diet (Fig. 4). Correlation of findings between two independent assessors of elastin fragmentation, both of whom were blinded to the treatment groups, was strong ($R^2=0.73$).

The effect of n-3 PUFA diet on expression of pro-inflammatory cytokines in infiltrating inflammatory cells was determined using immunohistochemistry. There was a significantly higher level of TGF-β1 immunoreactivity in the suprarenal aorta of mice that were fed the high, compared to the low n-3 PUFA diet (Table 2). Expression levels of MCP-1 and IL-1β were unaffected by diet.

Discussion

AAA is a degenerative cardiovascular disease characterised by permanent dilation of the abdominal aorta [1, 2]. The dominant histological features of AAA include medial and adventitial
inflammation and degeneration [1]. MMPs contribute to the pathogenesis of AAA through the
destruction of the structural components of the aortic wall, elastin and collagen [7]. The long chain n-3
PUFAs EPA and DHA are considered cardioprotective, namely through their anti-inflammatory
effects, and the pro-resolving activity of their metabolites [14, 18-20]. This study focussed on
characterising the effect of a diet fortified with n-3 PUFAs on the intensity of immunoreactive
staining for MMP-2, MMP-9 and TIMP-1 in endothelial cells lining the vessel lumen, smooth muscle
cells of the media, and adventitial inflammatory infiltrates in an ApoE<sup>-/-</sup> AngII-infused mouse model
of pre-AAA.

MMP-9 causes proteolysis of elastin and collagen, resulting in diminished elasticity and
reduced tensile strength of the aorta [7]. In acute dissected human thoracic aortas, MMP-9 was
strongly associated with infiltrating neutrophils within the aortic wall [21]. Gene deletion of MMP-9
reduces AAA development in mice that are subjected to peri-aortic CaCl<sub>2</sub> application, or elastase
perfusion [10, 22]. In the present study, MMP-9 immunoreactivity was lower in endothelial cells and
inflammatory cells infiltrating the suprarenal aorta, and in inflammatory cells infiltrating the
infrarenal aorta of mice fed the high, compared to the low n-3 PUFA diet. Systemic activity of latent
MMP-9 was also lower in ApoE<sup>-/-</sup> mice that were fed the high, compared to the low n-3 PUFA diet.
The localisation of inflammatory cells to the suprarenal aorta is consistent with previous studies that
have used angiotensin II-infused ApoE<sup>-/-</sup> mice [17]. Our study also identified the presence of
inflammatory cells in the infrarenal aorta, in association with regions of aortic dissection. Our
findings showing lower MMP-9 immunoreactivity in mice that were fed the high n-3 PUFA diet for
eight weeks is in line with a recent report by Wang <i>et al</i>., [15] which showed reduced MMP-9
expression and activity in a murine CaCl<sub>2</sub>-model of AAA following four day administration of EPA.
Together, the findings suggest that dietary supplementation with n-3 PUFAs provide some protection
against tissue destruction in AAA by down-regulating MMP-9. In contrast with the study by Wang <i>et al</i>., [15]
which also showed reduced MMP-2 expression, our study revealed no effect of n-3 PUFAs
on MMP-2 immunoreactivity in endothelial cells, smooth muscle cells or inflammatory cells. The
disparate findings could be attributed to differences in the animal model (peri-aortic application of
CaCl<sub>2</sub> versus AngII infusion in ApoE<sup>-/-</sup> mice), or the type and amount of LC n-3 PUFA used. The
dietary intake of EPA was much higher in the previous study (10%, [15]), compared to the current study in which mice were supplemented with a diet containing only 0.3% DHA and 0.07% EPA. The omega-3 index calculated using erythrocyte membrane phospholipids for ApoE−/− mice fed the high n-3 PUFA diet for eight weeks in this study was 13.03%. This level of n-3 PUFA incorporation can be achieved in hyperlipidaemic humans who ingest 1 g EPA and 0.672 g DHA per day for 12 weeks (omega-3 index, 12.44%, [23]).

MMP in its active form may be complexed with an inhibitor such as TIMP, preventing its ability to degrade elastin and collagen [7]. In this study, higher TIMP-1 immunoreactivity of inflammatory cells infiltrating the suprarenal aorta was found in mice fed the high, compared to low n-3 PUFA diet. TIMP-1 expression was unaffected by EPA supplementation in the CaCl2-model of AAA [15]. It is possible that the type of n-3 PUFA (only EPA compared to predominantly DHA, where differential effects of EPA and DHA have been described previously [24]), dose and duration of treatment, or animal model used contributed to the differences.

The inflammatory cells that were found to express MMP-9 included neutrophils, as evidenced by histological staining (not shown). Inflammatory cell infiltration and degradation of elastin are early events in AAA development, occurring within two days of angiotensin II infusion [17]. Since MMP-9 is known to degrade elastin, and MMP-9 immunoreactivity was reduced, and TIMP-1 immunoreactivity was increased in mice fed the high n-3 PUFA diet, we hypothesised that supplementation of mice with the high diet might also lead to protection against elastin degradation. Our findings showed a trend for reduced elastin fragmentation, although this did not reach significance.

Although the role of TGF-β in development of AAA is somewhat contentious [25], several recent studies have suggested a protective effect of TGF-β against abdominal aortic inflammation and progression of abdominal aortic aneurysm in angiotensin II-infused mice [12, 26]. The administration of a TGF-β-neutralising antibody to C57BL/6 and ApoE−/− mice was associated with increased infiltration of macrophages into the vessel wall, increased latent MMP-9 gelatinolytic activity, increased degradation of elastin, and increased mortality that was associated with AAA rupture [12]. The findings in the current study show increased TGF-β immunoreactivity in the suprarenal aorta of
mice that were fed a high n-3 PUFA diet and suggest a possible mechanism for reduction in MMP-9 expression.

In conclusion, this study showed reduced MMP-9, and increased TIMP-1 expression in mice that are supplemented with a high, compared to a low n-3 PUFA diet, and that this was associated with a trend for reduced elastin fragmentation. The silent nature of AAA means that patients are often not diagnosed until advanced stages of the disease. Further studies are required to determine if AAA development is attenuated when an n-3 PUFA supplementation begins after disease onset. This could be achieved using the ApoE<sup>−/−</sup> AngII-infused mouse model of AAA, with dietary supplementation beginning at the time of AngII-infusion. Approximately 10% of mice die during AngII infusion, with these deaths typically occurring with the first 10 days [17]. Since it can take up to eight weeks of n-3 PUFA supplementation for full incorporation of EPA and DHA into cell membrane phospholipids [27], sample size would have to be increased to ensure a sufficient number of mice survive to the terminal experiment.

**Acknowledgement:** This work was supported by a Faculty Seed Grant to FDR.
Table 1: Fatty acid composition of low and high omega-3 polyunsaturated fatty acid diets

<table>
<thead>
<tr>
<th>Nutritional Parameters</th>
<th>% (Low Diet)</th>
<th>% (High Diet)</th>
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</thead>
<tbody>
<tr>
<td>Protein</td>
<td>20.00</td>
<td>20.00</td>
</tr>
<tr>
<td>Total fat</td>
<td>4.70</td>
<td>4.70</td>
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<tr>
<td>Crude fibre</td>
<td>4.90</td>
<td>4.90</td>
</tr>
<tr>
<td>Digestible energy</td>
<td>14.4 MJ/kg</td>
<td>14.4 MJ/kg</td>
</tr>
</tbody>
</table>

**Fatty Acid Composition**

Total polyunsaturated fats 1.36 1.95

    Total LC n-3 PUFAs 0.14 0.70
        Eicosapentaenoic acid (EPA) 0.0 0.07
        Docosahexaenoic acid (DHA) 0.0 0.30

Total LC n-6 PUFAs 1.23 1.24

    Arachidonic acid (AA) 0.03 0.02

Total monounsaturated fats 2.52 1.68

Total saturated fats 0.60 0.77

LC n-3 PUFAs, long chain omega-3 polyunsaturated fatty acids; LC n-6 PUFAs, long chain omega-6 polyunsaturated fatty acids. Data is according to manufacturers’ specification sheets (Specialty Feeds, Glen Forrest, WA).
Table 2  Stain intensity for transforming growth factor-β1 (TGF-β1 active), monocyte chemoattractant protein-1 (MCP-1), and interleukin-1β (IL-1β) in inflammatory cells that infiltrated the suprarenal and infrarenal regions of the abdominal aorta. Apolipoprotein E-deficient mice were fed a low or high omega-3 polyunsaturated fatty acid diet for 8 weeks and then infused with angiotensin II (1000 ng/kg/min) for two days.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Suprarenal</th>
<th></th>
<th></th>
<th>Infrarenal</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low diet</td>
<td>High diet</td>
<td></td>
<td>Low diet</td>
<td>High diet</td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>37.4±2.5</td>
<td>44.1±1.4</td>
<td><em>P</em> =0.032</td>
<td>43.5±3.2</td>
<td>44.5±0.9</td>
<td><em>P</em> &gt;0.05</td>
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<tr>
<td>MCP-1</td>
<td>72.3±5.1</td>
<td>80.2±2.2</td>
<td><em>P</em> &gt;0.05</td>
<td>86.8±5.1</td>
<td>90.2±3.3</td>
<td><em>P</em> &gt;0.05</td>
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<tr>
<td>IL-1β</td>
<td>34.1±6.0</td>
<td>31.1±2.1</td>
<td><em>P</em> &gt;0.05</td>
<td>40.2±4.5</td>
<td>38.2±2.7</td>
<td><em>P</em> &gt;0.05</td>
</tr>
</tbody>
</table>

Data are arbitrary units, expressed as mean±SEM.
Figure 1: Detection of matrix metalloproteinases MMP-2 and MMP-9, and tissue inhibitor of metalloproteinase 1 (TIMP-1) immunoreactivity within endothelial cells (asterisks), smooth muscle cells (diamonds) and inflammatory cells (arrows) within the infrarenal aorta of apolipoprotein E-deficient (ApoE<sup>−/−</sup>) angiotensin-II (AngII) infused mice fed a diet low (A-C, E) or high (D, F) in omega-3 polyunsaturated fatty acids (n-3 PUFAs). Tissue sections were incubated with antibodies to MMP-2 (A), MMP-9 (C, D) or TIMP-1 (E, F). Control section in which the primary antibody was substituted for antibody diluent (B). Sections were developed with metal enhanced 3,3′-diaminobenzidine (DAB) and viewed with a Nikon Eclipse Ti microscope. Scale bars represent 50 µm.

Figure 2: Effect of omega-3 polyunsaturated fatty acids (n-3 PUFAs) on matrix metalloproteinases MMP-2 and MMP-9, and tissue inhibitor of metalloproteinase 1 (TIMP-1) immunoreactivity within endothelial cells (ECs), smooth muscle cells (SMCs) and inflammatory cells (ICs) infiltrating the adventitial region of the suprarenal (S) and infrarenal (I) aortas of apolipoprotein E-deficient (ApoE<sup>−/−</sup>) mice. Mice were fed a diet low (open bars) or high (filled bars) in n-3 PUFAs for 8 weeks, before being infused with angiotensin-II (AngII; 1000 ng/kg/min in 0.9% saline) for 2 days. *, P < 0.05; †, P < 0.01. Data are presented as mean ± SEM.

Figure 3: Latent MMP-9 activity determined in plasma using zymography. Plasma was obtained from apolipoprotein E-deficient (ApoE<sup>−/−</sup>) mice that were fed a diet low (open bar) or high (filled bar) in omega-3 polyunsaturated fatty acids (n-3 PUFAs) for 8 weeks, before being infused with angiotensin II (AngII, 1000 ng/kg/min in 0.9% saline) for 2 days. Latent MMP-9 activity was lower in ApoE<sup>−/−</sup> mice that were fed a high, compared to a low n-3 PUFA diet (*, P<0.05). Data are presented as mean ± SEM.
Figure 4: Elastin fragmentation in aortas of apolipoprotein E-deficient (ApoE<sup>−/−</sup>) mice fed a diet with low or high omega-3 polyunsaturated fatty acid (n-3 PUFA) content. Elastin fragmentation was visualised using van Gieson staining of 4 μm tissue sections (A, arrows). Some regions of some aortas from mice on the low n-3 PUFA diet showed evidence of total loss of elastin and replacement with fibrotic tissue (B). A trend for fewer fragmented elastin fibres was evident in both suprarenal (S) and infrarenal (I) aortic segments (C), with strong correlation between two independent assessors (R<sup>2</sup> = 0.73; D). Scale bars represent 50 μm.
References


Figure 2

**Immunoreactivity ECs (Arbitrary units)**

![Bar chart showing immunoreactivity of ECs with values for S and I conditions for MMP-2, MMP-9, and TIMP-1](chart ECs)

**Immunoreactivity SMCs (Arbitrary units)**

![Bar chart showing immunoreactivity of SMCs with values for S and I conditions for MMP-2, MMP-9, and TIMP-1](chart SMCs)

**Immunoreactivity ICs (Arbitrary units)**

![Bar chart showing immunoreactivity of ICs with values for S and I conditions for MMP-2, MMP-9, and TIMP-1](chart ICs)
Figure 3

Low diet                      High diet

Band area (arbitrary units)

Latent MMP-9
Figure 4

A and B: Images showing the elastin breaks per mm². The arrows indicate the areas of interest.

C: A chart showing the elastin breaks per mm² for S and I conditions. The bars represent the mean and standard error, with the numbers 10, 9, and 10 indicating the counts for each condition.

D: A scatter plot showing the relationship between elastin breaks per mm² for Examiner 1 and Examiner 2.