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# PPARγ as an indicator of vascular function in an experimental model of metabolic syndrome in rabbits

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ABSTRACT

*Background and aims:* Underlying mechanisms associated with vascular dysfunction in metabolic syndrome (MetS) remain unclear and can even vary from one vascular bed to another.
*Methods:* In this study, MetS was induced by a high-fat, high-sucrose diet, and after 28 weeks, aorta and renal arteries were removed and used for isometric recording of tension in organ baths, protein expression by Western blot, and histological analysis to assess the presence of atherosclerosis.
*Results:* MetS induced a mild hypertension, pre-diabetes, central obesity and dyslipidaemia. Our results indicated that MetS did not change the contractile response in either the aorta or renal artery. Conversely, vasodilation was affected in both arteries in a different way. The aorta from MetS showed vascular dysfunction, including lower response to acetylcholine and sodium nitroprusside, while the renal artery from MetS presented a preserved relaxation to acetylcholine and an increased sensitivity to sodium nitroprusside. We did not find vascular oxidative stress in the aorta from MetS, but we found a significant decrease in PPARγ, phospho-Akt (p-Akt) and phospho-eNOS (p-eNOS) protein expression. On the other hand, we found oxidative stress in the renal artery from MetS, and PPARγ, Akt and p-Akt were overexpressed. No evidence of atherosclerosis was found in arteries from MetS.
*Conclusions:* MetS affects vascular function differently depending on the vessel. In the aorta, it decreases both the vasodilation and the expression of the PPARγ/Akt/eNOS pathway, while in the renal artery, it increases the expression of PPARγ/Akt signalling pathway without decreasing the vasodilation.

## 1. Introduction

Metabolic syndrome (MetS) is a condition involving a set of risk factors for cardiovascular disease and diabetes, such as central adiposity, decreased high-density lipoprotein cholesterol (cHDL) plasma concentration, hypertriglyceridaemia, hypertension and dysglycaemia. Diagnosis of MetS requires the presence of at least three of them [1].

Vascular disorders are central in this condition, and endothelial dysfunction, involving a decrease in nitric oxide (NO) bioavailability is classically associated with cardiovascular risk factors. Endothelial NO, synthesized from L-arginine by endothelial nitric oxide synthase (eNOS), preserves vascular homeostasis through its vasodilator effect, and prevents platelet adhesion and aggregation [2]. Moreover, NO has anti-inflammatory actions and inhibits smooth muscle cell proliferation [3]. Oxidative stress can decrease NO bioavailability and consequently lead to endothelial dysfunction. In the vascular wall, NADPH oxidase (Nox) is the main source of reactive oxygen and nitrogen species (RONS) by producing superoxide anion ($O_2^-$), which can be degraded by superoxide dismutase (SOD), or interact with NO to generate peroxynitrite (ONOO−), reducing NO bioavailability [4]. Nox activity mainly depends on Nox4 in the vascular wall [5], and Cu/Zn-SOD is the major antioxidant defence in blood vessels [6].

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Vascular function can also be affected by the peroxisome proliferator-activated receptor-γ (PPARγ) activity, a member of nuclear receptor super-family with anti-inflammatory effects [7]. It is involved in glucose and lipid metabolism, by enhancing insulin sensitivity and promoting lipogenesis. PPARγ is expressed in vascular cells and has a protective role on the vasculature [8,9]. PPARγ agonists have been demonstrated to improve endothelial function by enhancing eNOS phosphorylation and activity [10], though the specific underlying mechanisms are unclear. The regulation of eNOS activity implies the phosphorylation on Ser-1177 and Thr-495 and it is a complex process involving protein kinases, such as AMP activated protein kinase (AMPK) and Akt, also called protein kinase B [11]. Indeed, Akt increases eNOS activation through the phosphorylation on Ser-1177 [12].

We hypothesise that vascular dysfunction induced by MetS can be associated with vascular oxidative stress and/or an impairment in PPARγ signalling pathway. Therefore, we first tested whether our model of MetS altered the vascular reactivity, and second, we explored the involvement of vascular oxidative stress and PPARγ/Akt/eNOS pathway. Since the mechanisms implicated in vascular dysfunction and their adaptive responses can vary depending on the vascular bed, we carried out a comparative study using the abdominal aorta and renal artery.

## 2. Materials and methods

### *2.1Ethical approval*

The Bioethics Committee of the School of Medicine of the University of Valencia, Spain approved the present study, in accordance with the European Directive 2010/63/EU. Granja San Bernardo, Navarra, Spain provided and transported the animals under conditions specified in the Real Decreto 53/2013 (Spain). Animals were housed according to University of Valencia guidelines (12:12 h light/dark cycle at a constant room temperature of 22 °C and 60 % humidity). Rabbits were euthanised with pentobarbital 100 mg $kg^{-1}$ IV.

### *2.2. Animals and study design*

Twenty-four male New Zealand white rabbits (*Oryctolagus cuniculus*) were used in the present study, and were randomly assigned to control (n = 12) or MetS group (n = 12). Rabbits were fed during 28 weeks with 120 g of standard rabbit chow (V2333-000, Ssniff, Soest, Germany), or with a high-fat and high-sucrose diet *ad libitum* to induce MetS (10 % hydrogenated coconut oil, 5 % lard; S9052-E020, Ssniff, Soest, Germany and high-sucrose 15 % dissolved in water). Control diet contained 23.4 % protein, 11.1 % fat and 65.5 % carbohydrates (2.7 kcal g-1). High-fat diet was composed by 15.7 % protein, 43.1 % fat and 41.2 % carbohydrates (3.7 kcal g-1), and the animals consumed 0.6 kcal mL-1 in the drinking solution. The diet used in this study has previously showed its efficacy in inducing the main clinical and biochemical manifestations of MetS [13], namely central obesity, hypertension, pre-diabetes, and dyslipidaemia with low HDL and high LDL levels and hypertriglyceridaemia (see Supplementary Table S1). After the sacrifice, arteries were removed and mounted for isometric recording of tension experiments, stored at −20 °C for the protein expression measurement, or in formalin for histological analysis.

### *2.3. Organ bath experiments*

Arteries were cleaned and cut into 3 mm length rings to study the vascular reactivity by isometric recording of tension. Two L-shaped stainless steel wires were introduced through the lumen of the vessel. One of them was fixed to the wall of the organ bath and the other one was connected to a force-displacement transducer (FT03; Grass Instruments, West Warwick, RI, USA). Changes in isometric force were recorded on a Macintosh computer (Apple Corp., Cupertino, CA, USA) using Chart, version 7 and a MacLab/8e data acquisition system (ADInstruments). Each arterial segment was suspended in a 4 mL bath containing modified Krebs–Henseleit solution composed of (mM) 115 NaCl, 4.6 KCl, 1.2 $MgCl_2 \cdot 6H_2O$, 2.5 $CaCl_2$, 25 $NaHCO_3$, 11.1 glucose and disodium 0.01 EDTA and equilibrated with 95 % $O_2$ and 5 % $CO_2$ to obtain a pH about 7.35. Temperature was held at 37 °C. The optimal resting tension for abdominal aorta was 3 g and for renal artery was 1 g. After 2 h accommodation period, concentration response curves to KCl (5–100 mM), noradrenaline ($10^{-9}$ – $3x10^{-5}$) and endothelin-1 ($10^{-12}$ -$10^{-7}$ M) were performed in order to study both agonist-independent and -dependent contractile response. To examine the vasodilator response, vascular rings were precontracted with noradrenaline ($10^{-7}$ to $3x10^{-7}$ M, to obtain a contraction around 75 % of the maximal contraction to KCl 100 mM of each vascular ring) and then, the concentration response curves to acetylcholine and sodium nitroprusside were performed for the study of endothelium-dependent and -independent vasodilator response, respectively.

### *2.4. Western blot analysis*

Vascular tissues were homogenized in lysis buffer (0.125 M Tris-HCl, pH 6.8, 2 % SDS, 19 % glycerol and 1 % v/v protease inhibitors) and centrifuged at 12,000*g* for 15 min at 4 °C. Protein concentration was determined using a modified Lowry method [14]. Then, 0.5 % (v/v) 2-mercaptoethanol and 1 % bromophenol blue were added and the samples were heated for 5 min at 90 °C. Proteins (25 μg) were separated on SDS-PAGE gels and transferred to nitrocellulose membranes in a humid environment using a transfer buffer (25 mM Tris, 190 mM glycine and 20 % methanol). Primary antibodies were incubated overnight at 4 °C. The primary antibodies used were polyclonal Nox4 antibody (NB110–58849SS; dilution 1:500; Norvus Biologicals); polyclonal Cu/Zn-SOD antibody (SOD1; dilution 1:500; catalogue no. ADI-SOD-101; Enzo Life Sciences, Farmingdale, NY, USA); monoclonal Mn-SOD antibody (SOD2; dilution 1:500; catalogue no. sc-137,254; Santa Cruz Biotechnology, Santa Cruz, CA, USA); polyclonal eNOS antibody (Ab-1177; dilution 1:1000; catalogue no. 21170–2; Signalway-Antibody, CollegePark, MD, USA); polyclonal p-eNOS antibody (p-eNOS Ser-1177 sc-12972; dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal PPARγ antibody (MA5-14889; dilution 1:1000; Thermo Fisher Scientific), monoclonal Akt1 antibody (sc-5298; dilution 1:500; Santa Cruz, CA, USA), and monoclonal p-Akt1 antibody (p-Akt1 Ser-473 sc-293,125; dilution 1:500; Santa Cruz, CA, USA). Then, membranes were washed three times with wash buffer TBS + Tween 20 and incubated for 1 h at room temperature with the secondary antibodies anti-rabbit IgG, HRP-linked antibody (dilution 1:1000; catalogue no. 7074; Cell Signalling Technology) or goat anti-mouse IgG (H + L) secondary antibody, HRP (dilution 1:10,000; catalogue no. 31430; Thermo Fisher Scientific, Waltham, MA, USA). We used for the antibody detection a chemiluminescence method (Amersham Biosciences, Barcelona, Spain). Autoradiography signals were assessed using the digital image system ImageQuant LAS 4000 (GE Healthcare, Little Chalfont, UK). We used as a control for the amount of protein the primary monoclonal antibody α-tubulin (B-7) (dilution 1:1000; catalogue no. sc 5286; Santa Cruz Biotechnology).

### *2.5. Histological analysis*

Tissue samples from both aorta and renal arteries were fixed in neutral-buffered formalin and processed by the standard procedure to be embedded in paraffin, sectioned, and stained with haematoxylin-eosin and with Masson's Trichrome Stain Kit Artisan (Agilent, Santa Clara, CA, US). The presence of macrophages was assessed by immunostaining using the avidin-biotin-peroxidase method and CD68 monoclonal antibody (Dako, Glostrup, Denmark).

### 2.6. Statistical analysis

Maximal effect (Emax) was expressed in mg for KCl concentration-response curves, or as a percentage of maximal contraction to KCl (100 mM) for noradrenaline and endothelin-1 concentration-response curves. Vasodilator response was expressed as the percentage of relaxation respect to the contractile tone induced by noradrenaline.

Sensitivity is expressed as $EC_{50}$, concentration of substance causing 50 % of the maximal effect, for KCl, and as $pD_2$, –log $EC_{50}$, for noradrenaline and endothelin-1. Relaxation is expressed as a percentage of inhibition of noradrenaline-induced contraction. Values are expressed as the mean ± SEM for organ bath experiments or mean ± SD for Western blot analysis. Values were compared by an unpaired *t*-test and one- or two-way ANOVA. Bonferroni correction was applied for pairwise comparisons. $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Effects of MetS on vascular reactivity

MetS did not change the concentration-response curves to KCl (5–100 mM), noradrenaline ($10^{-9}$-3x$10^{-5}$ M) and endothelin-1 ($10^{-12}$-$10^{-7}$ M), indicating that MetS did not modify any contractile mechanism, dependent on or independent of agonist in either the aorta or renal artery (Fig. 1 and Supplementary Tables S2 and S3).

In the aorta, the presence of MetS shifted the concentration-response curve to acetylcholine to the right, diminishing $pD_2$ (from 7.51 ± 0.24 to 6.60 ± 0.29, $p$= 0.034 for control and MetS, respectively) and Emax (from 88 ± 1 % to 69 ± 9 %, $p$= 0.028 for control and MetS, respectively) (Fig. 2A). MetS also shifted the relaxation curve to sodium nitroprusside to the right without changing $pD_2$ but decreasing Emax (from 98 ± 1 % to 88 ± 3 %, $p$= 0.039 for control and MetS, respectively, Fig. 2C). On the other hand, in renal rings, MetS did not change the relaxation to acetylcholine (Fig. 2B) but increased the sensitivity to sodium nitroprusside ($pD_2$ varied from 7.34 ± 0.13 in control to 7.72 ± 0.07 in MetS, $p$=0.030), without changes in the Emax (98 ± 1 % *vs* 100 ± 1 %, $p$= 0.160 for control and MetS, respectively, Fig. 2D). (Supplementary Tables S4 and S5).

### 3.2. Effects of MetS on Nox4, Cu/Zn-SOD, Mn-SOD, PPARγ, Akt, p-Akt, eNOS and p-eNOS protein expression

MetS did not alter Nox4, Cu/Zn-SOD and Mn-SOD protein expression in the aorta, however, in renal artery, MetS increased Nox4 (from 0.42 ± 0.07 in control to 1.59 ± 0.27 in MetS, $p$= 0.005) and decreased Cu/Zn-SOD protein expression (from 1.28 ± 0.17 in control to 0.24 ± 0.16 in MetS, $p$= 0.011) without changes in Mn-SOD (Fig. 3).

Fig. 4 shows the changes induced by MetS in aorta. MetS significantly decreased PPARγ (from 1.25 ± 0.21 in control to 0.72 ± 0.12 in MetS, $p$= 0.008), p-Akt protein expression (from 0.59 ± 0.06 in control to 0.34 ± 0.08 in MetS, $p$= 0.026), and p-eNOS (from 0.55 ± 0.22 in control to 0.10 ± 0.07 in MetS, $p$= 0.003) without modifying Akt and eNOS protein expression. The ratios between p-Akt/Akt (0.82 ± 0.06 in control and 0.45 ± 0.04 in MetS, $p$=0.009) and p-eNOS/eNOS (0.86 ± 0.14 in control and 0.34 ± 0.05 in MetS, $p$=0.001) were lower in the aorta from MetS.

Fig. 5 shows the changes induced by MetS in renal artery. In this vessel, MetS increased the protein expression of PPARγ (from 0.99 ± 0.13 in control to 2.12 ± 0.28 in MetS, $p$= 0.006), Akt (from 0.74 ± 0.06 in control to 0.93 ± 0.03 in MetS, $p$= 0.011) and p-Akt (from 0.52 ± 0.13 in control to 0.91 ± 0.10 in MetS, $p$= 0.014). The ratio between p-Akt/Akt (0.68 ± 0.09 in control and 0.96 ± 0.11 in MetS, $p$=0.02) was higher in the renal artery from MetS. However, MetS did not alter the expression of eNOS, p-eNOS or the ratio between p-eNOS/eNOS in the renal artery.

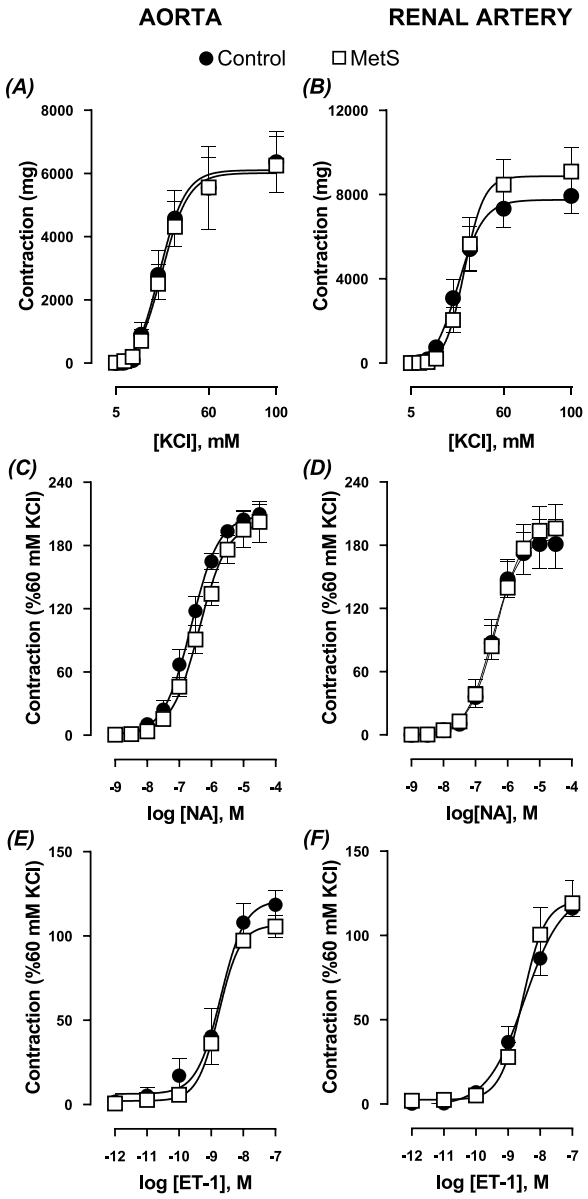


**Fig. 1.** Concentration response to contractile agonists.
Concentration–response curves to KCl (5–100 mM), noradrenaline (NA) ($10^{-9}$ to 3 x $10^{-5}$ M), and endothelin-1 (ET-1) ($10^{-12}$ to $10^{-7}$ M) (A, C, and E for aorta and B, D and F for renal artery). Data are shown as mean ± SEM.

### 3.3. Histological analysis

No evidence of atherosclerotic lesions was observed by histological procedures and there were no sign of pre-atherosclerotic changes as immunostaing of CD68 was negative in all the samples (Supplementary Fig. S1).

## 4. Discussion

Our model of MetS represents an early stage of the disease, with mild hypertension, pre-diabetes, central obesity, and alterations in the lipid profile (an increase in triglycerides and LDL, a decrease of HDL, and no changes in total cholesterol) [13], that begins damaging the aorta and triggering compensatory mechanisms in the renal artery. In addition, our findings underline that the model represents an incipient stage in the development of atherosclerosis and support that the changes in the

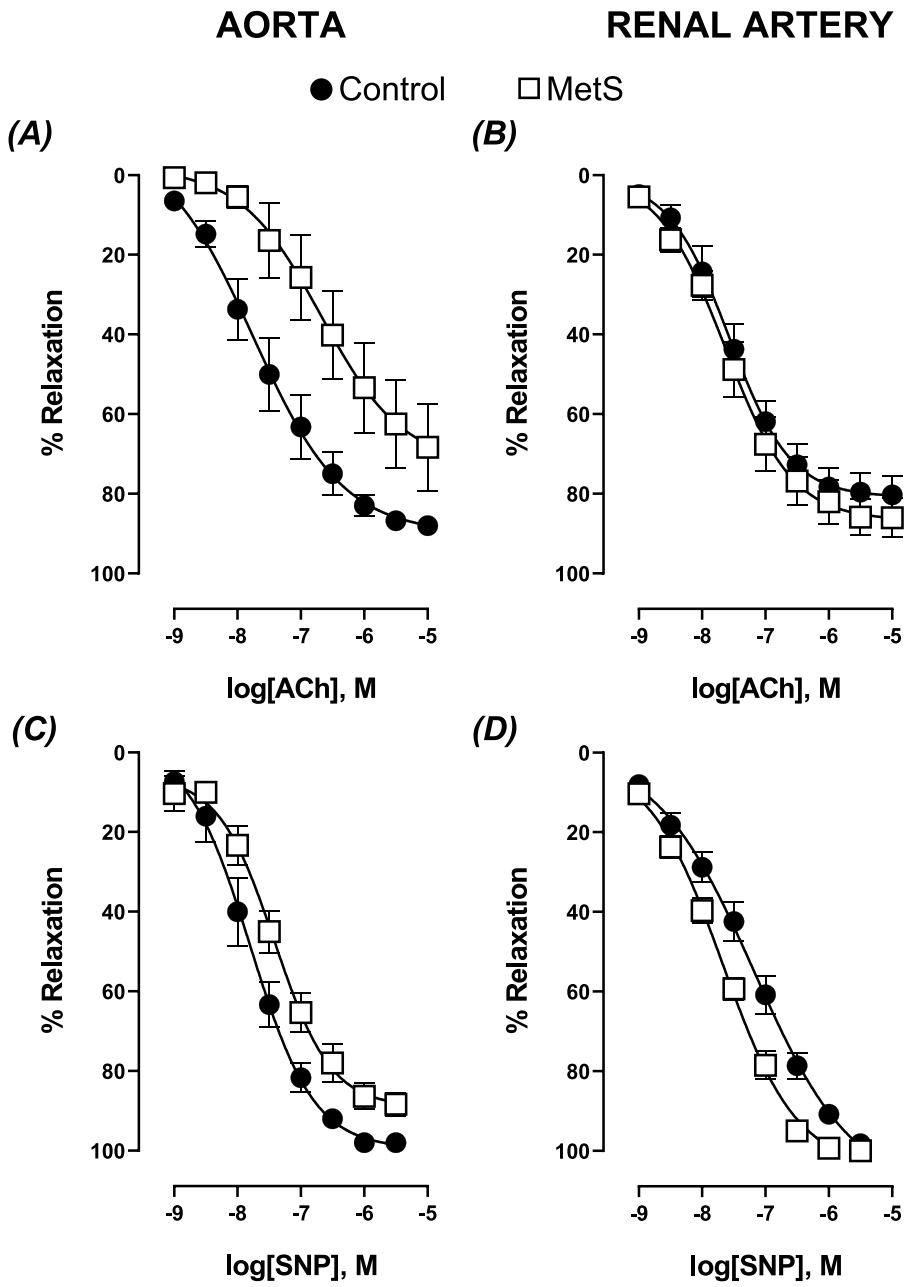


**Fig. 2.** Concentration response to vasodilator agonists.
Relaxation–response curves to acetylcholine (ACh) ($10^{-9}$ to 3 x $10^{-5}$ M) and sodium nitroprusside (SNP) ($10^{-9}$ to 3 x $10^{-6}$ M) (A and C for aorta, and B and D for renal artery). Data are shown as mean ± SEM.

vasodilator response and alterations detected at the molecular level occur before the histological changes become evident. Some diets capable of inducing vascular alterations develop early compensatory mechanisms [15]. The advantage of studying the initial stages of a disease is to be able to detect the pathways that are damaged earlier, since they constitute promising therapeutic targets to slow the progression of the disease.

Vascular alterations induced by our model were limited to relaxation. Controversy exists in the literature regarding vascular alterations induced by MetS. Whereas some authors found that a high-fat diet increased contractile response to noradrenaline, KCl, and angiotensin II in thoracic aorta from rabbits [16], others found a lesser response to noradrenaline in the thoracic aorta, and to noradrenaline, KCl and U46619 in small mesenteric arteries in mice [17]. In line with our results, a highly palatable cafeteria diet did not change the contractile response but reduced both endothelium-dependent and -independent vasodilation in rat mesenteric arteries [18]. These differences can be attributed to the vessel studied, animal species, and the experimental model.

In the aorta, our results showed that MetS induced vascular dysfunction, since it decreased both pD2 value and maximal effect for the concentration-response curve to acetylcholine compared to the control group. This vascular dysfunction was not only an endothelial dysfunction; it was partly caused by a lower vascular smooth muscle capacity to vasodilate, as the sodium nitroprusside experiments indicated. On the contrary, the renal artery from MetS developed an

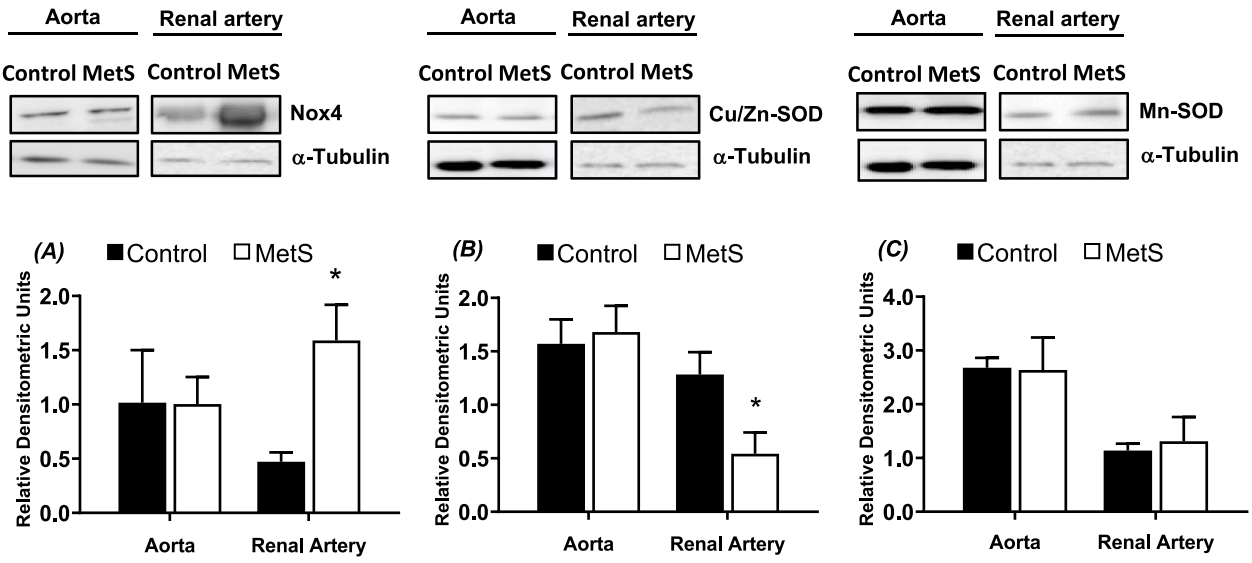


**Fig. 3.** Protein expression levels of Nox4, Cu/Zn-SOD and Mn-SOD.
Protein expression levels of (A) Nox4, (B) Cu/Zn-SOD and (C) Mn-SOD in aorta and in renal artery from control and MetS. α-tubulin was used as internal control and the signal intensity was plotted as the ratio of target protein to α tubulin. Data are mean ± SD of 4 independent experiments. *$p < 0.05$ *vs* control.

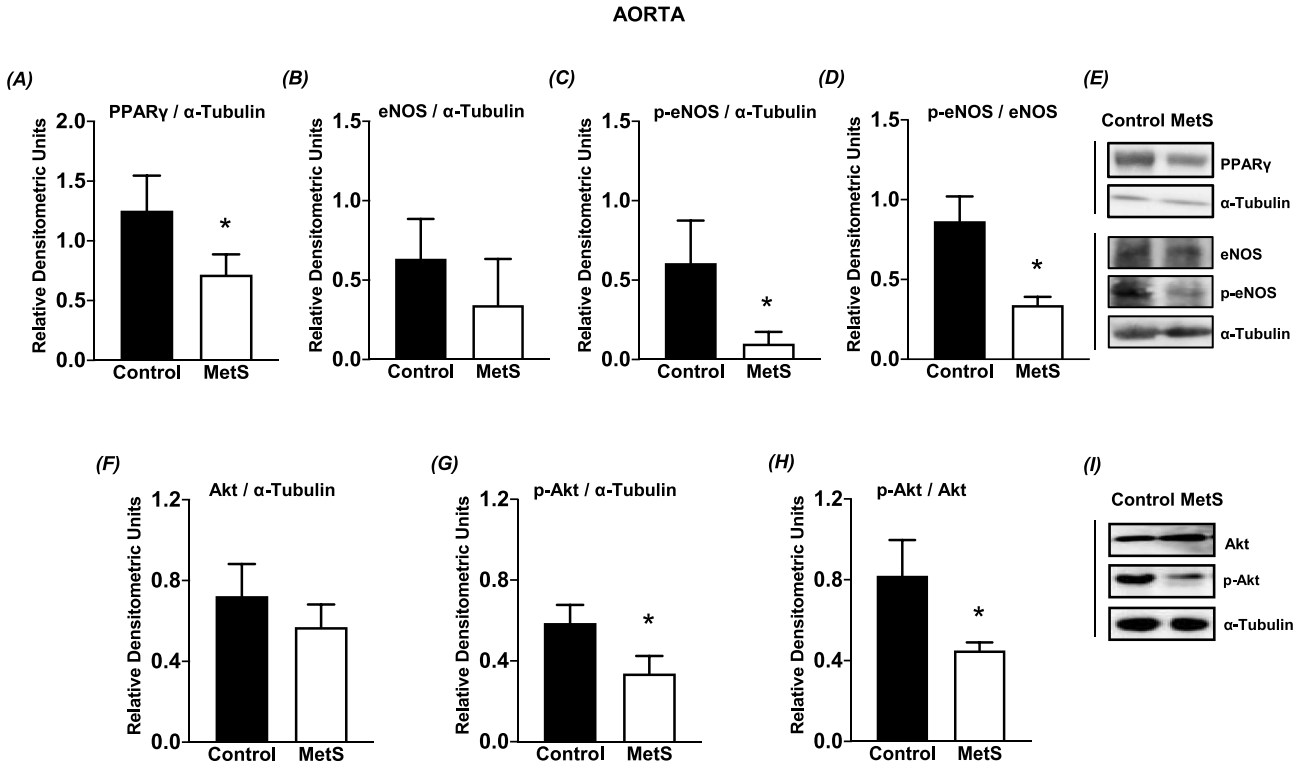


**Fig. 4.** Protein expression levels of PPARγ, eNOS, p-eNOS, Akt and p-Akt in aorta.
Protein expression levels of (A) PPARγ, (B) eNOS, (C) p-eNOS, (F) Akt and (G) p-Akt in the aorta from control and MetS, and representative western blots for each antibody (E and I). α-tubulin was used as internal control and the signal intensity was plotted as the ratio of target protein to α-tubulin. (D and H) represents the ratio of p-eNOS/eNOS and p-Akt/Akt. Data are mean ± SD of 4 independent experiments. *$p < 0.05$ *vs* control.

adaptive mechanism to preserve the vasodilation, keeping the relaxation to acetylcholine, and increasing the response to sodium nitroprusside. This result is noteworthy, because relaxation to NO donors enhances when there is an impairment of the eNOS-NO pathway. In this regard, Moncada et al. [19], found that sodium nitroprusside induced an increase in cyclic guanosine monophosphate (cGMP), which is known to induce smooth muscle relaxation through NO, and a supersensitivity to nitrovasodilators can occur when the NO pathway is impaired [19]. This important adaptive mechanism allows the maintenance of vascular tone. In line with this argument, we hypothesise that, in the renal artery from MetS, the increased sensitivity of vascular smooth muscle cells to NO would prevent this vessel from endothelial dysfunction, which seems consistent with an early stage of vascular adaptation.

MetS is related to oxidative stress [20,21], a situation due to an

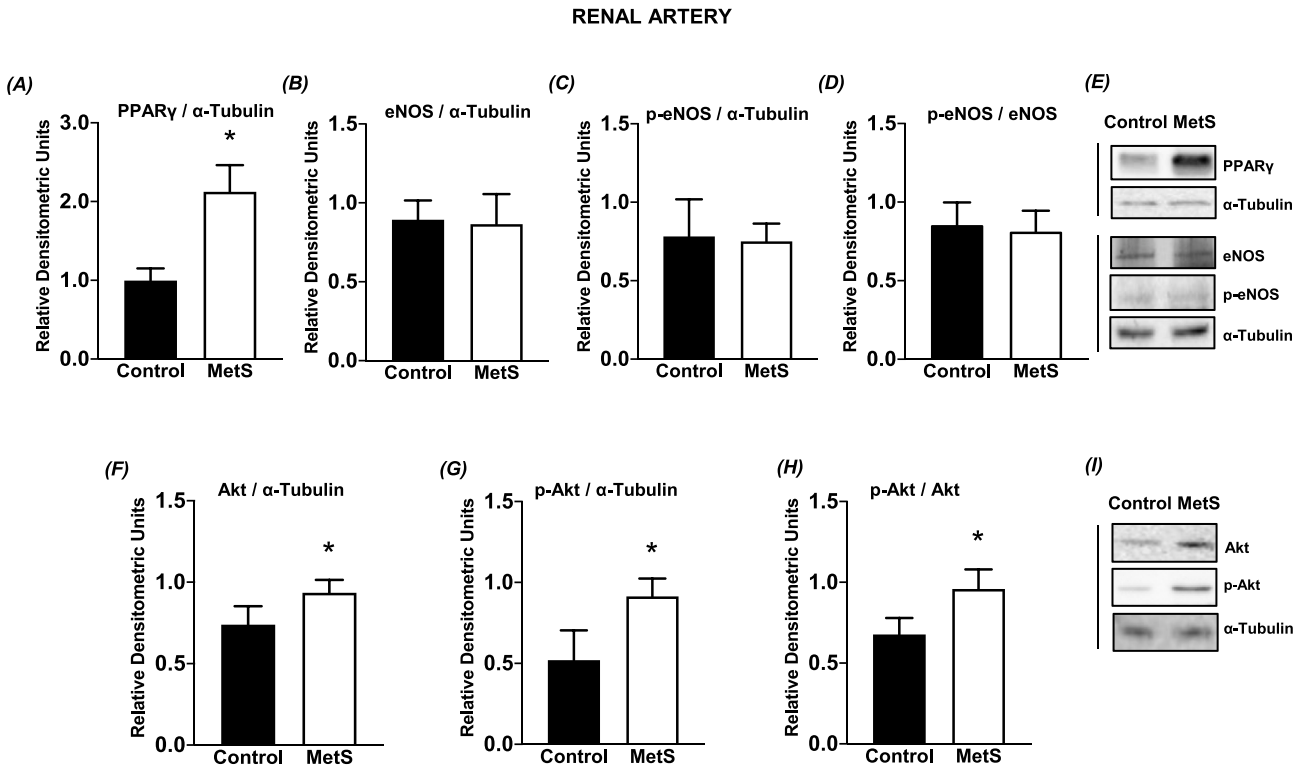


**Fig. 5.** Protein expression levels of PPARγ, eNOS, p-eNOS, Akt and p-Akt in renal artery.
Protein expression levels of (A) PPARγ, (B) eNOS, (C) p-eNOS, (F) Akt and (G) p-Akt in the renal artery from control and MetS, and representative western blots for each antibody (E and I). α-tubulin was used as internal control and the signal intensity was plotted as the ratio of target protein to α-tubulin. (D and H) represents the ratio of p-eNOS/eNOS and pAkt/Akt. Data are mean ± SD of 4 independent experiments. *$p < 0.05$ *vs* control.

imbalance in the production between pro-oxidants and antioxidants in favour of the former, which is also associated with vascular dysfunction. To evaluate if MetS induces oxidative stress in the vascular wall, we measured the protein expression of Nox4, Cu/Zn-SOD and Mn-SOD and we found vascular oxidative stress only in the renal artery from MetS. Therefore, the vascular dysfunction induced by MetS in the aorta is not related to oxidative stress. Surprisingly, in the renal artery from MetS the endothelium dependent vasodilation is the same that the one obtained for control group, and the endothelium-independent vasodilation is even enhanced. Consequently, we can say that the renal artery from MetS has a preserved vasodilation. One can expect that the oxidative stress found in the renal artery from MetS leads to a dysfunction, but we also found a PPARγ overexpression, with the beneficial vascular effects previously described by others [8–10] that, on the basis of our results, prevails over the possible negative effects of oxidative stress.

PPARγ is considered a cardiovascular protective agent and an alteration in its function is involved in many diseases such as diabetes or obesity [22]. PPARγ activation regulates AMPK/eNOS pathway, probably via Akt phosphorylation [23], and the inhibition of PPARγ significantly suppresses AMPK phosphorylation, eNOS expression and nitric oxide production. Akt is a protein kinase that contributes to phosphorylate different proteins and has important metabolic effects; consequently, its downregulation is associated with several pathologies including cancer, diabetes and neurocardiovascular diseases. Some kinases are known to be capable of activating Akt through the phosphorylation on Ser-473 [24]. Once Akt is turned into p-Akt, it can phosphorilate eNOS on Ser-1177, thus activating the enzyme and producing more NO [25]. According to our results, the aorta from MetS showed a reduction in PPARγ, p-Akt/Akt and p-eNOS/eNOS ratios. PPARγ can activate several signalling pathways implicating kinases which could phosphorylate Akt [24]. Consequently, if PPARγ was underexpressed, p-Akt could decrease without changes in Akt, as our results showed. Hence, a lower activation of eNOS through phosphorylation on Ser-1177 would lead to a lower p-eNOS/eNOS ratio and consequently a decrease in NO production. Thus, we propose that in aorta from MetS the impaired vasodilation induced by acetylcholine is partly due to a downregulation in PPARγ, which can reduce downstream the activity of eNOS.

As we discussed above, in the aorta, MetS not only significantly shifted the concentration-response curve to acetylcholine to the right, but also decreased Emax to sodium nitroprusside, indicating that there is another mechanism affecting vascular function, which is endothelium-independent. Qu et al. [26] demonstrated that some PPAR agonists act independently of endothelial NO. Halabi et al. [27] demonstrated that transgenic mice overexpressing the same dominant negative mutation in PPARγ which causes hypertension in humans, exhibited an impaired of both nitric oxide endothelium-dependent and -independent relaxation when compared to non-transgenic mice and wild-type PPARγ mice. Therefore, PPARγ, as a transcription factor, would modulate multiple signalling pathways including endothelium-dependent and -independent.

In the renal artery, where MetS increased the sensitivity of vascular smooth muscle cells to NO, mechanism that can prevent this vessel from endothelial dysfunction [19], we found an increase in the protein expression of PPARγ and p-Akt/Akt ratio. Thus, we propose that the activation of the PPARγ/Akt signalling pathway could explain the compensatory mechanism to keep vasodilation in this vessel, indicating that the positive effects derived from the activation of this pathway prevails over negative effects induced by oxidative stress. Despite overexpression of the PPARγ/Akt signalling pathway in the renal artery from MetS, it did not modify eNOS phosphorylation on Ser-1177, indicating that eNOS activation is not increased in this group. This is consistent with the unchanged vasodilation response to acetylcholine observed in the renal artery from MetS. The fact that an increase in

p-AKt in the renal artery from MetS does not result in an increase in eNOS phosphorylation is not surprising, because of the complicated regulation of eNOS activity by phosphorylation, which implies multiple protein kinases and phosphatases. Accordingly, eNOS phosphorylation on Ser-1177 activates the enzyme whereas phosphorylation on Thr-495 inactivates eNOS. For instance, Akt, cAMP-dependent protein kinase (PKA), AMP-activated protein kinase (AMPK) and the protein serine/threonine phosphatase 1 (PP1) activate eNOS by phosphorylation on Ser-1177 (the first three) or dephosphorylation on Thr-495 (the last one). Conversely, protein kinase C (PKC) or the protein serine/threonine phosphatase 2 (PP2) inhibit eNOS activity by phosphorylating Thr-495 and dephosphorylating Ser-1177, respectively [11]. Therefore, and because of the highly coordinated regulation of eNOS activity, MetS could also be affecting any of these mechanisms, and hence eNOS phosphorylation, which could explain why an overexpression in PPARγ/Akt signalling pathway in the renal artery from MetS does not cause an increase in eNOS activation. In addition, these mechanisms can vary from one vascular bed to another. More studies are needed to delve into the vascular alteration mechanism underlying MetS.

Finally, our results are in concordance to previous studies demonstrating that rabbits only develop atherosclerosis when their diet consists of 4 %–8 % fat per kilogram of weight and cholesterol is added (0.3 %–2 %). Indeed, rabbits, as herbivores, are very sensitive to cholesterol and fats, and when diet contains up to 2 % cholesterol, a rapid rise in plasma cholesterol occurs, leading to atherosclerosis [28]. In our study, the diet inducing metabolic syndrome did not contain cholesterol; therefore, we expected rabbits to develop an early state of metabolic syndrome without atherosclerosis.

It should be considered that our experimental protocol cannot discriminate between endothelial and smooth muscle cells when we determine the protein expression by Western blot from the total vascular tissue. What we can discriminate is if vasodilation is associated with endothelial NO or exogenous NO when we perform experiments in the organ bath (using acetylcholine or sodium nitroprusside, respectively) and both responses are altered in MetS. PPARγ signalling pathway could be connected with both, endothelial and smooth muscle cells function. Although in our study we focus on the PPARγ/Akt/eNOS pathway, which implies endothelial cells, we cannot rule out the non-endothelial effects of PPARγ activation. Our results show interesting correlations between an overexpression in PPARγ and an improvement in vasodilation, indicating the more PPARγ expression in the vessel, the more endothelium-dependent and endothelium-independent vasodilatation occurs. However, more research would be needed to demonstrate a direct relationship.

In conclusion, downregulation of PPARγ/Akt signalling pathway can inactivate eNOS through decreasing phosphorylation on Ser-1177, leading to endothelial dysfunction. Furthermore, this pathway could further activate endothelium-independent mechanisms to keep the vascular homeostasis. Accordingly, in the aorta from MetS, where the PPARγ/Akt/eNOS pathway is underexpressed, both endothelium-dependent and -independent vasodilation are impaired; on the other hand, the renal artery from MetS, where PPARγ/Akt signalling pathway is overexpressed, preserves endothelium-dependent vasodilation and even increases endothelium-independent vasodilation.

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## CRediT authorship contribution statement

**Sol Guerra-Ojeda:** Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Patricia Marchio:** Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Marc Gimeno-Raga:** Investigation, Visualization, Writing – review & editing. **Óscar Julián Arias-Mutis:** Validation, Investigation, Writing – review & editing. **Teresa San-Miguel:** Investigation, Visualization, Writing – review & editing. **Soraya Valles:** Investigation, Writing – review & editing. **Martin Aldasoro:** Investigation, Resources, Writing – review & editing. **José M. Vila:** Investigation, Data curation, Writing – review & editing. **Manuel Zarzoso:** Conceptualization, Methodology, Validation, Resources, Project administration, Funding acquisition, Writing – review & editing. **Maria D. Mauricio:** Conceptualization, Methodology, Data curation, Writing – original draft, Supervision, Project administration, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2021.08.006.

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