ORIGINAL RESEARCH

In Vivo Magnetic Resonance Imaging-Based Detection of Heterogeneous Endothelial Response in Thoracic and Abdominal Aorta to Short-Term High-Fat Diet Ascribed to Differences in Perivascular Adipose Tissue in Mice

Anna Bar, PhD; Anna Kieronska-Rudek, MSc; Bartosz Proniewski, PhD; Joanna Suraj-Prażmowska, PhD; Krzysztof Czamara, PhD; Brygida Marczyk, MSc; Karolina Matyjaszczyk-Gwarda, PhD; Agnieszka Jasztal, MSc; Edyta Kuś, PhD; Zuzanna Majka, MSc; Agnieszka Kaczor, PhD; Anna Kurpińska, PhD; Maria Walczak, PhD; Elsbet J. Pieterman, B.Sc; Hans M. G. Princen, PhD; Stefan Chlopicki, MD, PhD

BACKGROUND: Long-term feeding with a high-fat diet (HFD) induces endothelial dysfunction in mice, but early HFD-induced effects on endothelium have not been well characterized.

METHODS AND RESULTS: Using an magnetic resonance imaging-based methodology that allows characterization of endothelial function in vivo, we demonstrated that short-term (2 weeks) feeding with a HFD to C57BL/6 mice or to E3LCETP mice resulted in the impairment of acetylcholine-induced response in the abdominal aorta (AA), whereas, in the thoracic aorta (TA), the acetylcholine-induced response was largely preserved. Similarly, HFD resulted in arterial stiffness in the AA, but not in the TA. The difference in HFD-induced response was ascribed to distinct characteristics of perivascular adipose tissue in the TA and AA, related to brown- and white-like adipose tissue, respectively, as assessed by histology, immunohistochemistry, and Raman spectroscopy. In contrast, short-term HFD-induced endothelial dysfunction could not be linked to systemic insulin resistance, changes in plasma concentration of nitrite, or concentration of biomarkers of glycocalyx disruption (syndecan-1 and endocan), endothelial inflammation (soluble form of vascular cell adhesion molecule 1, soluble form of intercellular adhesion molecule 1 and soluble form of E-selectin), endothelial permeability (soluble form of fms-like tyrosine kinase 1 and angiopoietin 2), and hemostasis (tissue plasminogen activator and plasminogen activator inhibitor 1).

CONCLUSIONS: Short-term feeding with a HFD induces endothelial dysfunction in the AA but not in the TA, which could be ascribed to a differential response of perivascular adipose tissue to a HFD in the AA versus TA. Importantly, early endothelial dysfunction in the AA is not linked to elevation of classical systemic biomarkers of endothelial dysfunction.

Key Words: endothelial function ■ high-fat diet-fed mice ■ magnetic resonance imaging ■ perivascular adipose tissue ■ thoracic and abdominal aorta
Endothelial dysfunction is a hallmark of various diseases including metabolic syndrome, and the assessment of NO-dependent vasodilation in the coronary or peripheral circulation predicts adverse cardiovascular events and poor long-term outcomes.

In experimental conditions, in various models of endothelial dysfunction, endothelium-dependent function in large and small vessels is classically studied in the myograph setups, and perivascular adipose tissue (PVAT) is usually removed for studies in isolated aorta preparation, even though this tissue has a well-documented influence on endothelial function.

Indeed, PVAT plays an important role in the regulation of vascular function. Under physiological conditions, PVAT releases a number of adipokines, such as adipocyte-derived relaxing factor, which have a beneficial anticontractile effect on vascular function and are essential for the maintenance of vascular homeostasis. Accumulation of PVAT, e.g., in obesity, was associated with impaired anticontractile function as well as perivascular inflammation.

Altogether, there is increasing experimental evidence to suggest that both structural and functional alterations in PVAT, in obesity and metabolic syndrome, might contribute to endothelial dysfunction, increasing the risk for atherosclerosis and hypertension development. Furthermore, there are numerous reports describing long-term effects (>8 weeks) of high-fat diet (HFD) feeding on endothelial function in mice.

However, to the best of our knowledge, there are no reports that have characterized the effects of short-term HFD feeding on endothelial function, taking into account heterogeneous characteristics of PVAT in various parts of the aorta and their distinct sensitivity to HFD-induced inflammation.

Regional differences in PVAT even within different regions of the same vessel, e.g., in the aorta, were reported. In rodents, fat surrounding the thoracic aorta (TA) has brown adipose tissue–like characteristics, whereas periaortic fat in the abdominal aorta (AA) has similar features to white adipose tissue. Generally, brown PVAT shows resistance to HFD-induced inflammation in the perivascular tissue in contrast to white PVAT, which is highly responsive to HFD with marked increases in macrophage infiltration and cytokine expression, leading to increases in oxidative stress.
However, it is not known whether regional differences of PVAT in the aorta between AA and TA influence development of endothelial dysfunction in the early phase of HFD.

Accordingly, the aim of the present study was to characterize a possibly distinct response of endothelium in the AA and TA in relation to PVAT characteristics (assessed by histology, immunohistochemistry, and Raman spectroscopy) after short-term (2, 4, and 8 weeks) feeding with a HFD in mice. To assess endothelial function in vivo, we used a 3-dimensional (3D) magnetic resonance imaging (MRI)-based method. This methodology does not have the limitations of ex vivo vascular studies and allows study of endothelial function in vivo in the presence of intact PVAT surrounding the studied vessel. To determine whether systemic effects of short-term HFD feeding might account for the observed differences in endothelium-dependent vasodilation in the TA and AA, we comprehensively assessed the systemic response to short-term HFD feeding by measuring insulin resistance, plasma concentrations of nitrite and nitrate, oxidative stress in red blood cells, and plasma concentration of various biomarkers of endothelial dysfunction.

METHODS

The data, analytic methods, and study materials will be made available on request to other researchers for purposes of reproducing the results or replicating the procedures.

Animals

Studies were performed in 6-week-old C57BL/6 male mice (from Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland), fed a HFD (HFD60%, 60 kcal% of fat +1% of cholesterol; ZooLab, Krakow, Poland) for 2, 4, and 8 weeks (measurements at 8, 10, and 14 weeks of age, respectively) in comparison to age-matched C57BL/6 male mice fed a control diet (AIN93G, ZooLab). Moreover, to confirm that regional differences in endothelial response in the aorta in response to a HFD can also be observed as early as after 2 weeks in another murine model, we chose APOE*3-Leiden, human Cholesteryl Ester Transfer Protein transgenic (E3L.CETP) mice (from the Netherlands Organisation for Applied Scientific Research, Leiden, Netherlands), an established and well-recognized model for hyperlipidemia and atherosclerosis. MRI experiments were performed using a 9.4 T scanner (BioSpec 94/20, USR). During the MRI experiment, mice were anaesthetized using isoflurane (Aerrane, Baxter Sp. z o. o., 1.5 vol%) in oxygen and air (1:2) mixture and imaged in the supine position. Heart function (rhythm and ECG), respiration, and body temperature (maintained at 37°C using circulating warm water) were monitored using a Monitoring and Gating System (SA Instruments, Inc.).

Assessment of Insulin Resistance Based on GTT

Before GTT was performed, mice were fasted for 4 hours with access to water. Baseline glucose level was measured using a standard glucometer in a drop of blood from the tail, cut at the end. Blood glucose concentration measurements were repeated after 15, 30, 45, 60, and 120 minutes of intraperitoneal glucose administration (2 g/kg body weight). Results are presented as a curve of blood glucose concentration in time and as an area under the curve.

Assessment of Acetylcholine-Induced Vasodilation In Vivo by MRI

MRI experiments were performed using a 9.4 T scanner (BioSpec 94/20, USR). During the MRI experiment, mice were anaesthetized using isoflurane (Aerrane, Baxter Sp. z o. o., 1.5 vol%) in oxygen and air (1:2) mixture and imaged in the supine position. Heart function (rhythm and ECG), respiration, and body temperature (maintained at 37°C using circulating warm water) were monitored using a Monitoring and Gating System (SA Instruments, Inc.).
Endothelial function in vivo was assessed by a previously described technique as an endothelium-dependent response to acetylcholine (Ach) administration. Response to injection of Ach (Sigma-Aldrich; 50 μL, 16.6 mg/kg, IP; dose of Ach was based on a previous study) was analyzed in the AA and TA.

Vasomotor responses were examined by comparing 2, time-resolved 3D images of the vessels before and 25 minutes after intraperitoneal Ach administration. The optimal time to measure Ach-induced vasorelaxation was chosen based on our previous work. 3D images of the aorta were acquired using the cine IntraGate FLASH 3D sequence and reconstructed with the IntraGate 1.2.b.2 macro (Bruker). Analysis was performed using ImageJ software (National Institutes of Health) and scripts written in MATLAB (MathWorks), in the hyperstack of the AA (10 slices in diastole, from the renal arteries down) and the TA (10 slices in diastole, from the celiac artery up). All cross-sectional areas of vessels at each slice were obtained using thresholding segmentation and exported to MATLAB, where vessel volumes were reconstructed and calculated.

Imaging parameters included the following: repetition time—6.4 ms, echo time—1.4 ms, field of view—30x30x14 mm³ for aorta, matrix size—256x256x35, flip angle—30°, and number of accumulations—15, reconstructed to 7 cardiac frames. Total scan time was about 12 minutes.

Assessment of Aortic Pulse Wave Velocity
Pulse wave velocity (PWV) measurements were performed using a Doppler flow velocity system (Indus Instruments, Scintica Instrumentation). During Doppler measurements, mice were anaesthetized using isoflurane (Aerrane; Baxter Sp. z o. o., 1.5 vol%) oxygen and air (1:2), mixture. Mice were taped supine to ECG electrodes incorporated into a temperature-controlled printed circuit board. The temperature of each mouse was monitored with a rectal probe and body temperature was maintained at 37°C. Before measurements, the chest and right hind limb of the mice were shaved.

The PWV, an index of arterial stiffness, was measured using two 20-MHz Doppler probes, by simultaneously recording velocity signals from 2 sites, representative for the TA and AA. The TA measurements were performed by measuring velocities in the aortic arch and in the descending aorta about 15 to 20 mm distal to the first probe. To obtain a signal from the descending aorta, the probe was placed just under the sternum and angled toward the heart at a depth of 4 mm. Measurements in the TA were performed by placing the second probe tip at the base of the left upper limb, oriented almost parallel to the board surface and aimed toward the upper mid-chest at a depth of 6 mm. The AA measurements were performed by measuring the velocities in the descending aorta and ~25 to 30 mm distal to the first probe in the right femoral artery by placing the second probe on the right hind limb of the mice and angled toward the murine femoral artery at a depth of 3 to 4 mm. Aortic PWV was calculated by dividing the separation distance by the difference in arrival times of the velocity pulse timed with respect to the ECG. Signal analysis was performed using Doppler signal processing workstation (version 1.625, Indus Instruments).

Blood Sampling and Tissue Collection
At the end of the experiment, mice were anaesthetized (100 mg/kg ketamine+10 mg/kg xylazine, IP) and blood was drawn from the heart and collected in tubes containing 10% solution of ethylenediaminetetraacetic acid dipotassium salt (Aqua-Med; 1 μL of EDTA/100 μL of blood). Next, blood was mixed with MS-SAFE Protease and Phosphatase Inhibitor (Sigma-Aldrich) in a ratio of 100:1. All samples were centrifuged at 664g, at a temperature of 4°C for 10 minutes to isolate plasma, as previously described. Obtained plasma samples were deep-frozen at −80°C for high-performance liquid chromatography measurements of nitrate (NO₃⁻) and nitrite (NO₂⁻) concentrations by ENO-20 NOx Analyser as well as for microLC/MS-MRM measurements of biomarkers of endothelial dysfunction. The remaining blood was deep-frozen at −80°C for measurements of glutathione concentration in red blood cells as described below. Aorta, PVAT, and liver were collected for further ex vivo assessments as described below. Moreover, liver, as well as perirenal adipose tissue and epididymal adipose tissue, were weighted after collection.

Histological Assessment of Liver Steatosis
Liver sections were stained with Oil Red O for lipids visualization, and at least 9 images of each liver were randomly obtained under x200 magnification. In detail, the image segmentation was performed using simple intensity thresholding. First, the source image was thresholded to extract the tissue image from the background, next within the tissue area another thresholding step was performed to obtain red, Oil Red O–stained lipids droplets mask. Finally, the area of the background, tissue, and lipid droplets were calculated. Liver steatosis was expressed as the percentage of fat area versus tissue area, calculated in the left liver lobe, using the Columbus Image Data
Characteristic of PVAT of the TA and AA by Immunohistochemistry

Formalin-fixed and paraffin-embedded thoracic and abdominal parts of the aorta with PVAT were cut into 5-μm slices. Antigen retrieval was performed according to the standard protocol. Area of the brown and white adipose tissue in PVAT was assessed by double staining using perilipin and UCP-1 (uncoupling protein 1) antibodies. To visualize the total PVAT area, a perilipin antibody (primary anti–perilipin-1 antibody, ab61682) was used, whereas the area of brown adipose tissue was identified by UCP-1 staining (primary anti–UCP-1 antibody, ab209483). Sections were incubated with the secondary antibodies FITC-conjugated donkey anti-goat IgG (705-454-003) and Cy3-conjugated donkey anti-rabbit (711-165-152). To characterize the functional differences between brown and white adipose tissue, the lectin I and phosphorylated endothelial NO synthase (Phospho-eNOS) were stained. To visualize glycoalx of microvessels in PVAT, biotinylated Lectin 1 (B-1105) was used. Phospho-eNOS—Ser1177 (9571) (primary Phospho-eNOS [Ser1177] antibody, #9571) was used to detect inflammatory processes in endothelium. Then, sections were incubated with the secondary antibody biotinylated-conjugated goat anti-rabbit IgG (111-065-003). Then the slices were incubated with VECTASTAIN Elite ABC-HRP Kit and diaminobenzidine (Sigma) to obtain the colour reaction. Subsequently, the cross-sections of the TAs and AAAs with PVAT were photographed (×100 magnification) and images were acquired using an AxioCam MRc5 digital camera and an AxioObserver 22 D1 inverted fluorescent microscope (Zeiss) or BX51 microscope (Olympus). Before analysis in the pictures with Lectin I and Phospho-eNOS nonadipose tissue (aorta, muscles, lymph nodes) fragments were manually excised. Image segmentation was performed using Ilastik (developed by the Ilastik team, with partial financial support of the Heidelberg Collaboratory for Image Processing, HHMI Janelia Farm Research Campus and CellNetworks Excellence Cluster). The pixels corresponding to the UCP-1 immunopositive area, as well as perilipin immunopositive area (FITC channel), were quantitatively determined using ImageJ software 1.46r as demonstrated in Figure S1. The mean number of pixels representing UCP-1 and perilipin were counted using ImageJ software. The results were expressed as the ratio of brown adipose tissue area (number of UCP-1 immunopositive pixels/PVAT area), area of mirovessels (number of Lectin I immunopositive pixels/PVAT area), or activated endothelium (Phospho-eNOS immunopositive pixels/PVAT area) and normalized to the respective control.

Characteristic of PVAT of the TA and AA by Raman Spectroscopy

Samples of the interscapular brown adipose tissue (iBAT), epididymal white adipose tissue (eWAT; from the adipose tissue attached to the epididymis and testicle), and PVATs of the TA and AA were isolated from wild-type C57BL/6J male mice fed an AIN93G or HFD for 4 weeks. Periaortic PVAT samples of the TA and AA were extracted from the descending aorta distal to the aortic arch and from the part of the artery lying in the abdominal cavity, respectively. For Raman spectroscopic measurements, all samples were rinsed in NaCl isotonic solution to wash traces of blood and put onto CaF2 slides as previously described. The Raman spectra of the iBAT, eWAT, and PVAT samples were acquired using a confocal WITec Alpha300 Raman spectrometer (WITec) equipped with a 532-nm laser, 20x air objective (Nikon S Plan Fluar, numerical aperture=0.45), UHTS 300 spectrograph (600 grooves·mm−1 grating), and CCD detector (Andor, DU401A-BV-352). For each sample, at least 5 single Raman spectra were collected using the maximum laser power at the sample position (~28 mW). The single spectra of 32 accumulations and the integration time of 0.5 second were normalized in the 1800 to 400 cm⁻¹ spectral range and averaged over all mice for each studied adipose tissue type.

All Raman spectra underwent routine preprocessing including baseline correction using an autopolynomial of degree 3 and an automatic cosmic ray removal procedure. Preprocessing was performed using the WITec Project Plus software. Raman spectra were averaged per type of adipose tissue and presented using the OriginPro 9.1 program. The OPUS 7.2 program was used for calculations of the integral intensity (I) of the bands at ca. 1660 and 1445 cm⁻¹, enabling estimation of the lipid unsaturation degree (I1660/I1445). The results were tested by ANOVA performed using Prism software (GraphPad Software, Inc.) to characterize the differences in unsaturation of lipids in various types of the adipose tissue in a quantitative manner.

Assessment of Endothelial NO Production in the Aorta Using Electron Paramagnetic Resonance

For measurements of eNOS-dependent NO production, electron paramagnetic resonance (EPR)
spin-trapping with diethylthiocarbamic acid sodium salt (DETC) was used ex vivo, as previously described,34 with minor modifications. The isolated aorta was divided into 2 parts: the abdominal and thoracic sections. Only half of the group of samples of the AA and TA were cleared from surrounding tissue, and the other half were analyzed in the presence of PVAT. Parts of the aorta were opened longitudinally and preincubated with 10 μmol/L of N6-(1-iminoethyl)-lysine, hydrochloride (L-NIL) in Krebs-HEPES buffer for 30 minutes at 37°C. Addition of L-NIL during the preincubation period allowed the direct measurements of NO produced by eNOS without the signal from NO produced by inducible NO synthase expressed by macrophages in atherosclerotic plaque,35 as previously described.30,34,36 Next, DETC (3.6 mg) and FeSO₄·7H₂O (2.25 mg) were separately dissolved under argon gas bubbling in two 10-mL volumes of ice-cold Krebs–Hepes buffer and were kept under gas flow on ice until use. After preincubation, a spin trap (125 μL of FeSO₄·7H₂O and 125 μL of DETC—final concentration of the colloid: 285 μmol/L) and calcium ionophore A23187 (the final concentration: 1 μmol/L) were added to the parts of aorta. Subsequently, incubation for 90 minutes at 37°C was started. Finally, dried aorta was weighed and frozen in liquid nitrogen (suspended in fresh buffer) into the middle of a 400-μmOD capillary (60.2 cm total length, 200 μm. Separation of the analytes took place in an uncoated fused-silica capillary (60.2 cm total length, 50 cm effective length, 50 μm ID, and 375 μm OD) thermostated at 25°C with a constant voltage of 25 kV (=6.5 μA). BisTRIS (75 mmol/L), boric acid (25 mmol/L) buffer adjusted to pH 7.8 by adding 1 mol/L NaOH, was chosen as a background electrolyte. Samples were introduced to the capillary by hydrodynamic injection for 20 seconds by 3.5 kPa, followed by injection of ultrapure H₂O for 2 seconds by 3.5 kPa. Between analytical runs, the capillary was rinsed with 1 mol/L of NaOH, ultrapure water, and background electrolyte, respectively (138 kPa; 2 minutes each). Obtained data were analyzed by PeakFit software (version 4.12, Systat Software).

Measurements of reduced glutathione and oxidized glutathione were performed as described by Hempe et al.37 Briefly, red blood cells were first separated from plasma by centrifuging whole blood. A hemolysate was prepared by adding to 50 μL of red blood cells, 200 μL of hemolyzing reagent (10 mmol/L KCN and 5 mmol/L EDTA in ultrapure H₂O). Then, samples were deproteinized by adding 100 μL of 5% metaphosphoric acid to 100 μL of hemolysate. After centrifugation (10 000g for 10 minutes at 4°C), the metaphosphoric acid extracts were diluted 1:4 with ultrapure H₂O and then used for capillary electrophoresis analysis. All chemicals except boric acid (J.T. Baker) and NaOH (VWR International) were purchased from Sigma-Aldrich.

### Assessment of Biomarkers of Endothelial Dysfunction in Plasma by MicroLC/MS-MRM

Assessment of 10 protein biomarkers of endothelial dysfunction was performed using a microLC/MS-MRM method as previously described.26,30,38–40 The panel included biomarkers of various aspects of endothelial dysfunction such as glycolalx disruption: syndecan-1 and endocan; endothelial inflammation: soluble form of vascular cell adhesion molecule 1, soluble form of E-selectin and soluble form of intercellular adhesion molecule 1; endothelial permeability: angiopoietin 2 and soluble form of fms-like tyrosine kinase 1; and hemostasis: von Willebrand factor, tissue plasminogen activator and plasminogen activator inhibitor 1.

A UPLC Nexera system (Shimadzu) connected to a highly sensitive mass spectrometer QTrap 5500 (Sciex) was used. During sample preparation, the murine plasma was subjected to proteolytic digestion using porcine trypsin to achieve unique and reproducible peptide sequences, applied as the surrogates of the proteins suitable for LC-MS/MS analyses. A detailed description of the targeted analysis of a selected panel of proteins was presented elsewhere.26,38,39

### Statistical Analysis

The study included separated mice groups to collect organs and tissue for further analysis after 2, 4, and 8 weeks of HFD feeding. Data are presented as mean and SD or in the case of a lack of distribution (Shapiro–Wilks test), as median and interquartile range. Statistical tests were performed using GraphPad Prism. Nonparametric (Kruskal–Wallis test
or Mann–Whitney U test) or parametric (1- and 2-way ANOVA with Tukey test, repeated measures ANOVA, unpaired t Student test) tests were performed. A value of P<0.05 was considered to be statistically significant.

RESULTS

General Characteristics of C57BL/6 Mice Fed a HFD (HFD60%) for 2 to 8 Weeks

Effects of feeding with an HFD60% were manifested by insulin resistance, as evidenced by an increased level of blood glucose concentration in GTT. In C57BL/6 mice fed an HFD60% for only 2 weeks, the area under the curve ≈2-fold higher in C57BL/6 mice fed a HFD60%, in comparison to C57BL/6 mice fed a standard diet, and was maintained at a similar level after 4 and 8 weeks of HFD60% feeding (Figure 1B).

HFD60% also induced mild liver steatosis that was similar after 2, 4, and 8 weeks of HFD60% (in a range between 9.1% and 14.7% as compared with 1.0% in mice fed a standard diet; Figure 1E). Early liver steatosis was not associated with changes in body weight (Figure 1A), liver mass (liver mass ≈1.0 g in all experimental groups), or changes in the mass of epididymal (Figure 1C) and perirenal (Figure 1D) adipose tissue. However, after 4 and 8 weeks of an HFD60%, the masses of epididymal adipose tissue and perirenal adipose tissue were ≈2- and 3-fold higher, respectively, and body weight also changed (by 20% in comparison to mice fed a standard diet).

Early Impairment of Acetylcholine-Induced Vasodilation and NO Production in the AA But Not in the TA in C57BL/6 Mice Fed HFD (HFD60%)—the Relationship With PVAT Characteristics

After 2 weeks’ feeding of C57BL/6 mice with an HFD60%, impairment of Ach-induced vasodilation in the AA (Figure 2B) was observed as paradoxical vasoconstriction (volume changes of the AA: −27.8% in comparison to 13.7% in age-matched control mice) with progression of endothelial dysfunction in HFD60% mice after 4 and 8 weeks of HFD60% feeding. In contrast, Ach-induced vasodilation in the TA was fully preserved throughout the 2- to 8-week period of HFD60% feeding (Figure 2A).

Moreover, 4 to 8 weeks of feeding with HFD60% induced arterial stiffness as evidenced by an increased value of the PWV in the AA (≈1.5-fold increase of PWV in comparison to control mice, Figure S2B), which was not observed in the TA (Figure S2A).

Differential response to HFD60% of the AA and TA was also confirmed by EPR measurements of NO production in aorta ex vivo (Figure 3A through 3D). In aorta with preserved PVAT, NO production level was maintained in the TA (Figure 3A) and decreased (≈30%) in the AA (Figure 3B) in HFD60%-fed mice in comparison to control mice. Furthermore, impaired NO production in the aorta with and without PVAT was observed in the AA (Figure 3D) but not in the TA (Figure 3C).

The difference in the AA and TA response to a short period of HFD60% was associated with distinct characteristics of PVAT in the TA and AA, related to brown and white adipose tissue, respectively. In fact, 2 weeks of HFD60% feeding induced a nonsignificant fall (P=0.2) in the amount of brown adipose tissue in the AA, which was even more pronounced and significant (≈2.5-fold decrease of brown adipose tissue amount) after 4 to 8 weeks of HFD60% (P=0.02 and P=0.03, respectively) (Figure 2D and 2F), whereas in the TA, HFD60% feeding did not induce any significant changes in the amount of brown fat tissue in PVAT (Figure 2C and 2E) after 2, 4, or 8 weeks of HFD feeding. Furthermore, 2 weeks of HFD60% feeding induced a significant increase in the lectin (Figure S3A and S3B) and a decrease in the Phospho-eNOS (Figure S3C and S3D) contents in PVAT of the aorta, with the latter change more pronounced in the AA (lectin: 12% higher, Phospho-eNOS: 11% lower in comparison to control mice; Figure S3B and S3D) than in the TA (lectin: 6% higher, Phospho-eNOS: 7% lower in comparison to control mice; Figure S3A and S3C), although the observed difference was not statistically significant.

To confirm AA and TA differential response to HFD60%, the lipid unsaturation degree was estimated by Raman spectroscopy in PVAT of the TA (Figure S4A) and AA (Figure S4B) in mice after 4 weeks of HFD60% feeding and compared with this parameter for the iBAT (Figure S4C) and eWAT (Figure S4D). HFD60% feeding induced a statistically significant (14%) decrease in lipid unsaturation in the PVAT of the AA that was qualitatively similar to a reduction of lipid unsaturation in the white adipose tissue in eWAT (20%). The decrease of lipid unsaturation was manifested as a decreased intensity of Raman bands at 1660 and 1268 cm−1 assigned to the C=C stretching and =C–H deformation vibrations, respectively, and increased intensity of the band at 1445 cm−1, attributable to the CH bending vibrations.41 Thus, the ratio of the integral intensities of bands at 1660 and 1445 cm−1 was chosen to assess the lipid unsaturation in the PVAT of the AA and in white adipose tissue.

Collectively, the Raman spectroscopy measurements showed similar biochemical response in PVAT in the AA and in white adipose tissue and
Figure 1. Effects of high-fat diet (HFD; HFD₀%₀) feeding on weight of mice, insulin resistance, epididymal and perirenal adipose tissue, and liver steatosis.

Changes in mice weight (A, Weight<sub>End</sub>−Weight<sub>Start</sub>/Weight<sub>Start</sub>), area under the glucose tolerance test (GTT) curve (B), mass of epididymal adipose tissue (EAT mass, C), and perirenal adipose tissue (PAT mass, D) as well as representative images of liver steatosis (E) in C57BL/6 mice fed an HFD (HFD₀%₀, white columns) for 2 (n=16, A through C; n=15, D), 4 (n=16), and 8 (n=18) weeks in comparison to age-matched C57BL/6 mice fed a control diet (black columns, after 2 [n=16, A through C; n=15, D], 4 [n=16], and 8 [n=16, B through D; n=15, A] weeks of feeding). Statistics: 2-way ANOVA followed by Tukey post hoc test (normality was assessed using the Shapiro–Wilk test): not statistically significant (ns) *P<0.05, **P<0.01, ***P<0.001. AUC indicates area under the curve.
Figure 2. High-fat-diet (HFD; HFD_{60%}) feeding reduces endothelial function in the abdominal aorta (AA) in association with decreased brown fat in perivascular adipose tissue.

Changes in end-diastolic volume of the thoracic aorta (TA; A, TA-Ach) and AA (B, AA-Ach) 25 minutes after acetylcholine (Ach) administration and ratio of brown adipose tissue area to total adipose tissue area as well as representative images of perivascular adipose tissue (PVAT) in the TA (C and E) and the AA (D and F) in C57BL/6 mice fed an HFD (HFD_{60%}; white columns) for 2 (n=6, E and F; n=5, A and B), 4 (n=6, A and E; n=5, B and F), and 8 (n=7, B; n=6, A; n=5, E and F) weeks in comparison to age-matched C57BL/6 mice fed a control diet (black columns, after 2 [n=7, B; n=6, A; n=5, E, and F], 4 [n=6, A and B; n=5, E and F], and 8 [n=8, F; n=7, E; n=6, A and B] weeks of feeding). Statistics: 2-way ANOVA followed by Tukey post hoc test (normality was assessed using the Shapiro–Wilk test): not statistically significant (ns), ***P<0.001.
Figure 3. Effects of high-fat diet (HFD; HFD<sub>60%</sub>) feeding on NO production in the aorta, nitrite concentration in plasma, and level of reduced glutathione (GSH) and oxidized glutathione (GSSG) in red blood cells.

Changes in NO production in the thoracic aorta (TA; A and C) and the abdominal aorta (AA; B and D), cleared (C and D) or not (A and B) from surrounding adipose tissue, measured by spin-trapping with DETC (sodium diethyldithiocarbamate, spin trap), changes in nitrite (E, NO<sub>2</sub><sup>-</sup>) concentration in plasma, and ratio of GSH and GSSG in red blood cells (F, GSH/GSSG) in C57BL/6 mice fed an HFD (HFD<sub>60%</sub>, white columns) for 2 (n=16, E; n=13, F; n=8, A and B; n=7, C and D), 4 (n=16, E; n=15, F; n=8, A through D), and 8 (n=19, E; n=18, F; n=9, A, B, and D; n=8, C) weeks in comparison to age-matched C57BL/6 mice fed a control diet (black columns, after 2 [n=16, E and F; n=8, A and B; n=7, D; n=6, C], 4 [n=16, E; n=15, F; n=8, A through D], and 8 [n=16, E; n=15, F; n=9, C; n=8, D; n=7, A and B] weeks of feeding). Statistics: 2-way ANOVA followed by Tukey post hoc test (normality was assessed using the Shapiro–Wilk test): not statistically significant (ns) *P<0.05, **P<0.01, ***P<0.001. EPR indicates electron paramagnetic resonance; and PVAT, perivascular adipose tissue.
similar response in PVAT of the TA and in brown adipose tissue. These results suggest a relative increase in saturated fatty acids in PVAT surrounding the AA, which did not occur in PVAT adjacent to the TA.

Lack of Early Changes in Plasma Concentration of Nitrite and Nitrate and of Biomarkers of Endothelial Dysfunction in C57BL/6 Mice Fed a HFD (HFD$_{60\%}$)

Feeding of C57BL/6 mice with a HFD from 2 to 8 weeks did not affect NO$\textsubscript{2}^{-}$ (Figure 3E) and NO$\textsubscript{3}^{-}$ concentration in plasma (average NO$\textsubscript{3}^{-}$ plasma concentration, throughout diet duration in C57BL/6 control mice: 36.1±7.7 μmol/L and HFD$_{60\%}$ fed C57BL/6 mice: 35.9±10.7 μmol/L), whereas a change in reduced glutathione/oxidized glutathione ratio (≈a 3-fold decrease in comparison to mice fed a standard diet; Figure 3F) was only seen after 8 weeks of HFD$_{60\%}$ feeding. Moreover, with the exception of increased plasma concentration of von Willebrand factor (concentration increased on average by ≈25%, in comparison to mice fed a standard diet, Figure 4H), there were no significant changes in plasma concentration of biomarkers of glycocalyx disruption (syndecan-1 and endocan; Figure 4A and 4B), endothelial inflammation (soluble form of vascular cell adhesion molecule 1, soluble form of intercellular adhesion molecule 1 and E-selectin; Figure 4C through 4E), endothelial permeability (soluble form of fms-like tyrosine kinase 1 and angiopoietin 2; Figure 4F and 4G), and hemostasis (tissue plasminogen activator and plasminogen activator inhibitor 1; Figure 4I and 4J) in HFD$_{60\%}$-fed mice as compared with mice fed a control diet.

Therefore, biomarkers of endothelial function were not sensitive to detect endothelial dysfunction after short-term HFD$_{60\%}$ feeding in contrast to the assessment of functional endothelial response measured in vivo.

Early Impairment of Acetylcholine-Induced Vasodilation in the AA But Not in the TA in E3L.CETP Mice Fed a HFD (HFD$_{32\%}$)

To confirm that regional differences in the early endothelial response in the AA and the TA to HFD is not limited to one murine model we also studied E3L.CETP mice. Similarly to HFD$_{60\%}$ feeding in C57BL/6 mice, 2 weeks of feeding with HFD$_{32\%}$ in E3L.CETP mice resulted in paradoxical vasoconstriction in the AA induced by Ach (Figure 5D volume changes of the AA: −4.2% in comparison to 5.3% in age-matched control mice). However, Ach-induced vasodilation in the TA was fully preserved (Figure 5C).

HFD$_{32\%}$ did not induce significant insulin resistance after short-term feeding (area under the curve: ≈1.8-fold higher in E3L.CETP mice fed with HFD$_{32\%}$ only after 16 weeks of feeding, in comparison to C57BL/6J mice fed standard diet; Figure 5A). Moreover, 2 weeks of HFD$_{32\%}$ feeding was not associated with the changes in body weight in the E3L.CETP mice (Figure 5B) and did not affect NO$\textsubscript{2}^{-}$ (Figure 5E) and NO$\textsubscript{3}^{-}$ (Figure 5F) concentration in plasma.

Altogether, in E3L.CETP mice fed a HFD$_{32\%}$, even though insulin resistance was not present, the impairment of endothelial function in the AA (but not in the TA) was clearly observed.

DISCUSSION

Using an MRI-based methodology to assess endothelial function in vivo,25 in vessels with intact PVAT, to the best of our knowledge, we demonstrated for the first time, that short-term feeding with a HFD resulted in endothelial dysfunction in the AA, whereas endothelial function in the TA was resistant to early HFD-induced dysfunction. These changes were independently observed in 2 different murine models, C57BL/6 and E3L.CETP mice, fed a HFD of different composition, and were thereby independent of systemic insulin resistance. Endothelial dysfunction in HFD-fed mice for 2 weeks was not associated with systemic changes in NO bioavailability and changes in reduced glutathione/oxidized glutathione ratio in red blood cells as the latter was only observed after 8 weeks of HFD$_{60\%}$ feeding, supporting an increase in systemic oxidative stress after 8 weeks but not after 2 weeks of HFD feeding. Furthermore, the concentration of biomarkers of glycocalyx disruption (syndecan-1 and endocan), endothelial inflammation (soluble form of vascular cell adhesion molecule 1, soluble form of intercellular adhesion molecule 1 and E-selectin), endothelial permeability (soluble form of fms-like tyrosine kinase 1 and angiopoietin 2), and hemostasis (tissue plasminogen activator and plasminogen activator inhibitor 1) were not significantly altered. In contrast, these biomarkers changed typically in other models of endothelial dysfunction in our previous studies.30,39,42 The heterogeneous endothelial response in the abdominal and thoracic parts of the aorta in response to short-term feeding with a HFD was ascribed to a distinct difference in the composition of white- and brown-like adipose tissue of PVAT in the AA and TA, as assessed by histology, immunohistochemistry, and Raman spectroscopy. Furthermore, the different endothelial response in the AA versus TA was mirrored...
by deterioration of arterial stiffness in the AA but not in the TA, underscoring the distinct functional consequences of PVAT-modulated endothelial response for the entire vascular wall. Altogether, our results support the notion of the important role of PVAT in the early phase of HFD-induced vascular dysfunction operating in some but not in all vessels that could not be detected on the basis of classical systemic biomarkers of endothelial dysfunction. These results underscore the insidious nature of early endothelial dysfunction in response to fat overload.

To assess endothelial function in vivo, in vessels with intact PVAT, a 3D MRI-based methodology was used. This methodology is well suited for detection and quantification, with good sensitivity and reproducibility, of NO-dependent endothelial response, and has been previously used to characterize endothelial function in the different murine models in vivo.25,29,30,43

In the present study, we focused on the short-term effects of a HFD, in particular after a 2-week period of HFD in C57BL/6 mice, a model that is characterized by the early development of insulin resistance and liver steatosis. The salient finding of this study was the detection of severe endothelial dysfunction after only 2 weeks of HFD60% feeding, as evidenced by a substantial impairment of Ach-induced vasodilatation in the AA, which was reverted to vasoconstriction, the magnitude of which was even more pronounced as compared with previously studied 28-week-old ApoE/LDLR−/− mice with advanced atherosclerosis.30

Our in vivo MRI-based approach revealed the robust impairment of endothelial function in the AA (but not in the TA) after 2 weeks of feeding with a HFD, which was barely detectable using ex vivo settings in previous work even after 4 weeks of a HFD,44,46 but was clearly present after 16 to 20 weeks of a HFD17,21 or an even longer feeding (eg, 8 months30). Indeed, previously, only a marginal effect was seen in isolated aorta ex vivo after a short period of 4 weeks of HFD feeding in the magnitude of Ach-induced vasodilatation, insulin-dependent vasodilatation, or magnitude of phenylephrine-induced vasoconstriction.34–46

We also demonstrated that HFD-induced endothelial dysfunction progressed into the development of arterial stiffness, which was observed as increased PWV in the AA after 4 to 8 weeks but not after 2 weeks of HFD feeding. Arterial stiffness of the TA did not change, again underscoring the regional differences between the AA and TA in response to a short-term HFD.

In addition to the assessment of PVAT characteristics in the AA and TA by histology and immunohistochemistry, Raman spectroscopy revealed that PVAT in the AA and TA displayed a different profile of lipid unsaturation, showing striking similarities between PVAT in the AA and eWAT as well as between PVAT in the TA and iBAT. These observations confirm the notion that PVAT in the AA and TA shows a predominantly white and brown phenotype, respectively, in line with our previous report using Raman spectroscopy–based assessment of PVAT in the aorta in ageing mice41 and with other studies showing distinct characteristics of PVAT in the TA and the AA in mice.12,22,47 Most importantly, a significant decrease of lipid unsaturation in PVAT of AA (and eWAT) was observed in response to HFD60% feeding, whereas PVAT of TA (and iBAT) was resistant to the increased amount of saturated lipids in the diet. The heterogeneous response of the AA and TA was also observed in EPR measurements in the ex vivo aorta, suggesting that the vasoprotective activity of PVAT was lost in the AA but not in the TA. Altogether, our results reveal that these 2 distinct PVAT depots have essentially antagonistic functions on endothelial function in the aorta in response to an HFD that either promote or prevent endothelial dysfunction in the AA and TA, respectively.

It should be underlined that there is not a clear division in pure brown adipose tissue or pure white adipose tissue between these 2 PVATs because infiltrations of one type of PVAT can be found in the other.2 White adipose tissue, which is composed of adipocytes with a large, single fat droplet, is presumed to be the main depot for lipid storage also in perivascular tissue48 that is highly responsive to HFD.23 Conversely, brown PVAT, which contains several smaller fat droplets and numerous mitochondria, displays vasoprotective activity,22 participates in the regulation of intravascular temperature49 and shows resistance to a HFD; thus, it protects the vascular wall against diet-induced inflammation, as well as against atherosclerosis.22,49

To confirm that heterogenous early effects of HFD feeding on endothelium in the TA and AA could be detected by MRI in vivo in another model as well,
Bar et al. Heterogeneous Endothelial Response in HFD-Fed Mice

A Biomarkers of glyocalyx disruption (SDC-1, ESM-1)

B Biomarkers of endothelial inflammation (sVCAM-1, sICAM-1, sE-sel)

C Biomarkers of hemostasis (vWF, t-PA, PAI-1)

D

E

F Biomarkers of endothelial permeability (sFLT-1, Angpt-2)

G

H

I

J

Downloaded from http://ahajournals.org by on January 12, 2021
Figure 5. Effects of high-fat diet (HFD; HFD32%) feeding on insulin resistance, weight of the mice, endothelial function, and nitrite and nitrate concentration in plasma in E3L.CETP mice. Blood glucose concentration 0, 15, 30, 45, 60, and 120 minutes after glucose administration (A), changes in mouse weight (B, WeightEnd−WeightStart /WeightStart), changes in end-diastolic volume of the thoracic aorta (TA; C, TA-Ach), and abdominal aorta (AA; D, AA-Ach) 25 minutes after acetylcholine (Ach) administration as well as changes in nitrite (E: NO\textsuperscript{2−}) and nitrate (F: NO\textsuperscript{3−}) concentration in plasma in E3L.CETP mice fed a HFD (HFD32% white columns with horizontal lines or triangles) for 2 (B through F) and 16 (A) weeks (n=8) in comparison to age-matched C57BL/6J mice fed a standard diet (black columns with horizontal lines or circles, n=6, A, B, E, and F; n=5, C and D). Statistics: (A): repeated measures ANOVA, (B, E, and F): Mann–Whitney U test, (C and D): Student t test (normality was assessed using the Shapiro–Wilk test): not statistically significant (ns) *P<0.05, **P<0.01.
we used a murine model for hyperlipidemia and atherosclerosis (E3L.CETP mice\(^{27,29}\)), which is based on the introduction of 3 human genes: apolipoprotein E*3 Leiden, apolipoprotein C1, and CETP.\(^{27,50}\) These mice exhibit a human-like lipoprotein metabolism\(^{51}\) and respond similarly to humans to pharmacological interventions modulating lipid metabolism.\(^{52,53}\) We demonstrated that in E3L.CETP mice fed a HFD\(_{32}\%)\end{equation}\end{figure}\end{center}\end{abstract}

**CONCLUSIONS**

We demonstrated here that short-term feeding with a HFD in 2 different mouse models resulted in the impairment of the vascular response in the AA, whereas this response was largely preserved in the TA. The heterogeneous endothelial response was ascribed to distinct PVAT composition, including white adipose tissue–like characteristics in the AA and brown adipose tissue–like characteristics in the TA, but was not associated with systemic insulin resistance or with classical systemic biomarkers of endothelial dysfunction. These data underscore the notion that PVAT plays a fundamental role in the modulation of endothelial function in mouse models of metabolic syndrome and obesity, which could also be relevant to mechanisms of early endothelial dysfunction induced by fat load in humans.\(^{57}\) Finally, our results also support the proposal that PVAT-targeted diagnosis\(^{58,59}\) and therapy\(^{60,61}\) might offer a novel approach to prevent residual vascular risks linked to the loss of PVAT-dependent homeostatic and vasoprotective function in response to excessive fat load, leading to endothelial dysfunction.\(^{62,63}\)

**REFERENCES**

1. McVeigh GE, Cohn JN. Endothelial dysfunction and the metabolic syn-

2. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation. 2000;101:1899–1906.

3. Halcox JP, Sichenke WH, Zaios G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KRA, Quyyumi AA. Prognostic value of coronary vascular endothelial dysfunction. Circulation. 2002;106:653–658.

4. Csányi G, Gadja M, Franczyk-Zarow M, Kostogryz R, Gwoźdz P, Mateuszuk L, Sternak M, Wojcik L, Zalewska T, Walski M, et al. Functional alterations in endothelial NO, PGI\(_2\), and EDHF pathways in aorta in ApoE/CDLDR-/ mice. Prostaglandins Other Lipid Mediat. 2012;98:107–115.

5. Smeda M, Kieronska A, Adamski MG, Proniewski B, Sternak M, Mohaisen T, Przyborowski K, Derszniak K, Kaczor D, Stojak M, et al. Nitric oxide deficiency and endothelial–mesenchymal transition of pulmonary endothelium in the progression of 4T1 metastatic breast cancer in mice. Breast Cancer Res. 2018;20:86.

6. Chiopecki S, Nilsson H, Mulvany M. Initial and sustained phases of myogenic response of rat mesenteric small arteries. Am J Physiol Heart Circ Physiol. 2001;281:H2176–H2183.

7. Gil-Ortega M, Somozo B, Huang Y, Gollasch M, Fernández-Alfonso MS. Regional differences in perivascular adipose tissue impacting vascular homeostasis. Trends Endocrinol Metab. 2015;26:367–375.

8. Gollasch M, Dubrovskova G. Paracrine role for perivascular adipose tissue in the regulation of arterial tone. Trends Pharmacol Sci. 2004;25:647–653.

9. Eringa EC, Bakker W, Smulders YM, Serné EH, Yucink JS, Stehouwer CD. Regulation of vascular function and insulin sensitivity by adipose tissue: focus on perivascular adipose tissue. Microcirculation. 2007;14:389–402.

**Affiliations**

From the Jagiellonian Centre for Experimental Therapeutics (JCET), Jagiellonian University, Krakow, Poland (A.B., A.K.-R., B.P., J.S.-P., K.C., B.M., K.M.-G., A.J., E.K., Z.M., A.K., A.K., M.W., S.C.); Chair of Pharmacology, Faculty of Medicine, Jagiellonian University Medical College (A.K.-R., B.M., S.C.) and Chair and Department of Toxicology, Faculty of Pharmacy (M.W.), Jagiellonian University Medical College, Krakow, Poland; Faculty of Chemistry, Jagiellonian University, Krakow, Poland (Z.M., A.K.); and Metabolic Health Research, Gaubius Laboratory, The Netherlands Organisation for Applied Scientific Research (TNO), Leiden, The Netherlands (E.J.P., H.M.P.).

**Acknowledgments**

The authors would like to thank Agnieszka Zakrzewska for expert technical assistance and measurements of nitrite and nitrate concentration in plasma, Zuzanna Kuryłowicz for help with immunohistochemical staining, and Krystyna Wandelz and Kristina Szczepanik for their excellent technical assistance in PWV measurements. Anna Kierońska-Rudek acknowledges the fellowship with the project No. POW.03.02.00-00-I013/16. Anna Bar and Krzysztof Czamara acknowledge the START scholarship, awarded by the Foundation for Polish Science (Foundation for Polish Science, START2020 program).

**Sources of Funding**

This work was supported by Polish National Science Centre grant: OPUS No. 2018/29/B/NZ7/01684 and by Team Tech–Core Facility program of the FPN (Foundation for Polish Science) co-financed by the European Union under the European Regional Development Fund (project No. POIR.04.04.00-00-5OAC/17–00).

**Disclosures**

None.

**Supplementary Material**

Figures S1–S4

**ARTICLE INFORMATION**

Received April 21, 2020; accepted August 26, 2020.

J Am Heart Assoc. 2020;9:e016929. DOI: 10.1161/JAHA.120.016929
24. Padilla J, Jenkins NT, Vieira-Potter VJ, Laughlin MH. Divergent phenotypic vessel function by peripheral adipose tissue and its potential correlation with adiposity/leptinometric dysfunction. Curr Pharm Des. 2007;13:2185–2192.

25. Marchesi C, Ebrahimian T, Angulo O, Paradis P, Schiffrin EL. Endothelial nitric oxide synthase uncoupling and peripheral adipose oxidative stress and inflammation contribute to vascular dysfunction in a rodent model of metabolic syndrome. Hypertension. 2009;54:1384–1392.

26. Somoza B, Guzmán R, Cano V, Merino B, Ramos P, Diez-Fernández C, Fernández-Alfonso MS, Ruiz-Gayo M. Induction of cardiac uncoupling protein-2 expression and adenosine 5-monophosphate-activated protein kinase phosphorylation during early stages of diet-induced obesity in mice. Endocrinology. 2007;148:924–931.

27. Westerterp M, Van Der Hoogt CC, De Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havenkes LM, Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in apoE−/−Leiden mice. Arterioscler Thromb Vasc Biol. 2006;26:2552–2559.

28. van den Hoek AM, van der Hoorn J, De Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havenkes LM, Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in apoE−/−Leiden mice. Arterioscler Thromb Vasc Biol. 2006;26:2552–2559.

29. Sternak M, Bar A, Adamski MG, Mohaisen T, Marczyk B, Kieronska A, Stojak M, Kus K, Tarjus A, Jaisser F, et al. The deletion of endothelial sodium channel α (ENaCα) impairs endothelium-dependent vaso-dilation and endothelial barrier integrity in endotoxemia in vivo. Front Pharmacol. 2018;9:178.

30. Bar A, Targosz-Korecka M, Suraj J, Prioniewski B, Jasztal A, Marczyk B, Sternak M, Przybyło M, Kupińska A, Walczak M, et al. Glycocalyx and multiple manifestations of endothelial dysfunction coincide in the early phase of endothelial dysfunction before atherosclerotic plaque development in apolipoprotein E/low-density lipoprotein receptor-deficient mice. J Am Heart Assoc. 2019;8:e011171. DOI: 10.1161/JAHA.118.011171.

31. Hartley CJ, Taffet GE, Michael LH, Pham TT, Entman ML. Noninvasive determination of pulse-wave velocity in mice. Am J Physiol. 1997;273:H494–H500.

32. Asmar R, Benetos A, Topouchian J, Laurent P, Pannier B, Brisaç AM, Target R, Levy B. Assessment of arterial distensibility by automatic pulse wave velocity measurement. Validation and clinical application studies. Hypertension. 1995;26:485–490.

33. Czamara K, Majka Z, Fuss A, Matjasik K, Pacia MZ, Sternak M, Chipicki S, Kaczor A. Raman spectroscopy as a novel tool for fast characterization of the chemical composition of peripheral adipose tissue. Analyst. 2018;143:5999–6005.

34. Przyborowski K, Prioniewski B, Czarny J, Smeda M, Sitek B, Zakrzewska A, Zoladz JA, Chipicki S. Vascular nitric oxide-superoxide balance and thrombus formation after acute exercise. Med Sci Sports Exerc. 2018;50:2905–2913.

35. Depre C, Havaux X, Renkin J, Vanoverschelde JL, Wijns W. Expression of inducible nitric oxide synthase in human coronary atherosclerotic plaque. Cardiovasc Res. 1999;41:465–472.

36. Prioniewski B, Kij A, Sitek B, Kelley EE, Chipicki S, Multiorgan development of oxidative and nitrosative stress in LPS-Induced Endotoxemia in C57Bl/6 mice: DHE-based in vivo approach. Oxid Med Cell Longev. 2019;2019:1–11.

37. Hempe JM, Ory-Ascani J. Simultaneous analysis of reduced glutathione and glutathione disulfide by capillary zone electrophoresis. Electrophoresis. 2014;35:967–971.

38. Suraj J, Kupińska A, Olkowski M, Niedziewska-Andres E, Smolik M, Zakrzewska A, Jasztal A, Sitek B, Chipicki S, Walczak M. Development, validation and application of a micro–liquid chromatography–tandem mass spectrometry based method for simultaneous quantification of selected protein biomarkers of endothelial dysfunction in murine plasma. J Pharm Biomol Anal. 2018;149:465–474.

39. Suraj J, Kupińska A, Zakrzewska A, Sternak M, Stojak M, Jasztal A, Walczak M. Early and late endothelial response in breast cancer metastasis in mice: simultaneous quantification of endothelial biomarkers using mass spectrometry-based method. Dis Model Mech. 2019;12:dmm036269.

40. Walczak M, Suraj J, Kus K, Kij A, Zakrzewska A, Chipicki S. Towards a comprehensive endothelial biomarkers profiling and endotheli-um-guided pharmaceutical therapy. Pharmacol Rep. 2015;67:771–777.

41. Czamara K, Majzerk N, Pacia MZ, Kochan K, Kaczor A, Baranska M. Raman spectroscopy of lipids: a review. J Raman Spectrosc. 2015;46:4–20.

42. Targosz-Korecka M, Jaglarz M, Malek-Zietek KE, Gregorius A, Raman spectroscopy of lipids: a review. Talanta. 2019;195:107613.

43. Westerterp M, Van Der Hoogt CC, De Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, Havenkes LM, Rensen PC. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in apoE−/−Leiden mice. Arterioscler Thromb Vasc Biol. 2006;26:2552–2559.
hepatic insulin resistance than an obesogenic high-fat diet. J Physiol. 2018;596:4597–4609.

47. Czamara K, Majka Z, Sternak M, Koziol M, Kostogrys RB, Chlipicki S, Kaczor A. Distinct chemical changes in abdominal but not in thoracic aorta upon atherosclerosis studied using fiber optic raman spectroscopy. Int J Mol Sci. 2020;21:1–14.

48. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nat Rev Immunol. 2011;11:85–97.

49. Chang L, Villacorta L, Li R, Hamblin M, Xu W, Dou C, Zhang J, Wu J, Zeng R, Chen YE. Loss of perivascular adipose tissue on peroxisome proliferator-activated receptor-γ deletion in smooth muscle cells impairs intravascular thermoregulation and enhances atherosclerosis. Circulation. 2012;126:1067–1078.

50. van den Maagdenberg AM, Hofker MH, Krimpenfort PJ, de Bruijn I, van Vlijmen B, van der Boom H, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein-E3-Leiden gene exhibit hyperlipoproteinemia. J Biol Chem. 1993;268:10540–10545.

51. Princen HM, Pouwer MG, Pieterman EJ. Comment on “Hypercholesterolemia with consumption of PFOA-laced Western diets is dependent on strain and sex of mice” by Rebholz S.L. et al. Toxicol. Rep. 2016 (3) 46-54. Toxicol Rep. 2016;3:306–309.

52. Zadelaar S, Kleemann R, Verschuren LD, Van Der Wei J, Van Der Hoorn J, Princen HM, Kooistra T. Mouse models for atherosclerosis and pharmaceutical modifiers. Arterioscler Thromb Vasc Biol. 2007;27:1706–1721.

53. Kühnast S, Fiocco M, Van Der Hoorn JW, Princen HM, Jukema JW. Innovative pharmaceutical interventions in cardiovascular disease: focusing on the contribution of non-HDL-C/IDL-C-lowering versus HDL-C-raising: a systematic review and meta-analysis of relevant preclinical studies and clinical trials. Eur J Pharmacol. 2015;763:48–63.

54. Kleemann R, Van Erk M, Verschuren L, Van Den Hoek AM, Koek M, Wielinga PY, Jie A, Pellis L, Bobeldijk-Pastorova I, Kelder T, et al. Time-resolved and tissue-specific systems analysis of the pathogenesis of insulin resistance. PLoS One. 2010;5:e8817.
SUPPLEMENTAL MATERIAL
Figure S1. Algorithm for assessment of brown and white adipose tissue content in perivascular adipose tissue of thoracic and abdominal aorta.

| Double staining of PVAT for UCP-1 and perilipin | UCP-1-immunopositive area (Cy3) | Perilipin -immunopositive area (FITC) |
|-----------------------------------------------|----------------------------------|--------------------------------------|
| ![Image A](image1.png)                       | ![Image B](image2.png)           | ![Image C](image3.png)               |
| ![Image D](image4.png)                       | ![Image E](image5.png)           |                                      |

The representative images of aorta cross-section with perivascular adipose tissue (PVAT) simultaneously stained for UCP-1 and perilipin (A). Separation of the channels for Cy3-based immunofluorescence of UCP-1 and FITC-based immunofluorescence of perilipin are shown in (B) and (C) respectively. For quantitative analysis, the images of cross-section of PVAT were segmented in Ilastik software to assess the ratio of brown adipose tissue (BAT) to total fat tissue area. The result of an Ilastik segmentation are shown in (D,E). Red (D) indicates BAT (UCP-1 positive area) while green (E) indicates WAT (perilipin positive area) and both corresponds directly to the immunopositive pixels in respective channels. The number of pixels representing UCP-1 and perilipin were counted using ImageJ software. The results were expressed as the ratio of brown adipose tissue area (number of UCP-1 immunopositive pixels) to the total adipose tissue area (sum of UCP-1+ perilipin immunopositive pixels), and normalized to the respective control.
Figure S2. Effects of high-fat diet (HFD$_{60\%}$) feeding on arterial stiffness measured as pulse wave velocity (PWV).

Pulse wave velocity (PWV) in the thoracic (TA, A) and the abdominal (AA, B) aorta in C57BL/6 mice fed a high-fat diet (HFD$_{60\%}$, white columns) for 2 (n=6, A; n=5, B), 4 (n=9, A; n=8, B) and 8 (n=7) weeks in comparison to age-matched C57BL/6 mice fed a control diet (black columns, after 2 (n=7), 4 (n=9) and 8 (n=7, B; n=6, A) weeks of feeding). Statistics: two-way ANOVA followed by Tukey’s post hoc test (normality was assessed using the Shapiro-Wilk test): ns - not statistically significant * $p<0.05$, ** $p<0.01$, *** $p<0.001$. 

Downloaded from http://ajhjournals.org by on January 12, 2021
Figure S3. Effects of high-fat diet (HFD_{60\%}) feeding on adipose tissue immunohistochemical characteristics.

C57BL/6  

C57BL/6 + HFD_{60\%}

A: Lectin-TA

Ratio of immunopositive pixels for lectin (A, B) and phosphorylated endothelial nitric oxide synthase (PeNOS, C, D) to total adipose tissue area in the thoracic (A, C) and the abdominal aorta (B, D) in C57BL/6 mice fed a high-fat diet (HFD_{60\%}, white columns) for 2 weeks (n=17, A; n=15, B; n=14, C; n=13, D) in comparison to age-matched C57BL/6 mice fed a control diet (black columns, n=18, D; n=17, A; n=15, B; n=14, C). Statistics: two-way ANOVA followed by Tukey’s post hoc test (normality was assessed using the Shapiro-Wilk test): ns - not statistically significant * p<0.05, ** p<0.01, *** p<0.001.
Figure S4. Effects of high-fat diet (HFD60%) feeding on adipose tissue chemical characteristics.

A: TA-PVAT

B: AA-PVAT

C: iBAT

D: eWAT

Raman Intensity

Wavenumber / cm⁻¹

Unsaturation of lipids (1600/1448)

C57BL/6 + AIN93G

C57BL/6 + HFD

ns

***

ns
Averaged Raman spectra and analysis of the lipid unsaturation degree \( \frac{I_{1660}}{I_{1444}} \) of the thoracic perivascular adipose tissue (A: TA-PVAT), abdominal perivascular adipose tissue (B: AA-PVAT), interscapular (C: iBAT) and epididymal adipose tissue (D: eWAT) in \textit{C57BL/6} mice fed a HFD\_60\% for 4 weeks (red columns and lines; \( n=5 \)) in comparison to age-matched \textit{C57BL/6} mice fed a control diet (blue columns and lines, \( n=5 \)). Statistics: Student’s t-test (normality was assessed using the Shapiro-Wilk test): ns - not statistically significant * \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \).