

**Interactions Between Vascular Wall and Perivascular Adipose Tissue Reveal Novel
Roles for Adiponectin in the Regulation of eNOS Function in Human Vessels**

Marios Margaritis MD^{1*}, Alexios S. Antonopoulos MD^{1*}, Janet Digby PhD¹, Regent Lee MBBS MS¹, Svetlana Reilly MD DPhil¹, Patricia Coutinho PhD¹, Cheerag Shirodaria MD MBA¹, Rana Sayeed FRCS PhD², Mario Petrou PhD², Ravi De Silva PhD², Shapour Jalilzadeh PhD¹, Michael Demosthenous MD³, Costas Bakogiannis MD³, Dimitris Tousoulis MD³, Christodoulos Stefanadis MD³, Robin P Choudhury FRCP DM¹, Barbara Casadei FRCP DPhil¹, Keith M Channon FRCP MD¹, Charalambos Antoniades MD PhD¹

¹Department of Cardiovascular Medicine, University of Oxford, United Kingdom

²Department of Cardiac Surgery, John Radcliffe Hospital, Oxford United Kingdom

³Department of Cardiology, Athens University Medical School, Athens Greece

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*Authors contributed equally to the study

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Corresponding author

Charalambos Antoniades MD PhD

Department of Cardiovascular Medicine, University of Oxford

John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

Tel: +44-1865-221870, Fax: +44-1865-740352

e-mail: antoniad@well.ox.ac.uk

Abstract

Background- Adiponectin (AdN) is an adipokine with potentially important roles in human cardiovascular disease states. We studied the role of AdN in the cross-talk between adipose tissue (AT) and vascular redox state in patients with atherosclerosis.

Methods & Results- The study included 677 patients undergoing coronary bypass surgery (CABG). Endothelial function was evaluated by flow mediated dilation of the brachial artery *in vivo* and by vasomotor studies in saphenous vein (SV) segments *ex vivo*. Vascular superoxide (O_2^-) and eNOS uncoupling were quantified in SV and internal mammary artery (IMA) segments. Local AdN gene expression and *ex vivo* release were quantified in perivascular (peri-SV and peri-IMA), subcutaneous and mesothoracic AT from 248 patients. Circulating AdN was independently associated with nitric oxide (NO) bioavailability and O_2^- production/eNOS uncoupling in both arteries and veins. These findings were supported by a similar association between functional polymorphisms in the AdN gene and vascular redox state. By contrast, local AdN gene expression/release in perivascular AT was positively correlated with O_2^- and eNOS uncoupling in the underlying vessels. In *ex vivo* experiments with human SVs and IMAs, AdN induced Akt-mediated eNOS phosphorylation and increased tetrahydrobiopterin bioavailability, improving eNOS coupling. In *ex vivo* experiments with human SVs/IMAs and AT, we demonstrated that peroxidation products produced in the vascular wall (i.e., 4-hydroxynonenal) up-regulate AdN gene expression in perivascular AT via a PPAR- γ -dependent mechanism.

Conclusions –We demonstrate for the first time that AdN improves the redox state in human vessels by restoring eNOS coupling, and identify a novel role of vascular oxidative stress in the regulation of AdN expression in human perivascular adipose tissue.

Key words: Tetrahydrobiopterin; Superoxide; eNOS coupling; Atherosclerosis; Endothelium; Adiponectin

Introduction

Circulating adiponectin (AdN), an adipokine involved in diabetes mellitus and insulin resistance, appears to be a link between obesity and atherosclerosis.^{1, 2} Reduced plasma AdN levels have been associated with increased cardiovascular risk³ and genetic variants decreasing plasma AdN levels increase the risk for diabetes mellitus⁴ and coronary heart disease,⁵ while AdN produced in perivascular adipose tissue may exert paracrine effects on the vascular wall.⁶ Although the expression of AdN gene in adipocytes is highly regulated by peroxisome proliferator-activated receptor gamma (PPAR- γ),⁷ the local mechanisms regulating AdN release and controlling its potential impact on vascular function in humans remain unclear.

Experimental studies suggest that AdN stimulates nitric oxide (NO) production in endothelial cell cultures.⁸ This is believed to be partly due to endothelial NO synthase (eNOS) activation through PI3 kinase/Akt-mediated phosphorylation.^{9 10} However, whether AdN exerts the same effects in the human vasculature remains to be established. Indeed, the biological role of AdN appears to be much more complex in human cardiovascular disease than in experimental models. In particular, the possible protective effect of high circulating AdN in healthy individuals is lost (or even reversed) in advanced cardiovascular disease states such as heart failure.¹¹

Under conditions of increased vascular oxidative stress observed in human atherosclerosis, eNOS is “uncoupled” mostly due to oxidation of its co-factor tetrahydrobiopterin (BH4), and produces superoxide radicals (O_2^-) instead of NO.¹²⁻¹⁴ In this biological setting, activation of eNOS by AdN may increase O_2^- generation from uncoupled eNOS¹⁵ if there is no parallel increase of vascular BH4. Therefore, the biological role of AdN in the regulation of eNOS functional status and activity in human atherosclerosis remains unexplored.

In this study we examine the impact of AdN and vascular NO bioavailability/redox state in patients with advanced atherosclerosis, and investigate, for the first time in humans, the relationship between local AdN synthesis in perivascular AT and O_2^- generation in human vessels. We then explore the molecular mechanisms by which AdN regulates eNOS activity and coupling in the human vascular endothelium, and describe a novel role of vascular oxidative stress in the regulation of AdN synthesis in human perivascular AT.

Methods

Population and Protocol of Study 1

The population of study 1 consisted of 677 patients (Table 1) undergoing elective coronary bypass surgery (CABG). The day before surgery, endothelium dependent flow mediated dilation (FMD) and endothelium independent dilatation (EID) of the brachial artery were determined (*see online Supplemental Methods*). Fasted blood samples were obtained on the morning of the surgery. During CABG, segments of saphenous vein (SV) and internal mammary artery (IMA) were obtained. Exclusion criteria were any inflammatory, infectious, liver or renal disease or malignancy. Patients with recent unstable coronary syndrome (within the previous 8 weeks), clinical heart failure syndrome or those receiving non-steroidal anti-inflammatory drugs, dietary supplements or antioxidant vitamins were also excluded.

In a subgroup of 248 patients, samples of perivascular AT surrounding the IMA (Peri-IMA-AT) and SV (Peri-SV-AT), subcutaneous AT (Sc-AT, from the site of the chest incision) and mesothoracic AT (Ms-AT, attached to the pericardium) were also obtained. AT samples from all sites were snap frozen and stored at -80°C for gene expression studies, or used for tissue culture experiments (Sc-AT, Peri-SV-AT and Ms-AT), as described below.

The study was approved by the Institutional Review Committee and all subjects gave written informed consent.

Blood Sampling and Adiponectin Measurements

Venous blood samples were obtained after 8h of fasting, on the morning of the surgery, and serum adiponectin was measured by enzyme linked immunosorbent assay (*See Online Supplemental Methods*).

DNA Extraction and Genotyping

Genomic DNA extraction from whole blood and genotyping were performed by standard methodology (*See Online Supplemental Methods*).

Vessel Harvesting and Vasomotor Studies

Human SV and IMA samples were obtained at the time of CABG and the vasorelaxations in response to acetylcholine (ACh) and sodium nitroprusside (SNP) were studied in an organ-bath setting as described previously (*See Online Supplemental Methods*).¹⁶

Vascular Superoxide Measurements

Vascular O₂⁻ production was measured in fresh, intact IMA and SV segments by using lucigenin (5 μmol/L)-enhanced chemiluminescence, as previously described.^{16, 17} (*See Online Supplemental Methods*)

Adipose Tissue Culture

Samples of Sc-AT, Ms-AT and Peri-SV-AT obtained from patients in study 1, were used to estimate AdN's biosynthetic rate, in an *ex vivo* bioassay (*See Online Supplemental Methods*). AT from these depots was routinely cultured for 4h, and culture supernatants were collected to estimate AdN's release

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Samples of Sc-AT, Ms-AT, peri-SV-AT and peri-IMA-AT were used for gene expression studies (*See Online Supplemental Methods*).

Measurement of Vascular Biopterins

Vascular BH₄, dihydrobiopterin (BH₂), and biopterin (B) levels were each determined separately from the same sample, using high-performance liquid chromatography followed by serial electrochemical and fluorescent detection, as we have previously described.¹² (*See Online Supplemental Methods*).

Population in Study 2 and Experimental Procedures

To examine the direct effects of AdN on the mechanisms regulating NO bioavailability and O₂⁻ production in human vessels, we used *ex vivo* models of human SVs and IMAs, as previously described.¹⁶ For these experiments we recruited 46 patients undergoing CABG (Table 1), using the same exclusion criteria as for Study 1. Serial SV/IMA segments were incubated *ex vivo* in the presence or absence of recombinant full-length AdN 10 µg/ml for 6h. The effect of AdN on vascular O₂⁻ (basal and LNAME-inhibitable O₂⁻) was quantified by lucigenin-enhanced chemiluminescence and visualized using oxidative fluorescent dye, dihydroethidium (DHE), staining (see below). The changes in Akt and eNOS phosphorylation status were determined by Western blotting; vascular biopterins were quantified by HPLC, as described above. In additional studies, SV and IMA segments were incubated with AdN 10 µg/ml in the presence and absence of wortmannin (100 nmol/L) to inhibit PI3 kinase/Akt signaling or 2,4-diamino hydroxyl pyrimidine (DAHP) to inhibit GTP-cyclohydrolase (the rate limiting enzyme in biopterins' biosynthetic pathway (*See Online Supplemental Methods*)).

To examine the impact of vascular oxidative stress on local AdN synthesis in perivascular AT, we first incubated peri-SV-AT and peri-IMA-AT with 4-hydroxynonenal (4-HNE, 30 $\mu\text{mol/L}$) for 16h, in the presence and absence of PPAR- γ inhibitor T0070907 (10 $\mu\text{mol/L}$ for Peri-SV-AT), and examined the changes in *ADIPOQ*, PPAR- γ and CD36 (which is downstream to PPAR- γ) gene expression. To examine whether 4-HNE is produced by human SVs and IMAs in the presence of oxidative stress, we quantified 4-HNE protein adducts content in 11 SVs and 18 IMAs and related these measurements to vascular O_2^- production from these vessels (*See Online Supplemental Methods*).

Oxidative Fluorescent Microtopography

In situ O_2^- production was determined in vessel cryosections by oxidative fluorescent dye dihydroethidium (DHE) staining, as previously described (*See Online Supplemental Methods*).

Western Blots

Western blots in human vessels for Akt/p-Akt (Ser473), eNOS/p-eNOS (Ser1177) and 4-HNE adducts were performed as described in the *Online Methods Supplement*.

Statistical Analysis

Continuous variables were tested for Normal distribution using the Kolmogorov-Smirnov test. Non-normally distributed variables were log-transformed for analysis.

Sample size calculations were based on previous data from our laboratory. For the clinical studies we estimated that a total number of 500 subjects would allow us to detect a 2.38 absolute difference in FMD between the highest and the lowest tertile of circulating AdN with $\alpha=0.05$, power 0.9 and assumed SD 2.3. For the adipose tissue experiments, we estimated that with 150 patients we would be able to detect a 0.13 difference in $\log(\text{O}_2^-)$ in SV samples between the extreme tertiles of AdN released from peri-SV-AT with $\alpha=0.05$,

power 0.9 and assumed SD 0.2. For the ex-vivo experiments, sample size calculations were performed based on our previous experience on this model,¹⁶ and we estimated that with a minimum of 5 pairs of samples (serial rings from the same vessel) we would be able to identify a change of $\log(\text{O}_2^-)$ by 0.48 with $\alpha=0.05$, power 0.9 and SD for a difference in the response of the pairs of 0.25.

In the clinical studies, continuous variables between 3 groups were compared by using one-way ANOVA followed by Bonferoni post-hoc test for individual comparisons, while comparisons between 2 groups were performed by unpaired t-tests. Categorical variables were compared by using chi-square test, as appropriate. Correlations between continuous variables were assessed by using bivariate analysis, and Pearson's coefficient was estimated. For the organ bath experiments, the effect of "circulating AdN tertile" on vasorelaxations in response to ACh was evaluated by using two-way ANOVA for repeated measures (examining the effect of "ACh or SNP concentration" x "circulating AdN tertile or genotype" interaction on "vasorelaxations"), in a full factorial model. For the *ex vivo* experiments (where serial rings from the same vessel were incubated with multiple interventions, we performed repeated measures ANOVA and paired t-tests for individual comparisons, followed by Bonferoni post-hoc correction for multiple testing as appropriate.

In the clinical studies, correlations between continuous variables were tested by calculating the Pearson's correlation coefficient. Linear regression was performed by using FMD or $\log(\text{vascular O}_2^-)$ as dependent variables, and as independent variables, $\log(\text{circulating AdN})$ and those of the clinical demographic characteristics (age, gender, diabetes, smoking, dyslipidemia, hypertension) that showed a simple association with the dependent variable at the level of 15%. A backward elimination procedure was then used by having $p=0.1$ as threshold to remove a variable from the respective model. All statistical tests were performed by using SPSS v20.0 and $P<0.05$ was considered statistically significant.

Results

AdN and NO-Mediated Vasorelaxations in Human Vessels

We first examined the association between circulating AdN and endothelial function (as evaluated by FMD and vasorelaxations of SV rings *ex vivo*). Circulating AdN was positively correlated with FMD (Figure 1). In multivariable analysis, the independent predictors of FMD were log(circulating AdN) (β (SE): 1.75(0.69), $P=0.012$), hypertension (β (SE): -2.16(0.47), $P=0.0001$) and smoking (β (SE):-1.51(0.31), $P=0.0001$). This finding was confirmed in organ bath studies, where the vasorelaxations of SV segments in response to ACh were significantly greater in vessels from patients in the highest tertile of circulating AdN compared to patients in the lowest tertile. There were no associations between circulating AdN and either EID of the brachial artery *in vivo* or vasorelaxations of SVs in response to SNP *ex vivo* (Figure 1).

Circulating vs Local AdN in Peri-Vascular Adipose Tissue And Vascular Superoxide

We next examined whether circulating AdN was related to vascular O_2^- generation in human arteries (IMA) and veins (SV). We observed that circulating AdN was closely related with basal O_2^- in both vessel types (Figure 2). In multivariable analysis, the independent predictors of log(O_2^-) in SV were log(circulating AdN) (β (SE):-0.2984(0.06), $P=0.0001$), smoking (β (SE):0.068 (0.024), $P=0.005$) and treatment with statins (β (SE):-0.219(0.044), $P=0.0001$). Similarly, the independent predictors of log(O_2^-) in IMAs were log(circulating AdN) (β (SE):-0.265(0.077), $P=0.001$), statin treatment (β (SE):-0.199(0.061), $P=0.001$), diabetes (β (SE):0.112(0.047), $P=0.019$) and smoking (β (SE):0.053(0.032), $P=0.097$).

In order to test for possible paracrine effects of local AdN secreted by perivascular AT, we quantified both the expression of *ADIPOQ* gene and local AdN protein secretion by peri-SV-

AT after 4h of culture *ex vivo*, and tested for their associations with vascular redox state. In contrast to what was observed with circulating AdN, increased vascular O_2^- in human SVs and IMAs was related to increased expression of *ADIPOQ* gene and local AdN secretion from the perivascular AT (Figure 2). These discordant relationships between vascular O_2^- production and circulating vs local AdN production in perivascular AT (confirmed in both human arteries and veins) suggest that local production of AdN in perivascular AT and circulating AdN are differentially regulated and imply that vascular O_2^- has the potential to stimulate local AdN production in the neighbouring perivascular AT.

Circulating vs Local AdN in Peri-Vascular Adipose Tissue And eNOS coupling

To assess whether there are interactions between circulating/local AdN and eNOS coupling, we examined the association between both circulating AdN and its local expression (in peri-SV-AT and peri-IMA-AT) and LNAME-inhibitable vascular O_2^- (which provides an estimate of eNOS uncoupling). We observed that low circulating AdN levels were related to greater LNAME-inhibitable O_2^- in both SV and IMA segments, which is indicative of more eNOS uncoupling (Figure 3). In multivariable analysis we observed that the independent predictors of LNAME-delta(O_2^-) in IMAs were log(circulating AdN) (β (SE): 4.37(0.73), $P=0.0001$) and treatment with statins (β (SE):2.73(0.57), $P=0.0001$) whereas the predictors of LNAME-delta(O_2^-) in SVs, were also log(circulating AdN) (β (SE): 2.12(0.54), $P=0.0001$) and statin treatment (β (SE): 2.25(0.39), $P=0.0001$). These findings suggest that increased circulating AdN and regular statin treatment are independently associated with improved eNOS coupling in these human arteries and veins. By contrast, we observed that higher LNAME-inhibitable O_2^- in either SVs or IMAs (indicative of more eNOS uncoupling) was related with higher *ADIPOQ* gene expression in the perivascular AT (Peri-SV-AT and Peri-IMA-AT) of these vessels, a finding compatible with the increased local AdN synthesis observed in peri-SV-AT from these patients (Figure 3).

Linking Circulating And Local AdN production in Adipose Tissue: The Role Of PPAR- γ

To further explore the possible contribution of the different AT depots to the circulating AdN pool, we examined the association between circulating AdN and its local release/*ADIPOQ* gene expression in Ms-AT, Sc-AT, Peri-SV-AT and Peri-IMA-AT. We observed a significant, albeit weak, correlation between local AdN release and *ADIPOQ* gene expression in Ms-AT and Ac-AT, suggesting that the circulating pool may be partly driven by AdN produced by these “remote” (non-perivascular) depots (Figure 4). By contrast, circulating AdN was not related with either local AdN release/*ADIPOQ* gene expression in Peri-SV-AT (Figure 4), or *ADIPOQ* gene expression in Peri-IMA-AT ($r=0.072$ $P=0.602$), implying that different, probably local, mechanisms control the release of AdN in perivascular AT.

To further explore the subcellular mechanisms controlling *ADIPOQ* gene expression in the various AT depots, we quantified the expression of PPAR- γ , which is known to regulate *ADIPOQ* gene expression in cell culture models.¹⁸ We observed a strong correlation between PPAR- γ and *ADIPOQ* gene expression in peri-SV-AT ($r=0.576$, $P<0.0001$), Peri-IMA-AT ($r=0.751$, $P<0.0001$), Ms-AT ($r=0.514$, $P<0.0001$) and Sc-AT ($r=0.344$, $P<0.0001$). To confirm that PPAR- γ gene expression provides a good estimate of PPAR- γ activity, we then quantified the expression of its downstream molecule CD36 (known to be highly regulated by PPAR- γ activity¹⁹) and confirmed co-linearity between the expression of PPAR- γ and CD36 genes in Peri-SV-AT ($r=0.976$, $P<0.0001$), Peri-IMA-AT ($r=0.956$, $P<0.0001$), Ms-AT ($r=0.933$, $P<0.0001$) and Sc-AT ($r=0.953$, $P<0.0001$). These results confirm that *ADIPOQ* gene expression remains under the direct control of PPAR- γ in all types of human AT studied.

Effects of AdN on Endothelial Function And Vascular Redox State by Using The Genetic Variability of ADIPOQ

To explore the discordant associations between vascular O_2^- and circulating levels vs local (perivascular) AdN biosynthesis, we searched for genetic SNPs with a known impact on AdN circulating levels. The genetically determined variability of AdN levels could then be used as a model system to test indirectly the effect of AdN on vascular NO bioavailability and vascular O_2^- generation in human vessels. We genotyped the entire population in Study 1 for two common genetic polymorphisms: rs17366568 in *ADIPOQ* and rs266717 in *ADIPOQ* gene promoter both known to affect circulating AdN levels in genome wide association studies.^{20, 21} These two polymorphisms were not in linkage disequilibrium (data not shown), so their effect on *ADIPOQ* gene expression could be independent from each other and therefore additive. Indeed, we observed that the number of rs266717T and rs17366568G alleles had an additive effect on local AdN biosynthesis in Ms-AT that was also reflected in circulating AdN levels (Figure 5). However, there was no effect of these SNPs on local AdN synthesis/ *ADIPOQ* expression in perivascular AT (peri-SV-AT or peri-IMA-AT, Figure 5). These findings suggest that local factors may overwhelm the influence of genetic variability that is observed in the non-perivascular AT tested.

The number of rs266717T + rs17366568G alleles was also positively associated with FMD, the *ex vivo* vasorelaxations in response to ACh and total/LNAME-inhibitable O_2^- in SV and IMA segments (indicative of eNOS uncoupling when the genetic background leads to lower circulating AdN). These findings document for the first time that genetically-determined hypo-adiponectinemia may actually lead to endothelial dysfunction and eNOS uncoupling in human vessels.

Direct Effects of AdN on Redox State And eNOS Coupling by Using Ex Vivo Models of Human Vessels

To further explore the discordant associations between vascular endothelial function/redox state and circulating/local AdN production in perivascular AT (in Study 1) we performed a number of mechanistic experiments in human IMAs and SVs *ex vivo* (Study 2). We firstly incubated segments of these vessels with/without AdN 10 µg/ml for 6h and examined its effect on basal and LNAME-inhibitable O_2^- . AdN induced a striking reduction of basal O_2^- by restoring eNOS coupling in both human SVs and IMAs (Figure 6). This effect was also confirmed by DHE staining of these vessels, where AdN rapidly reversed LNAME-inhibitable O_2^- in the endothelium (Figure 6).

To further explore the mechanisms by which AdN affects eNOS physiology in the human endothelium, we explored its impact on eNOS phosphorylation in these vessels. *Ex vivo* incubation of human SVs and IMAs with AdN increased Akt phosphorylation at Ser473 and eNOS phosphorylation at Ser1177 (Figure 7). The effect of AdN on eNOS phosphorylation was blocked by wortmannin, an inhibitor of PI3-mediated Akt phosphorylation, suggesting that AdN induces a PI3-Akt-mediated phosphorylation of eNOS, resulting in its activation. The ability of AdN to induce eNOS phosphorylation remained unchanged in the presence of DAHP (an inhibitor of GTP-cyclohydrolase, blocking the biopterins' biosynthetic pathway), suggesting that a reduction in vascular BH4 levels does not modify the effect of AdN on eNOS phosphorylation.

However, activation of eNOS by Ser1177 phosphorylation does not necessarily increase NO synthesis, since phosphorylated eNOS in the presence of BH4 deficiency may even lead to increased O_2^- generation by the uncoupled enzyme.¹⁵ Therefore, we examined whether circulating AdN was related to vascular BH4 content. In a subgroup of 176 patients from

Study 1, circulating AdN was positively correlated with BH4 and the BH4:total biopterins ratio in both SV and IMA segments (Figure 7). Previous reports suggest that BH4 administration increases circulating AdN in an animal model,²² but it is unclear whether circulating AdN has any direct effect on vascular BH4 bioavailability in humans. We observed a significant but weak association between circulating AdN and vascular biopterins in Study 1 (Figure 7). To further explore this association, we incubated human SVs and IMAs with AdN (10µg/ml) for 6h. AdN increased vascular BH4 and BH4/total biopterins ratio (Figure 7), whereas inhibition of GTP-cyclohydrolase with DAHP resulted in the expected reduction of vascular BH4 levels, even in the presence of AdN. Taken together, these findings suggest that AdN improves NO and reduces O₂⁻ bioavailability in human vessels through a combined effect on eNOS activation (via PI3/Akt phosphorylation) and coupling (by increasing BH4 bioavailability).

Effects Of Vascular Oxidative Stress On Adiponectin Expression In Perivascular Adipose Tissue

To explore the positive association observed between O₂⁻ production in human SVs/IMAs and AdN release/*ADIPOQ* expression in the respective perivascular AT, we then examined whether products of peroxidation released from the vascular wall may regulate *ADIPOQ* expression in perivascular AT. Recent data suggested that 4-HNE (a product of lipid peroxidation) may up-regulate *ADIPOQ* gene expression in both primary human adipocytes¹⁸ and skeletal muscle cells;²³ therefore we hypothesised that 4-HNE may be involved in the cross-talk between vascular wall and perivascular AT in humans. To examine whether increased vascular O₂⁻ leads to increased 4-HNE production in human vessels, we first quantified the levels of 4-HNE protein adducts in SV (n=11) and IMA (n=18) segments from our cohort in Study 1 and demonstrated a good correlation between vascular O₂⁻ and 4-HNE protein adducts in both SVs (r=0.720, P=0.042) and IMAs (r=0.489, P=0.039) (representative

examples are shown in Figure 8), suggesting that there is increased production of 4-HNE in human arteries and veins in the presence of increased vascular oxidative stress. We then incubated peri-SV-AT (n=6) and peri-IMA-AT (n=6) with 4-HNE 30 μ M for 16h, and observed that *ADIPOQ* gene expression was up-regulated by ~2 fold in both peri-SV-AT (Figure 8) and peri-IMA-AT (by 1.9 ± 0.3 fold $p<0.05$ vs control). This was accompanied by a parallel up-regulation of PPAR- γ in peri-SV-AT (Figure 8) and peri-IMA-AT (by 1.7 ± 0.4 fold $P<0.05$ vs control), whereas in the presence of T0070908 at 10 μ M (an inhibitor of PPAR- γ activity) the effect of 4-HNE on *ADIPOQ* gene expression in peri-SV-AT was abolished (Figure 8). As “positive control” in these experiments we used CD36 (a downstream molecule whose expression is under the direct control of PPAR- γ) that showed similar responses to those of *ADIPOQ* in both peri-SV-AT (Figure 8) and peri-IMA-AT (up-regulated by 1.8 ± 0.4 fold after incubation with 4-HNE). These experiments suggest that 4-HNE produced in human SVs and IMAs in the presence of increased vascular oxidative stress may up-regulate the expression of *ADIPOQ* gene in perivascular AT, through a PPAR- γ -dependent mechanism.

Discussion

In the present study we examine the role of both circulating and locally produced AdN in the regulation of vascular redox state in patients with atherosclerosis. We demonstrate for the first time in humans that in addition to its association with NO bioavailability, circulating AdN is also inversely related with vascular O_2^- (derived from uncoupled eNOS) in human arteries and veins, independent of atherosclerosis risk factors. These findings are also confirmed by linking the genetic variability of *ADIPOQ* with vascular redox state and NO bioavailability, and by using *ex vivo* experiments with human arteries and veins. We further

demonstrate that the effect of AdN on eNOS coupling is mediated by its combined impact on PI3/Akt-mediated phosphorylation of eNOS and vascular BH4 bioavailability. By contrast, increased vascular O_2^- is associated with increased local AdN release/*ADIPOQ* gene expression in perivascular AT, implying that local mechanisms related to the vascular redox state may control AdN synthesis in perivascular AT in patients with atherosclerosis. In additional *ex vivo* experiments in human vessels and perivascular AT, we demonstrated that vascular oxidative stress induces the release of products of lipid peroxidation (i.e. 4-HNE) that up-regulate *ADIPOQ* gene in the perivascular AT via a PPAR- γ -dependent mechanism. This cycle of cross-talk between the vessel and perivascular AT may represent a novel defence mechanism of the human vascular wall against oxidative stress (Figure 8).

AdN and Endothelial Dysfunction

AdN is an adipokine with antiatherogenic properties in experimental models, but has a controversial role in the clinical setting.¹ Although circulating AdN is reduced in obesity²⁴ and is related with reduced cardiovascular risk in the general population,³ increased circulating AdN is inversely correlated with mortality and the overall clinical outcome in advanced cardiovascular disease states such as heart failure.¹¹ Moreover, pharmacological treatments that improve endothelial function and reduce cardiovascular risk (such as lipophilic statins) appear to reduce circulating AdN in patients with hypercholesterolaemia.²⁵ ²⁶ This discordance between experimental data and clinical observations has introduced the concept that AdN may behave as a “rescue hormone” in advanced disease states,¹ although the mechanisms behind this hypothesis have not been explored in the clinical setting.

Experimental evidence suggests that AdN stimulates eNOS-derived NO in endothelial cells through stimulation of PI3/Akt-mediated eNOS phosphorylation.^{9, 27} In keeping with these first observations from cell culture models, AdN knock-out mice exhibit reduced eNOS

phosphorylation status (at Ser1177) and impaired endothelial function.¹⁰ However, the relevance of these observations to humans is totally unknown. In clinical studies, circulating AdN is directly related with endothelial function in the general population,²⁸ but this association is reversed in patients with type 2 diabetes mellitus and diabetic nephropathy,²⁹ introducing further controversy into the role of AdN in different clinical disease states.

In the present study we demonstrate that circulating AdN is an independent predictor of endothelial function in a well phenotyped cohort of patients with coronary artery disease without renal or heart failure. In a first attempt to prove a causal link between AdN and endothelial function, we examined whether genetic polymorphisms known from genome wide association studies to regulate circulating AdN levels (rs17366568 in *ADIPOQ* and rs266717 in *ADIPOQ* promoter), had an impact on endothelial function. We observed that genetically-determined lower circulating AdN levels were related with worse endothelial function. To further investigate the molecular mechanisms underlying this observation, we exposed human SVs and IMAs to AdN *ex vivo* and observed a rapid PI3/Akt-mediated increase in eNOS phosphorylation at Ser1177, which is known to activate the enzyme.¹⁵ Although eNOS is the source of NO in the vascular endothelium, deficiency of its co-factor BH4 leads to its uncoupling, turning it into a source of O₂⁻ instead of NO.³⁰ We demonstrate for the first time that AdN increases vascular BH4, a critical co-factor necessary for eNOS enzymatic coupling in human vessels.¹² Although the biological significance of circulating AdN in the regulation of vascular BH4 bioavailability in humans *in vivo* needs further validation, our finding is compatible with the strong association we observe between circulating AdN and eNOS coupling in human arteries and veins. Therefore it is likely that even this rather “weak” effect of AdN on vascular biopterins may have a significant impact on eNOS coupling *in vivo*. The combined phosphorylation of eNOS and increase of its

enzymatic coupling also explains the impact of circulating AdN on vascular NO bioavailability in humans.

AdN and Vascular Superoxide Generation

Evidence from cell culture models suggests that activation of eNOS via Ser1177 phosphorylation, leads to either increased O_2^- or NO, depending on the underlying coupling status of the enzyme.¹⁵ Given that vascular BH4 is oxidised by free radicals and the balance between its synthesis and oxidation defines its net bioavailability,¹² any change in vascular redox state could drive eNOS uncoupling and trigger a pro-atherogenic vicious cycle.

We demonstrate for the first time in humans that circulating AdN is an independent predictor of vascular O_2^- and eNOS coupling in arteries and veins from patients with coronary atherosclerosis. By using the genetic variability of *ADIPOQ* and *ex vivo* models of human arteries and veins, we also demonstrate that AdN reduces vascular O_2^- by its combined impact on BH4-mediated eNOS coupling and PI3/Akt-mediated activation of the enzyme.

Effects of Vascular Oxidative Stress On AdN Expression In Perivascular Adipose Tissue

Evidence suggests that ischaemia/reperfusion injury leads to increased AdN expression in brain vessels,³¹ a vascular bed where uncoupled eNOS is a key contributor to vascular O_2^- generation.³² Moreover, it has been recently shown that 4-HNE, a product of peroxidation released from tissues with high oxidative burden, up-regulates *ADIPOQ* in primary human and 3T3 adipocytes,¹⁸ as well as in skeletal muscle.²³ These findings support the notion that AdN may behave as a “rescue hormone”, being up-regulated in conditions of increased oxidative stress.

In our study, vascular O_2^- production (in both arteries and veins) was paradoxically positively correlated with AdN release and *ADIPOQ* gene expression in perivascular AT. We

have shown that peroxidation products (i.e. 4-HNE, produced in the vascular wall in the presence of increased vascular oxidative stress), up-regulate *ADIPOQ* gene in perivascular AT in a PPAR- γ -dependent mechanism. These findings introduce the concept of a novel, local defence mechanism of the vascular wall against oxidative stress that relies on the continuous cross-talk between the vessel and its perivascular fat.

Given that both vessel types used in our study (IMA and SV) were free of atherosclerosis, it is unclear whether these findings are applicable to the human coronary arteries, especially in the presence of coronary atherosclerosis. Although our *ex vivo* models use human vessels obtained from patients with advanced atherosclerosis (therefore chronically exposed to a pro-atherogenic environment), the absence of atherosclerotic lesions in these vessels is a potential limitation of the study. Moreover, the extent to which AdN produced in perivascular adipose tissue is sufficient to effectively control redox state in the human vascular wall *in vivo* is hard to estimate, given the complexity of the mechanisms implicated in redox state regulation in the human vascular wall. Finally, it is unclear whether our findings are applicable to human arteries and veins from healthy individuals, as these vessels are only harvested during CABG surgery, and these patients are also under multiple pharmacological treatments.

Conclusion

This is the first study demonstrating that AdN has a direct impact on redox state in human arteries and veins, through its combined effect on BH4-mediated improvement of eNOS coupling and PI3/Akt-mediated phosphorylation of eNOS. It also introduces the novel concept that increased oxidative stress in the vessel wall leads to the release of peroxidation products (i.e. 4-HNE) that up-regulate AdN gene expression in perivascular adipose tissue, via a PPAR- γ -dependent mechanism. These findings propose for the first time a bi-

directional cross-talk between the human vascular wall and perivascular AT, with potentially important implications in vascular biology.

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Legend to the Figures

Figure 1. Flow mediated dilation (FMD) was higher in patients with elevated circulating adiponectin (A), but endothelium independent dilation (EID) was not related with circulating adiponectin (B) in the entire population of Study 1. Patients with high adiponectin levels had significantly better vasorelaxations of SV segments (n=230) in response to acetylcholine (ACh, C) but not to sodium nitroprusside (SNP, D). Values expressed as mean±SEM.

Figure 2. Patients with high circulating adiponectin (AdN) had significantly lower vascular superoxide (O_2^-) in both saphenous veins (SV, n=396, Panel A) and internal mammary arteries (IMA, n=257, Panel B). On the contrary, high *ADIPOQ* gene expression in peri-SV (Panel C) or peri-IMA (Panel D) adipose tissue (AT) was related with high vascular O_2^- in SV and IMA segments respectively. Similarly, high release of AdN from peri-SV-AT (after 4h culture, Panel E) was also related with high O_2^- in human SVs. Values are expressed as median[25th-75th percentile].

Figure 3. Patients with low circulating adiponectin (AdN) had significantly more LNAME-inhibitable vascular superoxide (O_2^-) in both saphenous veins (SV, n=244, Panel A) and internal mammary arteries (IMA, n=182, Panel B). On the contrary, high *ADIPOQ* gene expression in peri-SV (Panel C) or peri-IMA (Panel D) adipose tissue (AT) was related with high LNAME-inhibitable O_2^- in SV and IMA segments respectively. Similarly, high release of AdN after 4h culture of peri-SV-AT (Panel E) was also related with greater LNAME-inhibitable vascular O_2^- in human SVs. Values are expressed as mean±SEM.

Figure 4. Circulating adiponectin (AdN) was significantly correlated with AdN release after 4h of *ex vivo* culture and *ADIPOQ* gene expression in mesothoracic (Ms-AT, Panels A and B) and subcutaneous (Sc-AT Panels C and D) but not peri-vascular (peri-SV-AT Panels E and F) adipose tissue. Pearson's r correlation coefficients are presented.

Figure 5. The number of G alleles for rs17366568G (polymorphism in *ADIPOQ* gene) and T alleles for rs266717 (polymorphism in *ADIPOQ* promoter region), had an additive effect on circulating adiponectin (AdN) levels (n=677, Panel A). A similar effect was also observed for AdN release by mesothoracic (Ms-AT, Panel B) but not perivascular (Peri-SV-AT, Panel C) adipose tissue (AT). The number of rs17366568G and rs266717T alleles was also positively associated with flow mediated dilation of the brachial artery (FMD, Panel D) and vasorelaxations of SVs (n=230) in response to acetylcholine (ACh, Panel E) but not to sodium nitroprusside (SNP, Panel F). The number of rs17366568G and rs266717T alleles was also related with less vascular superoxide (O_2^- , Panel G) and less LNAME-inhibitable O_2^- (Panel H) in both SVs (n=396) and internal mammary arteries (IMAs, n=257). Values expressed as median [25th-75th percentiles] for Panels A, B, C and G or mean±SEM for panels D, E, F and H.

Figure 6. *Ex-vivo* incubation of serial human saphenous vein (SV, n=9 pairs, Panel A) and internal mammary artery (IMA, n=5 pairs B) rings with adiponectin (AdN 10µg/ml for 6h, induced a significant reduction of vascular superoxide (O_2^- , Panels A and B) and an improvement of LNAME-inhibitable O_2^- (Panels C and D) as measured by lucigenin chemiluminescence. This finding was also confirmed by using DHE staining (Panels E and F), where we observed a reduction of endothelium-derived O_2^- and reversal of endothelium-derived LNAME-inhibitable O_2^- in both SVs and IMAs with AdN (representative images of 10 patients). Values are expressed as median[25th-75th percentile] (Panel A) or mean±SEM (Panel B). The dotted line in Panels E and F defines the endothelium.

Figure 7. *Ex-vivo* incubation of serial saphenous vein segments (SV, 11 patients, Panels A and B) and internal mammary artery segments (IMA, 5 patients, Panels C and D) with adiponectin (“A” 10µg/ml for 6h) increased Akt and eNOS phosphorylation at Ser437 and Ser1177 respectively, while these changes were prevented by wortmannin (“W”, 100nmol/L,

a PI3/Akt inhibitor). DAHP (“D”, an inhibitor of GTP-cyclohydrolase blocking BH₄ biosynthesis), did not modify the effects of AdN on Akt or eNOS phosphorylation (Panels A to D). Circulating AdN was significantly correlated with vascular tetrahydrobiopterin (BH₄, Panel E) and BH₄/total biopterins ratio (Panel F) in both SVs and internal mammary arteries from 173 patients in Study 1. *Ex vivo* incubation of serial SV (from 13 patients) and IMA (from 7 patients) rings with adiponectin 10 µg/ml for 6h, significantly increased vascular BH₄ (Panels G and H) and BH₄/total biopterins ratio (Panels I and J), while both vascular BH₄ and BH₄/total Biopterins ratio were significantly reduced by DAHP 1 mM. Values are expressed as mean±SEM (Panels A to D, I and J) or as median [25th-75th percentiles] (Panels G and H). *P<0.05 vs control; +P<0.05 vs adiponectin. t-Akt= total Akt; p-Akt= phosphorylated Akt at Ser473; t-eNOS= total endothelial nitric oxide synthase; p-eNOS= phosphorylated eNOS at Ser1177.

Figure 8. The vascular content of 4-hydroxynonenal (4-HNE) protein adducts was evaluated in SVs and IMAs with known O₂⁻ generation from cohort 1. Examples of blots for 4-HNE adducts in SVs with high (8.1 RLU/ Sec/mg) vs low (2.9 RLU/ Sec/mg) O₂⁻ and IMAs with high (6.7 RLU/ Sec/mg) vs low (0.41 RLU/ Sec/mg) O₂⁻ (Panel A) are presented. Incubation of peri-SV-AT (n=6) with 4-HNE 30 µM for 16h up-regulated *ADIPOQ*, PPAR-γ and CD36 genes (Panel B). The up-regulation of *ADIPOQ* and CD36 (but not PPAR-γ) genes was prevented by the PPAR-γ inhibitor T0070907 (10 µM) in the Peri-SV-AT (Panel B). A proposed novel cross-talk between perivascular adipose tissue and the vascular wall is presented in panel C. Values expressed as mean±SEM; *P<0.05 vs control. AT:Adipose tissue; BH₄=Tetrahydrobiopterin; eNOS=Endothelial nitric oxide synthase; PI3K= Phosphoinositide 3-kinase ; PPAR-γ = peroxisome proliferator-activated receptor -γ; ROS= Reactive oxygen species

Table 1. Demographic characteristics of study participants

	Clinical Studies (Study 1)	<i>Ex vivo</i> studies (Study 2)
Participants (n)	677	46
Age (years)	66.1±0.3	63.2±1.5
Males gender (%)	563 (83)	35 (78)
Hypertension, n(%)	465 (68)	36 (80)
Hyperlipidaemia, n(%)	433 (64)	22 (49)
Diabetes mellitus, n(%)	221 (33)	12 (27)
Smoking (active/ex) , n(%)	161/325 (24/48)	8/20 (18/44)
BMI (Kg/m ²)	27.6±0.17	29.3±0.72
Cholesterol (mg/dl)	177.7±2.0	155.8±16.3
HDL (mg/dl)	38.6±0.5	36.3±3.9
Triglycerides (mg/dl)*	124[94-166]	107[56-128]
Medication (%)		
ACEi/ARBs	65	62
Beta blockers	73	71
Aspirin/clopidogrel	80	95
Statins	76	79
CCBs	32	24

BMI: Body mass index; ACEi: Angiotensin converting enzyme inhibitors; ARBs: Angiotensin receptor blockers; CCBs: Calcium channel blockers. Values are expressed as means±SEM; *Values expressed as median[25th-75th percentiles].

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SUPPLEMENTAL MATERIAL

Supplemental methods

Assessment of Pre-Operative Endothelial Function

FMD and endothelium-independent vasodilatations of the brachial artery were measured the day before surgery by using a linear array transducer, and automated off-line analysis (Vascular Analyser, Medical Imaging Applications LLC), as previously described by our group.¹ Briefly, brachial artery diameter was recorded before, and sixty seconds after a five minutes forearm blood flow occlusion. A further measurement was made three minutes after a sublingual spray of glyceryl trinitrate (400 µg). FMD and endothelium-independent dilatation (EID) of the brachial artery were defined as the %change in vessel diameter after forearm ischaemia or sublingual GTN, respectively.

Blood Sampling and Adiponectin Measurements

Venous blood samples were obtained after 8 hours of fasting, on the morning of surgery. After centrifugation at 2000 g at 4°C for 15 min, plasma or serum was collected and stored at –80 °C until assayed. Whole blood was also collected for genotyping. Serum adiponectin was measured by enzyme linked immunosorbent assay (BioVendor, Brno, Czech Republic).

DNA Extraction and Genotyping

Genomic DNA was extracted from whole blood using standard methods (QIAamp DNA blood Midi kit, Qiagen). Genotyping for the rs17366568 (functional polymorphism in ADIPOQ gene) and rs266717 SNPs (functional polymorphism in ADIPOQ gene promoter) was performed using TaqMan probes (Applied Biosystems; Assay IDs: C-33187752-10 and C-8288442-10 respectively). The assay was run according to manufacturer's conditions, on an ABI StepOne Plus PCR system.

Vessel Harvesting

IMA and SV samples were harvested with a “no touch” technique with their perivascular tissue (peri-IMA-AT and peri-SV-AT) at the time of CABG as we have described previously.^{15, 16} Vascular segments were transferred into oxygenated (95% O₂ / 5% CO₂) ice-cold Krebs Henseleit buffer and the vessel lumen was flushed gently by using an insulin syringe to remove blood. Each vessel was separated from its adipose tissue in the lab, under magnification by the same operator, to limit the between-patients variability. The same anaesthetics were used in all cases, and each sample was always obtained at the same stage of the operation, to limit the between-patients variability.

Vasomotor Studies

Vasomotor studies were performed in SV segments obtained during CABG as we have previously described.^{2, 3} Vessels were equilibrated in the organ bath for 60 minutes, in oxygenated (95% O₂/5% CO₂) Krebs-Henseleit buffer at 37°C to achieve a resting tension of 3g. Contractile responses were tested by exposure to Krebs-Henseleit buffer containing potassium chloride (60mM). Four rings from each vessel were pre-contracted with phenylephrine (3x10⁻⁶M); then endothelium-dependent relaxations were quantified using acetylcholine (ACh, 10⁻⁹M to 10⁻⁶M). Finally, relaxations to the endothelium-independent NO donor sodium nitroprusside (SNP, 10⁻¹⁰M to 10⁻⁷M), were evaluated in the presence of the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME; 100μM), as we have previously described.^{2, 3}

Vascular Superoxide Measurements

Vascular O₂⁻ production was measured in fresh, intact IMA and SV segments by using lucigenin (5 μmol/L)-enhanced chemiluminescence, as we have described previously.^{4,15, 16} Vessels were opened longitudinally to expose the endothelial surface and equilibrated for 20 minutes in oxygenated (95% O₂/5% CO₂) Krebs-HEPES buffer (pH 7.4) at 37°C. The contribution of uncoupled nitric oxide synthase (NOS) to vascular O₂⁻ production was quantified as the change of O₂⁻ from

baseline to 20 minutes after incubation with the NOS inhibitor L-NAME (100 μ mol/L), and presented as delta-LNAME O₂- as we have previously described.^{4,15, 16}

Adipose Tissue Culture

Samples of Sc-AT, Ms-AT and Peri-SV-AT obtained from patients in study 1, were used to estimate AdN's biosynthetic rate, in an ex vivo bioassay. Briefly, adipose tissue was isolated after removing the adventitia and washed in sterile phosphate buffer saline. Samples of ~500mg tissue of each type were transferred to the lab within 30 minutes of harvesting. The samples were then cut in ~1-2mm³ cubes, washed and equilibrated for 1h at 37 °C in Medium-199 containing HEPES 25 mM, gentamycin 50 μ g/ml and fatty acid-free bovine serum albumin 1%, in the presence of protease inhibitor (Roche Applied Science, Indianapolis, IN) in a cell culture incubator with 5% CO₂ atmosphere. At the end of the equilibration period the media was changed (1 ml per 200mg tissue) and incubated for 4 hours under the same conditions, as previously described.⁵ At 4h, AT culture supernatants were collected, filtered and stored at -80°C until analysis. This allowed the generation of a unique bio-resource of adipose tissue culture supernatants. AdN levels were then quantified in AT culture supernatants by using a high sensitivity ELISA kit (BioVendor).

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Samples of Sc-AT, Ms-AT, Peri-SV-AT and Peri-IMA-AT were snap frozen in QIAzol (Qiagen, Stanford, CA) and stored at -80°C until processed. RNA was extracted by using the RNeasy Micro or Mini kit (Qiagen). Ribonucleic acid was reverse transcribed (Quantitect Reverse Transcription kit - Qiagen), and used for qPCR using TaqMan probes (Applied Biosystems, Foster City, CA; Assay IDs ADIPOQ: Hs00605917_m1; PPIA: Hs04194521_s1, PPAR- γ : Hs01115513_m1, CD36: Hs01567185_m1). The reactions were performed in triplicate in 384-well plates, using 5 ng of cDNA per reaction, on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The efficiency of the reaction in each plate

was determined based on the slope of the standard curve; relative expression of AdN was calculated using the Pfaffl method,⁶ with PPIA (cyclophilin) as housekeeping gene.

Measurement of Vascular Biopterins

Vascular BH₄, dihydrobiopterin (BH₂), and biopterin (B) levels were each determined separately from the same sample, using high-performance liquid chromatography followed by serial electrochemical and fluorescent detection, as we have previously described.⁷ Total biopterin levels are the result of the sum of BH₄, BH₂, and B individual levels. Biopterin levels were expressed as pmol/g in vascular tissue.

Population in Study 2 and Experimental Procedures

To examine the direct effects of AdN on the mechanisms regulating vascular NO bioavailability and O₂⁻ production in human vessels, we used a well validated ex vivo model of human SVs and IMAs, as we described in the past.^{15, 16} For these experiments we recruited 46 patients undergoing CABG following the same exclusion criteria as for study 1. Briefly, serial rings from the same vessel were incubated in oxygenated (95%O₂/5%CO₂) Krebs-HEPES Buffer in the presence or absence of recombinant full-length AdN 10 µg/ml (BioVendor) for 6 hours. The effect of AdN on vascular O₂⁻ (resting and LNAME-inhibitable O₂⁻) was quantified by lucigenin (5 µmol/L) enhanced chemiluminescence (as described above) and visualized by oxidative fluorescent dye dihydroethidium (DHE) staining (as described below). The changes in Akt and eNOS phosphorylation status were determined by western blotting. Vascular biopterins (BH₄, BH₂ and B) were quantified by HPLC, as described above. In additional studies, samples from these patients were incubated with full-length AdN 10 µg/ml in the presence and absence of wortmannin (100 nmol/L) to inhibit PI3 kinase/Akt signaling or 2,4-diamino-6-hydroxypyrimidine (DAHP; 1 mmol/L) to inhibit GTP cyclohydrolase, the rate-limiting enzyme of BH₄ biosynthesis, as indicated in the text.

To prove that endogenous oxidative stress regulates 4-HNE generation in vascular tissue, the 4-HNE protein adducts content of human SVs (n=11) and IMAs (n=18) was determined by using western blotting, and linked with vascular O₂⁻ production in these vessels.

Ex Vivo Incubation Of Perivascular Adipose Tissue With 4-HNE

To clarify the role of oxidative stress in the regulation of ADIPOQ gene expression in adipose tissue, samples of Peri-SV and Peri-IMA AT were exposed to the lipid peroxidation product 4-hydroxynonenal (4HNE, 30 µmol/L) in the presence or absence of the PPAR-γ inhibitor T0070907 (10 µmol/L – for peri-SV AT only, due to limited availability of peri-IMA AT). Briefly, samples were collected as described above, separated into three (peri-SV AT) or two (Peri-IMA-AT) parts, each part was then cut into small pieces (~1mm³) and allowed to equilibrate for 2h in medium-199 containing HEPES 25 mM, gentamycin 50 µg/ml and fatty acid-free bovine serum albumin 1%, in the presence of protease inhibitor (Roche Applied Science, Indianapolis, IN) in a cell culture incubator with 5% CO₂ atmosphere, as described above. One of the 3 peri-SV AT pieces was exposed to T0070907 during the equilibration period (2h), in order to inhibit PPAR- γ activity. Then, medium was changed and the samples exposed to 4HNE alone or 4HNE+T0070907 (for Peri-SV AT) for 16 hours. At the end of the incubation period, samples were snap frozen for gene expression studies, as described above.

Oxidative Fluorescent Microtopography

In situ O₂⁻ production was determined in vessel cryosections with oxidative fluorescent dye dihydroethidium (DHE), as previously described.^{1, 8} Serial SV and IMA rings were incubated with/without AdN 10µg/ml for 6 hours, and they were snap frozen in OCT. Cryosections (30µm) were incubated with DHE (2µmol/L for 5 minutes) in Kreps-Hepes buffer, with or without L-NAME (100µmol/L). Fluorescence images of the endothelium (x63, Zeiss LSM 510 META laser scanning

confocal microscope) were obtained from each vessel quadrant. In each case, segments of vessel rings (\pm L-NAME) were analysed in parallel with identical imaging parameters in a blinded fashion.

Western Blots For Vascular Akt/p-Akt, eNOS/p-eNOS and 4HNE Protein Adducts

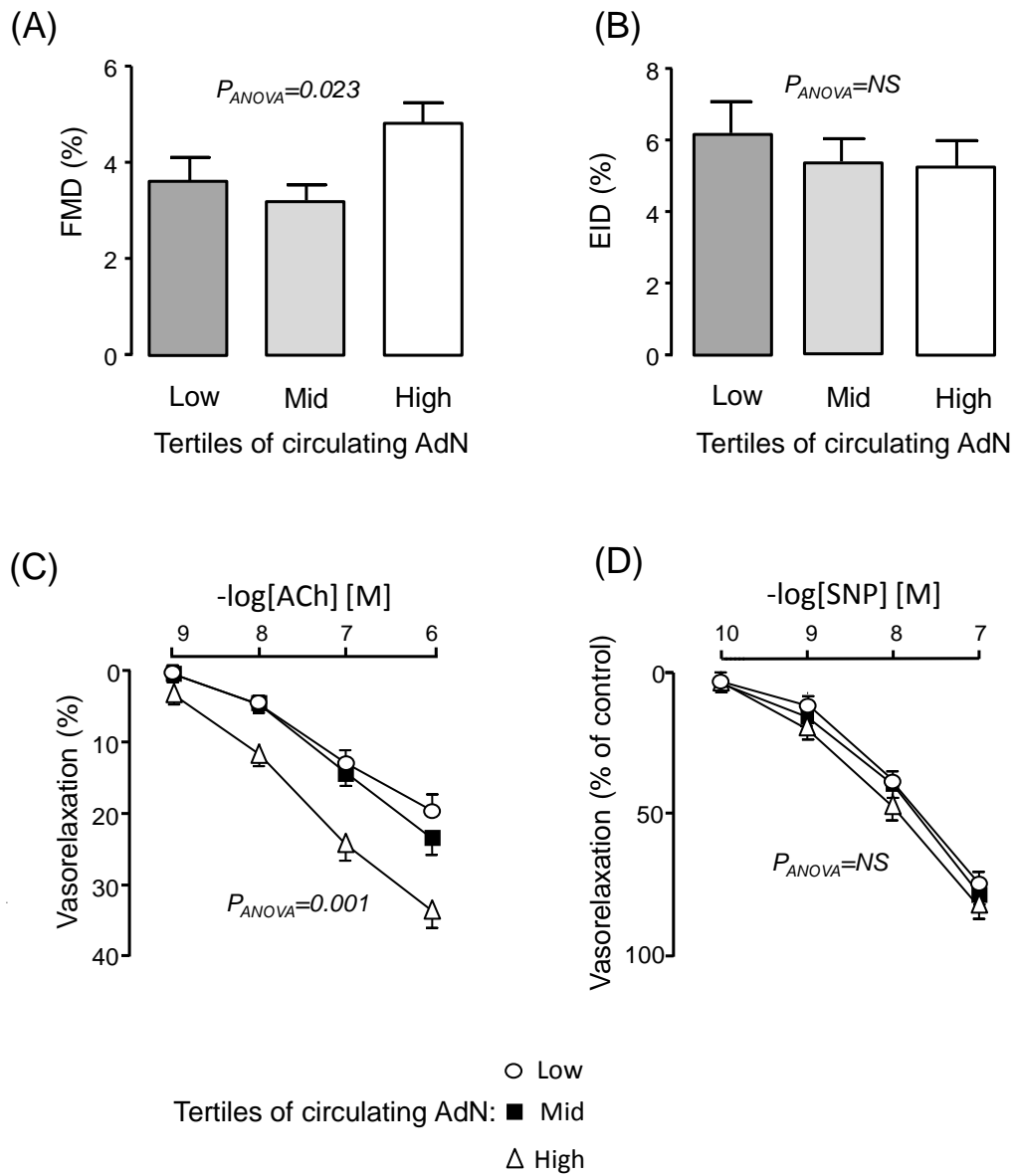
To examine the direct effects of AdN on Akt phosphorylation at Ser473 and eNOS phosphorylation at Ser1177, as well as quantify the levels of 4-HNE adducts, vascular tissue samples (SVs and IMAs) were homogenized for 30 seconds using a pre-cooled electric homogenizer Polytron in 220 μ l of lysis buffer (Invitrogen, UK) or 150 μ l of RIPA buffer (Cell Signaling Technologies, Danvers, MA) containing a protease inhibitor cocktail (Roche Applied Science). Homogenates were spun at 13,000 rpm for 10 minutes, at 4 °C.

The protein concentration of the supernatants was measured using the BCATM Protein Assay kit (Pierce, UK). Protein lysates were separated on 4-12% gradient SDS-NuPAGE or 10% Bis-Tris/SDS gel(Invitrogen, UK), and proteins transferred to nitrocellulose membranes (Amersham, UK Ltd.), and blocked with 5% powdered skimmed milk. The membranes were incubated with anti-eNOS antibody (BD Transduction Laboratories, CA), anti-phospho-eNOS (Ser1177), anti-phospho-Akt (Ser473), anti-pan-Akt (Cell Signaling Technologies) or anti-4HNE antibody (R&D Systems, Minneapolis, USA) as stated. Immunodetection of the primary antibodies was performed with horseradish-peroxidase-conjugated secondary antibodies (Promega) and enhanced chemiluminescence (Amersham Bioscience UK Ltd.) and quantified in relation to the house-keeping protein, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

Supplemental references

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Figure 1



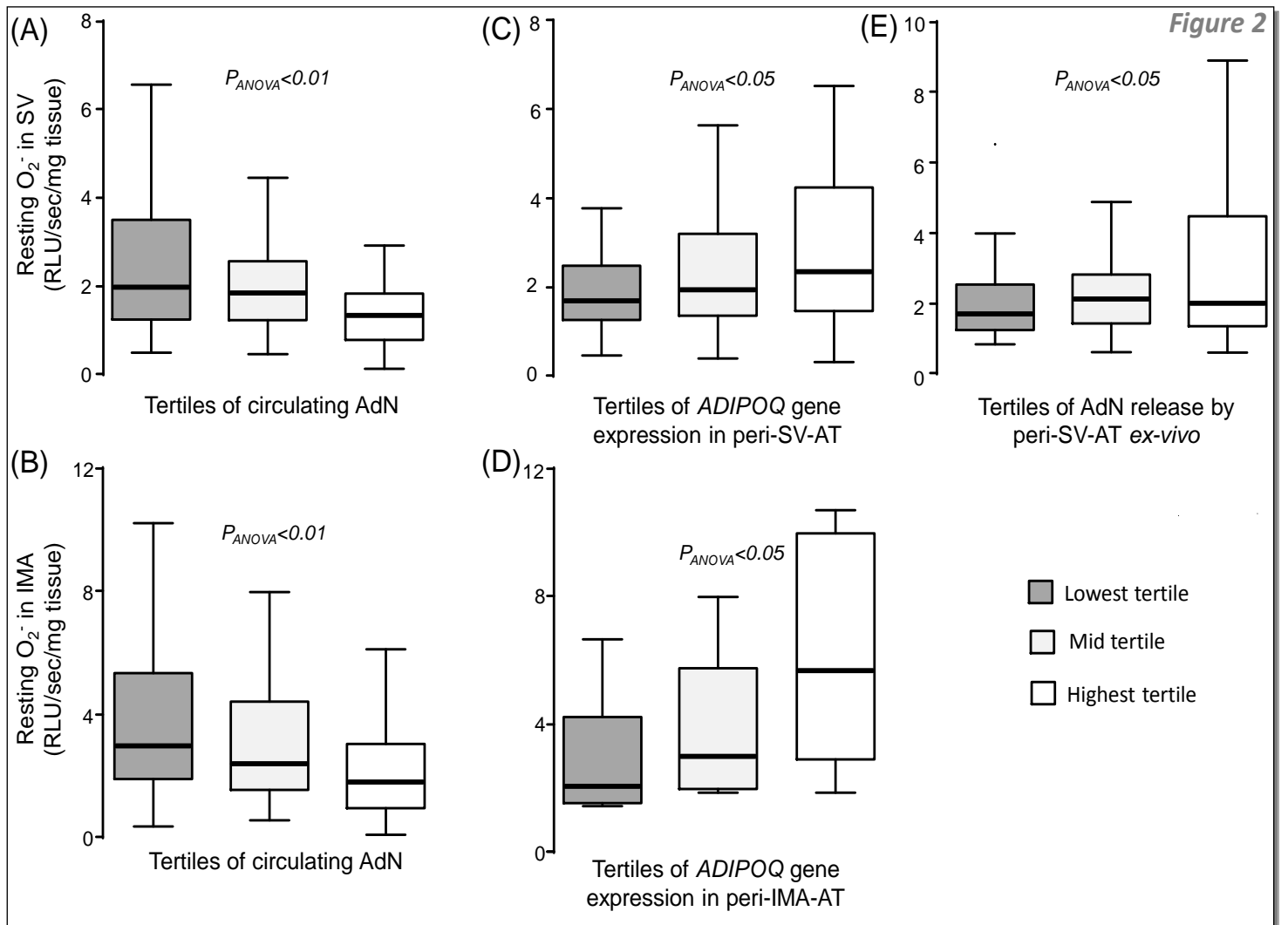
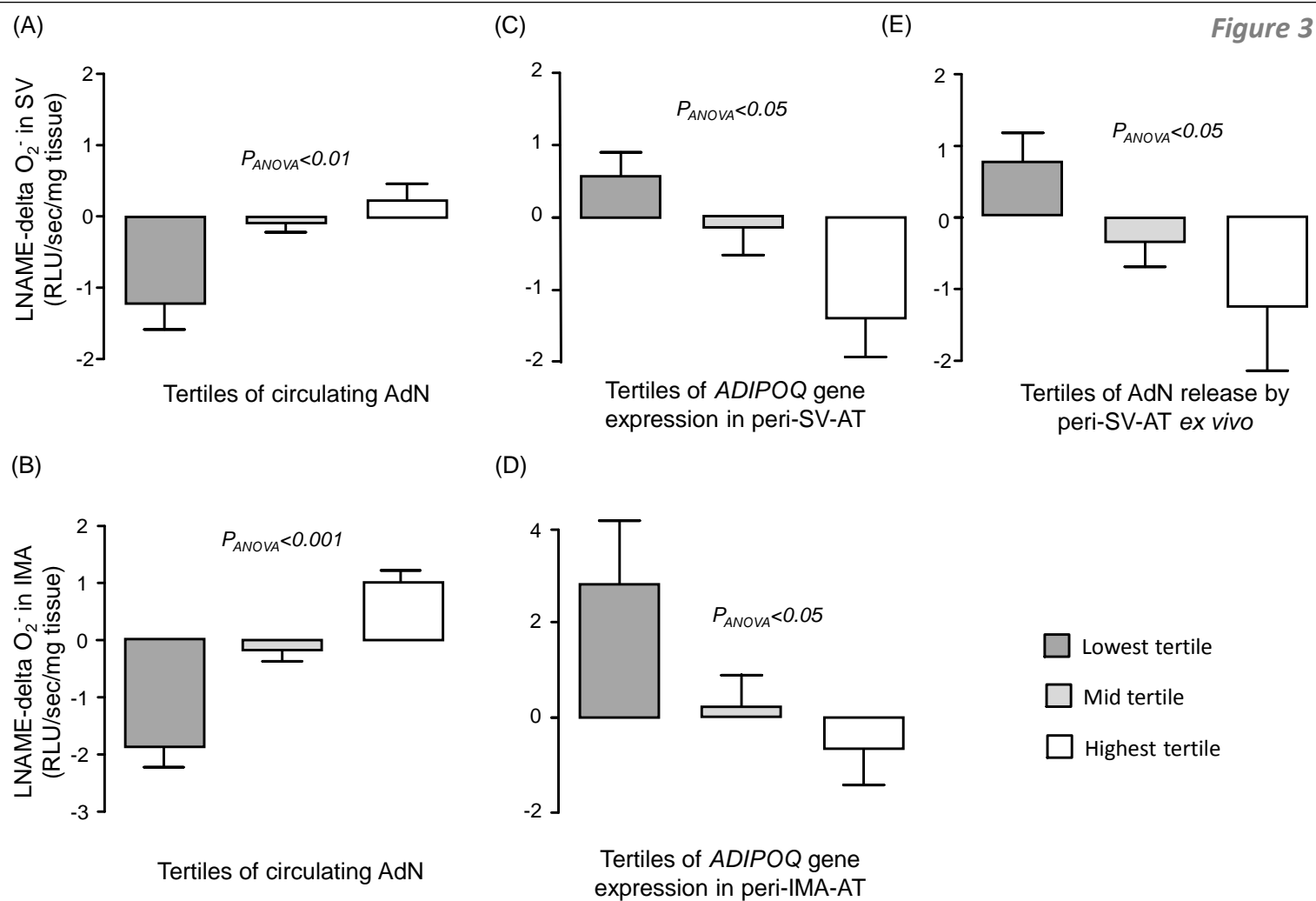


Figure 3



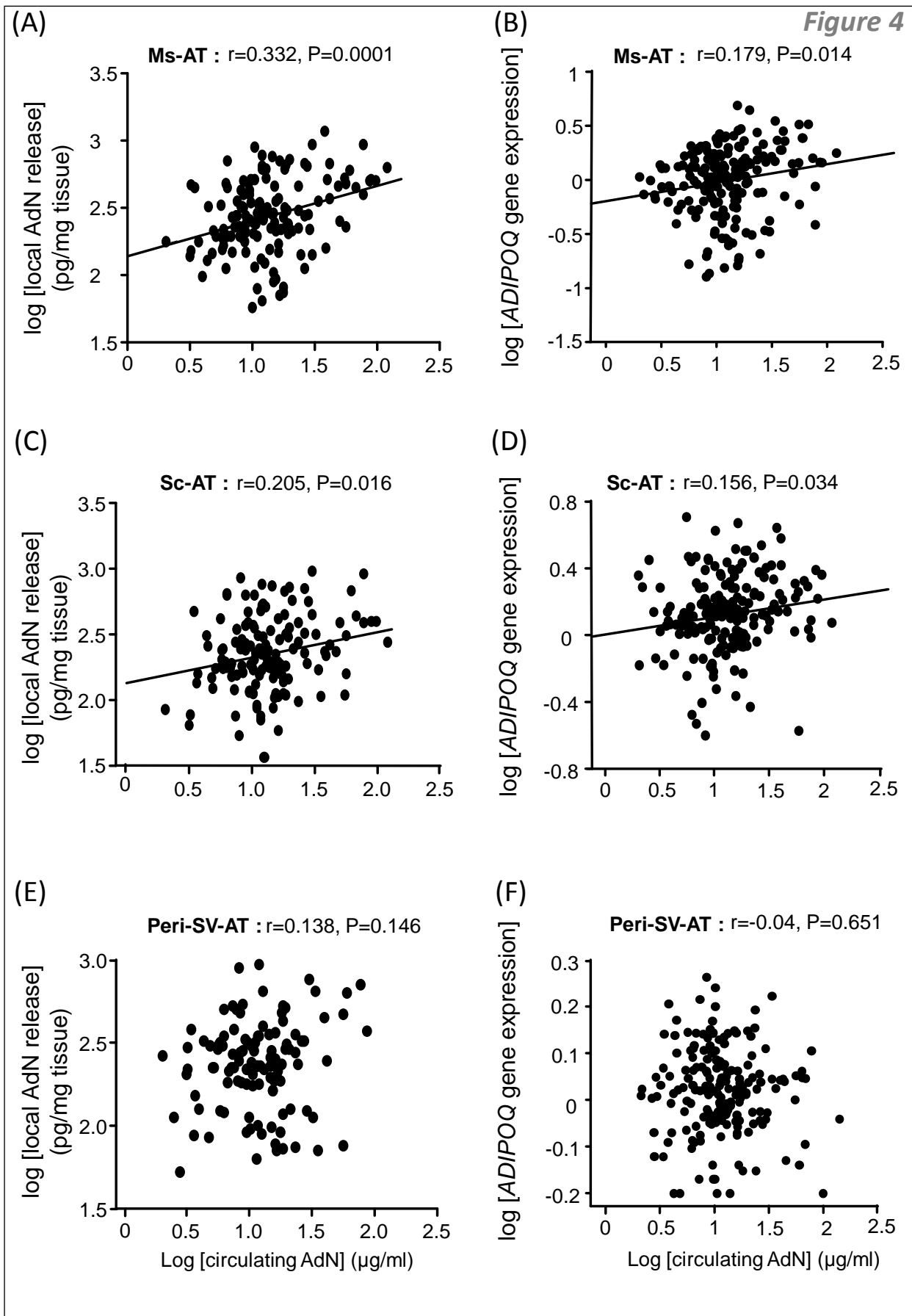


Figure 5

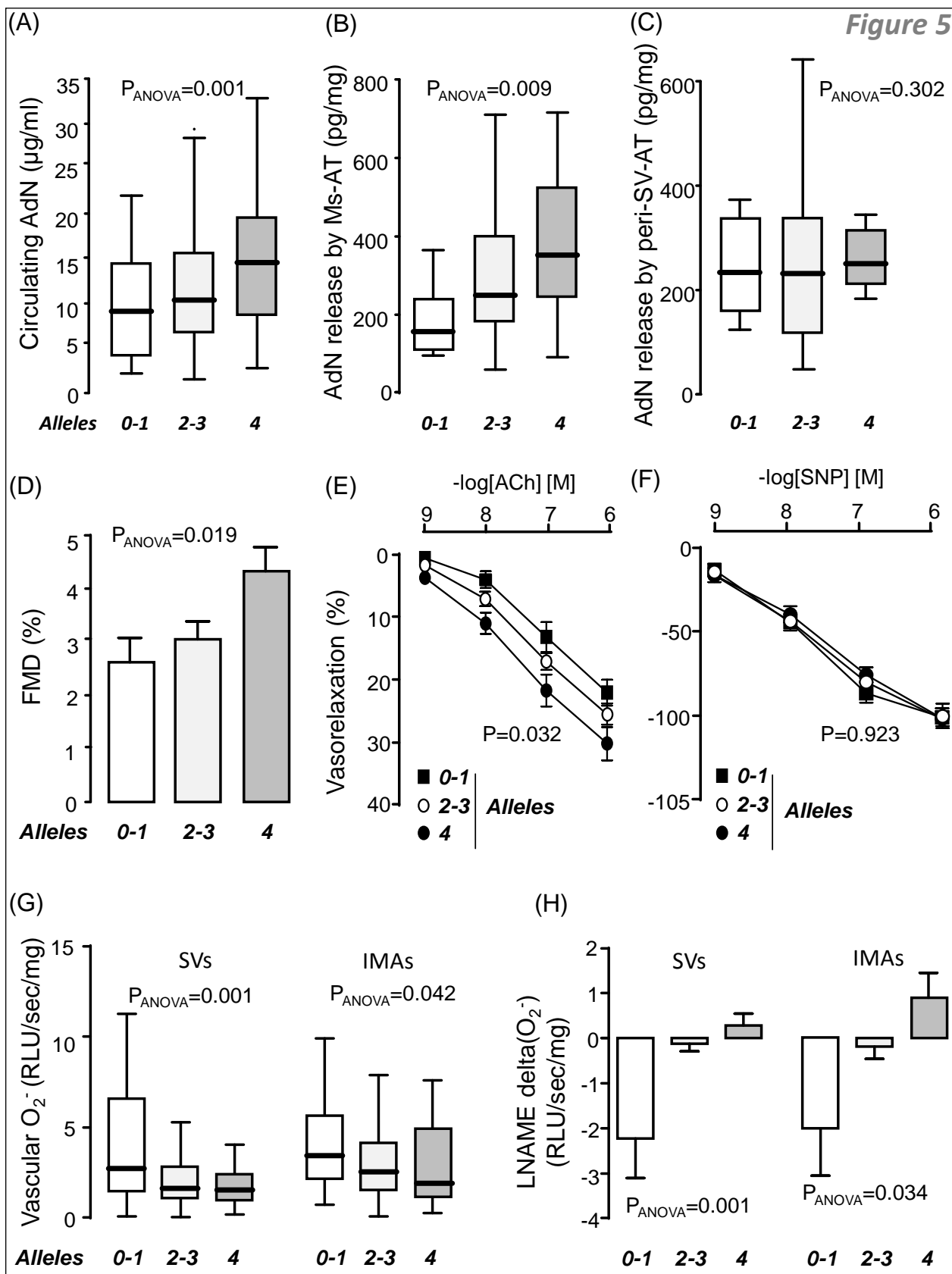


Figure 6

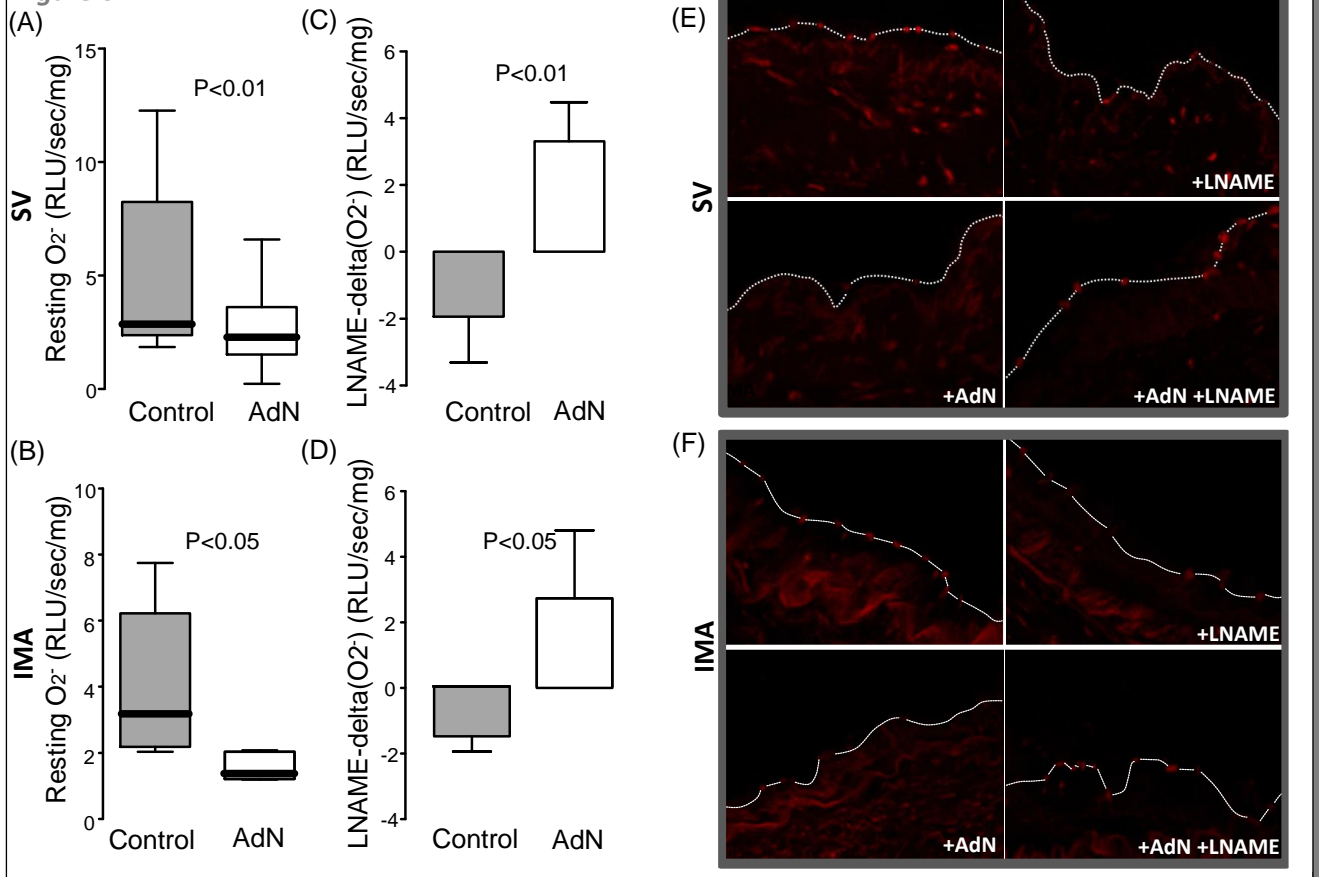


Figure 7

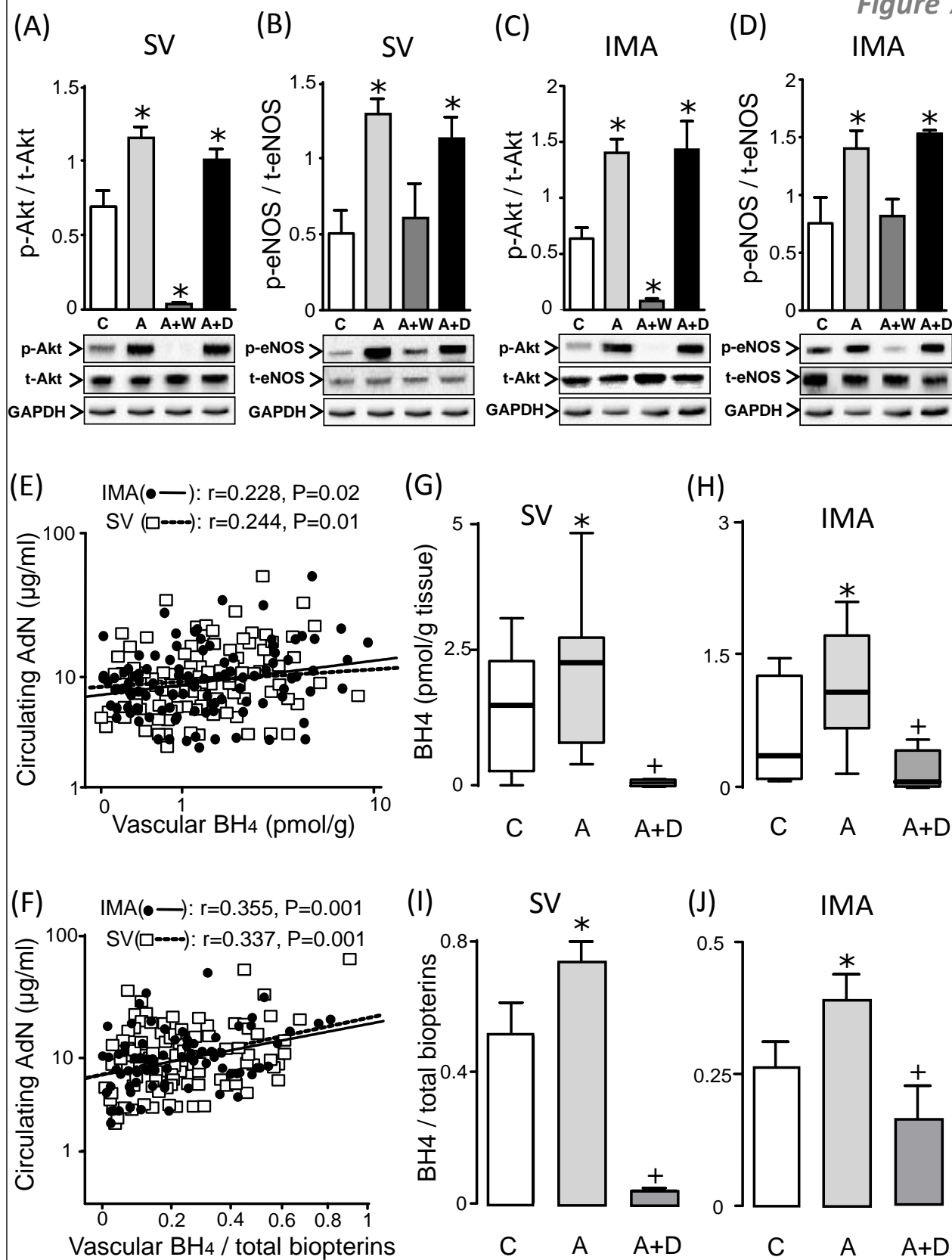


Figure 8

