



## Peroxisome proliferator-activated receptor ligands reduce aortic dilatation in a mouse model of aortic aneurysm<sup>☆</sup>

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### ABSTRACT

**Objective:** Osteopontin (OPN) is associated with human abdominal aortic aneurysms (AAA) and *in vitro* studies suggest that this cytokine is downregulated by peroxisome proliferator-activated receptor (PPAR) ligation. We examined the effect of two PPAR ligands within a mouse model of aortic aneurysm.

**Methods:** At 11 weeks of age apolipoprotein E deficient (ApoE<sup>-/-</sup>) mice were given pioglitazone ( $n=27$ ), fenofibrate ( $n=27$ ) or vehicle ( $n=27$ ) in their drinking water. From 13 weeks of age mice received angiotensin II (1  $\mu\text{g}/\text{kg}/\text{min}$ ) infusion via subcutaneous pumps until death or 17 weeks when the aortas were harvested and maximum aortic diameters were recorded. Suprarenal aortic segments were assessed for OPN concentration and macrophage accumulation. Saline infused mice served as negative controls ( $n=22$ ).

**Results:** Angiotensin II induced marked dilatation in the suprarenal aorta (>2-fold increase compared to controls) associated with upregulation of the cytokines OPN and macrophage infiltration. Suprarenal aortic expansion was significantly reduced by administration of pioglitazone (mean diameter  $1.61 \pm 0.11$  mm,  $p=0.011$ ) and fenofibrate (mean diameter  $1.51 \pm 0.13$  mm,  $p=0.001$ ) compared to the vehicle control group (mean diameter  $2.10 \pm 0.14$  mm). Immunostaining for macrophages was reduced in mice treated with pioglitazone (median staining area 6.2%, interquartile range 4.1–7.2,  $p<0.001$ ) and fenofibrate (median staining area 4.0%, interquartile range 2.2–6.1,  $p<0.001$ ) compared to mice receiving vehicle control (median staining area 13.2%, interquartile range 8.4–20.0).

**Conclusion:** These findings suggest the potential value of peroxisome proliferator-activated receptor ligation as a therapy for human AAAs.

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### 1. Introduction

Abdominal aortic aneurysm (AAA) is a common cause of death in Western countries [1]. The widespread use of imaging and the introduction of ultrasound surveillance programs in some regions have been associated with large numbers of asymptomatic small AAAs being detected [2]. Early elective surgery of small AAAs

has not been demonstrated to reduce mortality [3]. In order to identify potential therapies for small AAA a number of studies have been carried out in animal models but many of the medications found to date would require prolonged development and testing in order to establish safe prescription in patients [4,5].

Peroxisome proliferator-activated receptors (PPAR) are a group of nuclear receptors which can be activated by endogenous and drug ligands [6,7]. PPAR $\gamma$  is ligated by the thiazolidinedione pioglitazone and PPAR $\alpha$  by the fibric acid derivative fenofibrate [6,7]. Both PPAR $\alpha$  and  $\gamma$  ligation have also been shown to reduce cytokine production and inflammation, including osteopontin (OPN) concentration, which we have previously linked with AAA [6–8]. In the present study we investigated the effect of pioglitazone and fenofibrate in slowing aortic dilatation in a mouse model of AAA.

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## 2. Methods

### 2.1. Mice

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Ethical approval was obtained from the local institutional committee prior to commencement of the study. Male apolipoprotein E (ApoE<sup>-/-</sup>) mice at 11 weeks of age (obtained from Animal Resources Centre, Canning Vale, Western Australia) received either vehicle control (0.1% carboxymethylcellulose), pioglitazone (50 mg/kg/day) or fenofibrate (100 mg/kg/day) in their drinking water. At 13 weeks of age mice were anaesthetised by intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). Osmotic minipumps (Model 2004, ALZET, Durect Corporation, Cupertino, CA, USA) placed into the subcutaneous space along the dorsal midline delivered 1 µg/kg/min of angiotensin II (Sigma–Aldrich, Castle Hill, Australia) dissolved in sterile saline over 28 days. Age-matched ApoE<sup>-/-</sup> male mice ( $n=22$ ) in which osmotic pumps delivered sterile saline (referred to as saline controls), served as controls. Mice were maintained on a normal laboratory diet. After the 28 day infusion (or death), mice were sacrificed by CO<sub>2</sub> asphyxiation, the aortas were perfused with PBS and harvested from arch to iliac bifurcation. Segments from the aortic arch (left atrium to left subclavian artery origin) were fixed for atheroma assessment. Segments from suprarenal aorta (diaphragm to the lowest renal artery) were divided transversely in half and stored at -20 °C for later analysis by histology, immunohistochemistry (mounted in OCT embedding medium) and ELISA.

### 2.2. Aorta morphometry

Aortas were placed on a black background and digitally photographed (Coolpix 4500, Nikon). Maximum diameter of the aortic arch, thoracic aorta, suprarenal aorta and infrarenal aorta was determined from the images using computer-aided analysis (Scion Image, Scion Corporation). We have previously established that these measurements could be repeated with good intra-observer reproducibility [9].

### 2.3. Suprarenal aortic OPN measurement

Protein was extracted from individual frozen suprarenal aortic segments by homogenising in buffer (10 mM cacodylic acid, 60 mM L-arginine, 0.25% Triton X-100 in PBS, pH 7.2) and centrifuging at 18,000 × *g* at 4 °C for 20 min. Supernatant protein was quantified by the Bradford technique (Protein Assay, Bio-Rad, Hercules, CA, USA). OPN concentration was measured by ELISA (Quantikine, R&D Systems MOST00 for OPN) and expressed as pg/mg of protein. We have previously reported excellent reproducibility of similar assays [10]. All mice in which the aorta did not rupture had segments of suprarenal aorta examined for these cytokines.

### 2.4. Histology and immunohistochemistry

Suprarenal aortic segments from 10 mice from each group were selected using a random number generator. Serial cryostat sections 7 µm thick were cut from each segment and stained for OPN and macrophages. For each stain, all 40 sections were stained simultaneously, using identical reagents and incubation times. Serial frozen sections were air dried, fixed in acetone for 10 min at -20 °C, air dried and rehydrated with PBS before being incubated in 3% H<sub>2</sub>O<sub>2</sub>/0.1% sodium azide/PBS to block endogenous peroxidase. For OPN detection, slides were incubated in 2% normal goat serum (Vector Laboratories) in PBS and endogenous avidin and biotin

blocked using Avidin/Biotin blocking kit (Vector Laboratories), then 2 µg/ml rabbit anti-mouse OPN (clone O-17, Immuno Biological Laboratories), biotinylated goat anti-rabbit IgG (Vector Laboratories) and Vectastain Elite ABC-HRP. Rabbit IgG (Vector Laboratories) was used as isotype control antibody. For macrophage detection, sections were blocked in 2% normal goat serum in PBS followed by staining using pan-macrophage antibody (clone MOMA-2, Abcam), and goat anti-rat HRP (Chemicon). Rat IgG (Sigma) was used as isotype control. Slides were incubated in the peroxidase substrate 3,3'-diamminobenzidine (ImmPACT DAB, Vector), counterstained in Mayer's Haematoxylin, dehydrated, cleared in xylene and mounted in Depex mounting medium. Stained sections were photographed using a Leica BMLB microscope fitted with a SPOTTM CCD Camera (Diagnostic Instruments, Inc., USA) and digital images captured to a PC supported with SPOT32TM software (version 2.1.2.; Diagnostic Instruments, Inc., USA). Identical exposure times and settings were used for all sections. Image analysis was performed on digital tiff images using Adobe Photoshop CS3 Extended software. For each section, the total tissue area and area of macrophage staining were measured using the "Selection Tool" and "Record Measurements" functions. Macrophage staining was expressed as a percentage of total tissue area, i.e. area macrophage staining/total tissue area × 100. Measurements were made by two independent readers and the reproducibility of results analysed using Pearson's correlation coefficient, coefficient of variation and Bland–Altman plots. Correlation coefficient was 0.98 and mean coefficient of variation was 8.8 ± 5.9%. Examples of staining including positive and negative controls are shown in [supplementary Figs. 1 and 2](#). Sections of atheroma from elderly ApoE<sup>-/-</sup> mice were used as positive controls.

### 2.5. Atheroma assessment

Aortic arches were analysed from only 12 of the mice infused with saline but for all the mice receiving angiotensin II and medication (vehicle control 27, pioglitazone 27, fenofibrate 27). After being photographed, aortic arch segments were fixed overnight in 10% neutrally buffered formalin, transferred to 70% ethanol and stained with Sudan IV (0.1% Sudan IV dissolved in equal parts acetone and 70% ethanol) in order to identify intimal atherosclerotic plaque, as previously described [11]. The arch was opened longitudinally, pinned out on sheets of white dental wax and digitally photographed (Coolpix 4500 Nikon camera, SMZ800 Nikon dissecting microscope). The amount of lesion formation was measured using computer-assisted analysis (Scion Image, Scion Corporation) and expressed as a percentage (Sudan IV stained area per total area of the aortic arch).

### 2.6. Statistical analysis

The primary aim of this study was to assess the effect of PPAR ligation by fenofibrate or pioglitazone on suprarenal aortic dilatation in angiotensin II infused ApoE<sup>-/-</sup> mice. In order to assess this aim suprarenal aortic diameter after angiotensin II infusion was compared between mice receiving pioglitazone or fenofibrate with those receiving vehicle controls. As a secondary aim we also assess macrophage staining area and suprarenal aortic concentration of OPN. Finally as a measure of atherosclerosis we compared aortic arch Sudan IV staining areas. Since data was not normally distributed non-parametric tests were used. Maximal aortic diameters, macrophage staining area, OPN concentrations and Sudan IV staining area were compared between treatment and control groups using Mann–Whitney *U*-test. The incidence of aortic rupture between treatment and control groups was compared by Fisher's exact test.

**Table 1**  
Aortic diameters and ruptures in mice receiving different drugs.

Group	Saline infused control	Vehicle control	Fenofibrate	Pioglitazone
Number	22	27	27	27
Aortic rupture	0	9	12	6
Suprarenal (mm)	0.99 ± 0.08	2.10 ± 0.14	1.51 ± 0.13*	1.61 ± 0.11*
Aortic arch Sudan IV staining area (%) <sup>a</sup>	2.05 ± 0.75	2.24 ± 0.53	3.83 ± 0.80	2.42 ± 0.88

Shown are mean ± standard error of maximum diameters from the suprarenal aortic segments or staining area; and rupture numbers.

<sup>a</sup> Aortic arches were analysed from 12 mice in the saline control group and all other 81 mice.

\*  $p \leq 0.01$  compared to vehicle control.

### 3. Results

#### 3.1. Fenofibrate and pioglitazone reduce suprarenal aortic expansion

Angiotensin II infusion caused suprarenal aortic expansion and aortic rupture, as previously described (Table 1) [4,9]. Suprarenal aortic expansion was reduced in mice receiving fenofibrate ( $p = 0.001$  compared to vehicle control) or pioglitazone ( $p = 0.011$  compared to vehicle control) (Table 1). The relative reduction in mean suprarenal aortic diameter was 28 and 24% in mice treated with fenofibrate and pioglitazone, respectively (Table 1). The incidence of aortic rupture was not affected by either medication (Table 1).

#### 3.2. Fenofibrate and pioglitazone reduce suprarenal aortic macrophage infiltration

Immunostaining area for macrophages within the suprarenal aorta was increased in mice receiving angiotensin II (median staining area 13.2%, interquartile range 8.4–20.0,  $n = 10$ ) compared to saline infused mice (median staining area 2.7%, interquartile range 2.5–3.9,  $n = 10$ ,  $p < 0.01$ ; Figs. 1 and 2). Mice receiving fenofibrate (median staining area 4.0%, interquartile range 2.2–6.1,  $n = 10$ ,  $p < 0.01$ ) or pioglitazone (median staining area 6.2%, interquartile range 4.1–7.2,  $n = 10$ ,  $p < 0.01$ ) had significantly smaller areas of

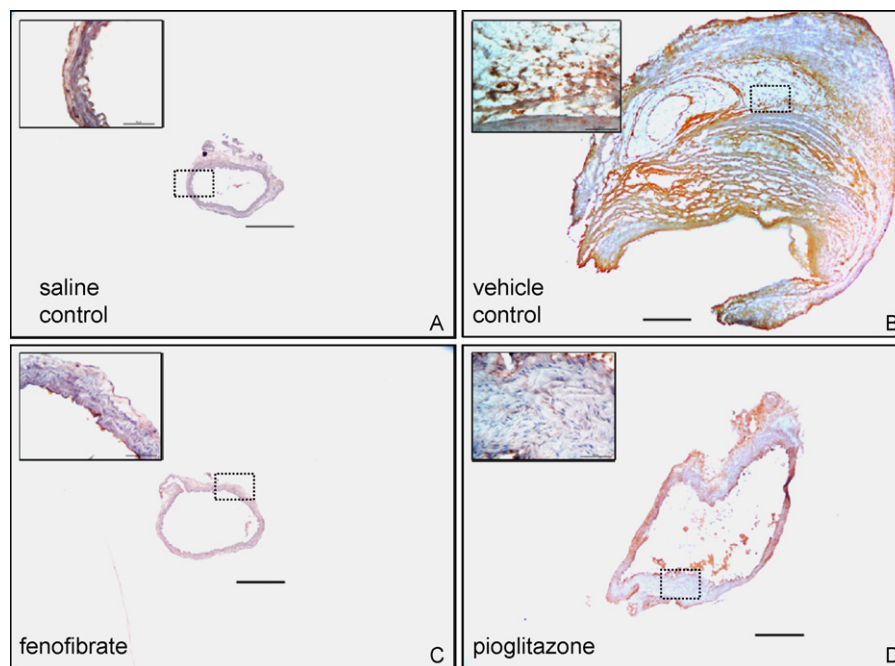
macrophage staining in their suprarenal aortas compared to those treated with vehicle control (median staining area 13.2%, interquartile range 8.4–20.0,  $n = 10$ ).

#### 3.3. Fenofibrate reduces suprarenal aortic concentration of OPN

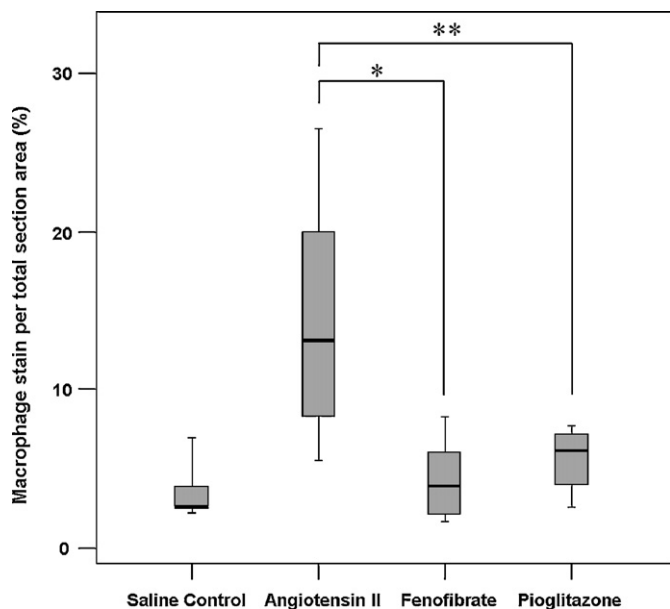
Fig. 3 illustrates the suprarenal aortic concentrations of OPN in relation to drug administration. Aortic OPN concentration was reduced in mice receiving fenofibrate (median in fenofibrate dosed mice 126 pg/mg compared to vehicle control 3198 pg/mg,  $p = 0.006$ ). Aortic OPN concentration was not significantly reduced in mice receiving pioglitazone (median in pioglitazone treated mice 1787 pg/mg compared to vehicle control 3198 pg/mg,  $p = 0.65$ ). Serial immunohistochemistry staining sections suggested that OPN was mainly distributed in areas of wall remodelling associated with macrophage accumulation, with reduction in staining for OPN in fenofibrate and pioglitazone treated mice (Fig. 4). The areas of atherosclerotic lesions in the aortic arch developing in these young mice was low (overall median area 1.26, interquartile range 0–4.02%) and not altered by any drugs (Table 1 and Fig. 5).

### 4. Discussion

The main findings from this study are that the PPAR ligands fenofibrate and pioglitazone reduce aortic expansion induced by angiotensin II in young ApoE<sup>-/-</sup> mice. These findings, together with



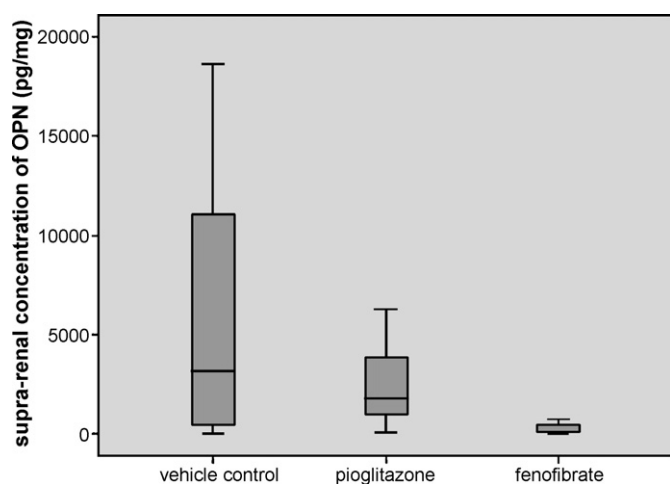
**Fig. 1.** Examples of macrophage staining in sections of suprarenal aorta from mice in relation to PPAR treatment. Examples of immunostaining for macrophages from 17-week-old ApoE<sup>-/-</sup> male mice who received (A) a 4 week saline infusion, (B) a 4 week angiotensin II infusion plus oral vehicle control, (C) a 4 week angiotensin II infusion plus fenofibrate, and (D) a 4 week angiotensin II infusion plus pioglitazone.



**Fig. 2.** Box plots showing staining area for macrophages in the suprarenal aorta of mice in relation to PPAR treatment. Ten harvested non-ruptured suprarenal aortic segments were randomly selected from each group for immunostaining for macrophages. Staining area was estimated by computer-aided analysis. Shown are box plots representing median and interquartile range of staining areas from mice for each group. Staining area for macrophages was reduced in mice receiving fenofibrate ( $*p < 0.01$ ) or pioglitazone ( $**p < 0.01$ ).

a recent report that another PPAR $\gamma$  ligand rosiglitazone reduces aortic expansion, suggest the potential value of PPAR ligands for treating AAAs [12]. The latter study also reported that rosiglitazone reduced the incidence of aortic rupture induced by angiotensin II in 12-month-old ApoE $^{-/-}$  mice. We found no effect of PPAR ligation on aortic rupture in the current study in which 3-month-old mice were used.

The angiotensin II infused ApoE $^{-/-}$  model is increasingly used in studies assessing the effect of pathways or medications in aortic aneurysm formation [1,4,5,9,10,12,13]. The model has some similarities to human AAA. Aneurysms are more common in male mice [14]; aortic dilatation is associated with inflammation and high concentrations of cytokines [9]; and aortic rupture occurs in larger



**Fig. 3.** Suprarenal aortic OPN concentration in relation to PPAR agonists. Shown are box plots representing median and interquartile range of supra-renal aortic OPN for mice receiving different treatments. The 27 mice who ruptured their aorta and thus fresh aortic samples were unavailable were excluded from analysis. The concentration of OPN was significantly reduced in mice receiving fenofibrate,  $p = 0.006$ .

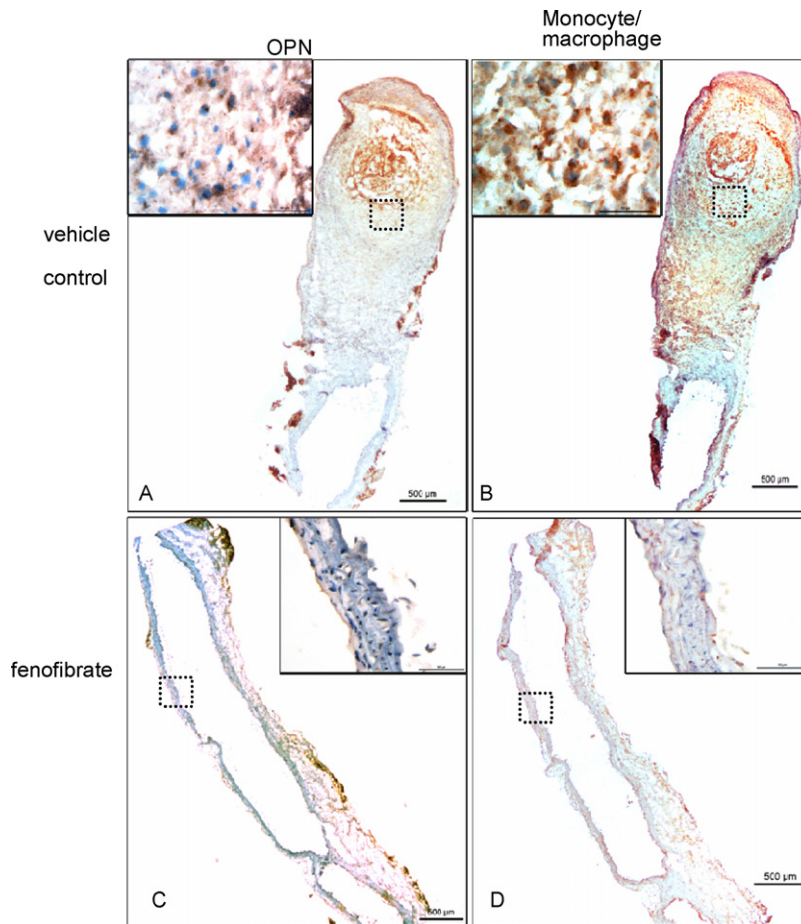
aneurysms [4,12]. Some of the features of the model are, however, disparate from human AAAs, including the predilection to the suprarenal aorta and the involvement of dissection as an important feature of the pathology [15].

The relative reduction in mean suprarenal aortic diameters induced by fenofibrate and pioglitazone was 28 and 24%, respectively. This degree of effect is comparable to that reported for other successful agents investigated in this and similar models including a chymase inhibitor (30%), a Rho-kinase inhibitor (28%), vitamin E (24%), 17 $\beta$ -estradiol (23%) and simvastatin (17%) [4,16–19]. Matrix metalloproteinase inhibition using doxycycline has also been demonstrated to reduce aortic dilatation in this model, but maximum aortic diameter measurements were not reported [20]. The effect of rosiglitazone reported in 12-month-old ApoE $^{-/-}$  mice appears to be greater than the latter studies but was measured in a very different way using MRI, and mice with ruptured AAAs were excluded thus comparison is difficult [12]. In addition rosiglitazone reduced AAA formation and rupture when given 1 week after angiotensin II infusion was commenced. The latter is suggestive that PPAR $\gamma$  ligation is able to inhibit AAA progression which we did not specifically investigate in this study.

Our findings suggest that PPAR ligands inhibit aortic expansion by reducing aortic inflammation. We found reduced infiltration of macrophages within the suprarenal aorta in mice treated with fenofibrate or pioglitazone. We also found lower concentrations of OPN within the aorta of mice treated with fenofibrate. OPN is a chemotactic cytokine for macrophages [21]. Circulating concentrations of OPN have been demonstrated to be increased in patients with AAA [8]. Mice deficient in OPN are resistant to angiotensin II induced AAA formation [22]. Thus, the ability of fenofibrate to downregulate OPN is a plausible mechanism which may have contributed to reduce macrophage infiltration and suprarenal aortic dilatation in mice receiving this medication. OPN deficiency has also reported to reduce angiotensin II induced progression of atheroma in ApoE $^{-/-}$  mice [22]. We did not find any reduction in aortic arch Sudan IV staining area in mice receiving fenofibrate. We suspect that this disparity in effects of fenofibrate on aortic dilatation and atheroma are related to the relatively small amount of intimal atherosclerosis in the young mice we used, as discussed further below. However, it is possible there maybe mechanisms other than OPN reduction which are important in the beneficial effects of PPAR ligands such as downregulation of angiotensin type 1 receptors and osteoprotegerin [12,23].

Further support for the potential value of PPAR ligands in human AAA comes from previous studies demonstrating the ability of these medications to reduce inflammation and proteolysis in atherosclerosis [7,24]. Unlike some other medications, such as statins, PPAR ligands are not presently indicated in most patients with AAA making it more straightforward to investigate these drugs in a randomised clinical trial in patients. The dose of pioglitazone and fenofibrate used in this study is similar to previous rodent studies in which they have been shown to ligate PPAR $\gamma$  and PPAR $\alpha$ , respectively [25,26]. They probably represent higher doses, based on the amount per weight, than currently used in patients where doses are in the range of 15–45 and 120 mg/day for pioglitazone and fenofibrate, respectively.

We also measured arch atheroma area in this study although this was not the primary aim of the work which was designed to look at AAA. Angiotensin II infusion has been reported to promote intimal atheroma in 6-month-old ApoE $^{-/-}$  mice [13]. In the current study using 13-week-old ApoE $^{-/-}$  mice we found no promotion of aortic arch Sudan IV staining area. Previous studies in ApoE $^{-/-}$  mice suggest the effects of angiotensin II are age and aortic region dependent [13,27,28]. Greater effects are seen within older mice and more distal aortic segments, such as the descending and abdominal aorta [13,27,28]. The current study was designed to look at AAA rather



**Fig. 4.** (A–D) Examples of OPN in relation to macrophage staining in sections of supragenital aorta from mice receiving vehicle control and fenofibrate. OPN and macrophage staining were mainly distributed in the area of the remodeling aneurysm.

than atheroma thus the mice used were quite young and more distal aortic segments were saved for effects on AAA not atherosclerosis. The amount of arch atherosclerosis present was small and not affected by angiotensin II in line with a previous report, which suggests there is a time lag between aortic arch atherosclerosis progression and angiotensin II infusion in young mice of up to 14 weeks [27]. Experiments in older aged mice on high fat diets are likely to be more useful for studying the effects of these medica-

tions on atherosclerosis rather than AAA, as previously reported [29,30]. Use of aortic sinus, descending or abdominal aortic samples may have demonstrated effects of angiotensin II even in the younger mice used in this study however we were unable to carry out these studies.

In conclusion, in this study we have demonstrated that the PPAR ligands fenofibrate and pioglitazone reduce aortic expansion in a mice model of AAA. These findings along with other recent studies suggest the potential value of these medications for treating human AAA.

**Conflict of interest**

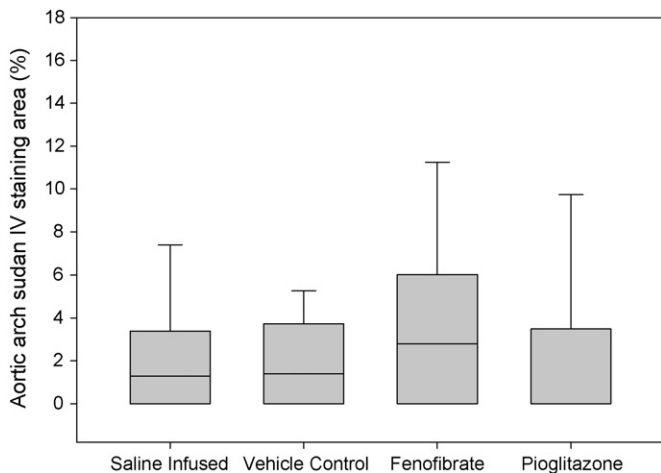
None declared.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2009.10.027.

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**Fig. 5.** Box plots showing the median and interquartile range of arch atheroma area in the mice in relation to drug treatment and angiotensin II infusion.

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