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# Adiponectin secretion by perivascular adipose tissue supports impaired vasodilation in a mouse model of accelerated vascular smooth muscle cell and adipose tissue aging

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ABSTRACT

*Objective:* Perivascular adipose tissue (PVAT) function during aging has not been investigated in detail so far and its effect on vasodilation remains to be fully elucidated. The aim of this study was to investigate endothelium-dependent vasodilation of thoracic aorta in a mouse model of accelerated, selective vascular smooth muscle and PVAT aging, induced by SM22α-Cre-driven genetic deletion of the endonuclease ERCC1 (SMC-KO mice) versus healthy littermates (LM). We hypothesized that PVAT enhances vasodilation in LM, possibly through adiponectin secretion, which might be compromised in SMC-KO animals.

*Methods:* Thoracic aorta was isolated from SMC-KO animals and LM and segments with and without PVAT were mounted in wire myography setups. The endothelium-dependent vasodilation was assessed via acetylcholine dose-response curves and pathway contribution was studied. Moreover, adiponectin secretion was measured after stimulating the aortic segments with PVAT with acetylcholine.

*Results:* Adiponectin, secreted by PVAT, led to increased NO-contribution to endothelium-dependent vasodilation in healthy LM, although this did not increase maximum relaxation due to loss of EDH. Endothelium-dependent vasodilation was decreased in SMC-KO animals due to reduced NO-contribution and complete EDH loss. Despite strong lipodystrophy the PVAT partially compensated for lost vasodilation in SMC-KO. LM PVAT contained acetylcholinesterase that attenuated acetylcholine responses. This was lost in SMC-KO.

*Conclusions:* PVAT-derived adiponectin is able to partially compensate for age-related decline in NO-mediated vasodilation, even during strong lipodystrophy, in conditions of absence of compensating EDH. In aorta with healthy PVAT acetylcholinesterase modulates vascular tone, but this is lost during aging, further compensating for decreased acetylcholine responsiveness. Thus, preservation of adiponectin levels, through relatively increased production in lipodystrophic PVAT, and reduction of cholinesterase might be regulatory mechanisms of the PVAT to preserve cholinergic vasodilation during aging.

## 1. Introduction

Perivascular adipose tissue (PVAT) surrounds blood vessels and gives structural and functional support [1]. It was shown that PVAT modulates vascular tone by secreting vasoactive factors that are classified into adipocyte-derived constricting factors and adipocyte-derived relaxing factors (ADRF) [2–4]. Healthy PVAT is therefore essential to maintain vascular function.

*Abbreviations:* ADRF, adipocyte-derived relaxing factors; BAT, brown adipose tissue; BKCa-channel, big conductance calcium-activated potassium channels; cGMP, cyclic guanosine monophosphate; EDH(F), endothelium-derived hyperpolarization (factor); eNOS, endothelial nitric oxide synthase; LM, littermates; NO, nitric oxide; PVAT, perivascular adipose tissue; VSMC, vascular smooth muscle cell; WAT, white adipose tissue.

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ADRF can activate different vasodilatory pathways. The main pathways contributing to vasodilation are the nitric oxide (NO) – cyclic guanosine monophosphate (cGMP) – pathway, endothelium-derived hyperpolarization (EDH) and the prostacyclin pathway [5]. Depending on the vascular bed, ADRF are on the one hand able to induce endothelial NO-production, and on the other hand they can act via potassium channel activation, which are involved in EDH [2,4,6]. By inducing vasodilatory pathways, ADRF counteract vascular smooth muscle cell (VSMC) constriction. This ADRF function is lost in diseases such as obesity, diabetes and metabolic syndrome [4,7,8]. One of these ADRF is adiponectin which has been shown to counteract vasoconstriction in rodent mesenteric arteries by stimulating NO-production in the vasculature [3,8,9].

Most studies focus on the anti-contractile effect of PVAT on VSMC, whereas there is little known about its effect on VSMC relaxation. Available data are inconclusive because results vary between species and vascular beds. Kagota et al. showed that PVAT enhanced vasodilation in mesenteric arteries of metabolic syndrome rats which was lost with age [10]. In further distinction, in adult Wistar rats, PVAT inhibited endothelium-dependent vasodilation in the thoracic aorta in a NO-dependent way [11,12]. A major difference between these studies are the different vascular beds that were investigated. Composition of PVAT significantly differs depending on the vascular beds: rodent mesenteric PVAT consists of white adipose tissue (WAT) while rodent thoracic aorta is surrounded by mainly brown adipose tissue (BAT) [7,13]. Moreover, resistance and conduit arteries have a differential function, namely regulation of flow velocity vs. flow uniformity during the cardiac cycle. Thus, it can be expected that both types of PVAT, of which BAT is relatively under-investigated, differ in function.

During aging vascular and adipose tissue function decline leading to increased inflammation and insulin resistance [14,15], which are systemically acting mechanisms. However, aging also takes place locally in tissues, which often occurs at different rates between the various organs with an individual pattern [16]. This depends on the rate of accumulation of unrepaired DNA during life. Tissue-selective aging can be dramatically accelerated by genetic introduction of DNA repair defects. This occurs e.g., in progeria syndromes, and can be modeled in mice by tissue-specific deletion of DNA repair genes like *Ercc1*, an endonuclease, leading to rapid, faithful reproduction of human-like, non-obstructive vascular aging [15,17,18]. Also, adipose tissue can be targeted, showing local adipose tissue aging features such as lipodystrophy and inflammation [19]. We hypothesize that local aging of VSMC and PVAT causes integrative dysregulation of vasomotor control. To this end we here investigate the interaction of aortic PVAT and VSMC in a Tagln1-promotor-Cre recombinase model of simultaneous *Ercc1* knockout in VSMC and adipocytes (SMC-KO mice).

Since ADRF counteract vasoconstriction, we hypothesized that the secreted ADRF by PVAT, in particular adiponectin, would enhance vasodilation in the thoracic aorta of healthy animals which might be compromised in the vascular aging mouse model. The aim of this study was to investigate the effect of PVAT on endothelium-dependent vasodilation in thoracic aorta of healthy mice and SMC-KO mice.

## 2. Methods

### 2.1. Animals

Animals were bred as described previously [15]. In brief, VSMC/PVAT-targeted *Ercc1* KO was achieved with Cre-loxP system by cross breeding of SM22α-promotor (Taglntm2(cre)Yec, c57Bl/6 background, The Jackson Laboratory) with Ercc1$^{flox/-}$ (Ercc1tm2Dwm, FVB background, Erasmus MC colony). Combined VSMC- and PVAT-targeted KO with the SM22α-promotor was already described previously which led to loss of PVAT and impaired VSMC function [20]. Corresponding Cre$^{+/-}$::Ercc1$^{flox/+}$ littermates (LM) were used as healthy control animals. Male and female animals (50:50, 6–11 animals per group) were housed in groups in ventilated cages with food (standard chow from SAFE®, France) and water ad libitum in 12 h dark/ light cycles at 20–22 °C. Animal experiments were performed at the EDC (Erasmus Laboratory Animal Science Center) following the guidelines from Directive 2010/63/EU. All studies were approved by the National Animal Care Committee and within Erasmus University Medical Center Rotterdam.

### 2.2. Ex-vivo vascular function

At the age of 22 weeks, animals were euthanized (exsanguination under 5% isoflurane) and thoracic aorta were collected in cold Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 2.5 mM $CaCl_2$, 11 mM glucose, 25 mM $NaHCO_3$, pH 7.4). The proximal part of the descending thoracic aorta was cleaned from PVAT (-PVAT), while the distal part was kept as it was with surrounding fat (+PVAT). 1,5–2 mm aortic segments were then mounted in DMT wire myograph setups (Denmark) filled with Krebs-Henseleit-buffer at 37 °C and aerated (95% $CO_2$, 5% $O_2$). After normalizing as described previously [15], viability of the segments was checked by adding 100 mM KCl. After washing, segments were pre-incubated for 15 min with endothelial nitric oxide synthase inhibitor L-NAME ($10^{-4}$ M) (Sigma-Aldrich), L-NAME and small and intermediate conductance calcium-activated potassium channels-inhibitors apamin ($10^{-7}$ M) (Sigma-Aldrich) and TRAM-34 ($10^{-5}$ M) (Sigma-Aldrich) respectively, or big conductance calcium-activated potassium channel-inhibitor iberiotoxin ($5 \times 10^{-8}$ M) to assess pathway contributions to vasodilation. In a separate cohort of littermate control mice, aortic segments with or without PVAT were pre-incubated for 30 min. With 10 μM of the acetylcholinesterase inhibitor neostigmine [21]. Vessel segments were then constricted with thromboxane A2 analogue U46619 ($10^{-8}$–$10^{-7}$ M) (Sigma-Aldrich) aiming for 50–100% of vasoconstriction induced by 100 mM KCl. After reaching the plateau, acetylcholine was added dose-dependently ($10^{-9}$–$10^{-5}$ M). All concentrations were chosen according to previous experiments [15].

To calculate pathway contributions the area under the curve (AUC) was assessed for each animal and condition separately using GraphPad Prism 8.0.1. Relative NO-contribution was calculated using the following formula: contribution [%] = ($AUC_{control}$-$AUC_{L\text{-}NAME}$)/$AUC_{control}$*100. EDH-contribution was assessed as difference between L-NAME and L-NAME/ apamin/TRAM34 curve: contribution [%] = ($AUC_{L\text{-}NAME}$-$AUC_{L\text{-}NAME/apamin/TRAM34}$)/$AUC_{control}$*100.

To investigate the involvement of adiponectin in PVAT signaling, +PVAT segments were incubated with anti-adiponectin antibody (5 μg/ml, ab22554) [22] for 1 h prior to pre-constriction and –PVAT segments were stimulated for 5 min with adiponectin peptide (1 μg/ml, R&D Systems) at a concentration where NO-production is induced, but only minimal relaxation by adiponectin itself takes place, [8,23]. After reaching the plateau of pre-constriction, acetylcholine was added in a dose-response curve for all segments as described in previous conditions. This approach allows detection of pharmacological additive or synergistic effects between adiponectin and acetylcholine.

### 2.3. ELISA

One –PVAT and one +PVAT segment per mouse were put in separate wells of 96-well plates, filled with 110 μl Krebs-Henseleit buffer and acclimatized to 37 °C for 5 min. After taking a baseline aliquot, $10^{-5}$ M acetylcholine, which induced the maximal response in the wire myography setups, was added to stimulate adiponectin secretion. The plate was incubated for another 5 min at 37 °C and a second aliquot was taken. Aliquots were measured with mouse total adiponectin ELISA (ALPCO) according to manufacturer's instructions. Baseline values were subtracted from values obtained in samples after stimulation to calculate total adiponectin secretion.

### 2.4. Molecular analysis

mRNA was isolated with TRIzol (ThermoFisher) from frozen abdominal aorta and cDNA synthesized (Maxima H Minus First Strand kit, ThermoFisher) according to the manufacturer's instructions. qPCR was performed with the CFX Opus Real-Time PCR System (Bio-Rad) using SYBR™ Green PCR Master Mix (Applied Biosystems™) with *gapdh*, *rpl13a* and *β-actin* as housekeeping genes. Results are presented as relative quantities ($2^{\Delta\Delta Ct}$ method). Used primers can be found in Table 1. Proteins from PVAT were isolated in homogenization buffer (0.3 M sucrose, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM sodium-orthovanadate, 50 mM sodium fluoride, 1 mM DTT, 1 mM PMSF, 1% (*v*/v) Triton x-100, phosphatase inhibitor cocktail 3 (Sigma-Aldrich) and cOmpleteTM Protease Inhibitor Cocktail (Roche)), loaded (25 μg) on gels (4–20% Criterion TGX Gel, Bio-Rad), followed by transfer to membranes (Trans-Blot Turbo 0.2 μm PVDF Transfer Pack, Bio-Rad) with the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with 5% (*w/v*) BSA or non-fatty milk, followed by overnight incubation in primary antibody (Table 2). After incubation in the secondary antibody (Table 2), membranes were visualized with Clarity Western ECL substrate (Bio-Rad) using an Amersham Al600. For adiponectin, the membrane was incubated for 10 min in PageBlue Protein Staining Solution (Thermo Scientific) as loading control, followed by de-staining in 5% (*v*/v) methanol, 7.5% /v/v) acetic acid overnight and visualization as described above. Quantification was performed with Image Studio software, applying background stain subtraction with the use of the build-in module.

### 2.5. Immunohistochemistry

Aortic segments with PVAT were embedded in paraffin, sectioned (4 μM), stained with adiponectin antibody (1:1000, ab22554) and biotin-conjugated horse anti-mouse antibody (Vector Laboratories) and visualized with the Bright-DAB kit (ImmunoLogic) according to the manufacturer's instructions. Pictures were taken with the NanoZoomer 2.0 (Hamamatsu).

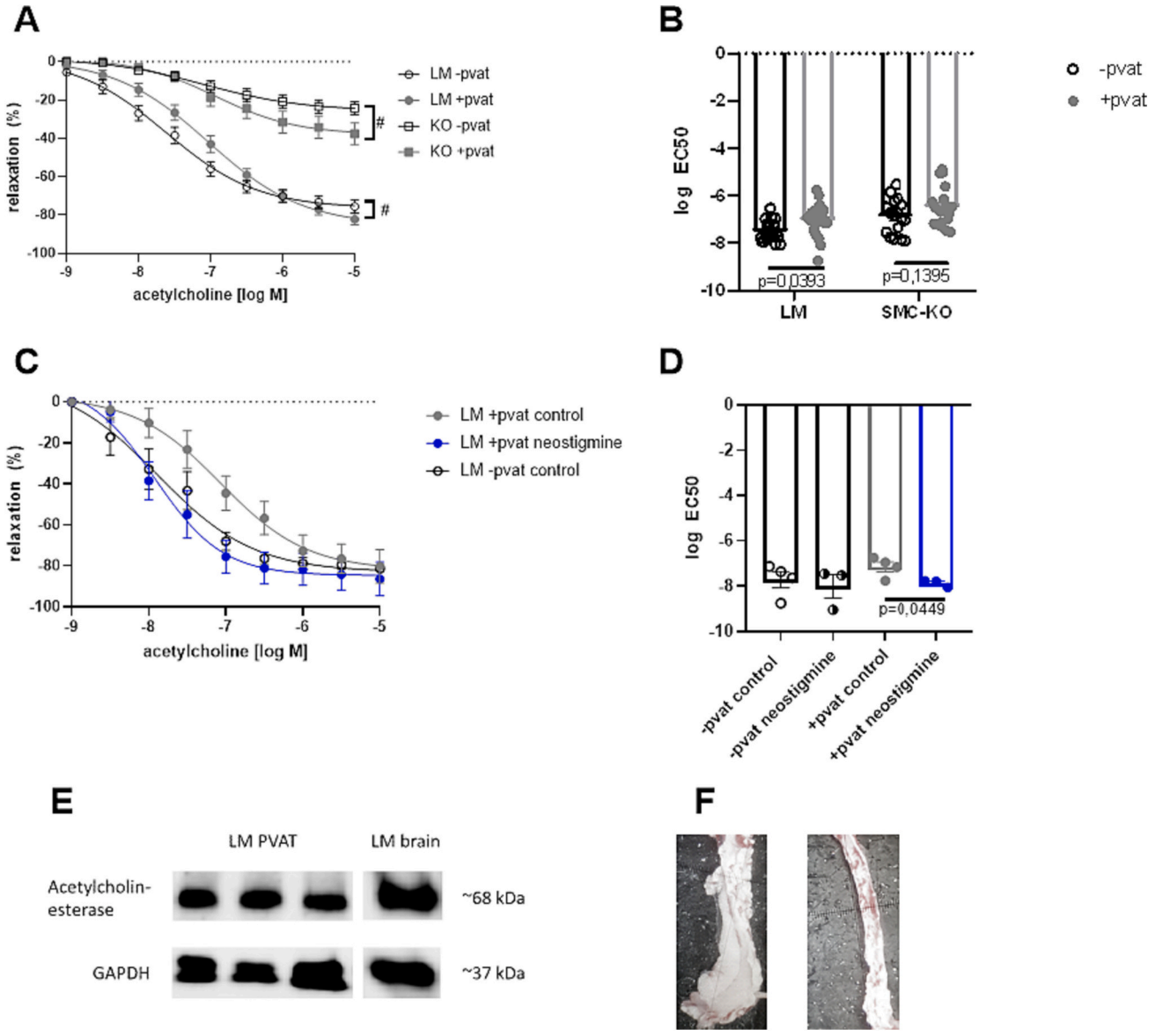


**Fig. 1.** Endothelium-dependent vasodilation of thoracic aorta of LM and SMC-KO animals. A: Acetylcholine-dose-response curve with or without PVAT and B: corresponding logEC50. C: Effect of acetylcholinesterase inhibitor neostigmine on acetylcholine-response of LM and D: corresponding logEC50. E: Western Blot for acetylcholinesterase in PVAT of LM animals with brain as positive control. F: Pictures of thoracic aorta with PVAT from LM (left) and SMC-KO (right). Dose-response curves were analyzed by GLM with repeated measures and bar graphs with 2-way ANOVA (B) or paired *t*-test (D). #: significant effect of PVAT ($p < 0.05$).

### 2.6. Statistics

Differences between dose-response curves were statistically analyzed by general linear model (GLM) with repeated measures (IBM SPSS Statistics 28.0.1.0). Column graphs were evaluated by paired student *t*-tests or 2-way ANOVA with post hoc Bonferroni's multiple comparisons test (GraphPad Prism 8.0.1).

## 3. Results

### 3.1. PVAT effect on vasodilation

We hypothesized that PVAT positively contributes to endothelium-dependent vasodilation in healthy mice (LM). However, although dose-dependent relaxations were significantly different between –PVAT and + PVAT rings, maximal vasodilation was not significantly altered (Fig. 1A). Instead, we observed a rightwards shift of the +PVAT curve leading to significantly shifted log EC50 Fig. 1B). This suggests that the presence of PVAT inhibited relaxations to acetylcholine, in contrast with our hypothesis.

PVAT could on one hand act as a physical barrier and delay acetylcholine response. However, our tracings indicate that the used protocol allows sufficient time to reach a maximal response and the presence of PVAT delayed the first response to acetylcholine from $10^{-8}$ M (-PVAT) to $10^{-7}$ M (+PVAT) (Suppl. Fig. 1), which is exemplarily for the other data. Alternatively, cholinesterase could be present in PVAT and reduce acetylcholine levels which was tested by pre-incubating with cholinesterase inhibitor neostigmine (Fig. 1C). Neostigmine significantly shifted the log EC50 in +PVAT segments, but had no effect on -PVAT segments (Fig. 1D). Furthermore, we detected acetylcholinesterase protein expression in PVAT from LM animals (Fig. 1E).

To test the effect of PVAT on vasodilation in a context of compromised VSMC function, we investigated vasodilation in SMC-KO mice which have decreased endothelium-dependent vasodilation [15] (Fig. 1A). Additionally, these mice show lipodystrophy resulting in loss of PVAT (Fig. 1E and Suppl. Fig. 2) and lower body weight compared to their healthy LM (Suppl. Fig. 3A). Functional decline of BAT and PVAT is also a characteristic of aging and therefore mimics natural aging in rodents and humans [14,24]. In contrast to LM, vasodilation was enhanced in +PVAT segments of SMC-KO mice, with increased maximal response and no significant rightward shift (Fig. 1A and B). We did not observe statistically significant differences in vascular responses with or without PVAT between males and females (Suppl. Fig. 3B and C). Therefore, all following results are presented for males and females together. In LM animals, the NO-pathway mainly contributes to vasodilation when compared to EDH. Furthermore, the relative contribution of NO was significantly enhanced in +PVAT segments (Fig. 2A and B). Although there was a trend in decreased EDH contribution by PVAT, the difference was not statistically significant because of high variation within the groups (Fig. 2B). There was a shift in NO-to-EDH ratio in –PVAT compared to +PVAT of 1.5 to 4.8. This reflects the well-known inverse interchange of both signaling pathways. In SMC-KO animals NO was the main contributor to vasodilation in –PVAT and + PVAT segments, while EDH could not be detected (Fig. 2C and D).

### 3.2. Adiponectin as ADRF

PVAT secretes various ADRF, and one of them is the adipokine adiponectin known to stimulate endothelial NO-production [3,8,9]. Therefore, we measured whether adiponectin is secreted from PVAT after stimulation with acetylcholine. In –PVAT samples, adiponectin levels were below the detection limit, whereas in +PVAT samples, adiponectin was already present at baseline and strongly increased after acetylcholine stimulation in LM and SMC-KO without statistical difference between the groups (Fig. 3A). Circulating adiponectin plasma levels were not statistically different between LM and SMC-KO (Fig. 3B).

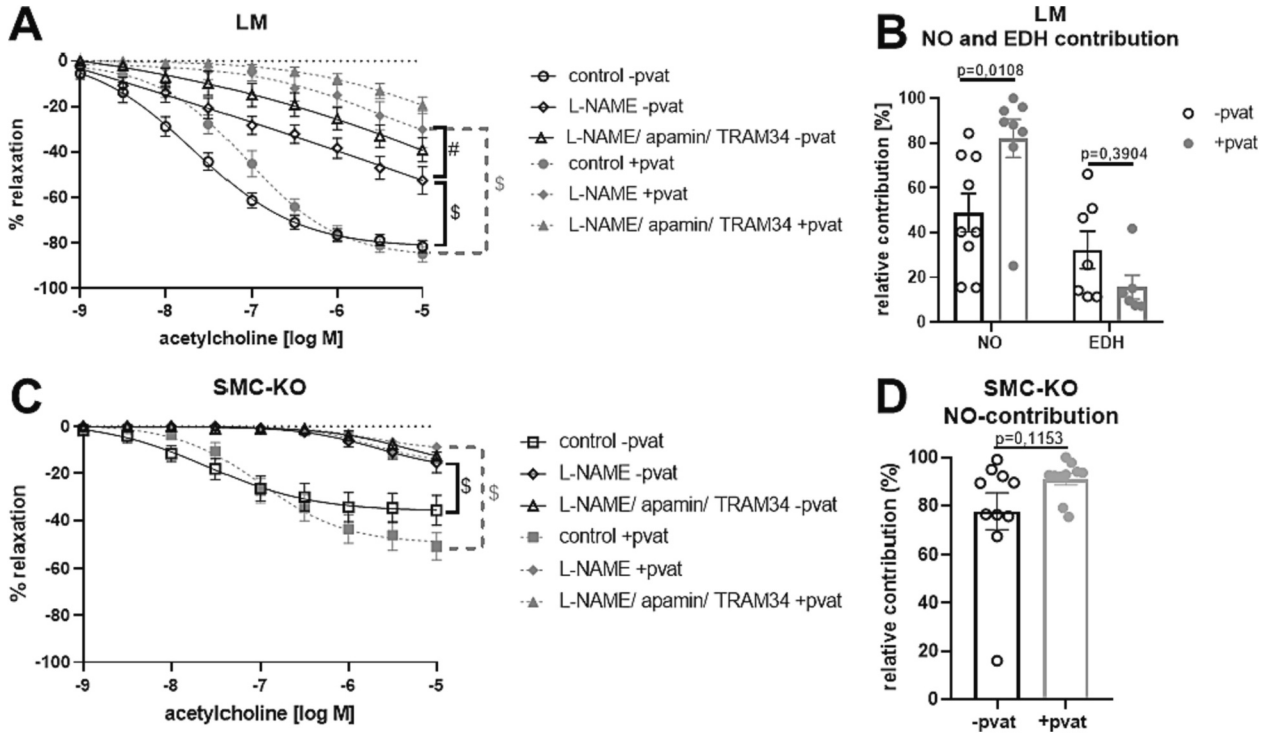


**Fig. 2.** Pathway contribution to endothelium-dependent vasodilation of thoracic aorta. A: NO- (L-NAME) and EDH- (L-NAME/ apamin/ TRAM34) contribution to vasodilation in LM animals and B: NO-contribution [%] to vasodilation in LM. C: NO- and EDH-contribution to vasodilation in SMC-KO animals and D: NO-contribution [%] to vasodilation in SMC-KO. Black symbols/lines were used for comparing -PVAT conditions and grey symbols/ grey dotted lines for +PVAT conditions. Dose-response curves were analyzed by GLM with repeated measures and bar graphs with 2-way ANOVA (B) or paired *t*-test (D). #: significant effect of PVAT ($p < 0.05$); $: significant effect of inhibitor ($p < 0.05$).

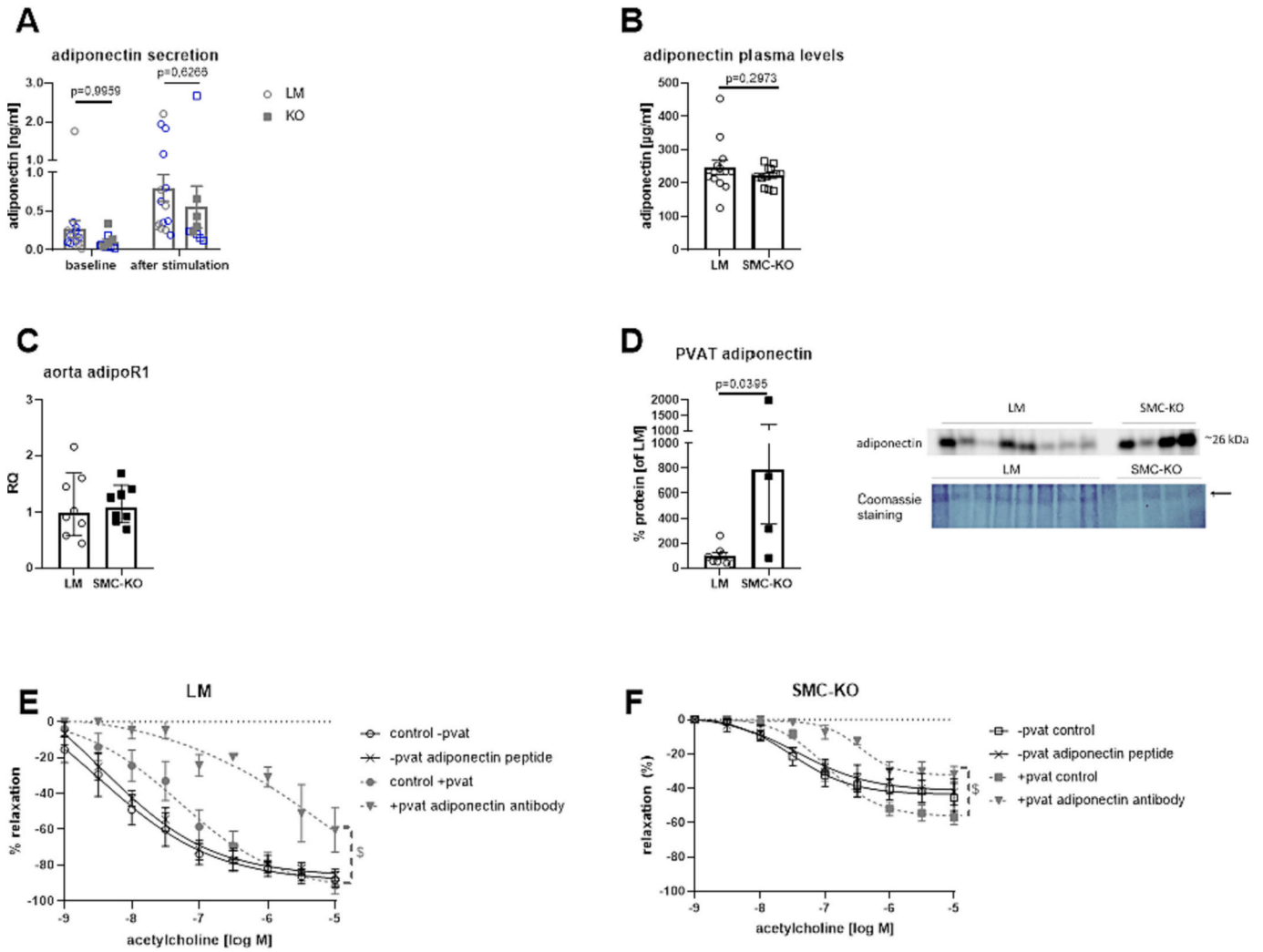


**Fig. 3.** A: Baseline adiponectin secretion and secretion after stimulation with acetylcholine from aortic segment with PVAT, blue: males, grey: females. B: Adiponectin blood plasma levels (evaluated by 2-way ANOVA). C: adiponectin receptor 1 mRNA expression in abdominal aorta. D: adiponectin protein expression in PVAT. Pictures show the adiponectin antibody staining (top) and the PageBlue protein stain (household gene correction with GAPDH or α-tubulin was not possible, see text and Suppl Fig. 4E. Arrow indicates the measured band). Effect of adiponectin-catching antibody and adiponectin peptide on endothelium-dependent vasodilation in LM (E) and SMC-KO (F), evaluated by GLM with repeated measures with $: significant effect of the antibody ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, we did not observe differences in adiponectin receptor 1 (AdipoR1) mRNA expression in abdominal aorta between the groups (Fig. 3C). However, protein expression of adiponectin in PVAT was higher in SMC-KO compared to their LM (Fig. 3D). Adiponectin protein expression was normalized to total protein, estimated by PageBlue (Coomassie) staining since GAPDH could not be used (similar mass as adiponectin) and α-tubulin as loading control showed significant differences between LM and SMC-KO which would affect the results (Suppl. Fig. 4E). Adiponectin immunohistological staining showed a similar trend of more intense staining in PVAT of SMC-KO compared to LM (Suppl. Fig. 2). Adiponectin secretion is influenced by sex-steroid hormones and it is known that adiponectin levels are higher in females compared to males [25]. We did not observe statistical differences between male and female mice regarding adiponectin secretion from PVAT Fig. 3A) or adiponectin plasma levels (Suppl. Fig. 3D) and therefore did not split the groups according to sex. Besides inducing NO-production, it is also known that noradrenaline-stimulated adiponectin secretion induces $BK_{Ca}$-channel signaling which counteracts vasoconstriction [6]. We therefore also checked the effect of $BK_{Ca}$-channel inhibitor iberiotoxin which showed a trend in -PVAT segments of SMC-KO animals, but was absent in +PVAT segments and there was no effect in LM animals (Suppl. Fig. 3E and F).

To further determine the involvement of adiponectin secretion in the acetylcholine response, we pre-incubated aortic +PVAT segments with an adiponectin which significantly decreased the acetylcholine response (Fig. 3E and F). However, incubation of –PVAT segments with adiponectin peptide right before adding acetylcholine did not affect acetylcholine response in LM or SMC-KO animals (Fig. 3E and F).

### 3.3. Molecular characterization

The abdominal aorta of SMC-KO showed increased expression of senescence markers which is characteristic for aging [26]. Here, we additionally analyzed mRNA expression of oxidative stress marker *nox2* which was increased in SMC-KO animals and protective marker *sirt-1* which was decreased in abdominal aorta. Moreover, there was a trend of decreased *eNOS* mRNA expression in SMC-KO animals compared to their LM (Fig. 4A-C). In PVAT, which has not been characterized in SMC-KO mice yet, we observed a trend of increased protein expression of senescence marker p21 and significantly elevated p16 (Fig. 4D-E). Furthermore, we detected dimers of eNOS for LM, whereas for SMC-KO both dimers and monomers were observed (Suppl. Fig. 4C).

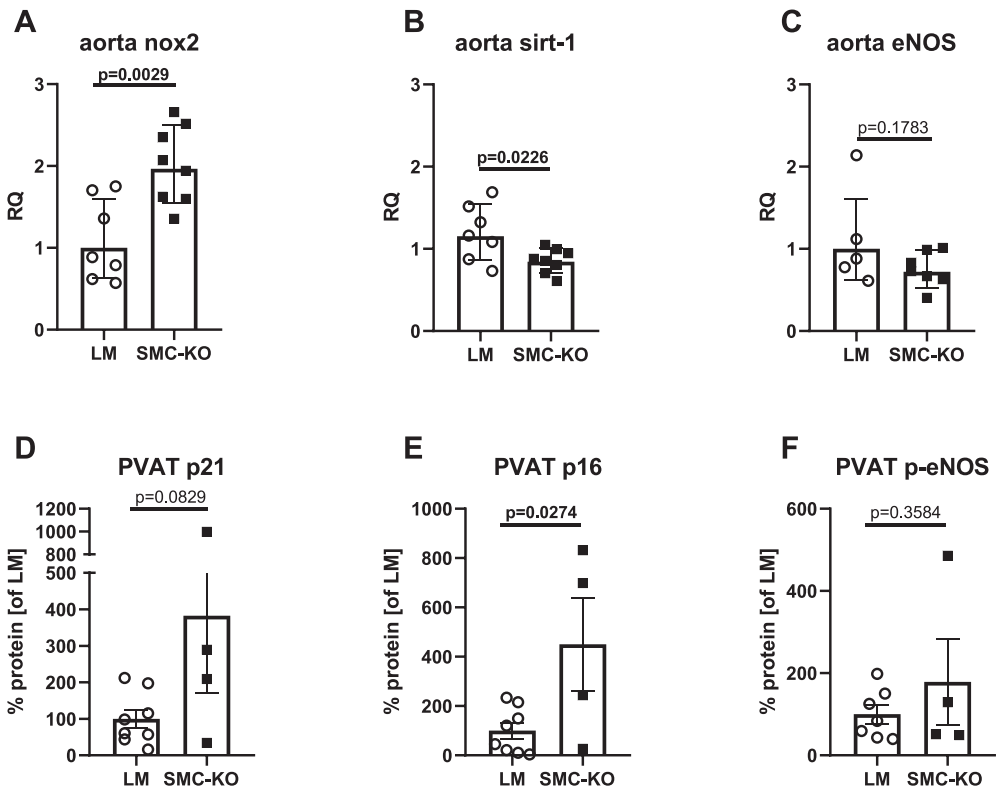


**Fig. 4.** mRNA expression in abdominal aorta (A-C) and protein expression in PVAT (D—F) of LM and SMC-KO animals evaluated by unpaired t-test with $p < 0.05$.

Phosphorylated eNOS (p-eNOS) protein expression was not different between the groups (Fig. 4F and Suppl. Fig. 4D).

## 4. Discussion

In this study we investigated the interaction of VSMC and PVAT in aorta of healthy mice (LM) and in a mouse model with accelerated development of impaired VSMC and PVAT function (SMC-KO), based on defective DNA repair due to genetic *Ercc1* knockout, a strategy used to rapidly mimic aging. Our results demonstrate that despite lipodystrophy of PVAT adiponectin release is preserved. Possibly this is due to an increased adiponectin production in remaining tissue. Also, there is a decrease of acetylcholinesterase in remaining adipose tissue, which may promote acetylcholine signaling. In SMC-KO this increases NO, and thus vasodilation. In wild-type LM NO is also increased, but compensatory loss of EDH, a well-known feedback mechanism [27], prevents increase of vasodilation to acetylcholine. This is further prevented by the presence of acetylcholinesterase in PVAT. The results suggest that aortic PVAT supports vasodilation in conditions that EDH is lacking, as demonstrated in SMC-KO. The proposed mechanism is summarized in Fig. 5.

Vascular aging is marked by decreased endothelium-dependent and -independent vasodilation [5], and this is mimicked in *Ercc1* mutant models at an accelerated rate [15,17,18]. Also, in alignment with previous reports, we show deregulation of aging markers p16, p21, sirt-1 and nox2 in Ercc1 mutant mice, features shared with aged wild-type mice [15,18,19,26,28]. In this novel study, we focused on the role of PVAT which has not been topic of studies yet. We showed that the aged PVAT of SMC-KO animals has similarities in aging marker changes observed in the aortic lamina media of SMC-KO animals [26] including increased p16 and p21 expression. This novel finding in PVAT is in line with previous studies in WAT and BAT of aged wild-type mice, and the accelerated adipose tissue aging mouse model of fabp4cre-mediated Ercc1 KO [19]. Thus, PVAT shows similar aging markers as WAT and BAT.

Another novel finding is that PVAT shows eNOS uncoupling, a feature also observed in aged endothelium [29]. The present results demonstrate that regardless the possibility that PVAT-located eNOS-mediated NO production should be compromised, there is still a contribution to acetylcholine-induced NO responses. The increase of adiponectin production in remaining PVAT and the possibility that this reaches the still intact endothelium seems a plausible explanation. The fact that we observed abundant adiponectin staining in endothelium is supportive to this paradigm. Nakladal et al. also reported that PVAT-derived NO compensated endothelial dysfunctions of aorta in aged pre-atherosclerotic apolipoprotein E (apoE)-deficient rats [30] which is in apparent agreement to the compensatory effect we observed in the SMC-KO mice. However, the finding in aged apoE rats was not directly connected to adiponectin-signaling, since mRNA adiponectin expression remained unchanged in PVAT, whereas eNOS mRNA was increased [30]. Therefore, our present study and the previous study by Nakladal et al. demonstrate two compensation mechanisms of aging PVAT: an increase in eNOS, and, if that should fail, an increase in adiponectin release. The appearance and result of these compensatory changes likely depends on the relative progression of PVAT vs. endothelial aging.

We here show for the first time that loss of aortic PVAT acetylcholinesterase helped to maintain acetylcholine responses. Previous studies indicated that only sympathetic nerves are present in mesenteric PVAT and that they act as reservoir for noradrenaline, preventing noradrenaline to reach the vessels and causing vasoconstriction [31]. However, transcriptome analysis of rat thoracic aorta PVAT indicated expression of acetylcholinesterase and related genes involved in the process of synaptic neurotransmission [32]. This observation is reminiscent of proposed varicosities of autonomic neurons in the perivascular plexus, which potentially release acetylcholine that can reach endothelial cells [33,34]. It was predicted that in large arteries, like aorta, neurotransmitters might not reach endothelial cells due to degradation along their path [34]. This seems to align with our present observations. The layout of the small wire organ bath setup, in which one halve of the lumen of the aortic ring is compressed on the other halve, might favor the

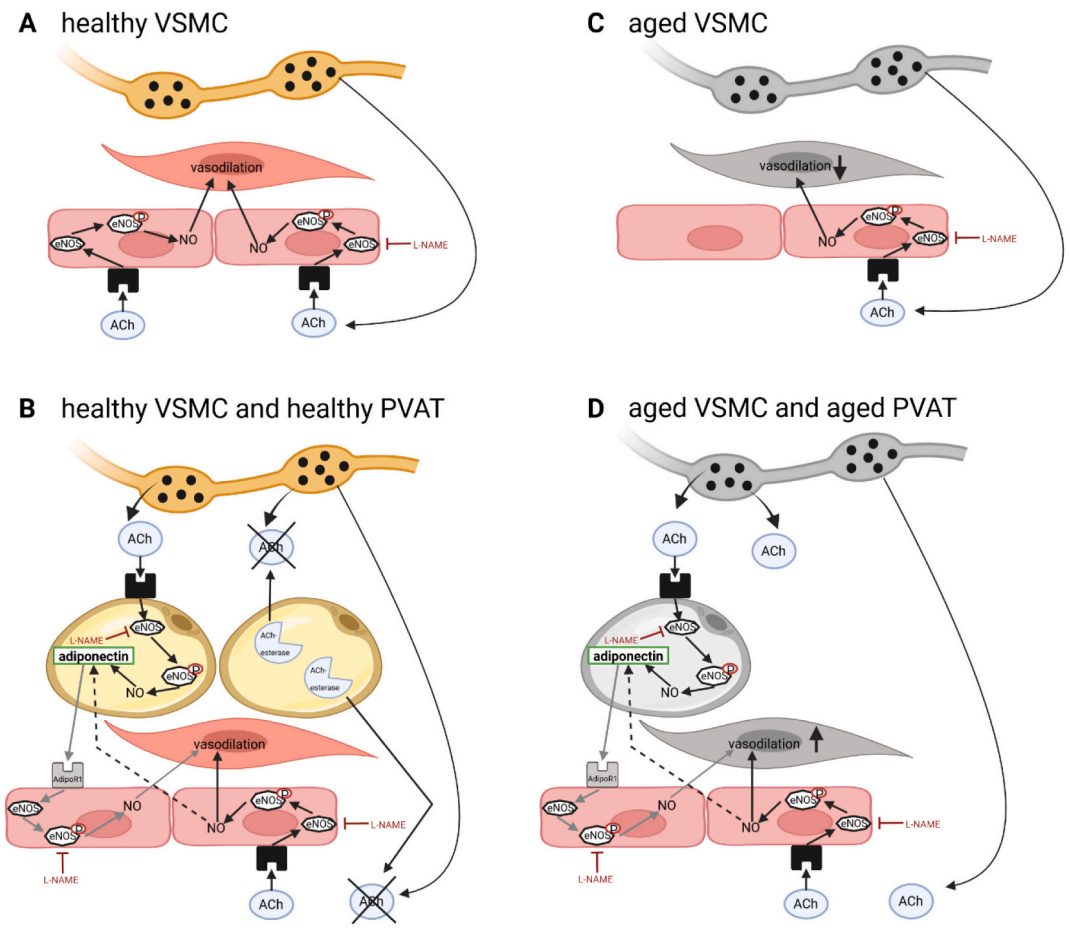


**Fig. 5.** Proposed mechanism for the effect of adiponectin and acetylcholinesterase on vasodilation in aorta of healthy animals (LM) (Panel A and B) and animals with aged VSMC and PVAT (SMC-KO) (Panel C and D). In LM vasodilation is mainly NO-dependent (A) which is decreased in SMC-KO animals leading to decreased maximal vasodilation (B). When the healthy PVAT remains around the aortic segments (C), adiponectin is secreted from the PVAT which increases the NO-contribution to vasodilation. Moreover, acetylcholine-esterase (ACh-esterase) is present in the PVAT decreasing the availability of acetylcholine which is released from autonomic nerve terminals within varicosities. The balance between the two results in no change of maximal vasodilation. In SMC-KO animals, the amount of PVAT is significantly decreased (D). However, adiponectin is still secreted from the PVAT leading to increased vasodilation compared to C. ACh-esterase seems to be absent. ACh: acetylcholine, AdipoR1: adiponectin receptor 1, (p-)eNOS: (phosphorylated) endothelial nitric oxide synthase, L-NAME: eNOS inhibitor. Created with BioRender.com.

approach of acetylcholine from the adventitial through the media to the endothelium. The observed fast responses to acetylcholine indicate swift diffusion in this well-stirred and large organ bath that provide copious amounts of acetylcholine. It is also conceivable that in a physiological situation acetylcholine could derive from varicosities in the PVAT, and it is in agreement with the high amount of acetylcholinesterase that is normally co-localized with the site of neurotransmitter release. Alternatively, acetylcholine might come from the vasa vasorum, the arteriolar structure in the PVAT that nourishes the adventitia. Apparently, during aging the PVAT is able to adapt to circumstance of reduced acetylcholine response by lowering the cholinesterase.

Adiponectin can have a similar effect as acetylcholine, activating eNOS through the adiponectin receptor 1 (AdipoR1) which can either be located on EC. eNOS activation by adiponectin can also occur in the PVAT itself, which could act as a backup source in case of decreased endothelial function [35,36]. This was visible in SMC-KO. In wild-type LM however, the NO increase is compensated by EDH loss, a well-known phenomenon in healthy vasodilation responses. To our knowledge this is the first study describing this NO-EDH interaction occurs due to combined acetylcholine and PVAT-derived adiponectin stimulation. The EDH-NO interchanges have been identified as the results of changes in endothelial Ca2+ handling [27]. Similar studies might be needed for adipocytes. In VSMC $BK_{Ca}$-channels are activated through AdipoR1, also leading to vasodilation which counteracts vasoconstriction [3,9,35,37,38]. $BK_{Ca}$-channel activation appeared not to be relevant for adiponectin relaxation in our mouse model.

Pre-incubation of +PVAT segments with an adiponectin-neutralizing antibody led to significantly decreased vasodilation in LM and SMC-KO animals. Therefore, the preserved secretion of adiponectin in SMC-KO mice seems to be a compensatory mechanism which could partially rescue vasodilation when the maximal response to NO is attenuated, such as in aging. In agreement, increased endothelial adiponectin was also associated with increased brachial artery flow-mediated dilation in elderly without changes in adiponectin plasma levels [39]. However, the effect of PVAT was not investigated in this study. Other protective effects in aging have also been reported. A recent study showed that in aged mice (140 weeks-old) overexpression of adiponectin preserved adiponectin plasma levels, which led to improved insulin sensitivity and decreased age-related inflammatory markers in adipose tissue depots compared to the control mice of same age [40].

To sum up, our data suggest a supporting effect of adiponectin on acetylcholine-induced vasodilation in the aorta of SMC-KO mice, which seems to be dependent on the presence of PVAT. In LM in contrast, there appears to be a balance between cholinesterase activity, EDH, and adiponectin-induced increased NO-contribution to vasodilation, whereas total vasodilation is not changed. The data were demonstrated

in aorta, where both EDH and NO are acting under healthy conditions, but NO and EDH are at least partly lost under accelerated aging conditions. Other vascular beds might act differently depending on the composition of the vasodilation signaling pathways and the PVAT itself [10–12].

In conclusion, we showed that aortic PVAT on the one hand promotes the NO-mediated part of relaxation responses to acetylcholine via adiponectin, but on the other hand contains cholinesterase activity which reduces the efficacy of this neurotransmitter. Furthermore, PVAT can increase maximal NO-response in conditions where this is reduced, such as in aging. Even severe lipodystrophy, as present in the SMC-KO mice, does not prevent this role. Therefore, stimulation of adiponectin release by PVAT could be an interesting target to support NO-mediated processes in aged conduit arteries.

## Authors statement

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## CRediT authorship contribution statement

**A.A. Jüttner**: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Visualization, Writing - original draft. **E. Ataei Ataabadi**: Investigation. **K. Golshiri**: Investigation. **R. de Vries**: Investigation. **I.M. Garrelds**: Investigation. **A.H.J. Danser**: Writing - review & editing, Supervision. **J.A. Visser**: Writing - review & editing, Supervision. **A.J.M. Roks**: Conceptualization, Supervision, Project administration, Funding acquisition, Writing - review & editing.

## Declaration of competing interest

J.A.Visser has received royalties from AMH assays, paid to the institute/laboratory with no personal financial gain.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2024.107281.

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