Received: 24 November 2017 | Revised: 30 January 2018 | Accepted: 15 February 2018

DOI: 10.1111/1755-5922.12322

ORIGINAL RESEARCH ARTICLE

WILEY Cardiovascular Therapeutics

Vascular dysfunction elicited by a cross talk between periaortic adipose tissue and the vascular wall is reversed by pioglitazone

Isabel Quesada[1] | Jimena Cejas[1] | Rodrigo García[2] | Beatriz Cannizzo[1] | Analía Redondo[1] | Claudia Castro[1]

[1]Vascular Biology Lab, Institute of Experimental Medicine and Biology of Cuyo (IMBECU) CONICET, School of Medical Sciences, National University of Cuyo, Mendoza, Argentina

[2]Laboratory of Cardiovascular Physiopathology, Institute of Experimental Medicine and Biology of Cuyo (IMBECU) - CONICET, Mendoza, Argentina

Correspondence
Claudia Castro, Institute of Experimental Medicine and Biology of Cuyo (IMBECU) - CONICET, Mendoza, Argentina.
Email: ccastro@fcm.uncu.edu.ar

Present address
Beatriz Cannizzo, Genzyme Argentina S.A., Boulogne, Argentina.

Funding information
This work was supported by grant 06/J431 2013-2015 from Secretary of Science and Technology, National University of Cuyo, and PIP-CONICET 2012-2014 (to C Castro). JC is a recipient of a fellowship from National University of Cuyo and RG, BC, and AR were granted by a doctoral fellowship from CONICET.

Summary

Aim: Perivascular adipose tissue (PVAT) is in intimate contact with the vessel wall and extravascular PVAT-derived inflammatory mediators may adversely influence atherosclerotic plaque formation and stability through outside-to-inside signaling. We sought to investigate the role of PVAT on the atheroma development in an experimental animal model of metabolic syndrome (MS) associated with oxidative stress and low-grade inflammatory state. We also studied the effect of pioglitazone an insulin sensitizer, on the aortic wall and its surrounding PVAT, considering a bidirectional communication between both layers.

Methods: Apolipoprotein E-deficient mice ($ApoE^{-/-}$) were fed with standard diet (CD, control diet) or fructose overload (10% w/v) (FD, fructose diet) for 8 weeks and treated with or without pioglitazone the latest 4 weeks.

Results: Biochemical variables show that glycemia and lipid peroxidation determined by thiobarbituric acid reactive species (TBARS) significantly increased in FD-fed $ApoE^{-/-}$ mice. FD significantly increased aortic PVAT expression of oxidative stress associated genes: $p22^{phox}$, Nox1, Nox2, Nox4 and $p47^{phox}$, and proinflammatory genes: Visfatin, MCP-1, and MMP-9. Pioglitazone diminished PVAT-oxidative damage elicited by fructose treatment and markedly downregulated proinflammatory markers. Even pioglitazone did not prevent the development of the aortic atheroma plaques stimulated by FD, significantly diminished VCAM-1 expression, MMP-9 expression and activity in aortic media wall and significantly reduced the accumulation of lipids and macrophages in atheroma plaques.

Conclusion: Our results support the fact that PVAT contributes to the development and progression of cardiovascular disease by underlying mechanisms elicited by "outside-in" signaling. Treatment with pioglitazone may offer a new effect on the whole vessel wall, promoting the stability of advanced atherosclerotic plaques.

KEYWORDS
adipose tissue, atherosclerosis, oxidative stress, pioglitazone

1 | INTRODUCTION

Perivascular adipose tissue (PVAT) is the adipose tissue adjacent to the artery wall, which produces various adipokines, growth factors and inhibitors with paracrine effects on the adjacent layers of the vasculature and thus, influencing vascular function and the pathogenesis of vascular disease.[1] Pathophysiological conditions such as obesity, diabetes type 2 (T2D), insulin resistance, vascular injury, aging, and infection could cause PVAT dysfunction that produces an imbalance between PVAT-derivate growth factors, leading to vascular proliferative disease, including atherosclerosis, restenosis, and hypertension.[2,3]

Evidence implicates adipokines derived from adipocytes as key regulators of the insulin resistance, exerting paracrine, and autocrine actions involved in cardiovascular disease (CVD).[4] Proinflammatory adipokines promote endothelial dysfunction through mechanisms that are linked to increase reactive oxygen species (ROS) generation by NADPH oxidase (Nox) system present in the vascular wall.[5] The "inside-out" model of the pathogenesis of atherosclerosis proposes that inflammation begins in the endothelium and radiates outwards. However, in recent years, PVAT has been extensively studied as responsible for initiating inflammation ("outside-in model").[6] Given the absence of separating lamina between PVAT and the associated vasculature, inflammatory mediators, adipokines, and PVAT-derivate ROS can actively communicate with the vessel wall and regulate vascular function. Pioglitazone is a thiazolidinedione (TZDs), a selective agonist of peroxisome proliferator-activated receptor gamma (PPAR- ɣ) used in the treatment of T2D, which works by decreasing insulin resistance in the periphery and in the liver by increasing glucose disposal.[7] An initial link between PPAR- ɣ and atherosclerosis was proposed based on the presence of these receptors in atherosclerotic plaques.[8] In vascular endothelial cells, activation of PPAR- ɣ inhibits endothelial inflammation by suppressing the expression of inflammatory genes, and therefore improves endothelial dysfunction.[9] In vascular smooth muscle cells (VSMCs), PPAR-ɣ activation inhibits proliferation and migration and promotes apoptosis.[10] In macrophages, PPAR-ɣ suppresses inflammation that regulates gene expression and cholesterol absorption and increases cholesterol efflux.[11] In vitro studies PPAR agonists reduce gene expression and secretion of proinflammatory cytokines, including tumor necrosis factor (TNF), interleukin (IL) -1 and IL-6,[12] and can reduce atherosclerosis by lowering the adhesion of monocytes to the vascular wall.[13] TZDs have a beneficial effect against ROS,[14] inflammation, and deregulation of adipokines,[15] however, its actions in aortic PVAT remains to be ascertained.

The aim of this study was to investigate in an experimental animal model of metabolic syndrome (MS) the role of PVAT on the atherogenesis and atheroprogression. We also addressed the effects of pioglitazone (PIO) on a bi-directional communication between the aortic wall and the surrounding PVAT. Our hypothesis is that a cross talk between aortic PVAT and the underlying vasculature induces a prooxidant and proinflammatory state that impact on the atheroma development, and the use of pioglitazone could contribute to reverse this proatherogenic state.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

All animals were cared for in accordance with the *Guiding Principles in the Care and Use of Animals* of the US National Institutes of Health (NIH). All procedures were approved by the Animal Research Committee of the National University of Cuyo (N° 38/2014, School of Medical Science, Mendoza, Argentina).

2.2 | Experimental protocol

Male C57/BL6J ApoE$^{-/-}$ mice, 2 months old (Jackson Laboratories, United State) were used for this study. The animals were maintained in a 22°C room with a 12-hours light/dark cycle, received drinking water ad libitum, and were fed with standard commercial chow (GEPSA, Argentina) diet. Animals were randomly divided into four groups (n = 8): (1)-control mice (Control diet, CD), with free access to tap water; (2)-fructose diet (FD) mice receiving fructose 10% (w/v) (Parafarm, Buenos Aires, Argentina) in their drinking water during 8 weeks, (3)-control mice treated with pioglitazone (PIO) (Pfizer; 20 mg/kg of body weight per day) in drinking water the last 4 weeks of protocol and (4)-ApoE$^{-/-}$ mice with FD treated with pioglitazone (FD-PIO) the last 4 weeks of protocol. This same protocol was repeated three times independently. Body weights were recorded weekly and blood was collected by cardiac puncture prior to euthanasia.

2.3 | Biochemical determinations

At the end of the experimental period, after 4 hours fasting, mice blood samples were collected from cheek puncture. The plasma glucose, cholesterol, and triglyceride concentrations were determined using commercial kits by enzymatic colorimetric methods (GT Lab, Buenos Aires, Argentina). Plasma malondialdehyde (MDA) was determined as an indicator of lipid peroxidation in terms of thiobarbituric acid reactive substrates (TBARS).

2.4 | Histomorphometric studies

Mice were euthanized by cardiac puncture under anesthesia and their aortas were perfused- in situ with chilled PBS. The extent of Oil Red O-stained atherosclerosis plaque in the aortic arch region up to the abdominal aorta was quantified using computerized morphometry. PVAT was isolated from the aorta, including arch, ascending, and descending thoracic aorta up to abdominal aorta. The clean remaining aorta was used when aortic tissue is mentioned.

2.5 | Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated with Trizol (Invitrogen) from surrounding perivascular adipose tissue (PVAT) and clean aortic tissue. 1 μg of total RNA was reversed transcribed, using random primer hexamers (Biodynamics, SRL) and M-MLV reverse transcriptase (Promega). Real-time qPCR was performed with cDNA samples and EVA Green (GenBiTech, Argentina) using a Rotor-Gene 6000 Series Software version 1.7 (Corbett). All samples were amplified in triplicate. The relative changes in the amount of transcripts in each sample were determined by normalizing with actin mRNA levels. The mRNA levels were expressed as a ratio, using delta-delta method for comparing relative expression results between treatments.

2.6 | Determination of tissue Nox-derived superoxide and vascular oxidant level

Tissue $O_2^{\cdot-}$ was calculated from the initial linear rate of SOD-inhibitable cytochrome *C* reduction quantified at 550 nm using the extinction coefficient of 21.1 (mmol L^{-1})$^{-1}$ cm^{-1} as previously described.[16] Briefly, isolated PVAT were homogenized in buffer containing 8 mmol L^{-1} potassium, sodium phosphate buffer, pH 7.0, 131 mmol L^{-1} NaCl, 340 mmol L^{-1} sucrose, 5 mmol L^{-1} MgCl2, 1 mmol L^{-1} EGTA, and protease inhibitors (Roche); and centrifuged at 10 000 rpm for 15 minutes at 4°C to remove unbroken cells, nuclei and debris. The assay of the supernatant was carried out using acetylated cytochrome *C* (0.2 mmol L^{-1}, Sigma-Aldrich), in buffer containing catalase (300U/mL) to prevent reoxidation of reduced cytochrome *C* by H_2O_2. After 5 minutes baseline measurement, NADPH (180 μmol L^{-1}) was added and $O_2^{\cdot-}$ production was measured at 550 nm using a UV-visible spectrophotometer (Mutiskan FC, Thermo Fisher). Superoxide production is expressed as nmoles $O_2^{\cdot-}$ min/mg protein using the extinction coefficient.

2.7 | Western blot analysis

Snap-frozen arterial wall or PVAT were pooled (n = 4 each group) for the preparation of whole cell extracts in ice-cold lysis buffer and electrophoretically separated on 10% acrylamide/Bis-acrylamide gels. Blots were incubated at room temperature with blocking solution (4% nonfat dry milk in PBS containing 0.1% Tween-20) for 30-40 minutes, followed by 1 hour incubation with the following primary antibodies: Anti visfatin (Santa Cruz Biotechnology 1/200), anti MMP-9 (Sigma Aldrich, St. Louis, MO, USA, 1/100); anti actin (Santa Cruz Biotechnology 1/200, sc-3035), and anti tubulin (Santa Cruz Biotechnology 1/200, sc-8035).

2.8 | MMP-9 activity by gelatin zymography

Soluble protein extracts were obtained by pulverizing 4 aortas from each treatment in liquid nitrogen, placed in lysis buffer without protease inhibitors and centrifuged at 21 000 *g* for 20 minutes at 4°C. Then, protein quantification was performed by Bradford method and samples were diluted with a buffer containing 12.5% 0.5 mol L^{-1} Tris HCl pH 6.8, 10% glycerol, 4% SDS, and 0.05% bromophenol blue, without mercaptoethanol to avoid denaturation and maintain the biological activity. Gelatin zymography was performed using an 8% SDS-polyacrylamide separating gels with 1% of gelatine (30% bis-acrylamide, 1.5 mol L^{-1} Tris HCL pH 8.8, 10% ammonium persulfate, 0.04% TEMED, 10% SDS). Electrophoresis was carried at 4°C using a constant current of 20 mA during 3 hours. Then, gels were washed twice in 2% Triton-x 100 for 15 minutes and three times with distilled water, incubated on buffer (Tris 0.25 mol L^{-1}, CaCl2 25 mmol L^{-1}, NaCl 1 mol L^{-1}, pH 7.6) for 48 hours at 37°C, stained with 0.1% Coomassie brilliant blue, 40% methanol and 10% acetic acid, for 1 hour, under gentle shaking, and distained for 20 minutes with 25% ethanol/8% acetic acid solution. Gels were photographed and the enzyme gelatinolytic activity was determined by densitometry and analyzed with Image J Software. Collagenase was used as a positive control.

2.9 | Features of plaque stability

Aortic arch samples embedded in paraffin were sectioned in microtome and stained with Masson's trichrome, photographed and the lipid core area was determined by quantifying acellular area (white area), which also contains cholesterol crystals. Macrophages were determined by immunohistochemistry of aortic arch sections, incubated with rabbit CD68-antibody (Biolegend) over-night, followed by secondary peroxidase-labeled antibody (Jackson) for 30 minutes. A computer-assisted morphometric analysis system (Image-J) was used for histopathological slides analysis.

2.10 | Statistical analysis

Results are reported as mean ± SEM Analyzes were performed by two-way ANOVA and Tukey's post hoc test or by student's *t* test using GraphPad Prism-6 software. Differences are considered significant if the *P* value is less than to .05.

3 | RESULTS

3.1 | Effect of pioglitazone on biochemical variables and lipid peroxidation

We first analyzed the effect of PIO on biochemical variables such as glucose, cholesterol, and triglycerides plasma levels of $ApoE^{-/-}$ mice treated with fructose (Fructose Diet, FD; Table 1). FD increased glucose plasma levels significantly compared with control diet group (CD) and treatment with PIO significantly decreased glycemia in FD-fed $ApoE^{-/-}$ mice. Cholesterol and Triglycerides were not modified by any treatment. To determine lipid peroxidation products, as indicative of oxidative stress, thiobarbituric acid-reacting substance (TBARS) were quantified (Table 1). Fructose administration significantly increased plasma concentration of TBARS compared

TABLE 1 Average plasma glucose, lipid levels and TBARS in ApoE$^{-/-}$ mice fed control diet or fructose diet with/without pioglitazone

	CD (n = 8)	FD (n = 8)	FD+PIO (n = 8)	PIO (n = 8)
Glucose (mmol L^{-1})	4.9 ± 0.5	9.8 ± 0.3***	5.8 ± 0.3$^{\#}$	4.1 ± 0.2$^{\#}$
Cholesterol (mg/dL)	279 ± 25	347 ± 21	351 ± 23	272 ± 17
Triglycerides (mg/dL)	159 ± 20	162 ± 17	149 ± 9	166 ± 13
TBARs (mmol L^{-1})	1.31 ± 0.15	2.43 ± 0.18**	1.32 ± 0.10$^{\#}$	1.49 ± 0.23$^{\#}$

Values are mean ± SEM.
*** P = .0007, ** P = .01 vs control diet (CD); $^{\#}$ P < .01 vs fructose diet (FD).

to control diet and this effect was significantly decreased by PIO. Increases in body weight were similar between all groups during the 8 weeks protocol (Appendix S1).

3.2 | Effect of pioglitazone on aortic PVAT

Considering the hypothesis that the formation of the atheroma plaque could be formed "outside in," that is, the release of proinflammatory adipokines together with the increase of oxidative stress in the PVAT would impact on the underlying layers of the artery wall, we decided to analyze the oxidative and inflammatory state in aortic PVAT.

3.3 | Pioglitazone reduces prooxidant state in PVAT

Given that NADPH oxidase contributes to basal vascular superoxide production, we first analyzed the oxidative state of PVAT by determining Nox-derived superoxide anion (O_2^-) by cytochrome C in homogenized PVAT and also NADPH oxidase subunits genes expression. We found that FD increased significantly the Nox-derived superoxide anion (O_2^-) in PVAT after NADPH stimulation, suggesting the involvement of oxidative stress in our metabolic syndrome (MS) model. Four weeks treatment with PIO significantly reduced O_2^- generation, in ApoE$^{-/-}$ mice PVAT fed with FD (Figure 1A). To elucidate the molecular mechanisms involved in the reduction of O_2^- in PVAT mediated by PIO, we evaluated the mRNA expression of different NADPH oxidase subunits. We showed that high fructose diet upregulated Nox1, Nox2, Nox4, p22^{phox}, and p47^{phox} in PVAT (Figure 1B). Four weeks pioglitazone treatment in fructose-fed animals could significantly diminish mRNA levels of these genes. These set of results suggest that pioglitazone is able to diminish oxidative stress in aortic PVAT and therefore could diminish the paracrine oxidative effect on the underlying aortic tissue.

3.4 | Pioglitazone reduces proinflammatory markers expression in PVAT

Inflammation in PVAT is closely linked to the development of atherosclerosis,[17] therefore, we examined the effects of pioglitazone on PVAT proinflammatory markers expression. We choose MCP-1 as an inflammatory factor, which accelerates the progress of atherosclerosis and plaque instability through the recruitment of macrophages and monocytes,[18] MMP-9 as a potential effector molecule capable of degrading stromal tissue and play an important role in atherosclerosis and plaque instability,[19] and Visfatin, a cytokine first identified as a growth factor derived from PVAT, which has proinflammatory properties associated with CVD and acute coronary syndromes.[20]

We found that high fructose diet induced a strong increase in MCP-1 expression and significantly raise the expression of MMP-9 and visfatin compared to control diet-fed mice, and pioglitazone reversed almost completely the effects produced by fructose diet (Table 2). We determined the protein expression of visfatin by western blot, in PVAT from FD ApoE$^{-/-}$ mice treated with or without PIO, and found a significantly diminished expression of FD-induced visfatin, triggered by PIO (Figure 2A). Our results suggest that the antiinflammatory effect of PIO results in a beneficial effect on the PVAT and therefore, less inflammation mediators could impact the underlying vasculature.

3.5 | Effect of pioglitazone on aortic wall

To determine whether the decrease in oxidative stress and proinflammatory adipokines expression in PVAT produced by PIO could have beneficial effects on the aortic wall, we decided to evaluate the development of the atherosclerotic plaque. First, we measured in the aortic wall by real-time qRT-PCR (Figure 2B) and western blot (Figure 2C), the expression of vascular cell adhesion molecule (VCAM)-1, an inducible protein released by the activated endothelium. We found that VCAM-1 expression increased significantly in FD ApoE$^{-/-}$ mice compared to controls. This effect was reversed by PIO treatment, suggesting that the antiinflammatory effect of PIO results in a beneficial effect on the aorta endothelial layer. We then quantified the lesion area of the atheroma plaques in the aortas of FD ApoE$^{-/-}$ mice. Eight weeks high-fructose diet triggers the development of atheroma plaque compared to control, as reported in our previous studies where the MS animal model was characterized[21,22], but nonsignificant changes were observed in the size of atherosclerotic plaques in mice treated 4 weeks with PIO (Figure 3A). As PIO was not sufficient to inhibit atheroma development in this MS model, we wonder if the generated plaques exhibited equal characteristics as those without PIO treatment. We measured plaque stability by analyzing protein expression of MMP-9 in aortas homogenates, by western blot and determined MMP-9 activity by gelatine zymography. PIO significantly inhibited FD-induced MMP-9 expression

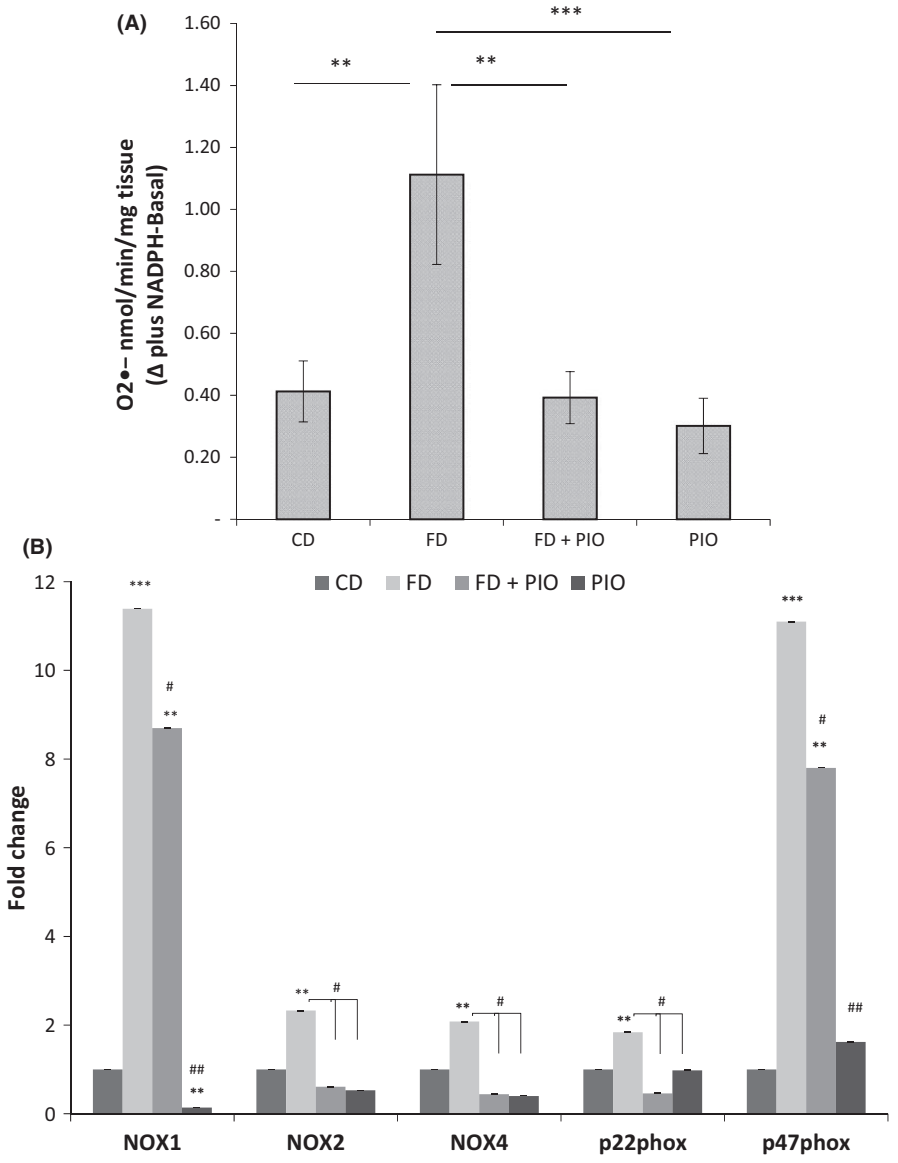

FIGURE 1 Effect of pioglitazone on superoxide (O_2^-) generation and oxidative stress-related genes on PVAT. A, Superoxide was measured by cytochrome C reduction assay in homogenates of PVAT. Production of O_2^- was calculated as a difference between basal and after adding reduced β-nicotinamide adenine dinucleotide. Results are means ± SEM, from three experiments. ** P = .0017; *** P = .0005. B, PVAT NADPH oxidase subunits mRNA expressions were determined by qRT-PCR. Gene expression level in control diet (CD) was set as 1. The corresponding ΔCt values are means ± SEM (for normally distributed data). ** P = .01; *** P = .0001 vs CD; ## P = .001; # P = .02 vs FD

TABLE 2 Effect of pioglitazone on inflammatory-related genes in PVAT

	Fold change			
Markers	CD (n = 8)	FD (n = 8)	FD+PIO (n = 8)	PIO (n = 8)
MCP-1	1.00 ± 0.18	12.24 ± 0.43***	2.45 ± 0.57*,#	0.83 ± 0.16#
MMP-9	1.00 ± 0.04	1.95 ± 0.15*	0.88 ± 0.04#	1.06 ± 0.13#
VISFATIN	1.00 ± 0.33	3.52 ± 0.43**	1.62 ± 0.04#	1.23 ± 0.28#

mRNA expression determined by qRT-PCR was analyzed in PVAT from ApoE$^{-/-}$ mice (n = 8) treated with control diet (CD), or fructose diet (FD) with or without pioglitazone (PIO). Gene expression level of the measured genes in CD was set as 1. The corresponding ΔCt values are means ± SEM. (for normally distributed data).

* P = .02; ** P = .01; *** P = .0001 vs CD; # P = .01 vs FD.

(Figure 3B). MMP-9 activity was enhanced by fructose-rich diet and this effect was inhibited by PIO (Figure 3C). To investigate the precise effect of PIO on plaque stability, the levels of lipid core and macrophages in plaques were detected. Masson's trichrome staining of aortic plaques revealed a significant decrease in lipid core area in PIO treated FD- *ApoE*$^{-/-}$ mice when compared with nontreated FD *ApoE*$^{-/-}$ mice (21.53% ± 4.03% vs 9.98% ± 2.15% P = .0003; Figure 3D). Similarly, PIO treated fructose-fed *ApoE*$^{-/-}$ mice showed less macrophage-positive area in aortic plaques when compared to that of FD *ApoE*$^{-/-}$ mice (P = .004; Figure 3E) Our results suggest that PIO could maintain the stability of atherosclerotic plaques decreasing the factors involved in the breakdown of the atheroma plaques.

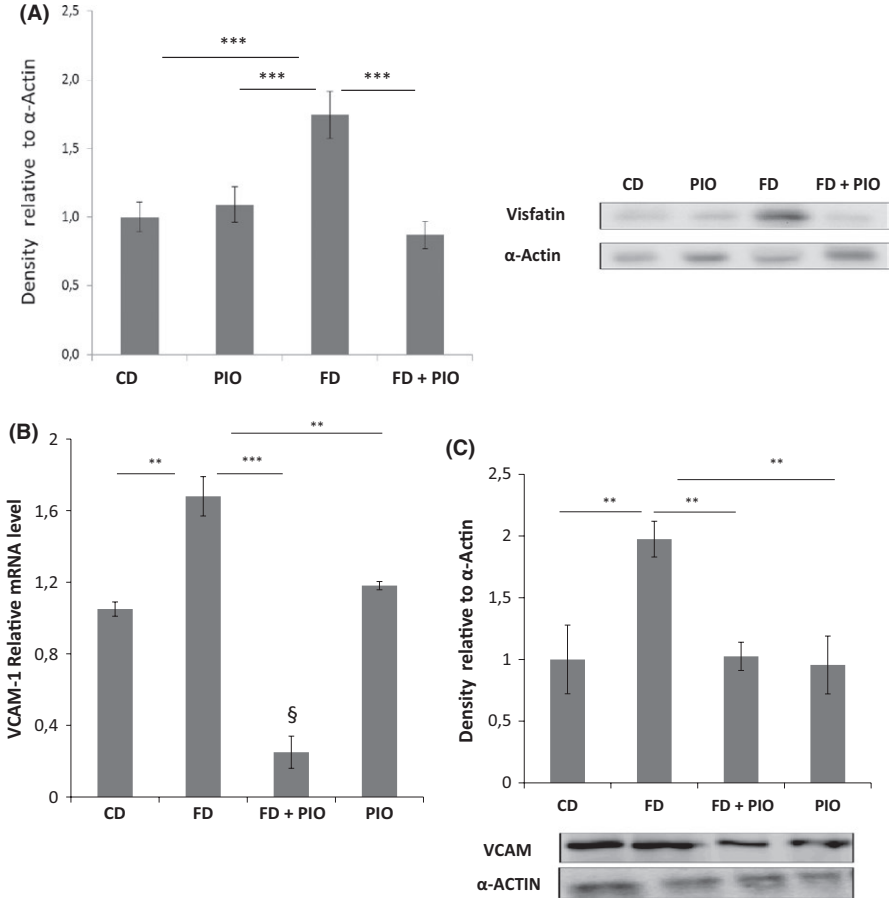

FIGURE 2 Effect of pioglitazone on visfatin and VCAM expression. A, Expression of visfatin determined by western blot in PVAT from ApoE$^{-/-}$ mice fed with control diet (CD) or fructose diet (FD) for 8 wk and treated with or without pioglitazone (PIO) the final 4 wk. Results are means ± SEM, from three independent experiments. *** $P < .0001$. B, Expression of VCAM-1 determined by qRT-PCR in aortic tissue from ApoE$^{-/-}$ mice fed a control diet (CD) or fructose diet (FD) for 8 wk and treated with or without pioglitazone (PIO) the final 4 wk. Results are means ± SEM, from three independent experiments. ** $P = .001$; *** $P < .001$; § $P = .001$ vs FD. C, Expression of VCAM-1 determined by western blot in the same groups mentioned above. α-Actin protein was used as control for relevant quantification. Results are means ± SEM, from three independent experiments (Density plot for n = 3 experiments). ** $P = .001$

4 | DISCUSSION

The cross talk between PVAT and the underlying vasculature is highly reported.[3,23-27] The lack of an anatomical separation between these layers promotes paracrine communications among them. For many years, it was considered that the onset of pathogenesis of atherosclerosis was in the inner layers of the vasculature, but more recently it has begun to consider that the adventitia could also be the initiator of the inflammatory and oxidative processes and that could impact the inner layers. According to Fuster et al the microenvironment of adipose tissue will affect the adipokine secretome, having actions on remote tissues, and adiposopathy in obese individuals is ultimately the consequence of a dysfunctional remodeling of the adipose tissue.[28] In any case, still is unknown what comes first and it seems probable that a bi-directional cross talk between PVAT and the underlying vasculature occurs. Antonopolus et al showed that this bi-directionality exists because PVAT senses oxidation products (or ROS), derived from underlying artery wall, and responds by increasing the expression of antiinflammatory adiponectin, which in turn decreases NADPH oxidase-derivate ROS, downregulating p22phox.[29] In this way, authors suggest the existence of a local protection mechanism to prevent vascular oxidation. Considering this phenomenon, we analyzed the expression of oxidative stress-related genes and proinflammatory genes in PVAT and arterial tissue obtain from a metabolic syndrome (MS) animal model, and evaluated the effects of PIO, an insulin sensitizer, on both interconnected layers of the aorta. In the treatment of MS, changes in lifestyle are the recommended initial therapy and, if this is insufficient, drugs may be indicated for individual risk factors.[30] Hyperglycemia in the MS is controlled according to the American Diabetes Association, with metformin as the first choice, and in the event that metformin does not work, PIO is being a second choice drug. Recent evidence appears to show that in patients with nonalcoholic fatty liver disease (NASH) and prediabetes or diabetes type 2 (T2D), PIO has the greatest treatment effects.[31] It targets not only liver histology, but also the underlying metabolic disturbances; in particular insulin resistance.[32] Histological improvement after PIO therapy is closely correlated with the reversal of adipose tissue insulin resistance and an increase in plasma adiponectin levels so early addition of PIO to the antidiabetic regimens should be considered after metformin therapy.[33]

In our study, PVAT from FD-fed ApoE$^{-/-}$ mice showed a misbalance in oxidative state evidenced by an increase of superoxide anion levels detected by cytochrome c assay and by the upregulation of various Nox genes subunits. In agreement with our results, Juha et al reported an upregulation of NADPH oxidase expression in PVAT of obese mice.[34] We also found that these effects triggered by high fructose diet were reversed by PIO, which decreased

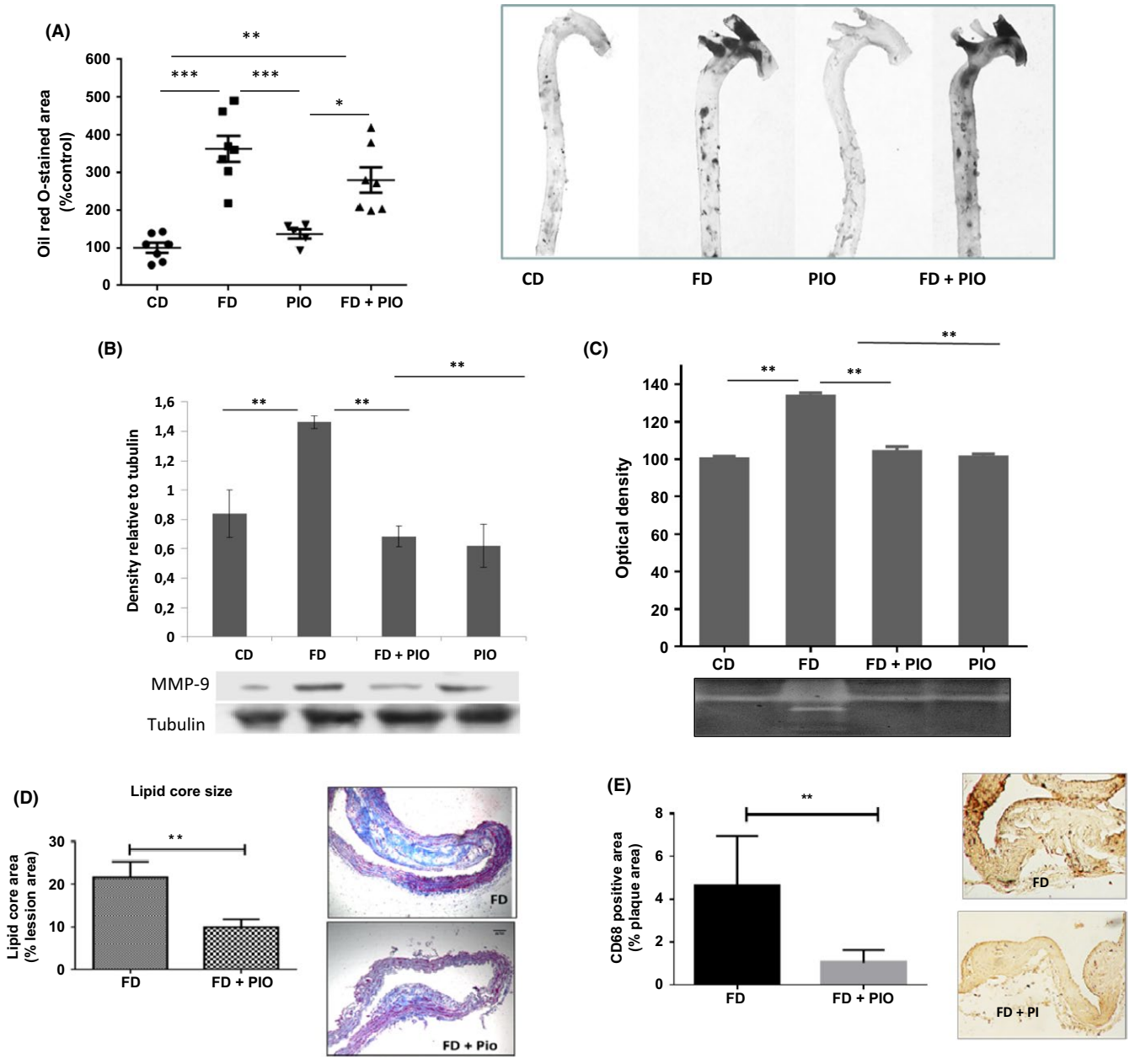

FIGURE 3 Effect of pioglitazone on atherosclerotic lesion in ApoE−/− mice. A, Atheroma development in the whole aorta (aortic arch up to abdominal aorta) was quantified by computerized morphometry. ApoE$^{-/-}$ mice were fed control diet (CD) or fructose diet (FD) during 8 wk and treated with or without PIO during last 4 wk. Arteries were stained with Oil red O and results represent the % area of lesion area relative to control group * $P < .01$, ** $P < .001$, *** $P < .0001$. B, Expression of MMP-9 determined by western blot in aorta arteries homogenates from ApoE$^{-/-}$ mice fed with control diet (CD), fructose diet (FD) during 8 wk and treated with or without pioglitazone (PIO) during last 4 wk. Tubulin protein was used as control for relevant quantification. Results are means ± SEM, from three independent protocols. ** $P < .001$. C, MMP-9 activity was measured by gelatine zymography in aorta arteries homogenates (pools from different animals; n = 4). The enzyme gelatinolytic activity was determined by densitometry and analyzed with Image J Software. Collagenase was used as a positive control. Results, expressed as % relative control, are means ± SEM ** $P < .001$. D, Lipidic core and (E) macrophage-positive areas in aortic plaques in ApoE$^{-/-}$ fructose-fed and treated with or without PIO. Results, expressed as % plaque area, are means ± SEM, ** $P < .01$

superoxide anion levels and down regulated mRNA Nox subunits. In accordance with our results, it was reported that PIO down-regulated Nox subunits p22^{phox} and p47^{phox} in rat mesangial cells cultured in a high glucose medium,[35] and reduced renal p47^{phox} expression and MDA levels, induced by chronic high fat, sucrose diet, and sodium intake.[36] The increase of oxidative stress in the PVAT would impact on the underlying layers of the artery wall and would contribute to the formation of the atheroma plaque. Brown et al discussed the accumulated knowledge regarding the roles of PVAT on the development of atherosclerosis, which indicate dual effects: proatherosclerotic and antiatherosclerotic.[2] Thermogenic properties of PVAT may reduce plasma triglyceride

levels, leading to reduced atherosclerosis, however high fructose intake in animals elicits inflammation of PVAT which is relevant to atherosclerosis.[37] These paradoxical effects nevertheless suggest that PVAT may be an attractive target for atherosclerosis interventions. The inflamed PVAT, characterized by macrophages infiltration, induces deregulation of adipokines expression and secretion, leading to insulin resistance and endothelial dysfunction. An increase of MCP-1 mRNA levels in PVAT of obese mice induces by 8 weeks high-fat diet consumption has been reported.[34] We found that PVAT from FD-fed mice was markedly inflamed, evidenced by an increase in MCP-1, MMP-9, and visfatin expression, which stimulate vascular smooth muscle cell proliferation and, in consequence, contribute to atherogenesis.[38] In our study, PIO was shown to have an antiinflammatory effect on a dysfunctional PVAT, induced by fructose diet in hypercholesterolemia mice, downregulating MCP-1, MMP-9, and visfatin mRNA levels. Although, in our experimental conditions, PIO did not affect atherosclerotic plaque development, perhaps due to the administration time of the drug; it turns out that PIO protects from endothelial dysfunction, downregulating VCAM-1 expression and promoting the stability of atherosclerotic lesions by decreasing the expression and activity of MMP9, and reducing macrophage and the lipid core in aortic plaques.

In summary, our results reaffirm that a cross talk between PVAT and vascular layers seems to be crucial in maintaining the normal function of the large arteries. Pioglitazone not only reduces traditional cardiovascular risk factors, but also modulates the functions of the different layers that compound the vascular wall.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

Conceived and designed the experiments: Claudia Castro. Performed the experiments: Isabel Quesada, Jimena Cejas, Rodrigo García, and Beatriz Cannizzo. Analyzed the data: Isabel Quesada and Jimena Cejas. Contributed reagents/materials/analysis tools: Analía Redondo. Wrote the article: Isabel Quesada and Claudia Castro.

ORCID

Claudia Castro http://orcid.org/0000-0002-8428-2484

REFERENCES

1. Chang K, Francis SA, Aikawa E, et al. Pioglitazone suppresses inflammation in vivo in murine carotid atherosclerosis: novel detection by dual-target fluorescence molecular imaging. *Arterioscler Thromb Vasc Biol*. 2010;30:1933-1939.
2. Brown NK, Zhou Z, Zhang J, et al. Perivascular adipose tissue in vascular function and disease: a review of current research and animal models. *Arterioscler Thromb Vasc Biol*. 2014;34:1621-1630.
3. Lastra G, Manrique C. Perivascular adipose tissue, inflammation and insulin resistance: link to vascular dysfunction and cardiovascular disease. *Horm Mol Biol Clin Investig*. 2015;22:19-26.
4. Kralisch S, Sommer G, Deckert CM, et al. Adipokines in diabetes and cardiovascular diseases. *Minerva Endocrinol*. 2007;32:161-171.
5. Galili O, Versari D, Sattler KJ, et al. Early experimental obesity is associated with coronary endothelial dysfunction and oxidative stress. *Am J Physiol Heart Circ Physiol*. 2007;292:H904-H911.
6. Padilla J, Vieira-Potter VJ, Jia G, Sowers JR. Role of perivascular adipose tissue on vascular reactive oxygen species in type 2 diabetes: a give-and-take relationship. *Diabetes*. 2015;64:1904-1906.
7. Forst T, Hanefeld M, Pfutzner A. Review of approved pioglitazone combinations for type 2 diabetes. *Expert Opin Pharmacother*. 2011;12:1571-1584.
8. Neve BP, Fruchart JC, Staels B. Role of the peroxisome proliferator-activated receptors (PPAR) in atherosclerosis. *Biochem Pharmacol*. 2000;60:1245-1250.
9. Dolezalova R, Haluzik MM, Bosanska L, et al. Effect of PPAR-gamma agonist treatment on markers of endothelial dysfunction in patients with type 2 diabetes mellitus. *Physiol Res*. 2007;56:741-748.
10. Redondo S, Ruiz E, Santos-Gallego CG, Padilla E, Tejerina T. Pioglitazone induces vascular smooth muscle cell apoptosis through a peroxisome proliferator-activated receptor-gamma, transforming growth factor-beta1, and a Smad2-dependent mechanism. *Diabetes*. 2005;54:811-817.
11. Nicholson AC, Hajjar DP. CD36, oxidized LDL and PPAR gamma: pathological interactions in macrophages and atherosclerosis. *Vascul Pharmacol*. 2004;41:139-146.
12. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature*. 1998;391:82-86.
13. Kintscher U, Goetze S, Wakino S, et al. Peroxisome proliferator-activated receptor and retinoid X receptor ligands inhibit monocyte chemotactic protein-1-directed migration of monocytes. *Eur J Pharmacol*. 2000;401:259-270.
14. Teissier E, Nohara A, Chinetti G, et al. Peroxisome proliferator-activated receptor alpha induces NADPH oxidase activity in macrophages, leading to the generation of LDL with PPAR-alpha activation properties. *Circ Res*. 2004;95:1174-1182.
15. Hulsmans M, Geeraert B, Arnould T, Tsatsanis C, Holvoet P. PPAR agonist-induced reduction of Mcp1 in atherosclerotic plaques of obese, insulin-resistant mice depends on adiponectin-induced Irak3 expression. *PLoS ONE*. 2013;8:e62253.
16. Molshanski-Mor S, Mizrahi A, Ugolev Y, Dahan I, Berdichevsky Y, Pick E. Cell-free assays: the reductionist approach to the study of NADPH oxidase assembly, or "all you wanted to know about cell-free assays but did not dare to ask". *Methods Mol Biol*. 2007;412:385-428.
17. Cejkova S, Kralova Lesna I, Fronek J, et al. Pro-inflammatory gene expression in adipose tissue of patients with atherosclerosis. *Physiol Res*. 2017;66:633-640.
18. Liu XL, Zhang PF, Ding SF, et al. Local gene silencing of monocyte chemoattractant protein-1 prevents vulnerable plaque disruption in apolipoprotein E-knockout mice. *PLoS ONE*. 2012;7:e33497.
19. Tan C, Liu Y, Li W, et al. Associations of matrix metalloproteinase-9 and monocyte chemoattractant protein-1 concentrations with carotid atherosclerosis, based on measurements of plaque and intima-media thickness. *Atherosclerosis*. 2014;232:199-203.
20. Liu SW, Qiao SB, Yuan JS, Liu DQ. Association of plasma visfatin levels with inflammation, atherosclerosis and acute coronary syndromes (ACS) in humans. *Clin Endocrinol (Oxf)*. 2009;71:202-207.
21. Cannizzo B, Lujan A, Estrella N, Lembo C, Cruzado M, Castro C. Insulin resistance promotes early atherosclerosis via increased proinflammatory proteins and oxidative stress in fructose-fed ApoE-KO mice. *Exp Diabetes Res*. 2012;2012:941304.

22. Cannizzo B, Quesada I, Militello R, et al. Tempol attenuates atherosclerosis associated with metabolic syndrome via decreased vascular inflammation and NADPH-2 oxidase expression. *Free Radical Res*. 2014;48:526-533.
23. Even SE, Dulak-Lis MG, Touyz RM, Cat AN. Crosstalk between adipose tissue and blood vessels in cardiometabolic syndrome: implication of steroid hormone receptors (MR/GR). *Horm Mol Biol Clin Investig*. 2014;19:89-101.
24. Abu Bakar H, Dunn WR, Daly C, Ralevic V. Sensory innervation of perivascular adipose tissue: a crucial role in artery vasodilatation and leptin release. *Cardiovasc Res*. 2017;113:962-972.
25. Gu PXA. Interplay between adipose tissue and blood vessels in obesity and vascular dysfunction. *Rev Endocr Metab Disord*. 2013;14:49-58.
26. Rajsheker SMD, Blomkalns AL, Chatterjee TK, Stoll LL, Weintraub NL. Crosstalk between perivascular adipose tissue and blood vessels. *Curr Opin Pharmacol*. 2010;10:191-196.
27. Antonopoulos AS, Sanna F, Sabharwal N, et al. Detecting human coronary inflammation by imaging perivascular fat. *Sci Transl Med*. 2017;9:eaal2658.
28. Fuster JJ, Ouchi N, Gokce N, Walsh K. Obesity-induced changes in adipose tissue microenvironment and their impact on cardiovascular disease. *Circ Res*. 2016;118:1786-1807.
29. Antonopoulos AS, Margaritis M, Coutinho P, et al. Adiponectin as a link between type 2 diabetes and vascular NADPH oxidase activity in the human arterial wall: the regulatory role of perivascular adipose tissue. *Diabetes*. 2015;64:2207-2219.
30. Cornier MA, Dabelea D, Hernandez TL, et al. The metabolic syndrome. *Endocr Rev*. 2008;29:777-822.
31. Bril F, Cusi K. Management of nonalcoholic fatty liver disease in patients with type 2 diabetes: a call to action. *Diabetes Care*. 2017;40:419-430.
32. Yau H, Rivera K, Lomonaco R, Cusi K. The future of thiazolidinedione therapy in the management of type 2 diabetes mellitus. *Curr Diab Rep*. 2013;13:329-341.
33. Gastaldelli A, Harrison SA, Belfort-Aguilar R, et al. Importance of changes in adipose tissue insulin resistance to histological response during thiazolidinedione treatment of patients with nonalcoholic steatohepatitis. *Hepatology*. 2009;50:1087-1093.
34. Ketonen J, Shi J, Martonen E, Mervaala E. Periadventitial adipose tissue promotes endothelial dysfunction via oxidative stress in diet-induced obese C57Bl/6 mice. *Circ J*. 2010;74:1479-1487.
35. Wang S, Ye SD, Sun WJ, Hu YY. Pioglitazone inhibits the expressions of p22(phox) and p47(phox) in rat mesangial cells in vitro. *ISRN Endocrinol*. 2014;2014:601352.
36. Kong X, Ma MZ, Qin L, et al. Pioglitazone enhances the blood pressure-lowering effect of losartan via synergistic attenuation of angiotensin II-induced vasoconstriction. *J Renin Angiotensin Aldosterone Syst*. 2014;15:259-270.
37. Rebolledo A, Rebolledo OR, Marra CA, et al. Early alterations in vascular contractility associated to changes in fatty acid composition and oxidative stress markers in perivascular adipose tissue. *Cardiovasc Diabetol*. 2010;9:65.
38. Spiroglou SG, Kostopoulos CG, Varakis JN, Papadaki HH. Adipokines in periaortic and epicardial adipose tissue: differential expression and relation to atherosclerosis. *J Atheroscler Thromb*. 2010;17:115-130.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Quesada I, Cejas J, García R, Cannizzo B, Redondo A, Castro C. Vascular dysfunction elicited by a cross talk between periaortic adipose tissue and the vascular wall is reversed by pioglitazone. *Cardiovasc Ther*. 2018;36:e12322. https://doi.org/10.1111/1755-5922.12322