EARLY EXPERIMENTAL OBESITY IS ASSOCIATED WITH CORONARY ENDOTHELIAL DYSFUNCTION AND OXIDATIVE STRESS.

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ABSTRACT

**Background:** Obesity is independently associated with increased cardiovascular risk. However, since established obesity clusters with various cardiovascular risk factors, configuring the metabolic syndrome, the early effects of obesity on vascular function are still poorly understood. The current study was designed to evaluate the effect of early obesity on coronary endothelial function in a new animal model of swine obesity.

**Methods:** Juvenile domestic crossbred pigs were randomized to either high fat/high calorie diet (HF) or normal chow diet for 12 weeks. Coronary microvascular permeability and abdominal wall fat were determined using electron beam CT. Epicardial endothelial function and oxidative stress were measured in vitro. Systemic oxidative stress, renin-angiotensin activity, leptin levels and parameters of insulin sensitivity were evaluated.

**Results:** HF pigs were characterized by abdominal obesity, hypertension, elevated plasma lysophosphatidylcholine and leptin in the presence of increased insulin-sensitivity. Coronary endothelium-dependent vasorelaxation was reduced in HF pigs and myocardial microvascular permeability increased as compared to normal pigs. Systemic redox status in HF was similar to normal, while the coronary endothelium demonstrated higher content of superoxide anions, nitrotyrosine and NADPH-oxidase subunits, indicating increased tissue oxidative stress.

**Conclusions:** The current study shows that early obesity is characterized by increased vascular oxidative stress and endothelial dysfunction, in association with increased levels of leptin and prior to the development of insulin-resistance and systemic oxidative stress. Vascular dysfunction is therefore an early manifestation of obesity and might contribute to the increased cardiovascular risk, independently of insulin-resistance.
KEY WORDS: coronary, endothelium, vasodilation, permeability, leptin.
INTRODUCTION

The prevalence of overweight and obesity is increasing in the Western world to epidemic proportions. Data from the Center for Disease Control and Prevention indicate the prevalence of overweight and obesity to be approximately 60 and 30%, respectively, in the United States adult population. Childhood and adolescence obesity, also increasing (49), is associated with vascular dysfunction in otherwise healthy young children (57) as well as with increased cardio-respiratory morbidity (56). Importantly, the presence of obesity and other cardiovascular risk factors in childhood and adolescence tends to persist and progress clinically in early adulthood (14) with high calorie intake being the predominant determinant of obesity in the Western societies (7).

Obesity is well-known to co-segregate with other cardiovascular and metabolic abnormalities, including hypertension, dyslipidemia, glucose intolerance/type 2 diabetes mellitus, in the so-called metabolic syndrome (22). The fundamental feature in the pathogenesis of the metabolic syndrome is considered insulin-resistance (10, 38); in addition, endothelial dysfunction, an early manifestation of atherosclerosis and an independent predictor of cardiovascular events (20, 30, 52, 53), has also been consistently associated with the metabolic syndrome (15, 48), in a complex interplay with insulin-resistance (10).

It has been previously demonstrated that obesity is an independent risk factor for coronary (1) and systemic (8) endothelial dysfunction. However, the mechanisms through which early obesity induces endothelial dysfunction are not clear. Obesity, in particular visceral obesity, is one of the main causes of the increased resistance to insulin. Therefore the presence of endothelial dysfunction in obese subjects or animal models is likely influenced by the insulin-resistance state, which per se, and independently from dysglycemia, has been demonstrated to induce oxygen reactive species production, leading to nitric oxide (NO) breakdown and endothelial
dysfunction(10). However, a possible effect of obesity in inducing endothelial-dysfunction before the development and independently from insulin-resistance, has been suggested in a rat model of diet-induced obesity(33). Various factors have been proposed to induce obesity-related endothelial dysfunction, including increased plasma levels of leptin(28) and free fatty acids(13). The current study was designed to test the hypothesis that the initial cardiovascular manifestations of obesity, including endothelial dysfunction, might start early and before the establishment of the fully developed metabolic syndrome. To this purpose, in a new experimental model of large animal early obesity we assessed coronary endothelial function, myocardial microvascular permeability as well as parameters of oxidative stress and metabolic homeostasis.
METHODS

**Animals:**
The Institutional Animal Care and Use Committee approved the study. Juvenile female domestic crossbred pigs (3 months old, initial weight 25-30 kg, Larson Products, Sargeant, MN) were placed on a high fat / high calorie diet (HF, n=6; 20% lard, 4.31kcal/g, TD.03358, Harlan Teklad, Madison, Wisconsin) or on a normal chow diet (N, n=6, 0.81 kcal/g) for 12 weeks. Content of carbohydrates, amino acids, minerals and vitamins was identical in both diet regimens. After the completion of the diet period, fasting blood samples were drawn, animals were anesthetized and scanned by electron beam computerized tomography (EBCT)(39) and thereafter euthanized with an overdose of pentobarbital sodium (10m mg/kg IV Sleepaway, Fort Dodge Laboratories, Fort Dodge, IA). Coronary arteries were harvested immediately after euthanasia.

**Systemic Measurements:**
At the end of the study period, body weight, abdominal wall fat thickness and intrabdomial fat (measured in EBCT images)(45, 46) were used as indicators of obesity. Blood pressure was recorded by an intraarterial catheter during the EBCT study. High sensitivity C-reactive protein (hsCRP), and plasma renin activity (PRA) were evaluated by standard procedures.

**Metabolic parameters:** Plasma lipid profile, glucose, and insulin were measured by standard methods. The glucose/insulin ratio and HOMA index (plasma glucose X insulin/22.5) were used as indicators of insulin sensitivity. Plasma leptin was measured by Multi-Species Leptin RIA assay (LINCO Research, Inc., St. Charles, MO). Lysophosphatidylcholine (LPC) 16:0 and 18:0 –
highly atherogenic products of lipid metabolism\cite{16, 29} were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS), using 17:0 Lyso-PC as internal standard, with an electrospray triple quadrupole MS (Sciex API 3000).

**Systemic oxidative stress:** was evaluated by plasma 8-Isoprostanes (8-Isoprostane EIA Kit, Cayman Chemical Company, Ann Arbor, MI) - a specific marker of free radical-induced damage\cite{21}, and oxidized-LDL (OxLDL, ELISA, Mercodia AB, Uppsala, Sweden).

**Plasma Nitric Oxide (NO) end-products:** Serum NO-derivates were quantified by a two step assay for the sum of both nitrites and nitrates using a commercially available kit (Nitric Oxide quantitation Kit, Active Motif, Carlsbad, CA) following manufacturer’s instructions.

**In Vivo Studies:**

**Myocardial microvascular permeability by EBCT:**

Each animal was anesthetized with 0.5 g of intramuscular ketamine (20 mg/kg) and xylazine (2 mg/kg), intubated, and mechanically ventilated. Anesthesia was maintained with a mixture of ketamine (0.2 mg/kg/min) and xylazine (0.03 mg/kg/min) in saline. Under sterile conditions and fluoroscopic guidance, intravascular catheters were then positioned in the suprarenal aorta for measurement of arterial pressure and in the right atrium for injections of contrast medium\cite{11, 39}. Animals were transferred to the EBCT (Imatron C-150, Imatron Inc. South San Francisco, CA) scanning gantry and allowed a 30-min recovery, during which saline (5 mL/min) was administered and a blood sample collected from the central venous catheter. Non contrast enhanced scans at the umbilical levels were used for evaluation of the subcutaneous and visceral fat. Heart localization scans were performed to identify cross-sectional images at two adjacent mid-left ventricular levels. As previously described\cite{6} for the assessment of coronary
microvascular permeability 40 consecutive ECG-triggered end-diastolic scans were obtained over the pre-selected levels at one to three heartbeat intervals after a bolus injection (0.3 ml/kg) of non-ionic, low-osmolar contrast agent iopamidol (Isovue-370, Squibb Diagnostics, Princeton, NJ) into the right atrium. The same acquisition sequence was repeated after intravenous infusion of adenosine (400 µg/kg per min) and dobutamine (15 µg/kg per min), in a randomized order.

**EBCT Data Analysis:**

For the measurements of coronary microvascular permeability, a parameter of microvascular endothelial function in vivo, regions of interest were traced in the anterior LV wall and chamber(31). Time-density curves were generated and the intra-vascular and extra-vascular transit of contrast medium was modeled, as previously described(31, 40). The area and first moment of each curve were calculated. Micro-vascular permeability (permeability index; arbitrary units) was calculated as: $60 \times 1.05 \times \frac{\text{slope}}{\text{area under input curve}} \times \frac{1}{\text{BV}}$, where slope is the maximal slope of the ascending arm of the extra-vascular curve which reflects vascular leakage of contrast medium(5), MTT represents the mean transit time, and blood volume (BV) was used as a surrogate for vascular surface area.

**Intra-abdominal adipose tissue quantification by EBCT**

For each pigs 5 EBCT-derived cross-sections (at the level of renal hilum) were analyzed to estimate the amount of intra-abdominal fat tissue(45, 46). Using an image analysis software (Analyze®, Biomedical Imaging Resource, Mayo Foundation, Rochester, MN) the density range of subcutaneous fat tissue was used to automatically threshold the adipose tissue in the entire image. Subsequently a region of interest was traced internally to the muscular wall of the abdomen (Figure 1). Intra-abdominal fat was expressed as percentage of the selected area.
In Vitro Studies:

Coronary Endothelial Function:
Coronary endothelial function was evaluated by organ chambers technique as previously described (5, 40). Briefly, arterial rings were pre-contracted with $10^{-7}$ mol/l endothelin-1 (Phoenix Pharmaceuticals, Mountain View, CA) and challenged by increasing doses of the endothelium-dependent vasodilator bradykinin ($10^{-11}$ to $10^{-6}$ mol/l, Sigma, St. Louis, MO), the non–receptor-mediated endothelium-dependent vasodilator calcium ionophore A23187 (10-11 to 10-6 mol/L, Sigma) was obtained, and the endothelium-independent vasodilator sodium nitroprusside ($10^{-9}$ to $10^{-4}$ mol/l, Sigma). Complete relaxation of each ring was tested by exposure to $10^{-3.5}$ mol/l papaverine and the response was calculated as a percent from complete relaxation. $ED_{50}$ was calculated as the effective dose required to reach 50% relaxation in each vessel and averaged.

Coronary oxidative stress:
1) Superoxide anions: tissue superoxide anions production was evaluated by the oxidative-sensitive fluorescence dye dihydroethidium (DHE)(50). Unfixed frozen sections of coronary arteries were cut into 30 µm-thick slices and placed on a glass slide. After incubating with dihydroethidium (10-6 mol/L) in light protected humidified chamber for 30 minutes at 37ºc, tissue sections were imaged with a laser scanning microscope (Zeiss Laser Scanning Microscope 5 Pascal, Version 3.2). An image analysis program (MetaMorph, Meta imaging series 4.6) was used to quantify the percentage of positively stained area in a blinded fashion.
2) Immunohistochemistry: coronary artery tissue slices were stained for NADH/NADPH, the major source of superoxide production in vascular tissues and nitrotyrosine, a marker of tissue protein oxidation. Briefly, coronary arterial slices were deparaffinized rehydrated and incubated
with equimolar 3% H$_2$O$_2$ to block endogenous tissue peroxidase activity. Primary antibodies (nitro-tyrosine 1:1000, Sigma; NADPH oxidase subunits p67 1:200, p47 1:200 and gp91 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA) were incubated overnight (4°C) and detected with the EnVision kit (Dako Corporation; Carpinteria, CA) in peroxidase-labeling technique with 3,3-diaminobenzidine tetra-hydrochloride (DAB) as the chromogen (Vector Laboratories Inc; Burlingame, CA), to yield a brownish reaction product. Incubation with a non-specific isotype antibody served as a negative control and the sections were counterstained with hematoxylin. A computer-assisted light microscopy and image analysis program (MetaMorph, Meta imaging series 4.6) was used to semi-automatically quantify the immunohistochemistry results as percent area positively stained.

Histology for coronary fibrosis:

Paraffin slides, 5µm-thick cut sections, were stained with standard Masson’s trichrome to evaluate perivascular fibrotic deposition in a similar fashion.

**Statistical analysis:**

Data are expressed as mean±SEM or as percent change from baseline (in-vitro endothelial function and EBCT permeability). Unpaired Student’s t test was used to compare groups. Statistical significance was accepted for a probability value <0.05.
RESULTS

Systemic measurements

As detailed in the table, pigs in the HF group were heavier, had significant accumulation of abdominal wall and intra-abdominal fat compared to N (Table 1 and Figure 1), and were hypertensive. In addition HF were characterized by a mild dyslipidemia, reflected by a significant increase in total and LDL cholesterol as compared to N, a tendency to increased HDL levels and unchanged triglycerides (Table 1).

Pigs in the HF group were characterized by non-significantly lower values of both plasma glucose and insulin. Interestingly, systemic insulin sensitivity in HF was increased, as demonstrated by the significantly higher glucose/insulin ratio and the tendency to lower HOMA index values (Table 1).

Systemic leptin levels were higher in the HF diet group than the normal diet group (Table 1).

Systemic oxidative stress markers 8-isoprostanes and OxLDL, as well as CRP, were similar in N and HF pigs (Table 1). Plasma LPC 18:0 was significantly higher in HF as compared to N, with no difference in the levels of LPC 16:0 (Table 1). In contrast, serum NO end-products levels were significantly lower in the HF group compared to the group on normal diet. Plasma renin activity was similar in the two groups.

Myocardial microvascular permeability by EBCT:

Basal permeability was similar in N and HF (1.5±0.1 and 1.3±0.4 AU, respectively; p=n.s.). As shown in Figure 2, myocardial microvascular permeability did not change significantly in N in response to either adenosine (+6.1±4.6%) or dobutamine (+18.8±26.6%). On the contrary, in HF pigs myocardial permeability increased significantly after both adenosine (+56.2±15.4%, p<0.05
vs. N) and dobutamine (+177.8±44.7%, p<0.05 vs. N), hence suggesting coronary microvascular endothelial dysfunction in HF pigs.

**Vascular Endothelial Function:**

The contraction to ET-1 was similar in the two groups. The maximal percent vasorelaxation to increasing doses of the endothelium-dependent vasodilator bradykinin was significantly attenuated in coronary vessels of HF (38.5±5.3%) compared with N (90.5±2.3%; p<0.001; Figure 3), and ED$_{50}$ was significantly higher in HF (logM -7.1±0.2 and -8.4±0.1, respectively, P<0.001). Also the maximal vasorelaxation to calcium ionophore was impaired in HF pigs (53.1±8.2%), compared to N (99.1±0.6%; p<0.001 vs. HF). Additionally, the dose-response curve to increasing doses of the endothelium-independent vasodilator sodium nitroprusside was mostly similar between the two groups, but at the highest dose HF showed a significantly lower vasodilation (53.2±4.6) as compared to N (82.2±4.1; p<0.01; Figure 3). However, ED$_{50}$ for vasorelaxation to sodium nitroprusside did not differ between HF and N (logM -4.4±0.2 and -5.0±0.2, respectively; p=n.s.).

**Vascular tissue measurements**

A marked increase in DHE fluorescence was found throughout the vascular wall of HF (12.98±0.66%) coronaries as compared to N (5.94±1.43%; p<0.05), reflecting an increase in superoxide anion production (Figure 4), which was localized mainly in the endothelial cells, and to a lesser degree in the adventitia.

Coronaries from HF pigs showed higher expression of NADPH oxidase subunits p67 (N, 0.6±0.2%; HF, 3.7±0.9%; p<0.05; Figure 5), and regulatory p47 (N, 0.4±03; HF, 4.2±0.5;
p<0.05; Figure 5), while no differences were observed between the groups in the expression of the catalytic subunit gp91\( (N, 0.05\pm0.01; HF, 0.07\pm0.03; p= \text{n.s.}; \text{data not shown}). \) HF pigs were also characterized by higher rate of protein nitration as demonstrated by the immunostaining for nitrotyrosine \( (N, 0.8\pm0.4; HF, 3.3\pm0.1; p<0.001; \text{Figure 5}), \) indicating interaction between superoxide and NO.

However, no morphological changes were observed in the vascular structure of the HF coronary arteries and they showed similar perivascular fibrotic deposition \( (N=11.4\pm0.7 \text{ and } HF=8.7\pm1\% \text{ area positively stained, } p=\text{n.s.}). \)
DISCUSSION

The present study demonstrates that the early phases of abdominal obesity are characterized by coronary endothelial dysfunction in association with vascular oxidative stress, hypertension and mild lipid profile abnormalities in the absence of a state of insulin-resistance. These changes are accompanied by a systemic increase in leptin and LPC levels and decreased NO end-products. In contrast, no systemic inflammation or oxidative stress were observed, suggesting that the early abnormalities induced by obesity are mainly localized at the vascular wall level.

The current study suggests that early obesity is associated with functional changes both in the epicardial arteries, as demonstrated by the impaired vasodilating response to the endothelium-dependent stimulus, as well as in the myocardial microcirculation, as demonstrated by the altered permeability response to cardiac challenge. These abnormalities may contribute to the progression of coronary atherosclerosis and cardiovascular events, prior to the development of insulin resistance, considered the center point of the metabolic syndrome(37).

The possible mechanisms involved in the pathogenesis of epicardial and microvascular coronary endothelial dysfunction are multifactorial and involve systemic and local factors.

**NO bioavailability**

Obesity-associated vascular dysfunction is often related to impaired biological activity of NO(23, 34). In the present study the reduced vasorelaxation to endothelium-dependent vasodilators in HF confirms the altered bioavailability of NO. This was also supported by the lower plasma levels of nitrites/nitrates in HF pigs. Indeed, in the vessels of HF pigs a significantly higher rate of tyrosine nitration is present as compared to N, as the result of the interaction between NO and
superoxide to form the actively nitrating substance peroxynitrite. A previous study in hypertensive rats showed that the exposure to oxidant agents leads to a reduction in plasma levels of nitrites/nitrates, which are restored by antioxidant treatment(55). Conceivably, in HF pigs the reduced levels of nitrites/nitrates might reflect the formation of other nitrated substances, such as nitrotyrosine, following the scavenging of NO by oxidative stress.

This is supported by the evidence of increased NADPH-oxidase subunits expression in the HF coronary endothelium. NADPH-oxidase is the main source of superoxide anions in atherosclerosis(47) and accordingly, we found increased levels of superoxide anions in HF endothelium. Since systemic levels of Ox-LDL and 8-isoprostone in the HF diet group were not increased as compared to N, these data implicate local vascular oxidative stress as a major determinant of early obesity-associated endothelial dysfunction.

**Leptin and toxic lipid-derived products**

Leptin, a hormone secreted by white adipose tissue, is elevated in obese individuals in proportion to the amount of adipose tissue(12). Evidence supports the involvement of leptin in the pathogenesis of obesity-induced cardiovascular risk, in particular hypertension(32, 44). In a recent study, Beltowski et al(3) demonstrated that pharmacological hyperleptinemia induces systemic and localized oxidative stress, decreases NO bioavailability, possibly due to its degradation by reactive oxidative species, and renal sodium retention that may contribute to leptin induced hypertension. Although associative, the current study suggests the possibility that a lower non-pharmacological increase in endogenous circulating leptin levels, although not sufficient to raise systemic oxidative stress, might be associated with increased vascular oxidative stress and endothelial dysfunction, as well as hypertension. Moreover, since leptin has
been associated with increased vascular wall stiffness(42), the increased leptin levels might account for the impaired vasodilating response to high dose endothelium-independent stimulus sodium nitroprusside. It is to be noted that other adipokines not assayed in the present work, including adiponectin, have been demonstrated to play a role in the modulation of several cardiovascular functions and may therefore participate in inducing the abnormalities we observed.

Interestingly, HF pigs showed a significant increase in the plasma levels of LPC 18:0 as compared to N, with no difference in LPC 16:0 levels. LPC is a highly atherogenic phospholipid(16) and, in animals, its plasma levels are regulated principally by the activity of the enzyme lecithin-cholesterol acyltransferase (LCAT), which catalyzes the transfer of fatty acids from phosphatidylcholine to cholesterol and leads to the formation of cholesterol esters and LPC(18). Long chain LPC (C>16:0) is known to increase endothelial permeability(25, 36, 59) and to induce endothelial dysfunction(17, 26, 41). Therefore, the increased levels of LPC 18:0 might account, at least partly, for the impairment in endothelial function and microvascular permeability, observed in the present study. Interestingly, in accordance with the results from our study, LPC was found to impair both endothelium-dependent and –independent vasorelaxation in porcine coronary arteries(41). Since LCAT plays an important role in the HDL-mediated transport of cholesterol from peripheral tissues to the liver(19), activation of the enzyme, possibly related to obesity(51) and/or high leptin levels(2), might lead, in these early phases of obesity, to an increase in the plasma levels of HDL-cholesterol (as observed in the present study, although not reaching statistical significance) and contemporarily to overproduction of LPC, participating to the impairment of endothelium-dependent, and possibly –independent vasorelaxation.
**Insulin-resistance**

Obesity is strongly associated with insulin resistance and this latter is considered the main mechanisms inducing local and systemic abnormalities observed in the metabolic syndrome(37). A complex interplay between insulin-resistance and endothelial function has been demonstrated(9), and studies have suggested that the endothelial dysfunction observed in obese patients is mediated by the reduced insulin mediated nitric oxide release(48). However, the present study suggests that insulin-resistance is unlikely to be the primary cause for the endothelial dysfunction in the early phases of obesity, since our porcine obesity model showed in fact increased insulin sensitivity. It might be speculated that the onset of insulin-resistance in obesity is a later event, and, as already proposed(9), can be induced or worsened by the presence of endothelial dysfunction, which on the contrary is an early feature of obesity.

**Inflammation**

The adipose tissue is not only a storage tissue but also an active endocrine organ and secretes numerous pro-inflammatory hormones and cytokines such as interleukin-6 and tumor necrosis factor-α(27). Furthermore, macrophages reside in the adipose tissue and further secrete pro-inflammatory mediators and up-regulate the secretory activity of the adipocytes(58). Hence, obesity is considered to be an inflammatory state that predisposes to atherogenesis in the long term. CRP, a highly sensitive marker of inflammation and an independent predictor of cardiovascular events, was not increased in HF pigs, arguing against the possibility that this early obesity model caused a systemic inflammatory reaction sufficient to explain the observed endothelial dysfunction. Moreover, while in obese subjects plasma leptin levels are correlated with inflammatory markers, particularly CRP(43), here we did not find such a relation. We might
speculate that the increased CRP levels are rather expression of more advanced stages of obesity, in association with the onset of insulin resistance. A limitation of the present study is represented by the lack of adipose tissue biochemical and histological characterization, which however has limited direct impact of on the function of large and small coronary arteries.

**Renin-angiotensin system and hypertension**

Brook et al proposed that increased angiotensinogen levels derived directly from adipocytes secretion might be an important link between uncomplicated obesity and vascular endothelial dysfunction(8). Adipose angiotensinogen gene expression is increased in obesity(54) and the subsequent increased in angiotensin II at the vascular tissue level may stimulate vascular tissue production of superoxide(4), a common factor in the etiology of endothelial dysfunction. The development of hypertension in HF pigs may suggest activation of the renin-angiotensin system. However, systemic PRA was similar in N and HF groups. Although ruling out the systemic activation of the renin angiotensin system, these data do not exclude an increased local tissue activity. Additionally, considering the early phase of obesity in our study, it is possible that the activation of the renin-angiotensin system establishes in subsequent stages, possibly in association with the onset of insulin-resistance(35). Another possible cause of hypertension in our animal model might be represented by the activation of the sympathetic nervous system associated with obesity. In particular, this seems plausible considering the increased levels of leptin found in HF pigs and the well established effect of leptin in inducing an overactivation of the sympathetic system(24).

The raised blood pressure levels observed as a part of the obesity syndrome, might be partly responsible for the impairment in endothelial function. However, in a previous study from our
group(40), coronary arteries from hypertensive pigs showed a milder reduction in the response to bradykinin and a normal response to calcium-ionophore. Therefore, hypertension does not seem to explain completely the vascular alterations found in HF.

Perspectives

The current study introduces a new model of experimental early obesity induced by high fat/high calorie diet. This model is associated with mild hyperleptinemia, increased vascular oxidative stress and decreased NO bioavailability, leading to endothelial dysfunction and hypertension. Moreover, a possible contributor to the observed impairment in endothelial function and permeability might be represented by the increased plasma levels of the atherogenic phospholipid LPC. Although associative, these results show a clustering a metabolic and cardiovascular abnormalities in the early phases of atherosclerosis in the absence of systemic insulin-resistance, oxidative stress or inflammation. The results of the present study lead to possibly important clinical implications, since in the initial phases of obesity, significant vascular functional alterations may occur, contributing to increase cardiovascular risk. The early intervention on obesity with dietetic and pharmacological approaches, as well as physical exercise might prevent or correct these abnormalities and the later onset of insulin-resistance which in turn leads to the vicious cycle of the metabolic syndrome. Importantly, these modifications are not associated with worsened insulin-sensitivity, systemic inflammation and systemic oxidative stress.
GRANTS

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Figure legend

**Figure 1:** Representative EBCT images at the umbilical level for the assessment of abdominal fat in N (A,B) and HF pigs (C,D). Left panel (A,C) show EBCT slices used to trace the intraabdominal cavity (dotted red line). Right panels (B,D) show the same slices after thresholding using the density range of the subcutaneous fat (red area). Intraabdominal fat was calculated as the percentage of red within the dotted red line. White and yellow arrows indicate subcutaneous and intraabdominal fat, respectively.

**Figure 2:** Percent change in myocardial microvascular permeability in response to adenosine (left) and dobutamine (right) in normal (N) and high-fat diet (HF) pigs. Data are presented as percent change ± standard error from baseline. * P<0.05 compared to N.

**Figure 3:** Coronary artery vasorelaxation response to endothelium-independent stimulus sodium nitroprusside (top panel) and endothelium-dependent stimuli bradykinin (middle panel) and calcium ionophore (bottom panel) in normal (N) and high-fat diet (HF) pigs. * P<0.001 compared to N.

**Figure 4:** Top: Fluorescence photomicrograph showing in situ superoxide anions in normal (N; left) and high-fat diet (HF; right) pigs coronaries (Original magnification 10X). Bottom: Bar graph showing quantification of superoxide anions as percent of the field that was positively stained. * P=0.002 compared to N. A, adventitia; I, intima; L, lumen.
**Figure 5:** Representative immunostaining nitrotyrosine (A,B), NADPH-oxidase p47 (C,D) and p67 (E,F) subunits in normal (A, C, E) and high-fat diet (B, D, F) pig coronary arteries. Brown staining (arrow) represents positive staining. Original magnification (20X). Bottom: Immunohistochemical localization of NADPH-oxidase p67 subunit in normal (C) and high-fat diet (D) pig coronary arteries. Brown staining (arrow) represents positive staining. Original magnification (20X)
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### Table 1. Systemic parameters (mean±SEM) in normal (N) and high fat diet (HF) pigs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N (n=6)</th>
<th>HF (n=6)</th>
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<tr>
<td>Abdominal wall fat thickness (cm)</td>
<td>1.35±0.13</td>
<td>1.78±0.05*</td>
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<tr>
<td>Intra-abdominal fat (%)</td>
<td>6.1±1.0</td>
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<td>Weight (kg)</td>
<td>62.3±1.38</td>
<td>70.5±1.5*</td>
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<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>109±11.1</td>
<td>130±11.3*</td>
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<tr>
<td>Total cholesterol (mmol/l)</td>
<td>1.96±0.21</td>
<td>2.63±0.17†</td>
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<td>HDL cholesterol (mmol/l)</td>
<td>0.93±0.12</td>
<td>1.20±0.12</td>
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<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>0.73±0.18</td>
<td>1.37±0.05†</td>
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<tr>
<td>Triglycerides (mmol/l)</td>
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<td>0.27±0.07</td>
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<td>Glucose (mmol/l)</td>
<td>7.6±0.9</td>
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<td>Insulin (µU/ml)</td>
<td>1.11±0.45</td>
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<td>Glucose/Insulin Ratio (mg/µU)</td>
<td>16.4±4.9</td>
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<td>HOMA index</td>
<td>0.43±0.23</td>
<td>0.06±0.02‡</td>
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<td>Leptin (ng/ml)</td>
<td>2.67±0.08</td>
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<td>Lysophosphatidylcholine 16:0 (µmol/L)</td>
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<td>32.9±3.3</td>
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<td>C-reactive protein (g/dL)</td>
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<td>0.02±0.001</td>
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<td>Serum nitrites/nitrates (µmol/L)</td>
<td>9.5±0.3</td>
<td>4.7±1*</td>
</tr>
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</table>

HDL, high-density lipoprotein; LDL, low-density lipoprotein; *, p<0.01; †, p<0.05; ‡, p=0.057.
Figure 2
Figure 3