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1 **Relationship of cardiac remodeling and perfusion alteration with**
2 **hepatic lipid metabolism in a prediabetic high fat high sucrose**
3 **diet female rat model**
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11
12 Running title: Heart-liver axis in prediabetic female rats.

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20 pierre.daude@univ-amu.fr; carole.lan@univ-amu.fr; frank.kober@univ-amu.fr; [**Abbreviations:** ACO: Acyl-CoA Oxidase; AUC: Area Under Curve; CPT1a: Carnitine Palmitoyl
Transferase 1A; CTRL: Control; CVD: Cardiovascular Disease; ECG: Electrocardiogram; FAS: Fatty
Acid Synthase; FFA: Free Fatty-Acids; HDL: High Density Lipoprotein; HFS: High-Fat-high-
Sucrose; HTLR: Heart-weight-to-Tibia-Length Ratio; IPGTT: Intraperitoneal Glucose Tolerance Test;
LCAD: Long Chain Acyl-CoA Dehydrogenase; LDL: Low Density Lipoprotein; LVDD: Left-
Ventricular Diastolic Dysfunction; LVEDV: Left Ventricular End-Diastolic Volume; LVEF: Left
Ventricular Ejection Fraction; LVM: Left Ventricular Mass; MAFLD: Metabolic-dysfunction-
Associated Fatty Liver Disease; MCAD: Medium Chain Acyl-CoA Dehydrogenase; MCP1: Monocyte
Chemoattractant Protein 1; MRI: Magnetic Resonance Imaging; NASH: Non-Alcoholic
SteatoHepatitis; PCR: Polymerase Chain Reaction; T2D: Type 2 Diabetes; TG: Triglycerides; WT:
Wall Thickness.](mailto:jean-</p></div><div data-bbox=)

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5

6 **Abstract**

7

8 **Background and Aims:** Cardiovascular disease (CVD) is known to be linked with metabolic
9 associated fatty liver disease and type 2 diabetes, but few studies assessed this relationship in
10 prediabetes, especially among women, who are at greater risk of CVD. We aimed to evaluate
11 cardiac alterations and its relationship with hepatic lipid metabolism in prediabetic female rats
12 submitted to high-fat-high-sucrose diet (HFS).

13 **Methods and Results:** Wistar female rats were divided into 2 groups fed for 5 months with
14 standard or HFS diet. We analyzed cardiac morphology, function, perfusion and fibrosis by
15 Magnetic Resonance Imaging. Hepatic lipid contents along with inflammation and lipid
16 metabolism gene expression were assessed. Five months of HFS diet induced glucose
17 intolerance ($p < 0.05$), cardiac remodeling characterized by increased left-ventricular volume,
18 wall thickness and mass ($p < 0.05$). No significant differences were found in left-ventricular
19 ejection fraction and cardiac fibrosis but increased myocardial perfusion ($p < 0.01$) and
20 reduced cardiac index ($p < 0.05$) were shown. HFS diet induced hepatic lipid accumulation
21 with increased total lipid mass ($p < 0.001$) and triglyceride contents ($p < 0.05$), but also
22 increased mitochondrial (CPT1a, MCAD; ($p < 0.001$; $p < 0.05$) and peroxisomal (ACO, LCAD;
23 ($p < 0.05$; $p < 0.001$) β -oxidation gene expression. Myocardial wall thickness and perfusion
24 were correlated with hepatic β -oxidation genes expression. Furthermore, myocardial
25 perfusion was also correlated with hepatic lipid content and glucose intolerance.

26 **Conclusion:** This study brings new insights on the relationship between cardiac sub-

1 clinical alterations and hepatic metabolism in female prediabetic rats. Further studies are
2 warranted to explore its involvement in the higher CVD risk observed among prediabetic
3 women.

4 **Key words:** Prediabetes; MAFLD; High-fat high-sucrose diet; Female sex; Cardiac MRI;
5 Lipid metabolism.

6

7 **1. Introduction**

8 Type-2 diabetes (T2D) continues to be one of the fastest growing public health
9 problems worldwide, with a constantly increasing prevalence, predicted to rise to 643 million
10 by 2030 according to the International Diabetes Federation [1]. Cardiovascular complications
11 remain the predominant cause of morbidity and mortality among diabetic patients [2], and a
12 sexual dimorphism has been enlightened for a few years now. Indeed, women with T2D have
13 a significantly greater cardiovascular risk than type 2 diabetic men [3]. In addition, recent
14 studies have shown that this sexual dimorphism can already be found at the prediabetic stage.
15 Prediabetes is an asymptomatic abnormal stage that precedes the development of clinically
16 evident diabetes defined by one of the following criteria: impaired fasting glucose (fasting
17 plasma glucose 5.6–6.9 mmol/l); impaired glucose tolerance (2 h glucose during a 75 g Oral
18 Glucose Tolerance Test 7.8–11.0 mmol/l); or HbA1c in the range of 39–47 mmol/mol (5.7–
19 6.4%) [4]. Its prevalence is constantly raising, especially in developed countries, as the
20 International Diabetes Federation estimated the prevalence in the adult population to be 7.5%
21 (373.9 million) in 2019 and projected an increase to 8.6% (548.4 million) in 2045 [1]. Cardiac
22 alterations and Cardiovascular Disease (CVD) risks are already present at this early stage [5–
23 7], and interestingly, a sexual dimorphism that is unfavorable for women with a greater
24 cardiovascular disease risk than prediabetic men has been found [8]. It is noteworthy that
25 prediabetic and type 2 diabetic women display similar risk for coronaropathies [8,9],

1 demonstrating that these cardiac alterations should then be considered especially for women
2 at the prediabetic stage. On the other hand, Metabolic-dysfunction-Associated Fatty Liver
3 Disease (MAFLD), formerly named nonalcoholic fatty liver disease (NAFLD), is the most
4 common chronic liver disease in developed countries affecting approximately 25% of the
5 adult population [10]. MAFLD diagnosis is based on histological (biopsy), imaging or blood
6 biomarker evidence of fat accumulation in the liver (hepatic steatosis) in addition to one of the
7 following three criteria: overweight/obesity, presence of T2D mellitus or evidence of
8 metabolic dysregulation such as prediabetes or high plasma triglycerides [10]. MAFLD and
9 T2D share a bidirectional relationship, being risk factors for each other with a causal pathway
10 notably mediated by insulin resistance [11]. As in T2D mellitus, the leading cause of death in
11 MAFLD patients is cardiovascular events [12]. Indeed, MAFLD is associated with an
12 increased CVD risk and may help cardiovascular risk stratification of patients since the
13 prediabetic state and presence of both MAFLD and prediabetes was associated with a worse
14 prognosis [13]. Furthermore, recent evidence suggests that MAFLD can be considered as an
15 independent cardiovascular diseases risk factor [14–17]. A meta-analysis including a total of
16 34,043 adult individuals showed a 64% higher risk of fatal and/or non-fatal cardiovascular
17 events for patients with MAFLD than those without MAFLD, with a proportional relationship
18 with MAFLD severity [18]. Interestingly, growing evidence suggest that a sexual dimorphism
19 also concerns MAFLD. Men display a higher prevalence of MAFLD than women across all
20 age intervals, but the association between MAFLD and diabetes or hypertension was
21 significantly stronger in women [19]. This higher susceptibility of women raises the question
22 about the role of MAFLD in the sexual dimorphism observed in cardiovascular risk associated
23 with T2D, especially at the prediabetic stage. In this context, our aim was to study the
24 relationship between cardiac sub-clinical and hepatic lipid metabolism alterations found in a
25 female prediabetic rat model. We therefore used multimodal cardiovascular Magnetic

1 Resonance Imaging (MRI) to explore cardiac morphology, function, perfusion and fibrosis
2 non-invasively. In parallel, we assessed hepatic steatosis and lipid metabolism gene
3 expression through biochemical and biomolecular analyses.

4 **2. Materials and Methods**

5 **2.1 Animal model and diets**

6 Seven-week-old female Wistar rats (n = 20) (Charles River, France) were housed in a
7 ventilated rack cabinet with monitored temperature (22–24 °C), light-dark cycle of 12:12 h
8 and environmental enrichment. All procedures were approved by the animal experiment
9 ethics committee of the University (APAFIS #10547-2017071009112930) and were carried
10 out in accordance with the Directive 2010/63/EU of the European Parliament on the
11 protection of animals used for scientific purposes. Animals had access to water and food *ad*
12 *libitum*. Rats were randomly assigned into the different groups. The control group was fed
13 standard diet (A04C-10, SAFE diets, Augy, France) for five months whereas the HFS group
14 was fed a High Fat High Sucrose (U8978 v19, SAFE diets, Augy, France) diet for 5 months.
15 The nutritional composition and energy contents of both diets are listed in Table I. Food
16 intake and animal weight were monitored weekly throughout the five months. Food efficiency
17 was estimated as the ratio of weight gain on food intake over the five months of diet.

18 **2.2 In vivo Magnetic Resonance Imaging**

19 In order to evaluate cardiac morphology, function, fibrosis and perfusion, magnetic resonance
20 imaging experiments were carried out at the 5th month. All acquisitions were performed using
21 a Bruker Pharmascan Magnetic Resonance system equipped with a 7 Tesla superconducting
22 magnet (Bruker Biospin, Ettlingen, Germany) and an actively decoupled Bruker 4 channel
23 receive surface coil array. Animals were sedated with 3.5% isoflurane and then placed prone
24 into a homemade cradle designed for rats. During the MRI investigations, anesthesia was

1 maintained at 1.5-2.5% isoflurane in 1L/min pure oxygen-flow to obtain regular breathing at a
2 rate of around 70 breaths per minute. Respiration rate, ECG signal and body temperature were
3 monitored throughout the whole exploration. An ECG-gated gradient-echo cine MRI
4 sequence (cine FLASH) was used to assess myocardial morphology, mass and systolic
5 function [20]. To evaluate myocardial fibrosis, a standard ECG- and respiration-gated spin-
6 echo pulse sequence was utilized using two echo times in order to create relaxation time maps
7 throughout the slice. The trigger timing was set such as to obtain images in the same cardiac
8 phase for both echo times [21]. *In vivo* myocardial perfusion was measured using a Look-
9 Locker Flow-sensitive Alternating Inversion Recovery Gradient-Echo (LLFAIRGE) Arterial
10 Spin Labelling (ASL) technique [22]. All imaging data were post processed using homemade
11 software developed in Python.

12 **2.3 IPGTT**

13 At the end of the five-months diet, an intraperitoneal glucose tolerance test (IPGTT) was
14 performed to evaluate glucose homeostasis. Animals were fasted for six hours prior to IPGTT
15 and had free access to water during the entire experiment. The extremity of the tail was
16 incised in order to collect small drops of blood for glycemia assessment using test strips of the
17 blood nano-glucometer ACCU-CHEK Performa (Roche, Bale, Switzerland). The first value
18 corresponded to fasting glycemia. Rats were then weighed, and a bolus of glucose was
19 injected into the intraperitoneal cavity at a dose of 1 g/kg. Glycemia was measured again at
20 15, 30, 60, 90 and 120 min post glucose injection.

21 **2.4 Biochemical analyses**

22 **2.4.1 Plasma analyses**

23 The colorimetric Free Fatty Acid Quantification Kit (MAK044-1KT, Sigma Aldrich)
24 was used to determine FFA concentrations in plasma. The colorimetric cholesterol assay kit

1 (ab65390; Abcam, Cambridge, United Kingdom) was used according to the manufacturer's
2 instructions to quantify HDL and LDL Cholesterol plasmatic concentrations.

3

4 **2.4.2 Liver analyses**

5 **2.4.2.1 Sample collection**

6 After sacrifice, liver was withdrawn and weighted, then samples were collected and
7 frozen in liquid nitrogen for further biochemical and histological analyzes.

8 **2.4.2.2 Total lipid mass**

9 Total lipid mass was determined using the Folch lipid extraction method. Briefly,
10 weighed liver samples were placed and ground in a chloroform/methanol (2/1) solution. After
11 centrifugation, the chloroform phase was collected and underwent another centrifugation. At
12 the end of the extraction, the lipidic phase (chloroform) was placed into pre-weighted glass
13 tubes and evaporated in order to have the total lipid content of the sample in the tube. Total
14 lipid mass was then normalized by the sample weight. Liver triglyceride concentration was
15 determined using the colorimetric GPO method Triglyceride assay kit (80019, Biolabo,
16 Maizy, France).

17 **2.4.2.3 Genes expression by RT-qPCR**

18 Total RNA from liver was extracted using TRIzol reagent according to the
19 manufacturer's instructions (Thermo Fisher, Courtaboeuf, France). cDNAs were then
20 synthesized from 1 µg of total RNA in 20 µL using random primers and Moloney Murine
21 Leukemia Virus reverse transcriptase (Thermo Fisher, Courtaboeuf, France). Real-time
22 quantitative RT-PCR analyses were performed using the AriaMX real time PCR system
23 (Agilent Technologies France, Les Ullis) as previously described [23], and data were

1 analyzed using the Aria Agilent software 1.8 (Agilent, Santa Clara, USA). All PCR reactions
2 were using a SYBR Green Master mix (PowerUp™ SYBR®, Thermo Fisher, Courtaboeuf,
3 France). The expression was quantified in duplicate, and the ribosomal protein 18S rRNA was
4 used as the endogenous control in the comparative cycle threshold (CT) method [24] in order
5 to express data as a relative expression ratio. Primers used for this study are listed in the Table
6 II.

7 **2.5 Liver histology**

8 Liver tissue samples were fixed by immersion in 10% formalin solution (approx. 4%
9 formaldehyde) under agitation during 24h. After washing in PBS, the samples were
10 dehydrated in a graded series of ethanol and embedded in paraffin blocks for light
11 microscopy. Paraffin-embedded samples were then rehydrated to enable staining with
12 hematoxylin and eosin (5 min Hematoxylin and 1 min Eosin) and then rehydrated again
13 following standard protocols. The images were captured by a light microscope (LEICA
14 DMRB, Leica Biosystems) using a 20 times magnification lenses and a ProgRes® color
15 camera (JENOPTIK, Jena, Germany).

16 **2.6 Statistical analysis**

17 Data are graphically provided as mean \pm SEM of absolute values. Graph Pad Prism software
18 9.0 (La Jolla, CA) was used for all statistical processing. Significant differences between
19 groups were determined using two-way analysis of variance (ANOVA) with repeated
20 measures over time for the time-dependent variables followed by Bonferroni post-hoc test. An
21 unpaired Student's t-test was used for the other parameters. Pearson test was realized to
22 evaluate correlations between observed cardiac *ex vivo*, *in vivo* parameters and hepatic
23 metabolism parameters. A *p* value of less than or equal to 0.05 was considered to indicate
24 significant difference.

1

2

3

4 **3. Results**

5 **3.1 High-fat high-sucrose diet effects on physiological parameters**

6 The HFS diet effects on physiological parameters are shown in Table III. After five months of
7 diet, no significant difference in animal weight was found between the groups. Food
8 efficiency was significantly higher in HFS versus CTRL at three and five months of diet
9 ($p<0.05$). HFS diet induced an increased fasting plasma glucose concentration compared with
10 CTRL ($p<0.01$). The IPGTT emphasized a significant glucose intolerance associated with an
11 increased AUC for HFS ($p<0.0001$). Non-fasting plasma free fatty acid as well as plasma
12 LDL cholesterol concentrations were significantly higher in HFS fed animals ($p<0.001$ and
13 $p<0.05$, respectively) but HFS diet had no effects on HDL cholesterol plasmatic
14 concentrations, compared to CTRL. Liver weight as well as liver weight to body weight ratio
15 were significantly higher in the HFS fed group compared to control animals ($p<0.001$ and
16 $p<0.05$, respectively). Plasma LDL cholesterol and FFA concentrations were positively
17 correlated ($r=0.8$, $p=0.01$), as shown in Table IVB. Interestingly, the heart weight normalized
18 to tibia length was significantly higher for HFS compared to CTRL ($p<0.001$).

19 **3.2 High-fat high-sucrose diet effects on *in vivo* cardiac morphology, function and** 20 **perfusion**

21 The effects of HFS diet on cardiac parameters are shown in Figure I. After five months of
22 high-fat high-sucrose diet, a significantly increased left ventricular volume ($p<0.05$, Figure
23 IA) associated with a significant thickening of the myocardial wall in systole ($p<0.05$, Figure

1 IB) were found in the HFS group compared with CTRL. These alterations were associated
2 with an increased left ventricular mass ($p < 0.05$, Figure IC) induced by the HFS diet. Heart
3 weight to tibia length ratio measured *ex vivo* was significantly correlated with myocardial wall
4 thickness ($r = 0.67$, $p = 0.009$) and ventricular mass ($r = 0.81$, $p = 0.047$) determined *in vivo*, as
5 shown in Table IVA. Five months of high-fat high-sucrose diet had no effect on left
6 ventricular ejection fraction (Figure ID) but led to a significantly reduced cardiac index
7 ($p < 0.05$, Figure IE) compared to CTRL. Myocardial perfusion was significantly higher in
8 HFS ($p < 0.01$, Figure IF) although the T2 measurements did not reveal myocardial fibrosis
9 (Figure IG) compared to CTRL.

10 **3.3 High-fat high-sucrose diet effects on hepatic steatosis state**

11 HFS diet effects on lipid contents are shown in Figure II. Five months of HFS diet induced
12 increased liver total lipid ($p < 0.05$, Figure IIA) as well as triglycerides concentrations ($p < 0.05$,
13 Figure IIB) compared with CTRL. Increased TG concentrations were associated with
14 histology assessment of lipid droplets in liver histological samples in HFS, as shown in Figure
15 IIC.

16 **3.4 High-fat high-sucrose diet effects on hepatic metabolism and inflammation**

17 **genes relative expression**

18 HFS diet effects on hepatic lipid metabolism and inflammation genes relative expressions are
19 shown in Figure III. Lipid metabolism gene expression analysis, as shown in Figure IIIA,
20 exhibited increased relative mRNA levels coding for carnitine palmitoyl transferase 1A
21 (CPT1a, $p < 0.001$), acyl-CoA oxidase (ACO, $p < 0.05$), long and medium chain acyl-CoA
22 dehydrogenase (LCAD ($p < 0.001$) and MCAD ($p < 0.05$), respectively). ACO mRNA levels
23 were correlated with CPT1a ($r = 0.7$, $p = 0.038$), MCAD ($r = 0.83$, $p = 0.006$), LCAD ($r = 0.83$,
24 $p = 0.005$) and TG levels ($r = 0.76$, $p = 0.03$). MCAD levels were correlated with CPT1a ($r = 0.71$,

1 $p=0.031$), LCAD ($r=0.75$, $p=0.019$) and hepatic total lipid mass ($r=0.69$, $p=0.04$), as shown
2 in Table IVB. Five months of HFS diet had no effect on the relative mRNA level of the fatty
3 acid synthase (FAS) and as shown in Figure IIIB, nor on the relative gene expressions of
4 Monocyte Chemoattractant Protein 1 (MCP1, also known as CCL2), Interleukin 6 (IL-6) and
5 Tumor Necrosis Factor α (TNF- α).

6 **3.5 Cardiac, glucose and lipid metabolisms parameters correlation analysis**

7 Interestingly, cardiac index was significantly correlated to hepatic TG contents ($r= -0.73$,
8 $p=0.039$) and ACO mRNA levels ($r= -0.73$, $p=0.037$). On the other hand, myocardial
9 perfusion was correlated with plasma glucose and FFA concentrations ($r=0.825$, $p=0.012$;
10 $r=0.816$, $p=0.014$, respectively), as well as IPGTT's area under curve ($r=0.815$, $p=0.014$).
11 Furthermore, LCAD and CPT1a mRNA levels were correlated with myocardial wall
12 thickness and perfusion ($r= 0.67$, $p=0.046$; $r= 0.75$, $p=0.02$; $r= 0.79$, $p=0.019$; $r= 0.71$,
13 $p=0.031$, respectively), as shown in Table IVC.

14 **4. Discussion**

15 The aim of the study was to explore the relationship between cardiac sub-clinical and hepatic
16 lipid metabolism alterations found in a female prediabetic rat model to further understand the
17 mechanisms involved in the increased CVD risk associated, especially for women.

18 Five months of HFS diet induced a prediabetic condition with impaired glucose
19 tolerance and mild fasting hyperglycemia as previously described [25]. *In vivo*, carbohydrate
20 metabolism alterations were associated with cardiac remodeling characterized by increased
21 left ventricular mass (also confirmed *ex vivo*) and volume without affecting Left Ventricular
22 Ejection Fraction (LVEF) at this stage. HFS diet also led to increased myocardial perfusion
23 after five months. Interestingly, these cardiac alterations were associated with a liver steatosis
24 condition characterized by histology, hepatic total lipid and TG accumulation as well as

1 hepatic metabolic gene expression involved in lipid oxidation and transport adaptations. To
2 alleviate type-2 diabetes associated alterations like diabetic cardiomyopathy, medical
3 intervention at the prediabetic stage is becoming increasingly relevant. Prediabetes is, indeed,
4 an interesting therapeutic window due to its reversibility at this stage, and, more importantly,
5 it is already associated with increased risk of all-cause mortality, coronary artery disease,
6 stroke and cardiac biomarkers [6,7], especially for prediabetic women [8,9,26]. However, this
7 increased cardiovascular risk is not exclusively associated with the prediabetic state but the
8 overall result of a systemic metabolic alteration. *In vivo*, we showed cardiac remodelling in
9 prediabetic females, characterized by increased left ventricular myocardial thickness, end-
10 diastolic volume and mass. These results are consistent with a previous study on this
11 prediabetic model [27]. Myocardial wall thickening, left ventricular volume and weight
12 increase observed here *in vivo* were associated with a significant increase in heart to tibia
13 length ratio *ex vivo*, a potential hypertrophy indicator [28]. This ratio was correlated with wall
14 thickness and ventricular mass determined *in vivo*, which, again, confirms the cardiac
15 remodeling observed, but not with left-ventricular volume. Since stroke volume and ejection
16 fraction were preserved also in the prediabetic group, one may speculate that the observed
17 cardiac remodeling may result from a metabolic compensation to sustain cardiac function.
18 These alterations were also found in other diet induced rodent models. Du Toit *et al* showed
19 cardiac hypertrophy as reflected by an increased ventricular weight, left ventricular posterior
20 wall thickness, associated with obesity in a Western diet induced prediabetic Wistar male rat
21 model after 16 weeks [29]. In a prediabetic high fat high sucrose diet male mouse model,
22 Philouze *et al.* demonstrated mild left-ventricular hypertrophy and impaired longitudinal
23 strain but preserved myocardial circumferential as well as global diastolic and systolic cardiac
24 function after 10 weeks [30]. Recent clinical studies also found this association between
25 prediabetes and concentric remodeling, relative wall thickness, left ventricular mass index and

1 hypertrophy, without assessing sexual dimorphism nor the impact of MAFLD [31–33]. We
2 also found increased myocardial perfusion in the HFS diet group. A similar and supposedly
3 compensatory response was also found in a previous work on a diet induced prediabetes rat
4 model after 6 weeks of diet [34] as well as in another earlier study on rats [25,35]. Myocardial
5 perfusion could be elevated as a consequence of the growing systemic insulin resistance,
6 which deprives the heart of glucose, its most oxygen-efficient substrate. In order to sustain
7 cardiac work, oxygen consumption may be augmented and therewith perfusion in order to
8 oxidize more lipids. In high-fat-diet fed rats, Cole *et al.* reported normal *in vivo* cardiac
9 function after three weeks but increased oxygen consumption and, therefore, decreased
10 cardiac efficiency associated with increased mitochondrial uncoupling compared to controls
11 [34]. We can suppose that this compensatory increase in myocardial blood flow will
12 eventually fade as overt diabetes is associated with reduced myocardial perfusion, and
13 previous work on T2D rodent models have previously shown this evolution [36,37].
14 Tomizawa *et al.* also reported a compensatory increase at first, followed by a decline of
15 myocardial blood flow during T2D progression in patients [38]. It is also important to note
16 that myocardial perfusion can be influenced by temperature, heart rate and isoflurane
17 concentrations [39]. Although no statistical difference in these three experimental parameters
18 was found between the groups, they could be involved in modulating myocardial perfusion.

19 As mentioned above, Metabolic Associated Fatty Liver Disease (MAFLD)
20 involvement in CVD is increasingly recognized [40,41], especially in association with type-2
21 diabetes [42–44]. These observations highlight the need for a multidisciplinary approach to
22 the diagnosis and management of patients with MAFLD and CVD to optimize clinical
23 outcomes, as there are still no Food and Drug Administration-approved nonalcoholic
24 steatohepatitis-specific therapies [45,46]. MAFLD is not fully recognized yet as a critical risk
25 factor for CVD by cardiologists, and it is not listed as a primary target of intervention for

1 CVD, Diabetes Mellitus (DM) or dyslipidemia [47]. However, the American Heart
2 Association recently recognized this association via a scientific statement affirming that
3 individuals with MAFLD have increased cardiovascular risks compared to those without it
4 [48]. Indeed, MAFLD is also closely linked to T2D and prediabetes, whose prevalence is in
5 constant rising, and CVD remains the leading death cause of MAFLD patients [49,50]. These
6 cardiac structural disorders represent another risk factor for cardiac arrhythmias and can be
7 attributed to myocardial energy metabolism alterations observable in MAFLD patients [51–
8 53]. In our prediabetic model, the cardiac alterations mentioned above were associated with
9 increased hepatic weight and hepatic steatosis characterized by significantly higher hepatic
10 TG, lipid levels and confirmed by histology with the presence of hepatic lipid droplets. HFS
11 fed animals presented a heavier liver associated with increased lipid concentration. Increased
12 liver weight as well as lipid content, associated with hepatic steatosis, were also found in
13 previous HFH fed rodent studies [54,55]. Other papers have shown an association between
14 MAFLD and cardiac structure alterations. Recently, Peng *et al.* found significantly higher
15 prevalence of left-ventricular diastolic dysfunction (LVDD) in patients with MAFLD and a
16 significant association with signs of cardiac remodeling such as increased interventricular
17 septum thickness, LV wall thickness and LV mass index [56]. Moderate to severe steatosis
18 patients had significantly higher risks for LVDD and cardiac remodeling [56]. Interestingly,
19 hepatic triglyceride concentrations negatively correlated with cardiac index *in vivo*. This
20 result is in line with Abdesselam *et al.* longitudinal study, which demonstrated an increase in
21 hepatic TG content by Magnetic Resonance Spectroscopy negatively correlated with cardiac
22 index in a HFS diet induced T2D male mice model [37]. The myocardial sub-clinical
23 alterations observed here may, therefore, be caused partially by lipotoxic effect of liver
24 steatosis, as already suggested above. Indeed, an imbalance between cardiac lipid uptake and
25 β -oxidation due to excess lipid availability may lead to the production of toxic lipid

1 intermediates [57,58], but also reactive oxygen species which will eventually cause impaired
2 calcium handling, cardiomyocyte hypertrophy and myocardial apoptosis [59–62]. However,
3 further studies are needed to uncover the mechanisms involved.

4 There is also a need for relevant animal models to understand and further explore the
5 mechanisms involved in MAFLD and prediabetes association with CVDs. In this context,
6 rodent diet induced models are thought to be one of the best options [63]. In addition, recent
7 data suggest that outbred strains should be used as they represent the polygenic variability
8 more reliably and showed no difference of trait stability compared to inbred strains [64]. The
9 combination of high-fat and high-sugar diet seems to be mandatory for achieving
10 hyperglycemia. In 10-week-old male Wistar rats fed High-Fat High-Fructose diet, impaired
11 fasting glucose (IFG) were observed after two months of diet [63]. HFS diet is then
12 commonly used to replicate prediabetes despite a high variability in species/strain, duration of
13 protocol, percentage of sugar and route of administration [63,65]. Here, after five months of
14 HFS diet, female Wistar rats exhibited a prediabetic profile with impaired glucose tolerance
15 and mild fasting hyperglycemia compared to controls, but not an overt diabetes
16 hyperglycemia *per se* (>1.25 mg/dL). Only a tendency to higher body weight was observed
17 for prediabetic females compared to controls. This could be primarily attributed to the group
18 heterogeneity inherent to outbred strain but also to the fact that female rodents are less prone
19 to become obese than their male counterpart [66]. However, with few exceptions [42,67],
20 most previous work on these models has only studied the progression of MAFLD and/or T2D
21 without assessing diet induced models in a more systemic way covering multiple organs.

22 In our prediabetic model, hepatic TG and total lipid content accumulation was
23 associated to higher hepatic lipid mitochondrial (CPT1a and MCAD) and peroxisomal (ACO
24 and LCAD) β -oxidation mRNA genes expression. In case of mitochondria lipid overload,
25 peroxisomal β -oxidation can contribute to lipid oxidation but is known to generate

1 considerable amounts of reactive oxygen species [68]. This oxidative stress will induce
2 systemic redox homeostasis and mitochondrial function alterations, *in fine* alleviating even
3 more this vicious cycle [68]. Interestingly, myocardial perfusion was here correlated with
4 hepatic metabolism mRNA levels (LCAD and CPT1a), IPGTT's area under curve and plasma
5 glucose and FFA concentrations which may suggest a compensatory process in the heart.
6 Such compensation by blood flow increase may be due to impaired redox homeostasis and
7 mitochondrial function, partly caused by altered glucose and hepatic lipid metabolism, as
8 suggested above. Indeed, LCAD is known to be a greater source of hydrogen peroxide than
9 other mitochondrial fatty acid enzymes [69]. LCAD and CPT1a mRNA levels were also
10 correlated with myocardial wall thickness. As mentioned above, hepatic steatosis induced
11 cardiac remodeling mechanisms are still scarcely elucidated, but oxidative stress seems to be
12 a main mediator of this relationship [70]. In addition, lipid peroxisomal β -oxidation enzyme
13 ACO mRNA levels were negatively correlated with cardiac index, strengthening the
14 hypothesis that the myocardial alterations observed may be caused by a lipotoxic effect
15 induced by hepatic steatosis. Further studies focused on oxidative stress and mitochondrial
16 impairment could help define the relationship observed here between cardiac sub-clinical and
17 hepatic lipid metabolism alterations. The prediabetic HFS group exhibited here an increased
18 plasma concentration of FFAs and Low-Density Lipoprotein (LDL) cholesterol as previously
19 described [27]. MAFLD is often associated with dyslipidemia characterized by high serum
20 TG and low high-density lipoprotein (HDL) cholesterol levels, as well as a predominance of
21 LDL particles [10,71]. In the present study we didn't assess blood TG levels as our previous
22 study on this model exhibited no significative difference between HFS diet-fed females and
23 controls [27]. LDL cholesterol is known to be a main risk factor for CVD, especially for its
24 important role in the pathogenesis of atherosclerosis [72–74]. Furthermore, a recent meta-
25 analysis reported a dose-dependent association between plasma LDL cholesterol levels and

1 CVD mortality [75]. Here, no significant difference was observed for HDL cholesterol levels,
2 suggesting that in this prediabetic model only an early stage of MAFLD was observed. Even
3 though plasmatic total cholesterol levels have not been addressed in this study, current
4 cardiovascular risk management guidelines for prediabetes are focused on LDL cholesterol
5 reduction rather than total cholesterol [76]. Histological analyses didn't exhibit any signs of
6 hepatocytes ballooning as well, suggesting again the absence of Non-Alcoholic
7 SteatoHepatitis (NASH), an advanced stage of MAFLD. Furthermore, hepatic inflammation
8 cytokines (MCP-1, TNF- α , IL-6) as well as de novo lipogenesis (FAS) gene expression,
9 which is known to be increased in advanced MAFLD and NASH [77], was not altered after
10 five months of HFS diet, compared to the control group.

11 **5. Limitations of the study**

12 One limitation of our study might be the lack of male groups, which could exhibit sex-specific
13 differences in the heart-liver axis relationship with cardiac alterations observed induced by the
14 HFS diet. However, our aim was to study this relationship among prediabetic women, which
15 firstly are still under-represented among studies in this context and secondly which represent a
16 higher concerning subject due to the higher cardiovascular risk since the prediabetic state.
17 Another limitation would be the lack of plasma insulin levels. As mentioned before, insulin
18 resistance is one of the main actors in the relationship between prediabetes and MAFLD
19 [11,78]. Plasmatic insulin levels could then bring insights on its role in the prediabetes/hepatic
20 steatosis found in our model. In the present study we didn't assess the role of adipose tissue,
21 which is known to have systemic effects associated with metabolic syndrome by the
22 intermediary of secreted adipocytokines which can be involved in a low-grade inflammation
23 [79]. This inflammatory state is known to induce oxidative stress, mitochondrial dysfunction
24 inducing *in fine* myocardial subcellular alterations and promoting maladaptive remodeling as
25 found in our model [80]. We also didn't explore the effects of estrogens in our female

1 prediabetic model, which are known to regulate hepatic lipogenesis and glucose
2 metabolism/homeostasis genes in a circulating estrogen level-dependent manner [81,82], *in*
3 *fine* protecting against hepatic steatosis [83]. Thus, plasmatic estrogen levels could help
4 evaluate the metabolic protection roles of estrogens in our model. However, further studies
5 using ovariectomized females should bring more insights on the cardioprotective and
6 metabolic protection role of estrogens in this context.

7 **6. Conclusion**

8 This female rat HFS diet-induced model exhibited after 5 months a prediabetic state
9 associated with cardiac sub-clinical alterations which were interestingly correlated with lipid
10 metabolism impairments. These results reinforce the hypothesis of a lipotoxic effect of
11 hepatic steatosis on cardiac parameters, potentially mediated by oxidative stress and
12 mitochondrial dysfunction. Increased myocardial perfusion as well as normal expression
13 levels of hepatic de novo lipogenesis and inflammation genes suggest an early stage of
14 MAFLD. Despite this early stage, cardiac alterations were already detectable, suggesting that
15 studies on reliable and representative animal models are needed to further explore the
16 relationship between the heart-liver axis and CV alterations associated with prediabetes. This
17 diet-induced model hints the relevance of such studies, which may provide useful elements in
18 the development of personalized therapeutic strategies, especially for women, who present an
19 overall unfavorable CVD profile compared to men in this context.

20 **Declarations**

21 **CRedit author statement:**

22 A.J: Conceptualization, Investigation, Writing-Original Draft, Visualization, Formal analysis;
23 K.H: Investigation, Visualization; I.V: Investigation, Writing - Review & Editing; J.S:
24 Investigation, Visualization; P.D: Software, Visualization; C.L: Investigation, Visualization
25 F.K: Methodology, Software, Writing – Review & Editing; JF. L: Resources, Writing –

1 Review & Editing; M.B: Resources, Writing – Review & Editing; M.D: Supervision,
2 Conceptualization, Project administration, Investigation, Writing – Review & Editing. All
3 authors read and approved the final manuscript.

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11 study design, in collection, analysis and interpretation of data, in the writing of the report, and
12 in the decision to submit the article for publication.

13 **Competing interests:**

14 The authors declare that they have no competing interests.

15 **Data availability**

16 Data will be made available on request.

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1 **Tables and figure captions :**

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Nutritional Composition (%)	Control	HFS
Nitrogen Free Extract	60.4	37.23
of which Starch	43.5	14.52
of which Sugars	3.2	20.25
Crude proteins	16.1	19.84
Crude Fat	3.1	35.92
Crude Ash	4.6	4.2
Crude Fiber	3.9	<0.5
Moisture	11.9	2.8
Energy Content (%)	Control	HFS
Energy from proteins	19.3	14.4
Energy from lipids	8.4	58.6
Energy from NFE	72.4	27

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12 **Table I: Nutritional composition and energy contents of control (SAFE, A04) and HFS**
13 **(SAFE, U8978 v19) diets.**

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Gene	Forward 3'-5'	Reverse 3'-5'
18S	GTGGGCCTGCGGCTTAAT	GCCAGAGTCTCGTTCGTTATC
Lipid metabolism	Forward 3'-5'	Reverse 3'-5'
<i>FAS</i>	GGCCACCTCAGTCCTGTTAT	AGGGTCCAGCTAGAGGGTACA
<i>ACO</i>	CACCTTCGAGGGAGAGAACA	CGCACCTGGTCGTAGATTTT
<i>CPT1a</i>	ACAATGGGACATTCCAGGAG	AAAGACTGGCGCTGCTCA
<i>MCAD</i>	CTTAGCTTCTGCCCTGTGGT	TGAGAGACACTTCTCAGGACCTT
<i>LCAD</i>	GCAGTTACTTGGGAAGAGCAA	GGCATGACAATATCTGAATGGA
Inflammation	Forward 3'-5'	Reverse 3'-5'
<i>TNF-α</i>	GCCCAGACCCTCACACTC	CCACTCCAGCTGCTCCTCT
<i>IL6</i>	CCCTTCAGGAACAGCTATGAA	ACAACATCAGTCCCAAGAAGG
<i>MCP1</i>	AGCATCCACGTGCTGTCTC	GATCATCTTGCCAGTGAATGAGT

6 **Table II: Primer sequences used for real-time RT-PCR experiments.**

7 18S: Ribosomal Protein S18; FAS: Fatty acid synthase; ACO: Acyl CoA oxidase; CPT1 a:
8 Carnitine pantoictransferase 1a (Liver); MCAD: Medium-chain acyl-CoA dehydrogenase;
9 LCAD: Long-chain acyl-CoA dehydrogenase; TNF- α : Tumor necrosis factor α ; IL-6:
10 Interleukin 6; MCP1: Monocyte chemoattractant protein 1.

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Physiological parameters	CTRL	HFS
Animal weight (5 months, g)	276.7 ± 13.65	328.6 ± 23.08
Food efficiency (3 months)	0.013 ± 0.0069	0.103 ± 0.0245 *
Food efficiency (5 months)	0.011 ± 0.0023	0.077 ± 0.01801 *
Fasting glycemia (mM)	5.617 ± 0.119	6.408 ± 0.1911 **
Fasting glycemia (g/L)	1.021 ± 0.022	1.165 ± 0.035 **
AUC IPGTT	625.4 ± 19.02	869.7 ± 22.67 ****
LDL-C (mg/dL)	13.41 ± 1.27	20.12 ± 2.03 *
HDL-C (mg/dL)	56.48 ± 5.41	53.24 ± 6.39
FFA (mM)	0.1600 ± 0.035	0.4487 ± 0.039 ***
Heart Weight/Tibia Length Ratio (g/cm)	0.198 ± 0.005	0.229 ± 0.005 **
Liver Weight (g)	7.5 ± 0.209	9.306 ± 0.364 ****
Liver Weight/ Body Weight	0.026 ± 0.0007	0.029 ± 0.001 *

Table III: High-Fat High-Sucrose diet effects on physiological parameters.

Physiological parameters: AUC IPGTT: IPGTT's Area Under Curve, FFA: Free Fatty Acid, LDL-C: Low-Density Lipoprotein Cholesterol, HDL-C: High-Density Lipoprotein Cholesterol. Data are means ± SEM. Unpaired Student's t t-test was performed on all parameters. * $p < 0.05$ HFS vs. CTRL; ** $p < 0.01$ HFS vs. CTRL; *** $p < 0.001$ HFS vs. CTRL; **** $p < 0.0001$ HFS vs. CTRL; CTRL: female control group; HFS: female high-fat high-sucrose diet group.

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A: Cardiac parameters		r	p
HTLR	LVM	0.81	0.009
HTLR	WT	0.67	0.047
HTLR	LVEDV	0.61	0.08
B: Hepatic and plasmatic parameters		r	p
MCAD	Total lipid content	0.69	0.04
ACO	TG	0.76	0.03
ACO	CPT1a	0.7	0.038
ACO	MCAD	0.83	0.006
ACO	LCAD	0.83	0.005
CPT1a	MCAD	0.71	0.031
CPT1a	LCAD	0.94	0.000145
LCAD	MCAD	0.75	0.019
LDL	FFA	0.8	0.01
C: Hepatic/plasmatic	Cardiac parameters	r	p
TG	CI	-0.73	0.039
ACO	CI	-0.73	0.037
CPT1a	WT	0.75	0.02
LCAD	WT	0.67	0.046
CPT1a	Perfusion	0.73	0.038
LCAD	Perfusion	0.79	0.019
Glycemia	Perfusion	0.83	0.012
IPGTT AUC	Perfusion	0.81	0.014
FFA	Perfusion	0.82	0.014

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4 **Table IV: Correlation analysis.**

5 A: *Ex vivo* and *in vivo* cardiac parameters correlations. HTLR: Heart weight to Tibia Length
6 Ratio; LVM: Left Ventricular Mass; WT: Wall Thickness; LVEDV: Left Ventricular End-
7 Diastolic Volume. B: Hepatic lipid contents, gene expression and plasmatic parameters
8 correlations. MCAD: Medium-chain acyl-CoA dehydrogenase; ACO: Acyl CoA oxidase; TG:
9 Triglycerides; CPT1a: Carnitine pantoictransferase 1a (Liver); LCAD: Long-chain acyl-
10 CoA dehydrogenase, LDL: Low Density Lipoprotein; FFA: Free Fatty-Acids. C: Hepatic and
11 cardiac parameters correlations. Pearson correlation test was performed on all parameters.

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2 **Figure I: High-Fat High-Sucrose diet effects on cardiac morphology, function, perfusion**
3 **and fibrosis assessed *in vivo* by MRI exploration.**

4 LVEDV: Left Ventricular End-Diastolic Volume (A), WT: Wall Thickness (B), LVM: Left
5 Ventricular Mass (C), LVEF: Left Ventricular Ejection Fraction (D), Cardiac Index (E),
6 Myocardial Perfusion and Myocardial Blood Flow maps examples (F) and Myocardial
7 Fibrosis (G). Data are means \pm SEM. Unpaired Student's t-test was performed on all
8 parameters. * $p < 0.05$ HFS vs. CTRL; *** $p < 0.001$ HFS vs. CTRL; CTRL: female control
9 group; HFS: female high-fat high-sucrose diet group.

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11 **Figure II: High-Fat High-Sucrose diet effects on hepatic steatosis state.**

12 Hepatic total lipid contents (A), Hepatic triglycerides concentrations (B), Hepatic histological
13 slice (HE staining), arrows showing lipid vacuoles (C). Data are means \pm SEM. Student
14 unpaired Student's t-test was performed on all parameters. * $p < 0.05$ HFS vs. CTRL; CTRL:
15 female control group; HFS: female high-fat high-sucrose diet group.

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17 **Figure III: High-Fat High-Sucrose diet effects on hepatic metabolism and inflammation**
18 **genes relative expressions.**

19 Hepatic mRNA relative expression of genes involved in lipid metabolism: CPT1a (relative
20 expression divided by three for visual convenience), ACO, LCAD, MCAD, FAS (A), hepatic
21 mRNA relative expression of inflammation genes: MCP1, IL-6, TNF- α (B). Data are means \pm
22 SEM. Unpaired Student's t-test was performed on all parameters. * $p < 0.05$ HFS vs. CTRL;
23 *** $p < 0.001$ HFS vs. CTRL; CTRL: female control group; HFS: female high-fat high-
24 sucrose diet group.

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