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## **Investigation of long chain omega-3 PUFAs on arterial blood pressure, vascular reactivity and survival in angiotensin II-infused Apolipoprotein E knockout mice**

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**Short title:** Effect of n-3 PUFAs in a mouse model of AAA

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### **Abstract**

Abdominal aortic aneurysm (AAA) is an inflammatory vascular disease. Long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) decrease inflammation and oxidative stress in an angiotensin II-infused apolipoprotein E-knockout (ApoE<sup>-/-</sup>) mouse model of AAA. We investigated the effects of LC n-3 PUFAs on blood pressure and vascular reactivity in fourteen angiotensin II-infused ApoE<sup>-/-</sup> male mice. Blood pressure was obtained using a non-invasive tail cuff method and whole blood was collected by cardiac puncture. Vascular reactivity of the thoracic aorta was assessed using wire myography and activation of endothelial nitric oxide synthase (eNOS) was determined by immunohistochemistry. A high LC n-3 PUFA diet increased the omega-3 index and reduced the n-6 to n-3 PUFA ratio. At day 10 post-infusion with angiotensin II, there was no difference in SBP or DBP in mice fed the high or low n-3 PUFA diets. The high LC n-3 PUFA diet resulted in a non-significant trend for delay in time to death from abdominal aortic rupture. Vascular reactivity and eNOS activation remained unchanged in mice fed the high compared to the low LC n-3 PUFA diet. This study argues against direct improvement in vascular reactivity in ApoE<sup>-/-</sup> mice that were supplemented with n-3 PUFA for 8 weeks prior to infusion with angiotensin II.

**Keywords:** Abdominal aortic aneurysm, Cardiovascular disease, Long chain omega-3 fatty acids, eicosapentaenoic acid, docosahexaenoic acid, wire myography.

## Introduction

Abdominal aortic aneurysm (AAA) is an inflammatory disease involving degradation of vascular elastin by matrix metalloproteinases (MMPs) and increased risk of rupture; a catastrophic event associated with a 74-90% mortality rate.<sup>1,2</sup> Importantly, no current medication is available to slow the progression of the disease, and small aneurysms are monitored by regular ultrasound imaging. The use of mouse models of AAA has been helpful in examining the progression of disease, in particular since human tissue samples acquired during repair surgery provide limited insight into the events preceding the development of late stage AAA.<sup>3</sup> Angiotensin II-infused, apolipoprotein E knockout (ApoE<sup>-/-</sup>) mice are an established animal model of human AAA.<sup>3,4</sup> Recently, Seto *et al.*,<sup>5</sup> reported that angiotensin II-infused ApoE<sup>-/-</sup> mice presented with increased arterial blood pressure, a hypersensitivity in contractile response of the thoracic aorta to KCl, and an impaired relaxant response to acetylcholine (Ach). This appeared to be due to reduced activation of endothelial nitric oxide synthase (eNOS), suggesting decreased nitric oxide (NO) bioavailability during AAA development.

Long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) and their metabolites have blood pressure lowering, anti-inflammatory and inflammation-resolving activities,<sup>6-11</sup> and may therefore be protective against the development of AAA. In support of this hypothesis, the incidence of AAA is lower in the Japanese population (1.7%),<sup>12</sup> which has a high dietary intake of eicosapentaenoic and docosahexaenoic acids (EPA+DHA, 950 mg/day)<sup>13</sup> compared to the European population (incidence, 4.3-4.9%),<sup>12</sup> which has a substantially lower dietary intake of EPA+DHA (250 mg/day).<sup>13</sup> We recently showed that angiotensin II-infused ApoE<sup>-/-</sup> mice fed a high LC n-3 PUFA diet showed no evidence of aortic dissection, reduced numbers of infiltrating neutrophils and macrophages, reduced oxidative stress, and an improved profile of MMP-9 to tissue inhibitor of matrix metalloproteinase (TIMP-1) when compared to angiotensin II-infused ApoE<sup>-/-</sup> mice fed a low LC n-3 PUFA diet.<sup>14,15</sup> Further, increased levels of the pro-resolving DHA metabolite resolvin D1, were observed in angiotensin II-infused ApoE<sup>-/-</sup> mice fed the high

compared to the low LC n-3 PUFA diet, indicating conversion of DHA to resolvin D1 during the acute inflammatory challenge.<sup>14</sup> However, the effects of LC n-3 PUFAs on blood pressure and vascular reactivity in an ApoE<sup>-/-</sup> mouse model of AAA have so far not been examined. We hypothesised that the infusion of angiotensin II into ApoE<sup>-/-</sup> mice would lead to increased arterial blood pressure and aberrant vascular reactivity, and that this might be corrected through dietary supplementation with LC n-3 PUFAs.

## Results

### **Weight gain monitoring, erythrocyte fatty acid profile and plasma resolvin D1 concentration**

Mouse body weight was monitored to ensure ongoing health of the animals and consistent ingestion of low or high n-3 PUFA feed for each treatment group. Weight was similar for mice in the low and high n-3 PUFA diet groups at the beginning of the study (Figure 1A). After 10 weeks, mouse weight significantly increased, regardless of the treatment group ( $p < 0.001$ , one-way ANOVA, low diet  $n=7$ , high diet  $n=6$ ), and there was no difference in final weight between groups (Figure 1A).

As expected, the n-3 index was significantly higher in mice fed the high, compared to the low n-3 PUFA diet (Figure 1B;  $p < 0.01$ , independent t-test, low diet  $n=7$ , high diet  $n=4$ ). The proportion of arachidonic acid (AA) to all other FAs detected in the erythrocyte membrane phospholipids (% arachidonic acid) was significantly higher in low compared to the high n-3 PUFA diet fed mice (Figure 1C;  $p < 0.001$ , independent t-test, low diet  $n=7$ , high diet  $n=4$ ). Additionally, mice fed the high n-3 PUFA diet showed a significantly lower n-6 to n-3 PUFA ratio when compared to the low n-3 PUFA diet fed mice (Figure 1D;  $p < 0.001$ , independent t-test, low diet  $n=7$ , high diet  $n=4$ ). There was also a non-significant trend for higher plasma resolvin D1 concentration in

mice that were fed the high n-3 PUFA diet when compared to mice fed the low n-3 PUFA diet (Figure 1E, 2-tailed independent samples t-test,  $p=0.129$ , low diet  $n=7$ , high diet  $n=5$ ).

**Figure 1:** Mouse body weight, measurement of the incorporation of long chain polyunsaturated fatty acids (LC PUFAs) into erythrocyte membrane phospholipids and plasma resolvin D1 levels in angiotensin II-infused ApoE<sup>-/-</sup> mice fed a low or high n-3 PUFA diet. Weight significantly increased after 10 weeks, regardless of the treatment (**A**) (one-way ANOVA, \*:  $p<0.001$ ). Initial and final weights were similar across treatment groups. Mice on the high diet presented with a higher n-3 index (**B**), lower percent arachidonic acid (**C**) and lower n-6 to n-3 PUFA ratio (**D**), than mice on the low n-3 PUFA diet (independent t-test, \*:  $p<0.01$ ). There was a non-significant trend for elevated plasma resolvin D1 concentration in mice fed the high compared to the low n-3 PUFA diet (**E**).

#### **Arterial blood pressure measurements and aortic rupture mortality**

SBP and DBP were similar for mice fed the low n-3 PUFA or high n-3 PUFA diet immediately before implantation of the osmotic mini pumps (Figure 2). Furthermore, there was no difference in the change in SBP and DBP after subtraction of baseline BP measurements in mice fed the high compared to the low n-3 PUFA diet (Figure 2A, B). In the current study, none of the mice in the low n-3 PUFA diet group died after infusion with angiotensin II. However, two mice died in the high n-3 PUFA diet treatment group. One of the deaths resulted from a ruptured thoracic aortic aneurysm (TAA) and a ruptured AAA caused the other. We have previously used this angiotensin II-infused, ApoE<sup>-/-</sup> mouse model of AAA and the collated data suggests that during 3 studies and for a total of 53 mice there was a non-significant trend for fewer deaths due to AAA rupture for mice fed the high n-3 PUFA diet (3 deaths;  $n=26$ ) compared to the low n-3 PUFA diet (7 deaths;  $n=27$ ,  $p=0.293$ ; two-sided Fischers exact probability test) (Figure 2C).

**Figure 2:** Arterial blood pressures and survival rates for angiotensin II-infused ApoE<sup>-/-</sup> mice fed a low or high n-3 PUFA diet. BP was measured prior to mini pump insertion and 10 days after implantation. At day 10 of the 14-day infusion, there was no difference in the change in SBP (A) or DBP (B) after subtraction of baseline BP measurements in mice fed the high (n=6) compared to the low n-3 PUFA diet (n=7; 2-tailed independent samples t-test). A trend for a delay in death from abdominal aortic rupture (C, collated data from 3 studies for a total of 53 mice) was observed in mice fed the high n-3 PUFA diet (n=26) when compared to mice fed the low n-3 PUFA diet (n=27).

### **Aortic reactivity using wire myography and endothelial nitric oxide synthase activation**

KCl caused concentration dependent contraction of isolated thoracic aortas of both saline and angiotensin II-infused mice. A leftward shift in response was observed in angiotensin II infused mice and this reached significance for the mice fed the low n-3 PUFA diet only (EC<sub>50</sub> saline, low n-3 PUFA diet: 31.28±2.66 mM, n=4; EC<sub>50</sub> angiotensin II, low n-3 PUFA diet: 20.02±2.83 mM, n=7; one-way ANOVA, p<0.05; EC<sub>50</sub> saline, high n-3 PUFA diet: 27.81±1.66 mM, n=5; EC<sub>50</sub> angiotensin II, high n-3 PUFA diet: 20.37±2.32 mM, n=5; Figure 3A). There was no difference in EC<sub>50</sub> values in angiotensin II infused mice that were fed the low or high n-3 PUFA diet (Figure 3A).

In mice fed the low n-3 PUFA diet, the magnitude of relaxant response to 0.32-3.2 μM Ach after pre-constriction with 60 mM KCl was greater after infusion with saline (n=4) compared to angiotensin II (n=5; 2-tailed independent samples t-test; p<0.05). The magnitude of response was similar in saline (n=4) and angiotensin II infused (n=5) mice fed the high n-3 PUFA diet (Figure 3B).

Immunoreactivity of phospho-eNOS (Ser1177) in sections of supra- and infrarenal aorta was similar for both low and high n-3 PUFA diet fed mice, indicating similar levels of eNOS activation in both treatment groups (Figure 3C-F, low diet n=7, high diet n=5).

**Figure 3:** Vascular reactivity in thoracic aortas and phospho-eNOS (Ser1177) immunoreactivity in supra- and infrarenal aortas from ApoE<sup>-/-</sup> mice fed a low or high n-3 PUFA diet. KCl induced dose-dependent contraction with a leftward shift in the response observed for mice infused with angiotensin II compared to saline infused mice (A). The relaxant response to acetylcholine in aortas that were pre-contracted with 60 mM KCl was greater in saline compared to angiotensin II infused mice fed the low n-3 PUFA diet (B, \* p<0.05, 2-tailed independent samples t-test, low diet saline vs. low diet angiotensin II). The relaxant response was similar in saline and angiotensin II infused mice fed the high n-3 PUFA diet (B). No immunoreactive staining was observed in endothelial cells where the primary antibody was omitted (C, arrows). Positive immunoreactive staining to phospho-eNOS (Ser1177) was observed in endothelial cells (D, inset and arrows). Endothelial phospho-eNOS levels were similar in suprarenal (E) and infrarenal (F) aortas of low and high n-3 PUFA diet fed mice. A.U., arbitrary units.

## Discussion

LC n-3 PUFAs have blood pressure lowering, anti-inflammatory and anti-thrombotic effects. In this study, the effect of dietary supplementation with LC n-3 PUFAs was investigated on blood pressure, vascular reactivity and eNOS activation in an angiotensin II-infused ApoE<sup>-/-</sup> mouse model of AAA.

The omega-3 index was measured after a 10-week intake of either a high or low LC n-3 PUFA diet. The index reflects long-term intake of LC n-3 PUFAs as well as increased incorporation of the LC n-3 PUFAs into other cell types, such as the myocardium and skeletal muscle.<sup>16,17</sup> Epidemiological studies conducted in the USA and Japan have shown that an n-3 index <4% is associated with a 10-fold increase in risk of sudden cardiac death compared to an n-3 index of >8%.<sup>16</sup> In the current study, 10-week consumption of a high LC n-3 PUFA diet increased the n-3 index to levels considered cardioprotective in humans (>8%). Increased consumption of LC n-3 PUFAs also lowers the n-6 to n-3 ratio and this may reduce mortality from cardiovascular disease.<sup>18</sup> In this study, the n-6 to n-3 ratio was markedly reduced with the high LC n-3 PUFA diet.

DHA is preferentially incorporated into the myocardium and erythrocytes and is effective in modulating heart rate and cardiac arrhythmias, suggesting it to be more biologically active in the cardiovascular system than EPA.<sup>19</sup> Beneficial effects of LC n-3 PUFAs may also in part be based on their conversion into biologically active metabolites. We previously showed a significantly higher plasma concentration of the DHA metabolite, resolvin D1, in ApoE<sup>-/-</sup> mice that had been fed a high, compared to a low LC n-3 PUFA diet and infused with angiotensin II for 2 days.<sup>14</sup> In the current study mice were infused with angiotensin II for 2 weeks and we observed a non-significant trend for increased plasma resolvin D1 concentration in the high LC n-3 PUFA diet mice. In line with previous studies, this suggests that conversion of DHA to resolvin D1 mainly occurs in the acute phase of the inflammatory challenge (for example, 2 day infusion of angiotensin II to ApoE<sup>-/-</sup> mice), and that this begins to return to baseline levels during a period of inflammation resolution (for example, the current study with a 2 week infusion with angiotensin II).<sup>11</sup>

No current medical treatments are available to slow the progression of AAA.<sup>21</sup> Collated data from three of our studies using 2-week angiotensin II infusion of ApoE<sup>-/-</sup> mice suggests a non-significant trend for delay in death from abdominal aortic rupture in mice fed the high compared to the low LC n-3 PUFA diet. A trend for a more pronounced delay in time to aortic rupture was observed when data was examined for death associated with ruptured abdominal aorta only, than death associated with rupture of either abdominal or thoracic aorta.<sup>14</sup> This finding suggests a localised benefit of LC n-3 PUFAs to the abdominal aorta. We have recently reported that a high LC n-3 PUFA diet protects against inflammatory cell infiltration and production of reactive oxygen species and matrix metalloproteinase in the abdominal aorta of angiotensin II-infused ApoE<sup>-/-</sup> mice.<sup>14,15</sup>

Increases in mean arterial pressure in angiotensin II-infused ApoE<sup>-/-</sup> mice have recently been reported.<sup>5</sup> In the present study, no significant difference was observed in the change in SBP or DBP after 10 days of angiotensin II infusion for mice that were fed a high or low n-3 PUFA diet.



KCl contracted isolated segments of thoracic aorta, and Ach relaxed KCl-pre-contracted vessels. The EC<sub>50</sub> value obtained for KCl-induced aortic contraction in this study (20.02-20.07 mM) is similar to that previously reported for angiotensin II-infused ApoE<sup>-/-</sup> mice (15.27 mM).<sup>5</sup> We hypothesised that the high LC n-3 PUFA diet may have a protective effect on endothelial function, and thereby modulate vasoreactivity to KCl and Ach. The findings showed no difference in the contractile and relaxant response of aortas, or activation state of eNOS in mice fed the high or low LC n-3 PUFA diet, indicating no protection from angiotensin II-induced endothelial dysfunction by the high LC n-3 PUFA diet in this model. This was of interest as some, but not all studies had reported beneficial effects of LC n-3 PUFAs on NOS expression and activation.<sup>22-25</sup> It is noteworthy that this study examined vascular reactivity in a large conduit artery. Examination of effects of LC n-3 PUFAs on small resistance arterioles, which have a major role in regulation of BP, presents an area of future investigation.

The diet used in this study contained a single, fixed ratio of EPA to DHA, and a different ratio could be used to further explore the effect of n-3 PUFAs on blood pressure and vascular reactivity of angiotensin II-infused mice. It would also be of interest for future studies to measure other resolving factors and inflammatory markers in this mouse model.

In this study, an angiotensin II-infused ApoE<sup>-/-</sup> mouse model of AAA was used to make inferences about human AAA. Unlike human AAA which develops primarily in the infrarenal region, the mouse model develops AAA mainly in the suprarenal region.<sup>3,4</sup> The difference has been attributed to differences in hemodynamics across species. Furthermore, dissection of the aortic wall in these animals leads to the development of a vascular haematoma, whereas the human disease results in the formation of a laminated thrombus.<sup>3</sup> Also, not all angiotensin II-infused ApoE<sup>-/-</sup> mice develop an AAA.<sup>4</sup> Notwithstanding the above limitations, this mouse model shares some common hallmarks of human AAA including infiltration of the media and adventitia by inflammatory cells as well as medial elastin degradation.<sup>3,4,14</sup>

In conclusion, the current study showed that a high LC n-3 PUFA diet raised the omega-3 index to levels deemed to be cardioprotective in human populations, with an associated trend for increase in plasma concentration of the pro-resolving metabolite, resolvin D1. A trend for delay in time to death from abdominal aortic rupture after angiotensin II infusion was observed for mice fed the high-, compared to the low n-3 PUFA diet. These beneficial effects are not mediated by improved vascular reactivity or increased NO bioavailability, warranting future research into other mechanisms of action of LC n-3 PUFAs in this animal model.

## **Methods**

### **Dietary supplementation**

Fourteen ApoE<sup>-/-</sup> 3-4 week old male mice were purchased from the Animal Resource Centre (Perth, Australia) and transported to the Herston Medical Research Centre (Brisbane, Australia). Mice were fed a standard cereal grain-based diet (0.02% EPA and 0.05% DHA; Specialty Feeds, WA, Australia) for 2 weeks before allocating them to either a high (n=7) or low n-3 PUFA diet (n=7) for 8 weeks. The low n-3 PUFA diet contained negligible amounts of EPA and DHA (total LC n-3 PUFAs, 0.14%), whereas the high diet contained 0.07% EPA and 0.30% DHA (total LC n-3 PUFAs, 0.70%).<sup>14</sup>

### **Subcutaneous angiotensin II infusion**

Mice were infused with angiotensin II (1000 ng/kg/min) for 2 weeks with continuation of the designated diets. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study was approved by the Animal Ethics Committee of the University of the Sunshine Coast (Permit

Number: AN/A/14/84) and endorsed by the University of Queensland Animal Ethics Committee (Permit Number: USC/061/14).

All surgery was performed under sodium pentobarbital anaesthesia (32.5 µg/g body weight in 0.9% saline, intraperitoneal injection) and animals received intraperitoneal analgesic (buprenorphine, 330 ng/g body weight). The interscapular region of each mouse was shaved and sterilized with 70% ethanol, allowing the alcohol to dry before making a 1 cm incision. A subcutaneous pocket was created by blunt dissection, and an osmotic mini pump (Alzet, model 1002; CA, USA), primed to deliver angiotensin II, was inserted into the pocket. The incision was closed using 3/0 sutures. All animals displayed normal mobility and feeding behaviour post-surgery.

#### **Blood processing and storage**

At the termination of the study period, animals were anaesthetised with pentobarbital (325 µg/g body weight in 0.9% saline) and whole blood was collected into 2 ml EDTA tubes by cardiac puncture. Samples were centrifuged (1500 x g, 15°C, 10 min), and plasma and erythrocytes were stored separately at -80°C until further analysis. Blood was not collected from two mice on the high n-3 PUFA diet that had died from a ruptured thoracic aorta and a ruptured abdominal aorta. Blood collected from another animal on the high n-3 PUFA diet did not yield enough erythrocytes to investigate the erythrocyte fatty acid profile, but did provide enough plasma to determine resolvin D1 concentration.

#### **Erythrocyte fatty acid profile**

To extract phospholipids from erythrocytes, 600 µl of methanol containing butylhydroxytoluene (BHT, 20 mg/100 ml) was added to 300 µl of erythrocytes and cells were homogenised by pulverization of sample using glass rods for 1 min. Homogenates were covered with nitrogen gas and stored on ice for 30 min before adding 600 µl of chloroform. Cells were homogenised

again for 1 min, stored on ice for 30 min and then centrifuged (3000 x g, 4°C, 5 min). The supernatant was withdrawn, placed in clean test tubes, covered with nitrogen gas and stored on ice until further addition of extracted lipids. The process was repeated twice, using reduced volumes of methanol with BHT and chloroform (300 µl), as well as reduced storage times on ice (10 min). To complete the extraction, 800 µl of chloroform and 460 µl of 0.05 M KCl was added to 1000 µl of the pooled lipid solution, mixed by vortex and centrifuged (3000 x g, 4°C, 10 min). The supernatant was discarded; the lipid fraction was transferred into GC vials and dried under nitrogen gas. To hydrolyse the extracted lipids, 500 µl of 9 M HCl:H<sub>2</sub>O:acetonitrile (1:1:18) solution containing BHT (25 mg/50 ml) was added, samples were covered with nitrogen gas and incubated overnight at 65°C. The hydrolysed samples were then dried under nitrogen gas and freeze dried for 15 min before adding 250 µl of hexane and 10 µl of derivatising agent (1-tert-butyldimethylsilylimidazole). Samples were covered with nitrogen gas, incubated at 37°C for 2 h and analysed using a PerkinElmer Clarus®580 gas chromatograph (GC) coupled to a PerkinElmer Clarus®SQ8S mass spectrometer (MS). Samples were run on an Elite-MS column (30 m x 0.25 mm x 0.25 mm film, PerkinElmer) with 70 eV ionization, to determine cellular uptake of EPA and DHA. The MS scan range was 45-450 Da. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The injection port was 300°C with the split ratio at 30:1. The oven program operated at 170°C and was ramped at 5°C/min until 310°C and held for 5 min for a total run time of 28.33 min. The n-3 index for each sample was calculated as a proportion of the combined integrated peak areas of DHA and EPA over the total peak area of fatty acids within the sample.

### **Plasma resolvin D1 concentration**

Plasma resolvin D1 concentration was measured using a resolvin D1 immunoassay kit according to the manufacturer's instructions (Cayman Chemical, MI, USA). Briefly, samples were diluted 1:1 in enzyme immunoassay sample buffer (Cayman Chemical) and compared against

resolvin D1 standards (3.3-200 pg/ml). The plate was read at 405 nm after 140 min (BioRad Model 680 Microplate Reader).

### **Non-invasive tail cuff blood pressure measurements**

Blood pressure was measured non-invasively by determining the tail blood volume with a volume pressure recording sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT). Animals were habituated to restrainers (Kent Scientific) for 2 weeks prior to commencement of the blood pressure measurement. Restrainers were placed on heated platforms (temperature in the restrainers was between 35-37°C) (Kent Scientific) and covered with dark towels to reduce stress of the animals. The protocol for blood pressure acquisition included 5 acclimatisation cycles, followed by 12 cycles of repeated blood pressure measurements with a 2 min break after the first 6 cycles. The cuff deflation time was 20 seconds. Systolic and diastolic blood pressure was determined as the mean of the repeat measurements. Pressure traces were assessed post-measurement and excluded if the arterial pressure did not exceed the cuff pressure. Final blood pressure measurements were not obtained for one mouse on the high n-3 PUFA diet that had died from a ruptured thoracic aorta.

### **Aortic reactivity using wire myography**

Following blood collection, the thoracic aorta was harvested from the mice and fat and connective tissue were removed under a dissecting stereo-microscope. Myograph studies were performed in a Multi Wire Myograph system (Model 610M, Danish Myo Technology (DMT), Denmark), using 6 ml of Krebs buffer solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 11 mM glucose and 1.8 mM CaCl<sub>2</sub>) per bath. Two 40 µm tungsten wires were passed through the lumen of the aorta, and the aorta was then mounted into the myography chamber containing Krebs buffer solution. The internal circumference of each vessel

was normalized (DMT Normalisation Module) and data was acquired using an ADI Powerlab Data Acquisition System (AD Instruments, Australia).

Concentration-response curves were constructed to KCl (10-60 mM). To examine the relaxation of aortas to Ach, aortas were pre-incubated with the cyclo-oxygenase inhibitor indomethacin (1  $\mu$ M) and the acetylcholinesterase inhibitor neostigmine (10  $\mu$ M), for 30 min. Vessels were pre-constricted with 60 mM KCl and once a plateau in response was achieved, the aortas were exposed to increasing concentrations of Ach (1 nM-10  $\mu$ M). The relaxation response was expressed as a percent of the maximal contractile response obtained for each vessel. For the low n-3 PUFA diet fed mice, 3 out of 5 curves were used to calculate the EC<sub>50</sub> value with the remaining two vessels showing no relaxant response to Ach. For the high n-3 PUFA diet fed mice, 4 out of 5 curves were used to calculate the EC<sub>50</sub> value with one vessel showing no relaxant response to Ach. Aortic reactivity was not determined for the two mice on the high n-3 PUFA diet; one that died from a ruptured thoracic aorta and the other from a ruptured abdominal aorta. Pre-contraction with KCl of two blood vessels from mice on the low n-3 PUFA diet was not achieved and so the relaxant response to Ach was not possible. One vessel from a saline-infused mouse on the high diet did not show a relaxant response to sodium nitroprusside and was excluded from the analysis.

#### **Aortic phospho-eNOS immunoreactivity**

The abdominal aorta was harvested after blood collection and separated into suprarenal and infrarenal segments. Tissues were fixed in 10% formalin and then dehydrated and embedded in paraffin. Tissue sections (4  $\mu$ m) were cut with a microtome and collected onto gelatin-coated microscope slides. Consecutive changes of xylene (10 min, 2x2min) and absolute ethanol (3x2 min) were used to deparaffinise the sections. Sodium citrate buffer was used to unmask antigenic sites (11.4 mM tri-sodium citrate, 0.05% Tween-20; pH 6.0 with HCl; 95-100°C, 20 min). The sections were cooled in the sodium citrate buffer at 22°C until the solution was ~45°C

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and then rinsed with distilled water. Sections were incubated with 3% H<sub>2</sub>O<sub>2</sub> (23°C, 20 min) and washed with Tris buffered saline (TBS) containing 1% Tween-20 (0.2 M Tris base, 1.37 M NaCl, 1% Tween-20; pH 7.6 with HCl, 23°C, 5 min). Sections were blocked with horse serum (1:20) in 0.01 M PBS (6.8 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.6 mM NaH<sub>2</sub>PO<sub>4</sub>; pH 7.2 with HCl) at 23°C for 20 min and then exposed to the primary antibody (rabbit anti-phospho eNOS; Ser1177; 1:50 in 0.01 M PBS, Cell Signaling) or PBS alone (23°C, 30 min). Sections were washed in TBS-Tween (23°C, 2x5min). Phospho-eNOS staining was revealed using a Vectastain Elite ABC kit and SIGMAFAST DAB with metal enhancer. Sections were mounted with coverslips and viewed using a Nikon Eclipse Ti microscope. Each whole aorta was photographed by stitching multiple images using a 20x objective lens. Stain intensity for all visible endothelial cells in the aortas was quantitated using Image J64 software. Aortic phospho-eNOS immunoreactivity could not be determined for two mice on the high n-3 PUFA diet that had died; one from a ruptured thoracic aorta and the other from a ruptured abdominal aorta.

### **Data analysis**

Data were analysed using 2-tailed independent t-tests or one-way ANOVA using IBM SPSS Statistics (version 21) software. Levene's test of equal variance was used to confirm homogeneity of the samples' variance. Data are expressed as mean±SEM.

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