Proinflammatory Phenotype of Perivascular Adipocytes
Influence of High-Fat Feeding


Abstract—Adipose tissue depots originate from distinct precursor cells, are functionally diverse, and modulate disease processes in a depot-specific manner. However, the functional properties of perivascular adipocytes, and their influence on disease of the blood vessel wall, remain to be determined. We show that human coronary perivascular adipocytes exhibit a reduced state of adipocytic differentiation as compared with adipocytes derived from subcutaneous and visceral (perirenal) adipose depots. Secretion of antiinflammatory adiponectin is markedly reduced, whereas that of proinflammatory cytokines interleukin-6, interleukin-8, and monocyte chemoattractant protein-1, is markedly increased in perivascular adipocytes. These depot-specific differences in adipocyte function are demonstrable in both freshly isolated adipose tissues and in vitro–differentiated adipocytes. Murine aortic arch perivascular adipose tissues likewise express lower levels of adipocyte-associated genes as compared with subcutaneous and visceral adipose tissues. Moreover, 2 weeks of high-fat feeding caused further reductions in adipocyte-associated gene expression, while upregulating proinflammatory gene expression, in perivascular adipose tissues. These changes were observed in the absence of macrophage recruitment to the perivascular adipose depot. We conclude that perivascular adipocytes exhibit reduced differentiation and a heightened proinflammatory state, properties that are intrinsic to the adipocytes residing in this depot. Dysfunction of perivascular adipose tissue induced by fat feeding suggests that this unique adipose depot is capable of linking metabolic signals to inflammation in the blood vessel wall. (Circ Res. 2009;104:541-549.)

Key Words: perivascular adipose tissue • adipocytes • adventitia • adipokines • cytokines

Atherosclerosis is traditionally viewed as a disease of the vascular intima, following a paradigm of endothelial cell dysfunction, inflammatory cell recruitment, and foam cell formation.1–4 More recently, dysfunction of medial smooth muscle cells and adventitial cells has also been demonstrated to play a role in the pathogenesis of atherosclerosis.5,6 Whereas reactive fibroblasts and inflammatory cells in the adventitia have been the focus of extensive investigations,6,7 very little is known about perivascular adipocytes that reside at the adventitial border of atherosclerosis-prone blood vessels. Adipocytes secrete numerous factors that could potentially modulate the development of vascular disease, including proinflammatory cytokines and adipokines, angiogenic molecules, and stem cell homing factors.8–10 Recent evidence indicates that the periadventitial adipose depot is a functional component of the vasculature, exerting paracrine influences on blood vessel contractility.11,12 Inflammatory cell infiltration is markedly increased in perivascular adipose tissue surrounding atherosclerotic human aorta as compared with nondiseased aorta.13 Moreover, inflammatory gene expression is upregulated14,15 and expression of adiponectin, an antiinflammatory adipokine, is downregulated,16 in perivascular adipose tissues surrounding human coronary arteries. The mechanisms underlying these observations are unknown. It is possible that perivascular adipose inflammation stems from disease of the blood vessel wall that passively extends into the surrounding adipose tissue. Conversely, depending on their functional properties, perivascular adipocytes could potentially initiate vascular wall inflammation. In this regard, emerging evidence suggests that anatomically separated adipose tissue depots are functionally diverse, originate from distinct precursor cells,17,18 and exert both systemic and local (paracrine) effects on tissue and organ function. Insulin sensitivity, and the balance of pro- and antiinflammatory adipokine and cytokine...
expression, varies widely among regional visceral and subcutaneous adipose depots. Moreover, high-fat feeding induces inflammation of visceral adipose tissues, which may contribute to insulin resistance and dyslipidemia. However, responses of perivascular adipose tissues to high-fat feeding have not been examined.

Accordingly, we characterized the molecular and functional properties of human perivascular adipose tissues and adipocytes and determined the effects of high-fat feeding on perivascular adipose tissues in a mouse model. Studies were conducted in adipose tissues of humans and mice devoid of atherosclerotic disease to eliminate confounding influences of preexisting vascular wall inflammation. Our results suggest that human perivascular adipocytes exhibit a reduced state of adipocytic differentiation, and a remarkably higher level of proinflammatory cytokine expression and release, as compared with adipocytes from other regional depots. Moreover, we observed that mouse perivascular adipose tissue is strikingly responsive to the effects of short-term high-fat feeding. Thus, by virtue of their unique functional and biochemical properties, we propose that perivascular adipocytes may play a primary role in establishing adventitial inflammation in atherosclerosis.

Materials and Methods

Animals
Eight-week-old male C57BL/6J mice, maintained on chow diet after weaning, were continued on chow diet or placed on a high-fat Western diet (Harlan Teklad, 42% calories from fat) ad libitum for 2 weeks before euthanasia. Subcutaneous, visceral (epididymal and perirenal), and perivascular (aortic arch) adipose tissues were dissected, rinsed, snap-frozen in liquid nitrogen, and stored at −80°C before use. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Human Adipose Tissue Collection and Processing
Human adipose tissue samples were collected from candidates for organ donation (for donor demographic information, see Table I in the online data supplement, available at http://circres.ahajournals.org). Institutional review boards at the University of Cincinnati and the University of Iowa approved our protocols. Adipose tissues were obtained from subcutaneous, visceral (perirenal), perivascular (coronary artery), omental, and epicardial (right ventricle) sites. Tissues were harvested, rinsed, placed in DMEM/F12, and rapidly transported to the laboratory. Tissues were separated for cell culture (see below) or fixed with 10% buffered formalin, paraffin-embedded, and stained with hematoxylin and eosin. Stained sections were examined microscopically, and cell diameters were determined using Leica image processing system.

Isolation and Differentiation of Human Preadipocytes

Tissues were minced and digested with collagenase type I (Worthington), after which, isolation and culture of preadipocytes was performed as previously described. Cells were maintained in DMEM/F12/10% FBS and passaged or subjected to the differentiation protocol when confluent. Adipocytic differentiation was accomplished by switching to a commercially available differentiation medium (Cell Applications), which was replaced every 4 days. Differentiated adipocytes were examined by DIC microscopy to visualize cytoplasmic lipid droplet accumulation. All experiments were performed on cells at passage 3 or less.

Oil Red O Staining and Spectrophotometric Quantification of Lipids

Differentiating adipocytes were washed with PBS, fixed in 10% formalin, stained with oil red O (Sigma) in 2-propanol, air-dried, and photographed. In some experiments, oil red O–stained cells were extracted with 2-propanol in 4% Nonidet P-40 (Sigma), and the color intensity was measured spectrophotometrically at 510 nm.

Table. Depot-Specific Differences in Tissue Adipocyte Diameter In Situ and Cellular Lipid Accumulation Within In Vitro–Differentiated Adipocytes

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<th>Perivascular</th>
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<tr>
<td>Tissue, adipocyte diameter (m)*</td>
<td>42.32±2.74</td>
<td>60.68±2.63†</td>
<td>22.53±1.53‡†</td>
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<td>Cells, oil red O–positive materials§</td>
<td>8053±180</td>
<td>4975±39†</td>
<td>3032±116†‡</td>
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*Data represent mean±SEM. †P<0.05 vs SQ; ‡P<0.05 vs PR. §Values are expressed as mean optical density at 510 nm/mg cellular protein±SEM.
Cellular protein was determined by a Micro BCA Protein Assay kit (Pierce), and data were expressed as optical density units per mg protein.

RNA Extraction and Quantitative RT-PCR
Total RNA from tissues and cells was extracted using RNeasy Lipid Mini kits (Qiagen). Quantitative RT-PCR was performed using Brilliant II QRT-PCR kits (Stratagene). The levels of acidic ribosomal phosphoprotein P0 RNA were used as endogenous controls for normalization of human and mouse RNA. The relative gene expressions were calculated using cycle threshold (Ct) values in accordance with the ΔΔCt method as described previously. Primer sequences used are available on request.

ELISA Determination of Adiponectin, Interleukin-8, Interleukin-6, Monocyte Chemoattractant Protein-1, and Leptin
Preadipocytes were placed in differentiation medium, as described above. At the designated time, 100 μL of medium was removed from each culture and stored at −30°C until assayed. Adiponectin, interleukin (IL)-8, IL-6, monocyte chemoattractant protein (MCP)-1, and leptin in the culture medium were quantified using ELISA kits (R&D Systems). Values were normalized to cellular protein.

Statistics
Data are presented as means±SEM. Differences between mean values were evaluated by ANOVA, followed by Student–Newman–Keuls testing. Statistical significance was defined as *P*<0.05.

Results
Morphological Features of Perivascular Adipocytes
Figure 1 shows representative images of coronary perivascular, perirenal, and subcutaneous adipose tissues. Perivascular adipose tissue is an integral part of the blood vessel wall, as noted by invasion of adipocytes into the adventitia (red arrows). Perivascular adipocytes are more irregularly shaped and smaller in size than perirenal or subcutaneous adipocytes. Comparative analysis of adipocyte size in situ is shown in the Table.

Preadipocytes From Perivascular Adipose Tissue Exhibit Reduced Capacity for Adipocytic Differentiation
Having observed that perivascular adipocytes are smaller than subcutaneous and perirenal adipocytes in situ, we next investigated the capacity of preadipocytes derived from these adipocyte depots to differentiate into mature adipocytes in vitro. All cell cultures were derived from patients without a preexisting history of cardiovascular disease, and the preadipocytes were isolated from each depot using identical techniques and culture conditions. For these and subsequent studies, perivascular adipocytes were cultured from adipose tissues overlying coronary arteries. The cultured, undifferentiated preadipocytes from each depot displayed similar morphological features when grown in standard medium (data not shown). When exposed to differentiating medium, subcutaneous and perirenal preadipocytes exhibited robust accumulation of cytoplasmic lipid droplets (an index of adipocyte differentiation) within 14 days. In contrast, relatively few perivascular adipocytes displayed cytoplasmic lipid droplets by 14 days after initiation of differentiation protocol, although approximately 50% to 70% of the cells formed lipid droplets by 28 days. A representative DIC microscopic picture of the live cells after 28 days of adipocytic differentiation is shown in Figure 1D through 1F. Oil red O staining confirmed that lipid accumulation was dramatically lower in differentiated perivascular adipocytes as compared with their subcutaneous and perirenal counterparts, as shown in Figure 1G through 1I and quantified in the Table.

Reduced Expression of the Adipocyte-Associated Genes PPARγ, C/EBPα, and FABP4 in Perivascular Adipocytes
We next compared the levels of expression of adipocyte-associated genes PPARγ, C/EBPα, and FABP4 in perivascular, subcutaneous and perirenal adipose tissues, and in differentiated adipocytes derived from these depots. In intact adipose tissues (Figure 2A) and in vitro–differentiated adipocytes (Figure 2B), the relative order of expression of all 3 adipocyte-associated genes was subcutaneous>perirenal>perivascular. Consistent with reduced lipid droplet formation in perivascular adipocytes, lower levels of expression of these genes, which are considered to be master regulators of adipogenesis, suggest that perivascular adipocytes exhibit a reduced state of differentiation as compared with subcutaneous and perirenal adipocytes. Moreover, the strong concordance in adipocyte-associated gene expression between in vitro–differentiated adipocytes and intact adipose tissues suggests that our cell culture system provides a valid model for investigating the molecular and biochemical properties of perivascular adipocytes.

Expression of Adipocytic Differentiation Markers Is Reduced in Perivascular Adipocytes
To provide further support for the hypothesis that adipocytic differentiation is reduced in perivascular adipocytes, we examined transcript levels of fatty acid synthase, glycerol 3-phosphate dehydrogenase 1 (GPDH), lipoprotein lipase, hormone-sensitive lipoprotein lipase, adipose triglyceride lipase, and perilipin (a lipid droplet–associated protein) in differentiated adipocytes isolated from subcutaneous, perirenal, and perivascular adipose tissues. As shown in Figure 3, these adipocyte differentiation/maturation-related genes tended to be expressed at lower levels in perivascular adipocytes. Likewise, adiponectin transcript expression was lower in perivascular as compared with subcutaneous and perirenal adipose tissues (Figure 4A), and in in vitro–differentiated perivascular adipocytes as compared with subcutaneous and perirenal adipocytes (Figure 4B).

The secretion of adiponectin protein by differentiated adipocytes from each depot was also compared. Baseline adiponectin release by subcutaneous, perirenal, and perivascular preadipocytes was very low (data not shown) and remained so early during the course of adipocytic differentiation. By day 8 after initiation of differentiation protocol, increased adiponectin release was detected from subcutaneous and perirenal cells, which peaked at day 12 and 16, respectively (Figure 4C). In contrast, the level of adiponectin released from perivascular cells remained very low throughout the course of the differentiation protocol. Taken together, these findings confirm that human perivascular adipocytes...
display reduced maturation/differentiation as compared with subcutaneous and perirenal adipocytes.

**Expression of Inflammatory Cytokines Is Increased in Perivascular Adipocytes**

Adiponectin possesses antiinflammatory effects, suggesting that perivascular adipocytes, which secrete very little adiponectin, could exhibit a heightened inflammatory state as compared with subcutaneous and perirenal adipocytes. Moreover, inflammation is an integral component of metabolic syndrome and insulin resistance\(^\text{23}\) and can both cause and result from adipocyte dysfunction. Therefore, we examined the expression of several proinflammatory cytokines associated with adipocyte dysfunction. IL-8 transcript expression was higher in perivascular, as compared with perirenal and subcutaneous adipose tissues (Figure 5A), and in differentiated perivascular adipocytes, as compared with their subcutaneous and perirenal counterparts (Figure 5B). Moreover, differentiated perivascular adipocytes released considerably more IL-8 (Figure 5C) and IL-6 (Figure 5D) into the medium as compared with subcutaneous and perirenal adipocytes.

MCP-1 is critically involved in the recruitment of macrophages to adipose tissues and in the subsequent development of insulin resistance.\(^\text{24,25}\) We therefore investigated MCP-1 release from differentiated perivascular, perirenal, and subcutaneous adipocytes. The level of MCP-1 protein released by subcutaneous and perirenal cells was low and remained stable throughout the 3-week course of the differentiation protocol (Figure 5E). In contrast, perivascular cells released substantial amounts of MCP-1 at baseline and throughout the
course of the study (approximately 10- to 40-fold higher than perirenal and subcutaneous adipocytes).

Epicardial fat may share a common embryonic origin with omental fat, which is known for its powerful proinflammatory properties and its important role in insulin resistance. We therefore examined MCP-1 release by differentiated omental adipocytes and epicardial adipocytes isolated from the surface of the right ventricle, away from the coronary arteries. As expected, differentiated omental and epicardial adipocytes released significant amounts of MCP-1, at levels exceeding those released by subcutaneous and perirenal adipocytes isolated from the same patients (Figure 5F). Nevertheless, differentiated perivascular adipocytes released approximately two- to three-fold more MCP-1 as compared to their omental and epicardial counterparts.

Leptin is an adipocyte-derived hormone whose secretion is stimulated by insulin. Hyperleptinemia is common in obesity and is independently associated with insulin resistance and cardiovascular disease. Considering that leptin stimulates MCP-1 production, and that plasma leptin levels correlate with inflammatory markers, we examined leptin expression in perivascular, subcutaneous and perirenal adipose tissues and in differentiated adipocytes derived from these depots.

Leptin mRNA levels were lower in perivascular adipocytes as compared to subcutaneous and perirenal adipocytes, respectively (Figure 5G and 5H). Likewise, perivascular adipocytes tended to release less leptin into the cell culture medium as compared to subcutaneous and perirenal adipocytes, although statistical significance was not met (Figure 5I). These data suggest that the proinflammatory phenotype of perivascular adipocytes is not related to increased leptin expression.

Expression of Brown Adipocyte–Related Genes and Development/Pattern-Forming Genes

Perivascular adipocytes have been suggested to be brown adipocytes, which exhibit distinct patterns of adipocyte-related gene expression and enhanced cytokine production. We therefore examined expression of PRDM16, PGC1α/β, UCP-1, and CPT1β, genes that are highly expressed in brown adipocytes, in freshly isolated subcutaneous, perirenal, and perivascular adipose tissues (Figure 6A) and in vitro–differentiated adipocytes (Figure 6B). As expected, subcutaneous adipose tissue and adipocytes exhibited low levels of expression of these genes. As compared with subcutaneous adipocytes, perirenal and perivascular adipocytes exhibited higher levels of expression of some
of the brown adipocyte-related genes; however, gene expression patterns were inconsistent, and levels of expression were several orders of magnitude lower than has been reported for brown adipocytes. For example, UCP-1, a prototypical marker of brown adipocytes, is expressed at ~1000-fold higher levels in brown versus white adipose tissues. Our results therefore suggest that human perivascular adipocytes are white rather than brown adipocytes.

Phenotypic differences in adipocytes could stem from developmental divergence of the precursor cells from which the mature adipocytes are derived. We therefore compared expression of developmental and pattern-forming genes En-1, Emx-2, and Hox-A10, whose expression levels differ dramatically between human subcutaneous versus omental preadipocytes. We found that expression of all 3 genes was markedly reduced in perivascular as compared with subcutaneous adipocytes (Figure 6C); interestingly, perirenal adipocytes displayed an intermediate pattern of gene expression (Figure 6C). These findings are consistent with the notion that adipocytes residing in these depots are derived from distinct precursor cells, which may underlie the phenotypic differences observed in our study.

**Mouse Perivascular Adipose Tissue Gene Expression: Response to High-Fat Feeding**

Having established that human coronary perivascular adipocytes exhibit reduced levels of adipocytic differentiation and a heightened proinflammatory state, we next investigated gene expression in mouse perivascular (aortic arch), subcutaneous, and visceral (perirenal and epididymal) adipose tissues. At 8 weeks of age, mice were either maintained on a normal chow diet or switched to a high-fat diet for 2 weeks. There was no significant difference in body weight between the 2 groups of mice at the completion of the study (chow, 25.6±1.1 g; high-fat, 27.8±4.4 g; n=6, P=0.09). In chow-fed mice, the transcript levels of adiponectin, PPARγ, FABP4, and leptin tended to be lower in perivascular adipose tissues as compared with the other adipose depots, whereas expression of the proinflammatory MIP1α gene was similar among all depots (Figure 7A). In mice fed a high-fat diet for 2 weeks, expression of adiponectin, PPARγ, and FABP4 was further reduced in perivascular adipose tissues as compared with chow-fed mice, whereas expression of the proinflamma-

tory leptin and MIP1α genes was markedly upregulated (Figure 7B). The responses were much more robust than was observed in the other adipocyte depots.

Because inflammatory cells can modulate adipocyte function, we examined macrophage and T-cell infiltration into the adipose depots by quantifying expression of CD68 (macrophage marker) and CD3 (T lymphocyte marker) mRNA. In chow-fed mice, CD3 expression was lower in perivascular as compared with subcutaneous adipose tissues (see supplemental Table II), whereas CD68 expression was similar among the various depots (data not shown). Thus, the reduced state of differentiation of perivascular adipocytes in chow-fed mice is not associated with increased inflammatory cell infiltration. Following 2 weeks of high-fat feeding, CD3 expression increased only in perivascular adipose tissues (see supplemental Table II) to levels comparable to subcutaneous adipose tissues, whereas CD68 levels remained unchanged in all depots, consistent with an earlier report (data not shown). Thus, increased inflammatory cell infiltration is not likely sufficient to account for the marked changes in perivascular adipocyte gene expression observed after 2 weeks of high-fat feeding.

**Discussion**

Perivascular adipose tissue inflammation has recently been observed in conjunction with atherosclerotic lesions. Here, we show for the first time that human perivascular adipocytes exhibit a heightened proinflammatory state and reduced adipocytic differentiation under basal conditions. We also report that murine perivascular adipose tissue is highly sensitive to the effects of high-fat feeding, which causes further reductions in adipocyte-associated gene expression while upregulating proinflammatory gene expression. Importantly, these characteristics were observed in perivascular adipose tissues from humans and mice without atherosclerotic disease. Taken together, these findings suggest that perivascular adipocytes are poised to play a primary role in development of adventitial inflammation, which in turn may contribute to atherosclerotic lesion development.

Obesity is increasing at alarming rates in industrialized societies, and abundant evidence indicates that it is an important risk factor for atherosclerosis. Obesity is associated with insulin resistance and dyslipidemia, as well as...
increases in circulating inflammatory factors, all of which may contribute to atherosclerosis. In this regard, the distribution of adipose tissue is thought to be important, with visceral adipose tissue generally viewed to be a stronger predictor of atherogenic risk than subcutaneous adipose tissue. Perivascular adipose tissue surrounding the great vessels (the focus of the present investigation) is not traditionally considered a visceral depot based on its anatomic location. However, epicardial fat thickness was shown to correlate with abdominal visceral fat and fasting insulin levels in humans, suggesting that it behaves like visceral fat. Nevertheless, the functional properties of the adipocytes that comprise epicardial and perivascular fat depots surrounding the great vessels have not been defined.

Here, we undertook a detailed comparison of human coronary perivascular, subcutaneous, and visceral (perirenal) adipocytes, performing morphological studies and examining gene expression profiles in intact adipose tissues and in vitro–differentiated adipocytes. The in vitro–differentiated adipocytes isolated from the various tissue depots displayed a similar profile of adipocyte-associated gene expression as compared with their in situ counterparts. Moreover, the smaller size of perivascular adipocytes as compared with subcutaneous and perirenal adipocytes in situ was paralleled by reduced lipid droplet accumulation in differentiated perivascular adipocytes in vitro. Cumulatively, our observations suggest that adipocytes differentiated in vitro under our defined culture conditions retain their depot-specific characteristics and provide a valid model to examine the characteristics of perivascular adipocytes.

We investigated the mechanisms responsible for decreased adipocyte-associated gene expression and reduced differentiation characteristic of perivascular adipocytes. Our data suggest that coronary perivascular adipocytes are white adipocytes that are derived from distinct precursor cells and that they inherently display a proinflammatory phenotype. Inflammation of adipose tissues occurs in diet-induced obesity, and cytokines such as tumor necrosis factor (TNF)α and MCP-1 present in inflamed adipose tissues are thought to trigger insulin resistance in the adipocytes. We observed that preadipocytes and differentiated adipocytes from all depots expressed very little TNFα mRNA, and TNFα release was below the detection limit of our ELISA (data not shown). Likewise, subcutaneous and perirenal preadipocytes produced very little IL-8 or MCP-1 during adipocytic differentiation, while secreting significant amounts of antiinflammatory adiponectin. In contrast, perivascular adipocytes produced very little adiponectin but released substantial amounts of proinflammatory IL-6, IL-8, and MCP-1, while exhibiting reduced adipocytic differentiation. These novel results suggest that inflammatory cytokine release by perivascular adipocytes could modulate insulin sensitivity and cellular function in an autocrine or paracrine manner while attracting macrophages to the depot, further exacerbating inflammation and adipocyte dysfunction.

In addition, in response to 2 weeks of high-fat feeding in mice, expression of adiponectin, PPARγ, and FABP4 in perivascular adipose tissue fell dramatically, whereas expression of the proinflammatory MIP1α and leptin genes were markedly upregulated. Interestingly, we were able to detect
upregulation of CD3, but not CD68, expression in perivascular adipose tissues after 2 weeks of high-fat feeding, suggesting infiltration of T cells, but not macrophages, in this short time period. However, the level of CD3 expression in perivascular adipose tissues did not exceed that detected in subcutaneous adipose tissues, suggesting that inflammatory cell infiltration per se is not sufficient to account for the dramatic changes in gene expression observed in perivascular adipose tissues. Together, these data suggest that perivascular adipose tissue is highly sensitive to the harmful influences of excess dietary fat. This may serve as a driving force for inflammatory cell recruitment to the vascular wall, which potentially could contribute to atherosclerosis.

Collectively, our study provides novel insight into the characteristics of perivascular adipocytes. Our data suggest that human perivascular adipocytes originate from distinct progenitor cells and exhibit reduced differentiation and a heightened proinflammatory state. We also report that high-fat feeding in mice causes further reductions in adipocyte-progenitor cell numbers and exhibit reduced differentiation and a heightened proinflammatory state. These results suggest that perivascular adipocytes may play a primary role in transducing adventitial inflammation in atherosclerosis.

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**Disclosures**

None.

**References**

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# AfA:African American; Cauc: Caucasian.

Online Table II. Depot-specific differences in CD3 mRNA level in mouse adipose tissues following two weeks of chow or high fat diet

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<td>PV</td>
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\textsuperscript{a} data are mean ± SEM of 4 mice in each diet group and expressed as percent SQ value of chow diet. \textsuperscript{b} p < 0.05 vs SQ of chow diet; \textsuperscript{c} p < 0.05 vs PV of chow diet.