HIGH FAT DIET INDUCES METABOLIC CHANGES AND REDUCES OXIDATIVE STRESS IN FEMALE MOUSE HEARTS

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ABSTRACT

After an acute myocardial infarction, obese patients generally have a better prognosis than their leaner counterparts, known as the "obesity paradox". In addition, female sex is associated with a lower risk of cardiac ischemic events and smaller infarct size compared to males.

The objective of the present work was to study the metabolic phenotype and mitochondrial function associated to female sex and short-term high fat diet.

^{1}H NMR spectra of mice heart extracts were analysed by mRMR variable selection and linear discriminant analysis was used to evaluate metabolic changes. In separate experiments, O_2 consumption and H_2O_2 production were measured from isolated mitochondria as well as serum oxidation susceptibility.

Fingerprinting showed that male hearts contained more myo-inositol, taurine and glutamate than female hearts. HFD reduced the levels of creatine, taurine citrate and acetate. Profiling showed increased alanine and fumarate in HFD suggesting altered glycolitic and Krebs cycle pathways. Female mice contained less glucose than males.

Female sex nor HFD altered mitochondria oxygen consumption but both conditions reduced the amount of H_2O_2 produced in an additive manner. Serum of females had lower oxidation susceptibility than serum from males but there were no differences associated with HFD.

In conclusion, female sex and short term HFD have an effect on the myocardial metabolic pattern and reduce the amount of H_2O_2 produced by mitochondria in an additive manner suggesting different mechanisms of action. This could explain, at least in part, the protection afforded by female sex and the "obesity paradox".

Highlights

Two weeks of high fat diet induces changes in the heart metabolic profile.

HFD and female sex reduce mitochondrial hydrogen peroxide production.

Changes in metabolic profile and oxidative state may explain the "obesity paradox".

Antioxidant capacity is higher in females but not affected by HFD.

1. INTRODUCTION

Cardiovascular risk is a leading cause of death and decreased quality of life despite improvements in treatment over the last decades [1]. Dyslipidemia is a modifiable risk factor associated to cardiovascular diseases through the accumulation of cholesterol esters in coronary atherosclerotic plaques that can lead to an acute cardiovascular event. In order to reduce the incidence of cardiovascular events, guidelines recommend the reduction of dietary lipids, in particular saturated fat, in order to reduce circulating levels of LDL cholesterol [2]. However, once an event has occurred, obese patients generally have a better short- and long-term prognosis than their leaner counterparts with equal cardiovascular burden, including myocardial infarction [3] and acute cardiovascular diseases [4]; this is the so-called "obesity paradox".

Female sex is associated with a reduced risk of developing cardiovascular disease when compared to males [5]. Moreover, studies in animal models of myocardial infarction show reduced damage in females than in males [6] and, in patients with ST-segment elevation myocardial infarction (STEMI), infarct size was smaller in women once corrected for confounding factors [7] highlighting an increased tolerance to ischemia in females as compared to males. This protective effect associated with female sex has been attributed to a hormone effect [8] and to several cellular signalling mechanisms [9]. In addition, there is evidence suggesting sex-related differences in cardiac metabolism [10].

Reactive oxygen species play an important role in the cardiovascular system patho-physiology including cardiac hypertrophy and myocardial infarction [11]. Also, serum oxidative susceptibility is associated with an increased risk of acute myocardial infarction [12]. Diet, and in particular western or high fat diet, is associated with increased oxidative stress [13] and is able to modify serum oxidative stress factors [14]. Sex also plays a role in serum oxidation susceptibility as men show a higher level of oxidation than women [15].

Metabolomics consists in the unbiased measure of the metabolites in a biofluid or tissue. The metabolome is downstream of the genome and transcriptome [16]. Also, because the metabolome is affected by environmental factors including diet, metabolomics has become an important tool to study dietary interventions [17]. ^{1}H NMR is a robust analytical approach that is easy to automate so it suited for its use in the clinical setting. There are two main approaches to analyse NMR spectra. Fingerprinting treats NMR spectra as curves in order to obtain classifiers able to differentiate between various conditions (e.g. cases and controls) without prior knowledge of the metabolites that appear in the spectra. The second approach, known as metabolic profiling,

requires the precise measure of metabolite concentration for each of the samples. The heart is a highly metabolic demanding tissue and has been extensively studied using metabolomics [18]; however, there is little information regarding the interaction of dietary fat and sex with metabolic profiles in myocardial tissue.

The objectives of the present work were to investigate whether metabolomics could be used to investigate the effects of short-term high fat diet on myocardial metabolism. The effects of high fat diet on mitochondrial function and its association with sex.

2. METHODS

2.1 Ethical approval

This study was approved by the Research Commission on Ethics of the Hospital Vall d'Hebron and conform to the ''Position of the American Heart Association on Research Animal Use'' and the European Directive on the Welfare of Research Animals (2010/63/UE). (Study Reference 30.15).

2.2 Mice

Metabolomic experiments were performed on 23 mice C57BL/6 (Charles River Laboratories) of 16 to 28 weeks of age. Half of the animals (7 males, 5 females) were given a high fat diet (HFD) consisting in 35% of fat (lard and soybean oil) accounting for 60% of the calories (Research Diets #D12492, New Brunswick, NJ) *ad libitum* for two weeks. The other half (6 males, 5 females) were given standard chow. A separate group of 24 animals (12 males, 12 females) were fed with either standard chow or HFD and used for mitochondria function experiments. All animals were housed under controlled conditions with a 12:12 hour light dark cycle.

After two weeks of high fat diet or standard chow, mice were euthanized by exsanguination under deep anaesthesia (100 mg/Kg Ketamine, 10 mg/Kg Xylacine). Blood was obtained by puncture of the left ventricle of the heart; then the heart was rapidly excised, excess blood removed in a bath of ice-cold phosphate saline buffer and used immediately for mitochondria purification or frozen in liquid nitrogen for metabolite extraction.

To obtain serum, blood was allowed to clot over ice and then centrifuged for 5 minutes at 1500g and 4C. Serum samples were kept at -80C until further processing.

2.3 Extraction

Metabolite extraction was performed according to the methanol:chloroform protocol as described previously [19]. Briefly, approximately 100 mg of tissue were pulverized with a mortar and pestle at liquid nitrogen temperature, transferred to a glass tube containing 3 ml of methanol:chloroform (2:1) mixture and left to stand on ice for 30 minutes with occasional mixing. The aqueous and organic phases were separated by the addition of 1.25 ml of chloroform and 1.86 ml of water. The remaining interface was re-extracted at room temperature and both aqueous phases pooled together.

The aqueous extract was lyophilized overnight and stored at -20C until NMR spectroscopy.

2.4 NMR

Prior to NMR, extracts were dissolved in 600 µl of deuterium oxide containing 1 mM of TSP as concentration and chemical shift standard. For serum spectra, 50 µl of serum was dissolved with 450 µl of deuterium oxide.

NMR spectroscopy was performed on a 9.7 T vertical bore magnet interfaced with a Bruker

Avance 400 spectrometer. Spectra from extracts consisted in the accumulation of 64 scans with a 1D NOESY pulse sequence with the mixing time adjusted to 100 ms. In the case of serum, spectra were acquired with a CPMG pulse sequence with an effective T2 delay of 32 ms. All spectra were acquired at 30C.

2.5 Metabolic Fingerprinting

The aliphatic part of the spectra, between 0.5 and 4.5 ppm was used for metabolic fingerprinting. Feature selection was done using a "minimal redundancy maximal relevance" (mRMR) method [20]–[23]. This approach provides good interpretability and the possibility of using a simple classifier as Fisher's discriminant analysis (LDA) from the selected variables.

For metabolic fingerprinting each sample spectrum is treated as a curve (that, in fact, is discretized to a high-dimensional vector). Then, the usefulness of the spectra to discriminate with respect to sex and diet is assessed via standard methods of supervised classification, combined with techniques of variable selection. In particular, this approach allows us to identify which variables in the spectra are more relevant for the factors under study.

2.6 Metabolic Profiling

Metabolite identification and concentration measurements were done using Chenomx Profiler 8.0 software (Chenomx Inc, Canada). Concentration data is given as micromols per gram of wet tissue.

2.7 Mitochondrial function

Fresh mitochondria were isolated as described previously [24]. Oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech, UK) at room temperature in the presence of malate 2mM, glutamate 5mM and ADP 1mM; oxygen consumption rates were normalized by citrate synthase activity. Hydrogen peroxide (H_2O_2) was determined as fluorescent dimer production of homovallinic acid after oxidation by H_2O_2 catalyzed by horseradish peroxidase at 440 nm using a fluorimeter (SpectraMax GeminiXS, Molecular Devices), using malate, glutamate or succinate substrates. Fluorescence values were normalized to values obtained at time zero (F_0).

2.8 Oxidation susceptibility

Oxidation susceptibility of the serum was measured according to the method described by Tynkkynen and cols [25]. Briefly, serum samples were treated with $CuS0_4$ 0.5mM for 6 hours at 37C. Lipid extracted using the chloroform:methanol approach. Lipid oxidation was measured with NMR spectroscopy and, each sample, compared to its own non-oxidized control. Results are given as % of oxidized lipids as compared to its control.

2.9 Statistics

Data are expressed as mean ± SEM. Two group, two treatment experiments were compared using a 2 Way ANOVA running in R software. Differences were considered significant when $p<0.05$.

RESULTS

At the end of the experiment, male mice were heavier than females (34.23 ± 4.00 g vs 25.17 ± 2.37 g; $p < 0.05$). In addition, the hearts of male animals were heavier than female hearts (Fig 1A). After two weeks of HFD the weight of the mice increased more than those on standard chow (5.16 g± 0.94 vs 2.30g ± 0.41; $p < 0.05$) (Figure 1B). However, there were no differences in the heart to weight ratio.

Serum spectra of HFD fed mice contained more lipoproteins, peaks at 0.80, 1.29 and 2.80 ppm, than the spectra of serum from animals fed with standard chow (Figure 1C-D).

3.1 Statistical analysis via pattern recognition

We have performed several supervised classification techniques, for sex and diet (high fat or normal), based on the ^{1}H NMR spectra of heart extracts. A first preliminary approach, intended just as a benchmark reference, has been made based on the raw data (that is, the original spectra with no dimension reduction technique or scale transformation applied on them). Since the standard linear classification method (LDA) cannot be directly used with the high-dimensional spectra data, we have employed the k-nearest neighbours (k-NN) classifier, with k=3. This is a "plain", assumption free, methodology with a minimal data processing. It resulted in a correct classification for 18 out of 23 data, according to sex, and only 11 out of 23 according to diet. It is worth mentioning that spectra normalization and variable scaling to unit variance did not improve the classification results. Table 1 shows the classification accuracy of the mRMR+LDA approach, classification errors were calculated using a leave-one-out methodology.

As a further, more sophisticated, alternative we have performed also classification on the "processed data", that is, on the result of applying on them a dimension reduction technique and then, in a second stage, using LDA. In the dimension reduction step we have in turn checked two methodologies: variable selection through the "minimal Redundancy Maximum Relevance" (mRMR) method [20], [21] and the Partial Least Squares (PLS) methodology [see,[26]] for a basic overview and further references. The mRMR method has been implemented in an improved version, as described in Berrendero et al. [23]. Let us note that as outcome of mRMR, a few “representative” variables were selected from each spectrum; so the method provides a dimension reduction in terms of some selected original variables. By contrast, the PLS methodology provides a few linear combinations of the whole set of original variables. These linear combinations are typically difficult to interpret. The usual leave-one-out methodology was used to assess the proportion of correct classification.

In short, both alternative approaches (mRMR+LDA and PLS+LDA) resulted in a greatly

increased classification success (with respect to classification based on the raw data with no dimension reduction). When the original data (with no normalization or scaling) are used, mRMR was able to correctly classify 21 out of 23 samples in both problems (diet and sex) while PLS did classify correctly 21 out of 23 diet and 20 out of 23 sex samples. Again, the normalization and scaling of the data did not improve things and in fact led to equal or slightly worse classification errors in both problems using both techniques. While there were no relevant differences in classification performance between mRMR and PLS, the use of mRMR for variable selection allows for an easier interpretation of the results. These methodological conclusions are in agreement with those reported by Szymanska and cols. [27] in the analysis of NMR based metabolomic data.

In the case of sex, the variables more frequently selected in the leave-one-out classification process were the ones corresponding to myo-inositol taurine and glutamate. In the case of diet, selected variables showed a decrease in creatine, taurine and citrate in HFD fed mice as compared to their control fed counterparts (Table 1). On closer inspection, it could be seen that the differences in diet arise mainly from male animals and, more interestingly, female hearts (both control and HFD) tend to cluster with hearts from male animals fed with HFD (Figure 2).

When measuring metabolite concentrations, it was found that high fat diet induced an increase in alanine both in female (from 1.35 ± 0.06 to 2.49 ± 0.17 μmol/gram fresh weight, $p<0.05$) and male mice (from 1.12 ± 0.15 to 1.61 ± 0.21 μmol/gram fresh weight, $p<0.05$). High fat diet also increased glucose content in males (from 0.89 ± 0.13 to 1.92 ± 0.30 μmol/gram fresh weight, $p<0.05$).

3.2 Mitochondrial function

Mitochondrial oxygen consumption rates of from all groups studied were similar, with no differences both in resting conditions (sate 2) or after maximal stimulation with ADP (state 3) (Figure 3 A, B).

Hydrogen peroxide production under malate and glutamate substrates was low, around 10% of the initial values, and equal in all groups tested, without differences between sexes or diets. However, under succinate incubation, mitochondria were able to produce large quantities of hydrogen peroxide (Figure 3 C-E).

Female sex and HFD were able to reduce the levels of H_2O_2 produced under succinate incubation, a standard two-way ANOVA analysis confirmed significant difference ($p<0.05$) for both factors (sex and diet) and the interaction between the two factors (Figure 3E).

3.3 Oxidation susceptibility

Lipid serum oxidation was significantly lower in females than in males as treatment with

$CuSO_4$ was able to oxidize 20 ± 3% of the lipids present in male mice serum but only 10 ± 4% of the lipids present in female samples ($p<0.05$). By contrast, there were no differences in the oxidation susceptibility of serum when comparing control and HFD (Figure 3 F-G) and the interaction between sex and diet was not statistically significant.

3. DISCUSSION

In the present work, we show that two weeks feeding with high fat diet is enough to induce changes in the heart metabolic profile and a reduction in mitochondrial hydrogen peroxide production under succinate stimulation. The reduction in hydrogen peroxide production observed in HFD fed and female mice are additive suggesting different mechanisms of activation. Moreover, female sex, but not HFD, show higher resistance to lipid serum oxidation. These changes could help to explain the previously described effects of female sex protection against ischemia-reperfusion and the "obesity paradox".

4.1 Metabolism

Analysis of the ^{1}H NMR fingerprint showed differences between hearts of mice treated with HFD for two weeks and control mice suggestive of a switch towards fatty acid oxidation and, hence, a less efficient energetic homeostasis in the hearts of HFD fed mice.

Metabolic profiling showed an increase in alanine content (and glucose in males) in HFD fed mice, this could be indicative that not all glucose could be incorporated into the Krebs cycle; in accordance with higher amount of Acetyl-coA originating from β-oxidation of lipids. Also, fumarate was increased in HFD relative to control diet; in the case of liver, it has been reported that an increase in fumarate correlates with a dysfunction in the Krebs cycle [28]. During ischemia fumarate accumulates through the activation of the malate/aspartate shuttle and the purine nucleotide cycle, then fumarate is reduced to succinate by the reversal of succinate dehydrogenase [29]. However, in non-ischemic conditions used in this work we could not detect differences in the amount of succinate consistent with the forward activity of succinate dehydrogenase. Mice fed with high fat diet show lower levels of taurine than controls. Although mammals are unable to oxidize taurine [30], it is an essential cofactor for energy production in the heart [31]. However, other relevant physiological roles for heart function have been attributed to taurine such as regulation of ROS production [32] and calcium handling [33].

When comparing sexes, metabolic fingerprinting suggests that male hearts contain more glutamate than female hearts, suggesting differential energy production mechanisms. The reduced amount of glutamate would be in agreement with a reduced mitochondrial content seen in female rat hearts [34]. When metabolite concentrations were measured, only glucose was found to be significantly reduced in females. This could be indicative of higher glycolytic rate in females. Glycolysis is more efficient, in terms of oxygen consumption, than β-oxidation and would allow females to maintain energy levels for longer during myocardial ischemia, in accordance with previous reports by Gabel and cols [35] showing that female hearts have increased ratio of

carbohydrate to fatty acid metabolism relative to males.

4.2 Mitochondrial Function

The rates of oxygen consumption during stages 2 and 3 of the respiration were not affected by short term high fat diet or sex. Literature reports show that there are no O_2 consumption differences between male and female animals [36]. Also, long term HFD does not alter mitochondrial O_2 consumption [37].

On the other hand, mitochondria from female animals produce less H_2O_2 than mitochondria from males as has been described in the literature [36]. Female sex is protective against ischemia-reperfusion injury [6]; the mechanisms proposed are mostly related to signalling pathways like TNFR2 [38] or oestrogen receptors [reviewed in [8], [10] [39]. Our results further support the hypothesis that ROS play a role in female cardioprotection.

We have also seen a reduction in H_2O_2 production associated to HFD in contradiction with published data [40]. However, the experimental conditions were significantly different regarding time of treatment (two weeks vs eight months) and animal model (mouse vs rat) and those differences between the models may explain the discrepancies.

The mechanisms leading to the protection offered by the "obesity paradox" are not fully understood. So far, the involvement of leptin [41] and the RISK pathway [42] have been described; both mechanisms have been shown to be interrelated with oxidative stress [43], [44]. Our findings show a direct involvement of ROS in the "obesity paradox".

Interestingly, the reduction in H_2O_2 production observed in female sex and HFD seem to be additive pointing to two different mechanisms of action. Furthermore, serum from females has higher resistance to oxidation than males while there were no differences associated to HFD. Together, this suggests that the protection offered by female sex is at the organism level while the protective effect of HFD would be cardiac specific.

Our results would be in agreement with the fact that once a myocardial infarction is established, female sex and HFD would provide a metabolic and oxidative background less susceptible to cellular damage. Also, serum spectra from mice in this study were similar to those of mice published in the literature [45] but the amount of lipids was reduced when compared to spectra obtained from humans after 12 hours of fasting [46]. It is thus possible that the effects seen on cardiac metabolism on our model system could be larger in the case of humans.

4.3 Considerations regarding pattern recognition approaches

Fingerprinting and profiling approaches have been able to discriminate between sex and diet

groups; however, the variables selected by the different approaches were not the same. It is worth noting that metabolite assignations in the case of fingerprinting are just tentative based on the chemical shift and resonances corresponding to other metabolites may appear in similar positions of those of the tentative assignations. There is also the possibility that the variables selected in the fingerprinting approach correspond to unknown compounds and, thus, are omitted in the profiling analysis.

Fingerprinting has the advantage that it facilitates data processing, as it does not require prior knowledge and it may allow to identify previously unknown biomarkers. On the other hand, profiling results are easier to interpret. Both strategies may take advantage of the fact that latent variables may give better results [47].

4.4 Study Limitations

This study has been performed using a mice model with relatively low number of animals. Further studies, with a larger number of cases, involving animals and humans, should be carried out to confirm these results.

Concluding remarks

Metabolomic analysis using variable selection combined with linear discrimination seems preferable (in terms of accuracy and interpretability) to dimension reduction via PLS.

Sex and short term high fat diet have an effect on the myocardial metabolic pattern. Female sex and HFD reduce the amount of H_2O_2 produced by mitochondria, this could explain, at least in part, the protection offered by female sex and the “obesity paradox”.

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FIGURES

Figure 1.- Panel A shows heart fresh weight. B corresponds to the increase in body weight during treatment (two weeks). C and D correspond to ^{1}H NMR spectra of serum from treated and control animals respectively. * and τ mark statistical differences ($p<0.05$) regarding sex and diet treatment respectively.

Figure 2.- Scatter plot showing the values of ^{1}H NMR peak height for creatine and myo-inositol (variables 161 and 54) chosen after mRMR+LDA analysis.

Figure 3.- Mitochondrial oxygen consumption and H_2O_2 production. Panels A and B correspond to mitochondrial oxygen consumption in states 2 (basal) and 3 (ADP induced) for control and HFD respectively. Time course of H_2O_2 production over time from isolated mitochondria after the addition of succinate in control (C) and HDF fed mice (D). In panel E, F/F0 corresponds to fluorescence values normalized vs. baseline values. Panel E shows the ratio corresponding to Ffinal/F0. * indicates significant differences ($p<0,05$) vs. male control diet group and τ indicates significant differences vs female control diet group. F and G correspond to the % of serum lipids oxidized after 6 hours of treatment with 0.5 mM $CuSO_4$ at 37C. * mark statistical differences ($p<0.05$).

TABLES

Table 1.- Classification accuracy for mRMR + LDA and list of metabolites responsible classification models able to differentiate sex and HFD.

	Accuracy	Elevated in Males	Elevated in Controls
Sex	21/23 (91%)	Myo-inositol, Taurine, Glutamate	
HFD	21/23 (91%)		Creatine, Taurine, Acetate

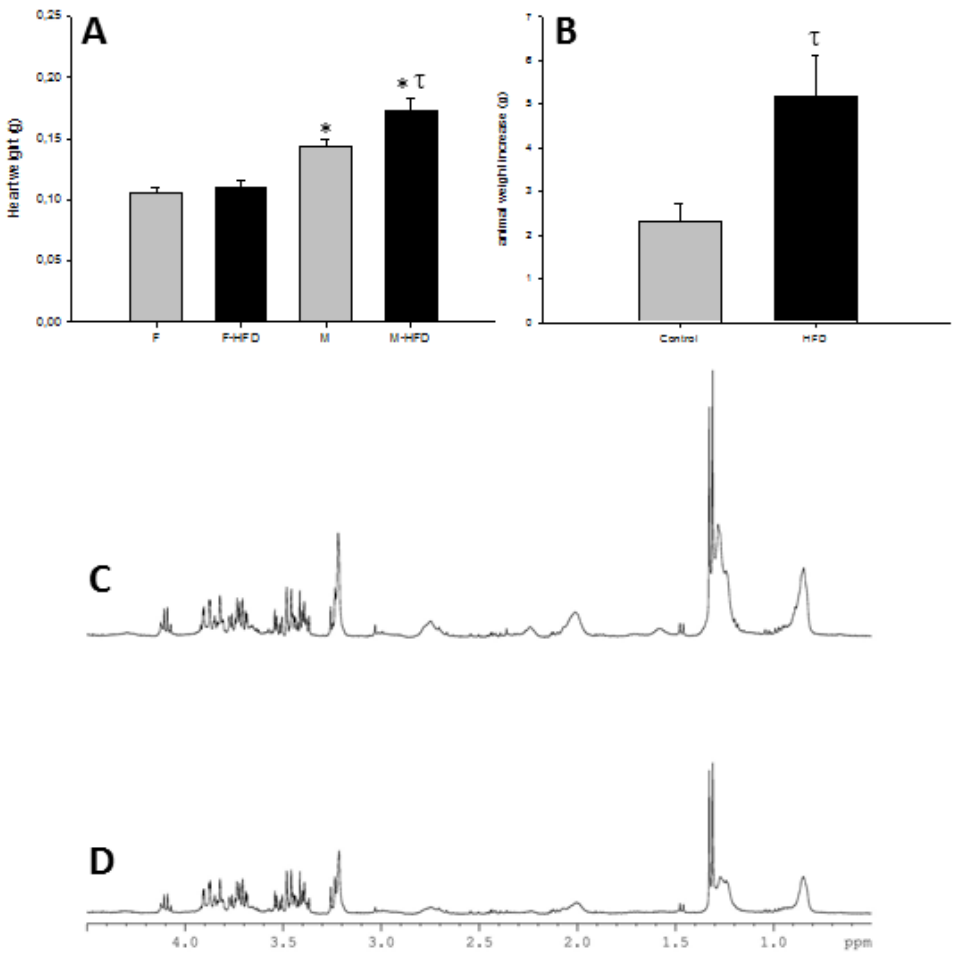
A
Heart weight (g)
0,25
0,20
0,15
0,10
0,05
0,00
*
* τ
F
F-HFD
M
M-HFD
B
animal weight increase (g)
τ
Control
HFD
C
D
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm

Figure 1

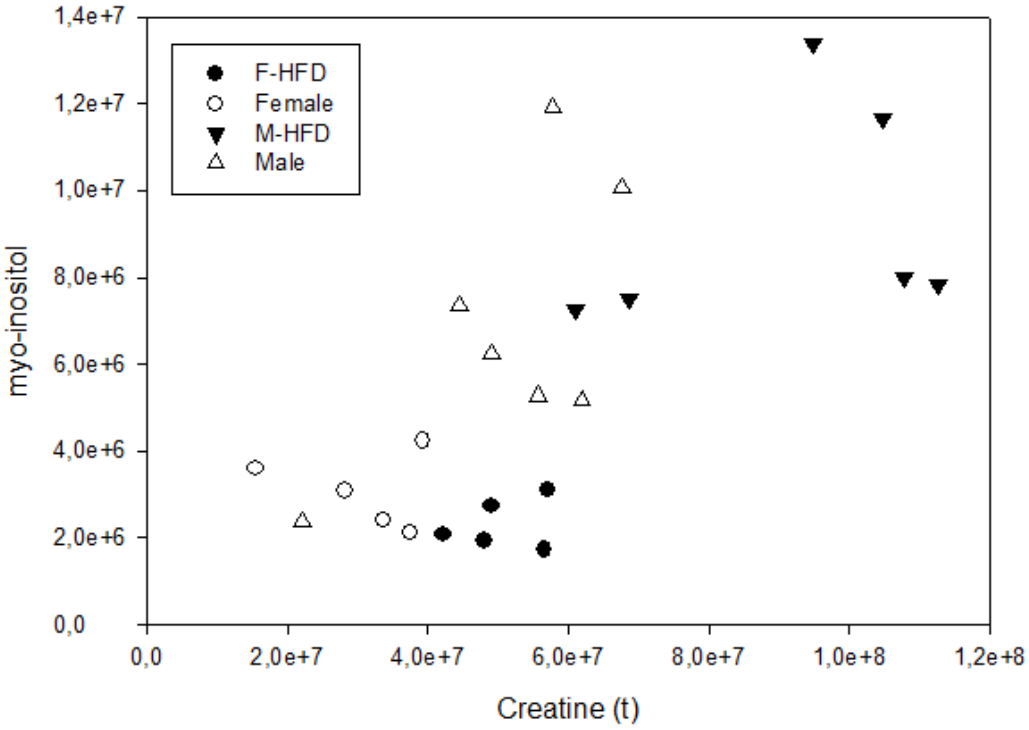

Figure 2

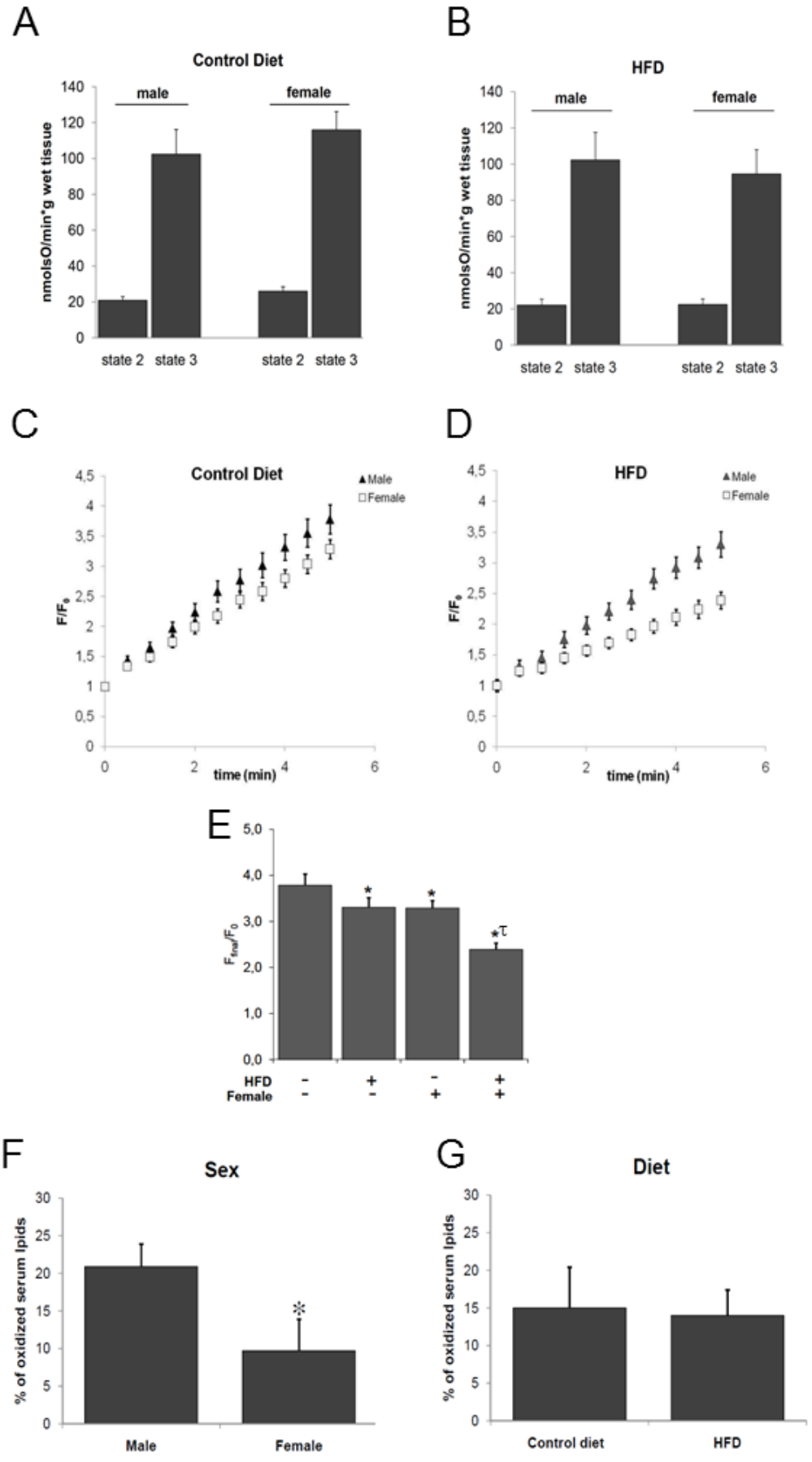
A
Control Diet
male
female
nmolsO/min*g wet tissue
state 2
state 3
B
HFD
C
Control Diet
Male
Female
F/F0
time (min)
D
HFD
E
F0
HFD
Female
F
Sex
% of oxidized serum lpids
Male
Female
G
Diet
Control diet
HFD

Figure 3