GALECTIN-3 PARTICIPATES IN CARDIOVASCULAR REMODELING ASSOCIATED WITH OBESITY

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SUPPLEMENTAL MATERIAL

METHODS

Study Population

Morbidly obese patients referred to bariatric surgery were consecutively recruited from the Obesity Care Unit of Fuenlabrada University Hospital, Spain. The selection of the patients was performed by Committee, which includes personnel from Endocrinology, general and upper gastroenterology surgery, internal medicine and cardiology services. Inclusion criteria: ≥18 years and universally accepted indications for bariatric surgery, long-term obesity (more than 4 years); body mass index (BMI) ≥40 despite other weight loss strategies or BMI ≥35 in the presence of obesity-related comorbidities (diabetes mellitus, obesity hypoventilation syndrome, obstructive sleep apnea syndrome, hypertension). Exclusion criteria were age >60 years and unacceptable surgical risk due to concomitant comorbidities. Volunteers (BMI ≤25) were recruited from staff of the Hospital. The study protocol was approved by the Ethics Committee, and all participants signed the informed consent. The study was conducted in compliance with Good Clinical Practice guidelines and the ethical principles stated in the Declaration of Helsinki.

Anthropometric Measurements

All participants underwent physical examination which included anthropometric measurements, blood tests, 12-lead electrocardiogram (ECG), and transthoracic echocardiogram. The height and weight of patients were recorded when taking the echocardiogram, with the patients wearing light clothing without shoes. We used wall scales. After 10min rest in a sitting position, a 12-lead ECG was taken and blood pressure was measured in the non-dominant arm. BMI was calculated according to the formula: weight (kg)/height squared (meters).

Echocardiogram

Echocardiography studies were performed using a commercially available unit Vivid I (GE Healthcare, Waukesha, WI, USA) equipped with a 2.5MHz probe. Transthoracic echocardiography study was performed according to the recommendations of the European Society of Echocardiography. The mass of the left ventricle (LV) in grams was calculated using the Devereaux et al. formula:

\[
\text{Mass of LV (g)} = 0.8 \times \left\{1.04 \times [(\text{LVEDD}+\text{dPW})^3 - \text{LVEDD}^3]\right\} \times 0.6
\]

where LVEDD is the LV end-diastolic diameter and dPW LV is the posterior wall thickness at end diastole.

The LV mass was indexed by the body surface area to the power of 2, in normoweight subjects and to the power of 2.7 in obese patients to minimize the interference of obesity in the estimate of ventricular mass. The relative parietal thickness (RPT) was calculated using the formula:

\[
\text{RPT} = \frac{\text{dIVS}-\text{dPW}}{\text{LVEDD}}
\]
where dIVS is the interventricular septal wall thickness in diastole.

An indexed value for LV mass $\geq 45 \text{g/m}^2$ for both male and female normoweight subjects and $\geq 51 \text{g/m}^2$ for both male and female obese patients, and $\text{RPT} \geq 0.45$ were considered to be defining values for concentric hypertrophy.

According to the RPT and the indexed mass for the LV, 4 LV geometric patterns were defined:

1. Normal: $\text{RPT} < 0.45$ and an indexed lv mass $< 51 \text{g/m}^2$.
2. Concentric remodelling: $\text{RPT} \geq 0.45$ and an indexed LV mass $< 51 \text{g/m}^2$.
3. Concentric hypertrophy: $\text{RPT} \geq 0.45$ and an indexed LV mass $\geq 51 \text{g/m}^2$.
4. Eccentric hypertrophy: $\text{RPT} < 0.45$ and an indexed LV mass $\geq 51 \text{g/m}^2$.

The volume of the left atrium (LA) was calculated according to validated formulas.$^1, 2$ Doppler analysis was performed according to standard recommendations.$^2$ The average of the measurements for 3 consecutive cardiac cycles was calculated for each value. The assessment of echocardiographic measurements was performed by a single observer under masked conditions. All measurements were performed in a post-processing workstation EchoPAC® (GE Healthcare, Waukesha, WI, USA).

**Laboratory Tests**

Blood samples were obtained following the clinical protocol approved for bariatric surgery. Briefly, venous blood samples (20 mL) were collected after an overnight fast (>10 hours) between 7:00 a.m. and 9:00 a.m. into vacutainers Rapid Serum Tubes. Serum was separated from whole blood by centrifugation (20 minutes at 300 g) and stored at $-80^\circ \text{C}$ until analysis. Routine hematologic and blood chemistry were performed with standard methods. C-reactive protein (CRP) serum levels were measured by EIA with highly sensitive latex based turbidimetric immunoassay on a Hitachi analyzer (Sigma Chemical). Serum levels of Pro-BNP (MilliplexMAP; Millipore Corporation), Gal-3 (BG Medicine), type I C-terminal collagen propeptide (CICP; Microvue bone health) and the metalloproteinase 2 tissue inhibitor of metalloproteinases 2 (MMP-2/TIMP-2; Complex (R&D Systems) were measured with specific EIA according to the manufacturer’s protocols. All samples were run in duplicate and analyzed on the same day to minimize day-to-day variation.

**Animal study**

Male Wistar rats of 150g (Harlan Ibérica, Barcelona, Spain) received either a high-fat diet (HFD, 33.5% fat; Harlan Teklad #TD.03307, MN, USA; n=16) or a standard diet (3.5% fat; Harlan Teklad #TD.2014, MN, USA; n=16) for 6 weeks. Half of the animals of each group received the Gal-3 activity inhibitor, modified citrus pectin (MCP; 100 mg/kg/day) in the drinking water for the same period as previously described.$^3$ The Animal Care and Use Committee of Universidad Complutense de Madrid approved all experimental procedures according to guidelines for ethical care of experimental animals of the European Community. Body weight was measured once a week. Food and water intake were determined throughout the experimental period. Blood pressure (SBP) was estimated basally, at mid-study and end-of-study through use of a tail-cuff plethysmograph (Narco Bio-Systems) in unrestrained animals as previously reported.$^4$
Evaluation of cardiac structure and function

Cardiac structure and function were evaluated by transthoracic echocardiography with a Philips CX50 (Philips, Netherlands) connected to a L12-3 MHz linear transducer in rats anesthetized with isoflurane (2%). 2D-guided M-mode recordings made from short axis views in order to measure LV chamber dimensions, interventricular septum (IVT) and posterior wall thickness (PWT) were measured from the bidimensional parasternal long-axis view. The mean measurements from several consecutive beats were used for data analysis.

Left ventricular ejection fraction (LVEF) was calculated according to the Teicholz Formula: 
\[
\text{LVEF} = \frac{\text{EDD} \times 7}{(2.4+\text{EDD})}
\]

LV systolic chamber function (pump function) was determined from LV endocardial fractional shortening (FS) = (EDD-ESD) / EDD x 100 and LV midwall fraction (MWFS) = (EDD/2+PWTd/2)-(ESD/2+PWTs/2) / (EDD+PWTd/2+IVT/2), where EDD is end-diastolic diameter in left ventricle, ESD is end-systolic diameter in LV, PWTd is posterior wall thickness in diastole and PWTs is posterior wall thickness in systole.

Morphological and histological evaluation

Cardiac and aorta tissue samples were dehydrated, embedded in paraffin and cut in 4 μm-thick sections. Both sections were stained with picrosirius red by routine methods. The area of cardiac interstitial fibrosis was identified as the ratio of interstitial fibrosis or collagen deposition to the total tissue area after excluding the vessel area from the region of interest. Aorta fibrosis was only quantified in the media layer as the ratio of interstitial fibrosis or collagen deposition to the total media area. For each sample, 10 to 15 fields were analyzed with a 40X objective under transmitted light microscope (Leica DM 2000; Leica AG, Germany). Myocytes (60–80 per animal) with visible nucleus and intact cellular membranes were chosen for determination of cross-sectional area in cardiac sections stained with hematoxylin and eosin. Quantitative analysis was performed using an analysis system (Leica LAS 4.3; Leica AG, Germany). A single researcher unaware of the experimental groups performed the analysis.

Real-time PCR

Total RNA was extracted with Trizol Reagent (Euromedex) and purified using the RNeasy kit, according to the manufacturer’s instructions (Qiagen). First strand cDNA was synthesized according to the manufacturer’s instructions (Roche). Quantitative PCR analysis was then performed with SYBR green PCR technology (ABGene) (Table S1).

Relative quantification was achieved with MyiQ (Bio-rad) software according to the manufacturer's instructions. Data were normalized by HPRT levels and expressed as percentage relative to controls. All PCRs were performed at least in triplicate for each experimental condition.

Western Blot

Total proteins were prepared as previously described from either cardiac or aortic homogenates from obese and control rats. Proteins were separated by SDS-PAGED on
10 % polyacrylamide gels and transferred to Hybond-c Extra nitrocellulose membranes (Hybond-P; Amersham Biosciences, Piscataway, NJ). Membranes were probed with primary antibody for Gal-3 (Thermo Scientific, Rockford, IL; dilution 1/2000), collagen I (AbDSerotec, Oxford, UK; dilution 1/1000), fibronectin (Millipore, Temecula, CA, USA; dilution 1/500), transforming growth factor β (TGF-β; Abcamplc, Cambridge, UK; dilution 1/500), connective tissue growth factor (CTGF; Torrey Pines BiolabsInc, East Orange, NJ; dilution 1/1000), osteopontin (OPN; Santa Cruz, CA, USA; dilution 1/500), monocyte chemotactic protein-1 (CC12; Santa Cruz, CA, USA; dilution 1/500) and α-tubulin (Sigma; dilution 1/10000) as a loading control. Signals were detected using the ECL system (Amersham Pharmacia Biotech). Results are expressed as an n-fold increase over the values of the control group in densitometric arbitrary units.

Detection of superoxide anion levels

Briefly, cardiac and aortic sections (14 μm) were equilibrated in Krebs-HEPES buffer (in mmol/L: NaCl 130, KCl 5.6, CaCl2 2, MgCl₂ 0.24, HEPES 8.3, glucose 11, pH 7.4). Fresh buffer containing DHE (5x10⁻³ mmol/L, 30 min, 37°C) was then added and viewed by either fluorescent laser scanning microscope (40X objective in a Leica DMI 3000 microscope) or inverted Leica TCS SP2 confocal laser scanning microscope with oil immersion lens (image size 379x379 μm). Quantitative analysis of O₂⁻ production was performed with image analyzer (LEICA Q550 IWB). Three sections per animal were quantified and averaged for each experimental condition. The mean fluorescence densities in the target region were analyzed. Results are expressed as an n-fold increase over the values of the control group in arbitrary units.

Statistical Analysis

Continuous variables are expressed as mean±standard deviation or median (interquartile interval) in case of asymmetry. Categorical variables are expressed in absolute values and percentages. The differences between categorical variables were analyzed using the chi-square test. Normality of distributions was verified by means of the Kolmogorov-Smirnov test. In the case of continuous variables, differences between two groups were analyzed by either unpaired Student's t-Test or Mann-Whitney as parametric and non-parametric tests, respectively. Specific differences between more groups were analyzed using Kruskall-Wallis followed by Dunns test for non-normal distribution variables. For normal distribution variables, one-way analysis of variance was used followed by Tukey test. Either Pearson or Spearman correlation analysis was used to examine association among different variables according to whether they are or not normally distributed, respectively. The factors associated with the left ventricle (LV) hypertrophy were addressed by a logistic model and their OR and 95% confidence interval were calculated. In order to find the factors associated with diastolic function as E/e’ ratio, the beta correlation coefficients (slope or mean difference, along with their 95% confidence intervals), were obtained using a linear regression model. In both cases, variables shown to have a statistical significance by univariate analysis and those clinically relevant were included to build the model. A value of P<.05 was used as the cut-off value for defining statistical significance. Data analysis was performed using the statistical program SPSS version 22.0 (SPSS Inc., Chicago, Il, USA).
References


## SUPPLEMENTAL TABLES

### Table S1: Primers used in rats in real time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>Gal-3</td>
<td>Forward</td>
<td>AGC CCA ACG CAA ACA GTA TC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGC TTC AAC CAG GAC CTG TA</td>
</tr>
<tr>
<td>Col 1a1</td>
<td>Forward</td>
<td>GCC TCC CAG AAC ATC ACC TA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATG TCT GTC TTG CCC CAA GT</td>
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<tr>
<td>Fibronectin</td>
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<tr>
<td>TGF-β</td>
<td>Forward</td>
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<td>Reverse</td>
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<tr>
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</table>
Figure S1. Impact of Gal-3 inhibition on superoxide anion levels in heart and aorta from control and obese rats. Heart and aorta from rats fed a standard diet (CT) or a high fat diet (HFD) treated with vehicle or with the inhibitor of Gal-3 activity (Modified Citrus Pectin, MCP; 100 mg/Kg/day) were analyzed with either fluorescent laser scanning microscope (40X) or inverted Leica TCS SP2 confocal laser scanning microscope (image size 379x379 μm). Representative microphotographs labeled with DHE and quantification in heart (A) and aorta (B). Bar graphs represent the mean ± SD of 6-8 animals. ***p<0.001 vs. control group. †††p<0.001 vs. HFD group.