CALL FOR PAPERS | Mechanisms of Diastolic Dysfunction in Cardiovascular Disease

Early cardiac changes induced by a hypercaloric Western-type diet in “subclinical” obesity

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Submitted 2 August 2015; accepted in final form 10 January 2016

Gonçalves N, Silva AF, Rodrigues PG, Correia E, Moura C, Eloy C, Roncon-Albuquerque R Jr, Falcão-Pires I, Leite-Moreira AF. Early cardiac changes induced by a hypercaloric Western type diet in “subclinical” obesity. Am J Physiol Heart Circ Physiol 310: H655–H666, 2016. First published January 13, 2016; doi:10.1152/ajpheart.00684.2015.—“Obesity cardiomyopathy” effects have been widely described; however, the specific contribution of metabolic changes and altered adipokine secretion are still uncharacterized. Moreover, a diagnosis based on body mass index might not be the most accurate to identify increased adiposity and its outcomes. In this study, we aimed to determine the impact of a Western-type diet [hypercaloric diet (HCD)] ingestion on biventricular structure and function, as well as the metabolic and endocrine changes that occur before the establishment of overt obesity. Wistar rats were fed for 6 wk with a regular diet or HCD. At the end of the protocol, metabolic tests, cardiac structure, and functional evaluation were performed, and blood and tissue samples collected to perform histological, molecular biology, and functional studies. The animals that ingested the HCD presented increased adiposity and larger adipocyte cross-sectional area, but similar body weight compared with the regular diet group. At the cardiac level, HCD induced biventricular cardiomyocyte hypertrophy, fibrosis, increased stiffness, and impaired relaxation. Galectin-3 plasma expression was likewise elevated in the same animals. The nutritional modulation also altered the secretory pattern of the adipose tissue, originating a proinflammatory systemic environment. In this study, we observed that before “clinical” overweight or frank obesity is established, the ingestion of a HCD-induced cardiac remodeling manifests by increased biventricular stiffness and diastolic dysfunction. The mechanism triggering the cardiac alterations appears to be the proinflammatory environment promoted by the adipose tissue dysfunction. Furthermore, galectin-3, a profibrotic molecule, might be a potential biomarker for the myocardial alterations promoted by the HCD before overweight or obesity.

OVERWEIGHT, OBESITY, AND THEIR metabolic consequences affect millions of people, particularly in developed countries, being nowadays considered a global epidemic (34). Sedentary lifestyles alongside with a cafeteria diet are the most important factors that contribute to increased body weight, metabolic impairment, and increased risk of developing cardiovascular diseases (CVD) (15). In the last years, the existence of “obesity cardiomyopathy” endorsed entirely or predominantly to obesity has been accepted, including features such as insulin resistance, inflammation, impaired myocardial relaxation, and diastolic dysfunction (8). However, the specific contribution of the metabolic changes and altered adipokine expression promoted by the expansion of adipose tissue (AT) are still undefined (36). Thus understanding these pathophysiological mechanisms would be particularly important to unravel potential strategies to target therapeutically early in the course conditions, where insulin resistance and inflammation are already present, before overweight or frank obesity is established.

Presently, most studies focus on the impact that “obesity cardiomyopathy” has on the left ventricle (LV) function and structure, overlooking its effects on the right ventricle (RV). Indeed, the impact on RV is highly conceivable, considering the obesity-induced proinflammatory systemic environment. A recent 2-yr follow-up study reported a twofold increased risk of heart failure (HF) in subjects without CVD but already presenting RV hypertrophy (16), highlighting the need to look to the “other” side of the heart.

Moreover, the diagnosis of overweight and obesity is still mostly based on body mass index (BMI), a debatable metric that might not be the most accurate, and early parameters to diagnose obesity and its early injuries are needed. In this study, we aim to evaluate the impact that a hypercaloric Western-type diet ingestion has on biventricular structure and function. Moreover, we investigated the metabolic and endocrine changes on myocardium and AT promoted by this regimen before the establishment of overt obesity.

NEW & NOTEWORTHY

After 6-wk ingestion of a Western-type diet, Wistar rats already presented biventricular cardiac remodeling and diastolic dysfunction despite no significant increase in body weight. Underlying the myocardial changes is a proinflammatory environment promoted by adipose tissue dysfunction. The profibrotic agent galectin-3 might be an important biomarker for these alterations.
Table 1. Diet composition

<table>
<thead>
<tr>
<th>Calorie Composition</th>
<th>Regular Diet (AO4%)</th>
<th>Hypercaloric Diet (F2685)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, %</td>
<td>16.1</td>
<td>20.5</td>
</tr>
<tr>
<td>Carbohydrate, %</td>
<td>3.2</td>
<td>34.7</td>
</tr>
<tr>
<td>Monosaccharides, g/kg</td>
<td>17.3</td>
<td>1</td>
</tr>
<tr>
<td>Disaccharides, g/kg</td>
<td>6.8</td>
<td>182</td>
</tr>
<tr>
<td>Polysaccharides, g/kg</td>
<td>26.1</td>
<td>157</td>
</tr>
<tr>
<td>Sodium, %</td>
<td>0.25</td>
<td>0.4</td>
</tr>
<tr>
<td>Fat, %</td>
<td>3.1</td>
<td>36.0</td>
</tr>
<tr>
<td>Saturated fat, g/kg</td>
<td>17.3</td>
<td>141</td>
</tr>
<tr>
<td>Monounsaturated fat, g/kg</td>
<td>6.8</td>
<td>162</td>
</tr>
<tr>
<td>Polysaturated fat, g/kg</td>
<td>26.1</td>
<td>40.2</td>
</tr>
</tbody>
</table>

METHODS

Animal Accommodation and Nutritional Modulation

All animal experiments were conducted in conformity with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 2011) and with the Portuguese law of animal welfare (DL 129/92, DL 197/96; P 1131/97). The Faculty of Medicine at the Universidade do Porto is a governmental institution granted approval by the Portuguese government to perform animal experiments as described in this study.

Animals were housed in groups of five rats per cage in a controlled environment under a 12:12-h light-dark cycle at a room temperature of 22°C, with a free supply of food and water. Male Wistar rats (Charles River Laboratories) weighing 100–150 g were randomly divided and fed with two types of diet for a period of 6 wk: regular diet (RD group; 2.9 kcal/g, AO4; Scientific Animal Food & Engineering) or a hypercaloric diet, rich in lipids, carbohydrates, and increased salt content (HCD group; 5.4 kcal/g, F2685; BioServed; detailed diet composition in Table 1).

Metabolic Evaluation

The metabolic tests were performed after 6 wk of nutritional modulation, namely glucose tolerance and insulin resistance. Glycemia was measured after 4 h of fasting at baseline, and 15, 30, 60, 90, and 120 min after administration of 1 g/kg glucose by gavage or a 0.5 U/kg intraperitoneal insulin injection, respectively. Blood was collected from the tail vein by puncture with a 25-gauge needle (n = 10 animals/group). The values obtained in each test were plotted and the area under curve, defined as milligrams per deciliter per hour, calculated.

Metabolic cages were used to assess the daily food consumption by the animals of each group (n = 8 animals/group).

Echocardiographic Assessment

Echocardiographic evaluation was performed using a 10-MHz transducer (GE Vivid 7). Animals (n = 6/group) were anesthetized with a combination of ketamine (75 mg/kg) and xylazine (10 mg/kg) and allowed to stabilize for 15 min. From the left parasternal short-axis view, two-dimensional guided M-mode tracings were made just below the mitral valve at the level of the papillary muscles for measurements of the interventricular septum (mm) and posterior wall thickness (mm). Data were indexed to body surface area, as described previously (10).

The myocardial performance or Tei index was retrieved from the following formula:

\[
Tei = (IVCT + IVRT)/ET
\]

where IVCT is the isovolumic contraction time, IVRT corresponds to the isovolumic relaxation time, and ET is ejection time.

Tricuspid annular plane systolic excursion (TAPSE) was assessed in M-mode as the base-to-apex shortening during systole of the lateral portion of the tricuspid annular plane.

The velocity at the mitral valve annulus during atrial contraction (A’) was measured in tissue Doppler evaluation, as well as tricuspid annular plane maximal systolic velocity (E’) and early ventricular filling velocity (E).

Hemodynamic Evaluation

After 6 wk of nutritional modulation, biventricular invasive assessment of cardiac function was performed, as previously described (10).

Briefly, the animals (n = 9/group) were anesthetized with sevoflurane (8% for induction and 2.5–3% for maintenance), intubated for mechanical ventilation, and placed over a heating pad (body temperature was maintained at 37°C). Under binocular surgical microscopy (Wild M651.MS-D, Leica), the right jugular vein was cannulated for fluid administration (prewarmed 0.9% NaCl solution, 10 ml kg⁻¹ h⁻¹) to compensate for perioperative losses. The heart was exposed through a median sternotomy, the aorta was dissected to place an ultrasonic flow probe, connected to a flowmeter (Transonic Systems), and a silk thread was passed around the inferior vena cava to perform transient occlusions and decrease preload. High-fidelity tip pressure-volume catheters were inserted into the LV and RV (SPR-847, 2F, and PVR-1045, 1F, respectively; Millar Instruments). After complete instrumentation, the animal preparation was allowed to stabilize for 15 min. Data were continually acquired digitally (MPVS 300, Millar Instruments), recorded at 1,000 Hz (ML880 PowerLab 16/30, Millar Instruments), and analyzed off-line by software PVAN 3.5 (Millar Instruments).

Hemodynamic recordings were made under basal and under isovolumetric contractions (vena cava and ascending aorta occlusion) with respiration suspended at end expiration. Analyzed LV and RV hemodynamic parameters include peak systolic pressure (Psys) and time constant of isovolumetric relaxation.

Conductance calibration. Parallel conductance values were obtained by the injection of ~100 µl of 10% NaCl into the right atrium. Calibration from relative volume units conductance signal to absolute volumes (µl) was undertaken using a previously validated method of comparison to known volumes in Perspex wells (37).

Sample Collection and Morphometric Analysis

Once the hemodynamic data collection was completed, the animals were euthanized by exsanguination, and blood and tissue samples were collected. The heart, lungs, liver, kidneys, AT (renal, gonadal, visceral, and subcutaneous), and right gastrocnemius muscle were excised and weighed. The right tibia was also removed and measured for normalization of the organs. The samples were collected, rinsed with NaCl 0.9%, frozen in liquid nitrogen, and stored at −80°C for molecular studies or fixed in 10% buffered formalin for histological procedures. Previously fixed tissue samples were dehydrated with graded ethanol, cleared with xylene, and included in paraffin blocks. Serial sections (4 µm of thickness) of paraffin blocks were cut by a microtome (RM2125RTS, Leica) and mounted on silane-coated slides.

Adipocyte Diameter

Section images of visceral and subcutaneous AT samples stained with hematoxylin and eosin were acquired with a light microscope (×400, LEICA DM4000B) and analyzed with an image software program (cellB life science basic imaging software, Olympus, CA). The average adipocyte diameter was determined measuring all adipocytes in 10 fields for each animal (n = 9/group).

Myocardium Extracellular Matrix Characterization

Cardiomyocyte cross-sectional area was determined at the nucleus level in 100 representative myocytes for each animal (n = 12/group) in hematoxylin and eosin-stained samples.
The percentage of fibrosis was calculated (ImagePro; MediaCybernetics) as the sum of all connective tissue areas divided by the sum of averaged connective tissue and muscle areas. Four representative fields of each picrosirisus red stained samples were analyzed, and areas of reparative and perivascular fibrosis were excluded (n = 6 animals/group).

Determination of the relative proportion between collagen type I and type III was made according to their different birefringences in five different images per sample with a polarized microscope (n = 8 animals/group).

Isolated Cardiomyocyte Measurements

Force measurements were performed in single, triton-permeabilized cardiomyocytes mechanically isolated as described previously (11) to detect systolic and/or diastolic dysfunction at the myofilament level.

Briefly, myocardial samples were defrosted in extraction solution (without Ca\(^{2+}\)), mechanically disrupted, and incubated for 5 min in relaxing solution supplemented with 0.2\% Triton X-100 to remove all membrane structures. The cardiomyocytes were then washed by consecutive centrifugations and attached between a force transducer and a motor transducer for different protocols.

Data acquisition was obtained after transferring the cardiomyocyte from relaxing [pCa (−log10[Ca\(^{2+}\)]) = 9.0] to activating solution (pCa = 4.5). The isometric force started to develop [total force (F\(_{\text{total}}\)], and, once a steady-state force level was reached, the cell was shortened within 1 ms to 80\% of its original length (slack test) to determine the baseline of the force transducer. The distance between the baseline and the steady force level is the F\(_{\text{total}}\). After 20 ms, the cell was restretched and returned to the relaxing solution, in which a second slack test of 10-s duration was performed to determine resting or passive force (F\(_{\text{passive}}\)). Active force (F\(_{\text{active}}\)) was calculated using the formula: F\(_{\text{total}}\) = F\(_{\text{active}}\) + F\(_{\text{passive}}\). On average, 30 cardiomyocytes were measured per group (~5 cardiomyocytes per animal in a total of 6 animals of each group).

The sarcomeric F\(_{\text{active}}\)-length relation was obtained by stretching the cardiomyocyte in relaxing solution from 1.8 to 2.3 μm of sarcomere length.

### Table 2. Primer sequence used in mRNA quantification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5' → 3'</th>
<th>Tissue Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TGGCCCTTCGCTTCTCCTACCC</td>
<td>Heart, AT</td>
</tr>
<tr>
<td>Reverse</td>
<td>CGCCCTTCGCTTCTACCTCCTCCTC</td>
<td></td>
</tr>
<tr>
<td>Collagen type I Forward</td>
<td>CTTGCGCTTCAGGTAGGAC</td>
<td>Heart</td>
</tr>
<tr>
<td>Reverse</td>
<td>CAATTGGCAGAGCCAGACGACGAC</td>
<td></td>
</tr>
<tr>
<td>Collagen type III Forward</td>
<td>GGGACAGGGGTCTCCTACTAAT</td>
<td>AT</td>
</tr>
<tr>
<td>Reverse</td>
<td>GATAGGAGGGGCCCCCTTGAGTAG</td>
<td></td>
</tr>
<tr>
<td>VEGF Forward</td>
<td>GTACCTCGCAGCACTGCAAGAT</td>
<td>AT</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCAATTAGGGGCAACGACGAC</td>
<td></td>
</tr>
<tr>
<td>TNF-α Forward</td>
<td>GCTCGGTGTTGGTGACTGAATGG</td>
<td>AT</td>
</tr>
<tr>
<td>Reverse</td>
<td>ACTGGCGGTGATGCTGACAAAAT</td>
<td></td>
</tr>
<tr>
<td>IL-1β Forward</td>
<td>ATATCCCTAAACTCTTTGGGTCTG</td>
<td>AT</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGGCCAACTCTGCTGGAATCTC</td>
<td></td>
</tr>
<tr>
<td>Resistin</td>
<td>TGGTGGGCTCATTGGAGAACT</td>
<td>AT</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGTACGCTGTGGCTTGCGTCTCGG</td>
<td></td>
</tr>
<tr>
<td>Leptin Forward</td>
<td>TCTCAGGACACAGTCTTTGGAAGG</td>
<td>AT</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCGCAGGGCGAGAGGGTCAC</td>
<td></td>
</tr>
</tbody>
</table>

AT, adipose tissue.

**RNA Quantification**

After total RNA extraction (Tripure, Roche) and reverse transcription, equal amounts of cDNA from every sample underwent real-time PCR (StepOne Plus, Applied Biosystems) experiments for each gene, using SYBR green as marker (Qiagen). Standard curves were obtained for each gene correlating the cDNA quantities in graded dilutions from a randomly selected tissue sample with the respective threshold cycles (second derivative maximum method). GAPDH was used as internal control since its cDNA levels were similar in the studied groups. Results are normalized for GAPDH [set as arbitrary unit (AU)]. Specific PCR primer pairs for the studied genes are listed in Table 2.

**Plasma Analysis**

At the end of the hemodynamic evaluation, venous blood samples collected from the RV in EDTA-containing tubes were centrifuged at 5,000 rpm for 15 min at 4°C, and plasma was collected and frozen at −80°C until analysis.

Plasma levels of galectin-3 were measured by an ELISA kit (MyBiosource), according to the manufacturer’s instructions (n = 7/group). Each sample was analyzed in duplicate. Absorbance was recorded at 450 nm using an ELISA plate reader (Perkin-Elmer), and linear regression was plotted and used to calculate galectin-3 concentration in the plasma samples.

The adipokine and inflammatory serum profile was assessed by a Proteome Adipokine Antibody Array (R&D Systems) in nitrocellulose membranes, according to the manufacturer’s instructions (n = 2/group).

**Statistical Analysis**

All data are presented as means ± SE, and n represents the number of animals. Differences between groups were analyzed with a t-test or Mann-Whitney after confirming normality and homogeneity of variance with Shapiro-Wilk and F-tests, respectively (GraphPad Prism version 6). Results were considered significantly different when P < 0.05.

**RESULTS**

Animal Morphometric and Metabolic Characteristics

After 6-wk ingestion of a HCD, the animals presented similar body weight (Table 3) but increased subcutaneous and visceral AT, as assessed by gonadal and renal AT normalized to tibia length. These findings were further supported by the hypertrophy of subcutaneous and visceral adipocytes from the HCD group (Fig. 1).

The weight of other organs or tissues, such as liver, lungs, or gastrocnemius, was similar (Table 3).

As observed in Table 4, at the end of the protocol, the HCD group ingested the same quantity of food daily, but the calories consumed were significant higher. Furthermore, these animals already presented metabolic dysfunction compared with the

### Table 3. Biometric data

<table>
<thead>
<tr>
<th></th>
<th>RD</th>
<th>HCD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>321 ± 8.1</td>
<td>336 ± 10.0</td>
<td>0.25</td>
</tr>
<tr>
<td>TL, cm</td>
<td>0.38 ± 0.003</td>
<td>0.38 ± 0.004</td>
<td>0.93</td>
</tr>
<tr>
<td>Heart/TL, g/cm</td>
<td>2.3 ± 0.05</td>
<td>2.5 ± 0.06*</td>
<td>0.008</td>
</tr>
<tr>
<td>Gonadal adipose tissue/TL, g/cm</td>
<td>5.4 ± 0.49</td>
<td>9.9 ± 0.64***&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Renal adipose tissue/TL, g/cm</td>
<td>6.2 ± 0.48</td>
<td>13.0 ± 0.65***&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Liver/TL, g/cm</td>
<td>26.6 ± 0.94</td>
<td>28.1 ± 0.90</td>
<td>0.28</td>
</tr>
<tr>
<td>Lungs/TL, g/cm</td>
<td>3.49 ± 0.096</td>
<td>3.47 ± 0.099</td>
<td>0.87</td>
</tr>
<tr>
<td>Gastrocnemius/TL, g/cm</td>
<td>5.3 ± 0.19</td>
<td>5.2 ± 0.13</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. TL, tibia length; RD, regular diet; HCD, hypercaloric diet. *P < 0.05 vs. RD; ***P < 0.001 vs. RD.
CARDiAC CHANGES IN “SUBCLINICAL” OBESiTY

Fig. 1. Adipocyte cross-sectional area (CSA) from subcutaneous (A) and visceral (B) adipose tissue (AT). Values are means ± SE; n = 9/group. *P < 0.05 vs. RD. Representative images of adipocytes from the regular diet (RD; C) and hypercaloric diet (HCD; D) groups are displayed.

RD group, as confirmed by significant hyperglycemia, insulin resistance, and glucose intolerance.

Echocardiographic and In Vivo Hemodynamic Analysis

Cardiac hypertrophy was already manifest after 6 wk of HCD consumption, as evidenced by the increased indexed heart weight (Table 3) and by enlarged cross-sectional area of cardiomyocytes (Fig. 2, A and B). Echocardiography further confirmed a concentric pattern of hypertrophy, as shown by the increased indexed thickness of interventricular septum and LV posterior wall, as well as decreased LV internal diameter (Table 5). Importantly, together with the echocardiographic evaluation, hemodynamic assessment evidenced biventricular (Table 5). Importantly, together with the echocardiographic evaluation, hemodynamic assessment evidenced biventricular functional changes induced by HCD consumption. The LV of the HCD group displayed subtle alterations, such as a significant decrease of ejection time (Table 5) and contractility reserve, the HCD group displayed subtle alterations, such as a significant functional changes induced by HCD consumption. The LV of the HCD group displayed subtle alterations, such as a significant functional changes induced by HCD consumption. The LV of the HCD group displayed subtle alterations, such as a significant increase of indexed thickness of interventricular septum and LV posterior wall, as well as decreased LV internal diameter (Table 5). Importantly, together with the echocardiographic evaluation, hemodynamic assessment evidenced biventricular (Table 5) and RV (Fig. 4A). At 2.2 μm, cardiomyocytes’ Factive was elevated between groups in the LV (Fig. 4B) and RV (Fig. 4D).

Force Measurements in Isolated Skinned Cardiomyocytes

Cardiac functional consequences of a short-term ingestion of HCD were also observed at the myofilamental level. Skinned cardiomyocytes from both ventricles of the HCD group presented increased myocardial stiffness reflected in higher Fpassive for all sarcomere lengths tested in the LV (Fig. 4A) and in the RV (Fig. 4C). At 2.2 μm, cardiomyocytes’ Factive was elevated between groups in the LV (Fig. 4B) and RV (Fig. 4D).

Extracellular Matrix Alteration

Besides biventricular cardiomyocyte hypertrophy (Fig. 2), HCD also induced significant fibrosis in both ventricles (Table 6). More precisely, collagen assessment using polarized microscopy revealed that, in the RV, HCD consumption induced a significant shift in collagen isoform composition toward a higher proportion of collagen III (Table 6). In the LV, no differences in collagen isoforms were detected between groups.

The molecular approach showed that the changes in extracellular matrix (ECM) composition were an on-going process with a significant elevation of collagen type III mRNA synthesis in the LV (HCD: 1.0 ± 0.17 AU vs. RD: 0.6 ± 0.09 AU, P = 0.02) and in the RV (HCD: 0.9 ± 0.06 AU vs. RD: 0.7 ± 0.04 AU, P < 0.001), consistent with the histological findings. Regarding the collagen type I expression, no differences were observed in the

Table 4. Metabolic parameters

<table>
<thead>
<tr>
<th></th>
<th>RD</th>
<th>HCD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>0.79</td>
</tr>
<tr>
<td>Food ingested in 24 h, g</td>
<td>16.0 ± 0.81</td>
<td>15.6 ± 1.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Kcal ingested in 24 h</td>
<td>46.5 ± 2.50</td>
<td>84.5 ± 5.80***</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glycemia, mg/dl</td>
<td>102 ± 2.4</td>
<td>127 ± 3.7***</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin resistance test, mg·dl·h⁻¹</td>
<td>9.338 ± 59.6</td>
<td>13,208 ± 531.7*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose tolerance test, mg·dl·h⁻¹</td>
<td>15.067 ± 654.9</td>
<td>20,139 ± 843.5*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. kcal, kilocalories. ***P < 0.001 vs. RD.
LV (HCD: 1.0 ± 0.10 AU vs. RD: 0.9 ± 0.11 AU, P = 0.45) nor in the RV (0.7 ± 0.07 AU vs. 0.6 ± 0.08 AU, P = 0.15).

**Plasma Characterization**

To have an overview of the potential mediators triggering the cardiac alterations herein described, we performed a rat adipokine plasma array in two representative animals of each group. The proteomic profile of the plasma adipokines and inflammatory molecules is described in Table 7, and the most important molecules involved in myocardial remodeling are demonstrated in the Fig. 5, left. Animals fed with HCD presented elevated levels of proinflammatory and chemotactic factors, with increased TNF-α, IL-6, monocyte chemotactic protein-1, endocan, and macrophage colony-stimulating factor expression. Molecules involved in cardiomyocyte hypertrophy and ECM remodeling, including resistin, tissue inhibitor of metalloproteinase-1, regulated on activation normal T-expressed and secreted, receptors for advanced glycation end-products, serpin-1, macrophage colony-stimulating factor, and lipocalin-2, were also elevated. The same pattern was observed in angiogenesis-related factors, VEGF, hepatocyte growth factor, and serpin-1. Only some of the abovementioned molecules reached statistical significance due to the small number of samples used in array experiments. Even though as early as 6 wk of HCD ingestion, a clear environment that promotes chronic sterile inflammation, cellular growth, and detrimental ECM remodeling was induced. Also, circulatory levels of galectin-3, a profibrotic molecule, were raised in the same animal group (Fig. 5).

**Visceral AT Alterations**

The HCD group already presented AT expansion and adipocyte hypertrophy after only 6 wk of nutrition modulation (Fig. 1). Thus we decided to analyze expression levels of key adipokines involved in inflammation, cardiac remodeling, and growth to verify its possible systemic impact. The results showed an overexpression of leptin, resistin, VEGF, as well the cytokines TNF-α and IL-1β. (Fig. 5, right).

**DISCUSSION**

In the present study, we describe the cardiac repercussions of a HCD ingestion in Wistar rats before the onset of frank obesity. We observed biventricular alterations in the ECM with increased collagen deposition, as well as cardiomyocyte hypertrophy. These modifications in myocardial structure result in impairment of diastolic function induced by HCD, despite similar body weight. Contributing to the myocardial changes are circulatory proinflammatory proteins like galectin-3, TNF-α, members of the IL family, as well as angiogenic-related factors. One of the sources instigating this chronic inflammatory state is the visceral AT, already hypertrophied and dysfunctional in the animals fed with a HCD for 6 wk.

**Effects of the HCD in Body Weight and Metabolic Status**

In our protocol, ingestion of a HCD for a period of 6 wk did not induce a significant increase in body weight (±7.2% vs. RD group, P = 0.2510). Through the last years, many studies have used diverse types of diet composition, protocol duration, and animal models, but, so far, no consensus in their repercussions was achieved. Some authors report an increase in body weight (24), while others reported no differences in this parameter (22), being these divergent outcomes are mostly due to the experimental protocol design. In fact, a diet combining high fat with high carbohydrates promotes more profound effects in the heart compared with high-fat diet alone (24). Other important aspects in the type of diet are the presence of saturated or unsaturated fat (21), as well as the type of sugar included in the diet (28). We used a diet that better reproduced the Western-type regime, enriched not only with fat (36%) and carbohydrates (35%), but also with excessive salt (0.4%) compared...
with the regular chow. This combination of nutrients and the inclusion of a high-salt content replicates the diets underlying the development of metabolic syndrome and the associated CVDs.

Although with the same body weight, the HCD animals already presented impaired metabolic function with elevated glycemia, glucose intolerance, and resistance to insulin. In the last years, special attention has been given to insulin resistance syndrome, pointed as a key pathophysiologic link between diastolic dysfunction and obesity (14). In fact, impaired diastolic function is already present in the prediabetic state, suggesting that insulin resistance is the first sign of myocardial changes (29). A recent population-based study supports this hypothesis, demonstrating that LV diastolic dysfunction is associated with insulin resistance and not with the presence of diabetes (12). Since insulin resistance influences not only the energy supply used, but also other pathways, such as fibrosis deposition and endothelial dysfunction, it might play an important role in promoting increased myocardial stiffness and diastolic impairment.

Structural Alterations in the Heart

It is acknowledged that overweight and obesity can directly and indirectly modulate the heart, either by promoting an increased hemodynamic overload, neurohumoral activation, as well as through secretion of proinflammatory adipokines. In fact, several studies have reported alterations in cardiac struc-

ture in animal models and patients with overweight or obesity (6, 8, 9, 24, 27). Carbone et al. (3) showed that, after 8 wk of ingestion of a high-sugar and high-fat diet, male and female mice exhibited 21% increase in body weight, as well as increased myocardial fibrosis. However, we observed that cardiac hypertrophy precedes the phenotypic manifestation of obesity, highlighting the importance of preventing the alterations associated with an unhealthy diet and to identify other parameters other than BMI to discriminate patients at risk. In our nonobese model, the animals presented early changes, such as collagen overexpression, cardiomyocyte hypertrophy, and an elevated heart weight, consistent with a pattern of concentric hypertrophy, as assessed by echocardiography.

Interestingly, in our experimental model, we also observed RV structural changes similar, but more severe, than those described for the LV in the HCD group, such as fibrosis and hypertrophy. Up until now, few studies explored the RV alterations and the underlying causes that occur during excessive energy intake. The MESA-Right ventricle study, enrolling more than 4,000 subjects, investigated the link between overweight, obesity, and RV structure and function (6). This study observed increased RV mass (overweight: 6%, obesity: 14%) and end-diastolic volumes, as well as lower ejection fraction in overweight and obese participants. Even after adjustment for LV parameters, the abovementioned differences were still significant in obese participants (obesity: 8%; P < 0.001), suggesting that the modifications in the RV are independent of LV parameters. The authors proposed several mechanisms to explain the cardiac effects of increased BMI, for instance, that the adipokines produced during obesity are different and able to promote cardiac remodeling. A smaller study with participants without cardiovascular risk factors investigated the association between RV hypertrophy and obesity (27). The data obtained demonstrated a positive correlation between BMI and RV mass in both men and women. Even after adjusting for LV diastolic dysfunction, one of the main factors responsible for RV enlargement, the association was present, suggesting that other mechanisms as altered adipokines secretion may mediate the RV alterations.

Another feature present in overweight and obesity models is cardiac fibrosis, whose degree depends on the animal species, the obesity trigger (genetic or diet induced), and the existence of other diseases (5). Previous reports state that, in these diet-induced models, the development of fibrosis is a late consequence, being necessary a prolonged exposure to the diet to induce a significant ECM remodeling. Quin et al. (26) reported diastolic dysfunction associated with LV cardiomyocyte hypertrophy and increased interstitial fibrosis, together with oxidative stress, in C57BL6J mice fed with a high-fat and high-sugar diet for 8 mo. Conversely, Calligaris et al. (2) described a similar amount of cardiac fibrosis in the same animal model but after 16 wk of HCD, demonstrating the variability of results by using different diets and protocol duration. In our study, the combination of a high-fat, high-carbohydrate, and high-salt diet promoted increased fibrosis deposition in a very short period of time. We detected biventricular increase of fibrosis, along with overexpression of collagen type III. Our results demonstrated that the alteration in the collagen content precedes the establishment of obesity, indicating that these parameters might be monitored and po-
tentially used to identify patients with increased cardiovascular risk.

One of the molecules involved in cardiac remodeling, especially in the promotion of fibrosis, is galectin-3. It has been reported that galectin-3 knockout mice submitted to transverse aortic constriction were unable to develop cardiac fibrosis and LV dysfunction (38), while its infusion promoted cardiac inflammation, fibrosis, hypertrophy, and dysfunction in rats (20).
Likewise, in diabetes mellitus type 2 and obese patients, elevated circulatory levels of galectin-3 have been reported, being the macrophages in visceral AT proposed to be the major source of its production (33). Similar to these data, the non-obese animals of the HCD group already presented increased plasma levels of galectin-3. However, the mRNA expression of galectin-3 in the visceral AT presented no significant differences between the two groups, suggesting that another source of this protein might be activated. Regardless of this result, a more specific macrophage analysis of the AT is needed to better understand its source and its role in cardiac remodeling.

**Impaired Relaxation of the Ventricles**

Several authors have already reported the effects of a Western type of diet in cardiac function. For instance, the study conducted by Carbone et al. (3) demonstrated that obese mice developed systolic and diastolic dysfunction after only 8 wk of nutritional modulation. In our protocol, even before body weight is significantly increased, rats fed with HCD already displayed impaired cardiac structure (cardiomyocyte hypertrophy and increased myocardial fibrosis). LV systolic function was preserved in the HCD group; however, some alterations in the myocardial function are only perceptible when the heart is subjected to a stress stimulus. This situation is particularly important to assess diastolic dysfunction, being frequently detected only under exercise stress tests (7, 18, 19). In this regard, we mimicked a stress condition by imposing an acute aortic occlusion and demonstrated that the LV of the animals subjected to a stress stimulus. This situation is particularly important to better understand its source and its role in cardiac remodeling.

**Inflammatory Environment and “Adiposopathy”**

The results observed in our animal model with concentric LV remodeling increased ECM deposition and impaired relaxation fit the description of an animal model of diastolic dysfunction. A recent new paradigm for HF with preserved ejection fraction (HFrEF) was suggested where the systemic inflammation promotes structural and functional changes in the myocardium (25). Several comorbidities can induce this pro-inflammatory environment, such as overweight, obesity, diabetes, and insulin resistance. Some of the mentioned features were present in our model, so we analyzed the adipokine and cytokine profile of the animals. The HCD group presented a systemic increase of mediators involved in cardiac remodeling and inflammation, such as resistin, TNF-α, members of the IL family, as well as other chemotactic and proliferative agents.

Several groups demonstrated that proinflammatory cytokines are able to directly promote diastolic dysfunction. One

**Table 6. Characterization of the extracellular matrix**

<table>
<thead>
<tr>
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<th>RD</th>
<th>HCD</th>
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<tr>
<td><strong>Left ventricle</strong></td>
<td></td>
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<tr>
<td>n</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>5.1 ± 0.46</td>
<td>12.3 ± 0.89*</td>
</tr>
<tr>
<td>Collagen I/total collagen</td>
<td>64.3 ± 2.14</td>
<td>64.4 ± 2.61</td>
</tr>
<tr>
<td>Collagen II/total collagen</td>
<td>35.7 ± 2.30</td>
<td>35.6 ± 2.61</td>
</tr>
<tr>
<td><strong>Right ventricle</strong></td>
<td></td>
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<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>5.6 ± 0.31</td>
<td>8.0 ± 0.56*</td>
</tr>
<tr>
<td>Collagen I/total collagen</td>
<td>81.9 ± 2.29</td>
<td>67.0 ± 3.08*</td>
</tr>
<tr>
<td>Collagen II/total collagen</td>
<td>18.1 ± 2.29</td>
<td>33.0 ± 3.08*</td>
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Values are means ± SE in percent; n, no. of rats. *P < 0.05 vs. RD.
of these molecules is TNF-α, whose circulatory levels are positively correlated with the degree of diastolic dysfunction and is a predictor of poor outcome (35). A recent study revealed that TNF-α suppresses SERCA2a gene expression and promotes increased diastolic Ca²⁺ and myocardial relaxation impairment (30).

Other cytokine that strengthens this hypothesis is IL1β. It has been reported that there are elevated plasma levels of IL-1β in HF patients (31), and that this molecule is able to impair myocardial contractility and relaxation (17). Additionally, preclinical studies showed improvement of cardiac function and exercise tolerance in patients with systolic and diastolic HF treated with anakinra, an IL-1 blocker (4, 31, 32).

One of the main “organs” mediating the inflammatory state is the AT through the secretion of adipokines. During excessive energy intake, this tissue hypertrophies, becoming dysfunctional with overproduction of factors involved in inflammation, cell proliferation, cell growth, and ECM remodeling, such as leptin and resistin (13). In fact, the term “adiposopathy” was proposed to designate the expansion and proliferation of adipocytes and AT alongside proinflammatory adipokine production that culminates in impaired metabolic status and increased CVD risk (1).

In our study, the HCD group exhibited similar body weight compared with the RD group; however, the fat depots were already enlarged. Both the gonadal and renal ATs presented elevated weight, and the subcutaneous and visceral adipocytes were hypertrophied. The phenotypic alterations in the AT were translated in endocrine and immune dysfunction. We observed an overproduction of the adipokines resistin and leptin, besides other proinflammatory and proliferative mediators.

The main goal of our study was to report the cardiac effects of a typical Western diet, hoping to contribute to unraveling of the complexity of “increased adiposity” underlying many of the common HFpEF-associated comorbidities. Despite the relevance of our data describing the early detrimental impact in the structure and function of both ventricles, a major limitation relates to the impossibility to determine what the triggering factor of cardiac remodeling is: AT impairment due to excessive calorie consumption, or a specific nutrient included in the HCD.

In conclusion, our study demonstrated that, even without “clinical” overweight or obesity, ingestion of a Western-type diet for a short period of time was able to stimulate myocardial structural and functional remodeling. The HCD promoted biventricular hypertrophy, cardiomyocyte stiffening, and fibrosis that resulted in impaired diastolic function and might lead to HFpEF. The mechanism stimulating these cardiac modifications appears to be the metabolic disarrangement, such as insulin resistance and the dysfunction of the AT endocrine role. Efforts should be made to change the lifestyle of the population, to establish more accurate parameters of excessive adiposity besides BMI, as well as to identify early the metabolic impairment to prevent the deterioration of the cardiovascular system and decrease the prevalence of CVD. Galectin-3, a profibrotic molecule, is an attractive biomarker for myocardial remodeling that needs more attention by the investigators. Its quantification and interventions to reduce its levels can be useful to prevent stiffening of the heart and, consequently, diastolic dysfunction.

ACKNOWLEDGMENTS

The authors thank Maria José Mendes for the cardiac histological evaluation and to Daniel Moreira-Gonçalves and Cristine Schmidt for support in performing experiments and drafting the manuscript.

GRANTS

This work was supported by the Portuguese Foundation for Science and Technology Grants UID/IC/00051/2013 financed with national funds by Fundação para a Ciência e Tecnologia and by Fundo Europeu do Desenvolvimento Regional through COMPETE 2020 - Programa Operacional Competitividade e Internacionalização; EXCL/BIM-MEC/0055/2012; and by European Commission Grant FP7-Health-2010, MEDIA-261409. This work was also supported by Portuguese Foundation for Science and Technology Grant SFRH/BD/66628/2009 (to N. Gonçalves).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Fig. 5. Adipocytokine profile in plasma (left) measured using ELISA (galectin-3; n = 7/group) or proteomic array (n = 2/group) and in the visceral adipose tissue (right) quantified by real-time RT-PCR (n = 7/group). VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; AU, arbitrary units. Values are means ± SE. *P < 0.05 vs. RD.
and I.F.-P. edited and revised manuscript; I.F.-P. and A.F.L.-M. approved final version of manuscript.

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