Supplementation with the prebiotic high esterified pectin improves blood pressure and cardiovascular risk biomarkers profile, counteracting metabolic malprogramming

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Figure 1

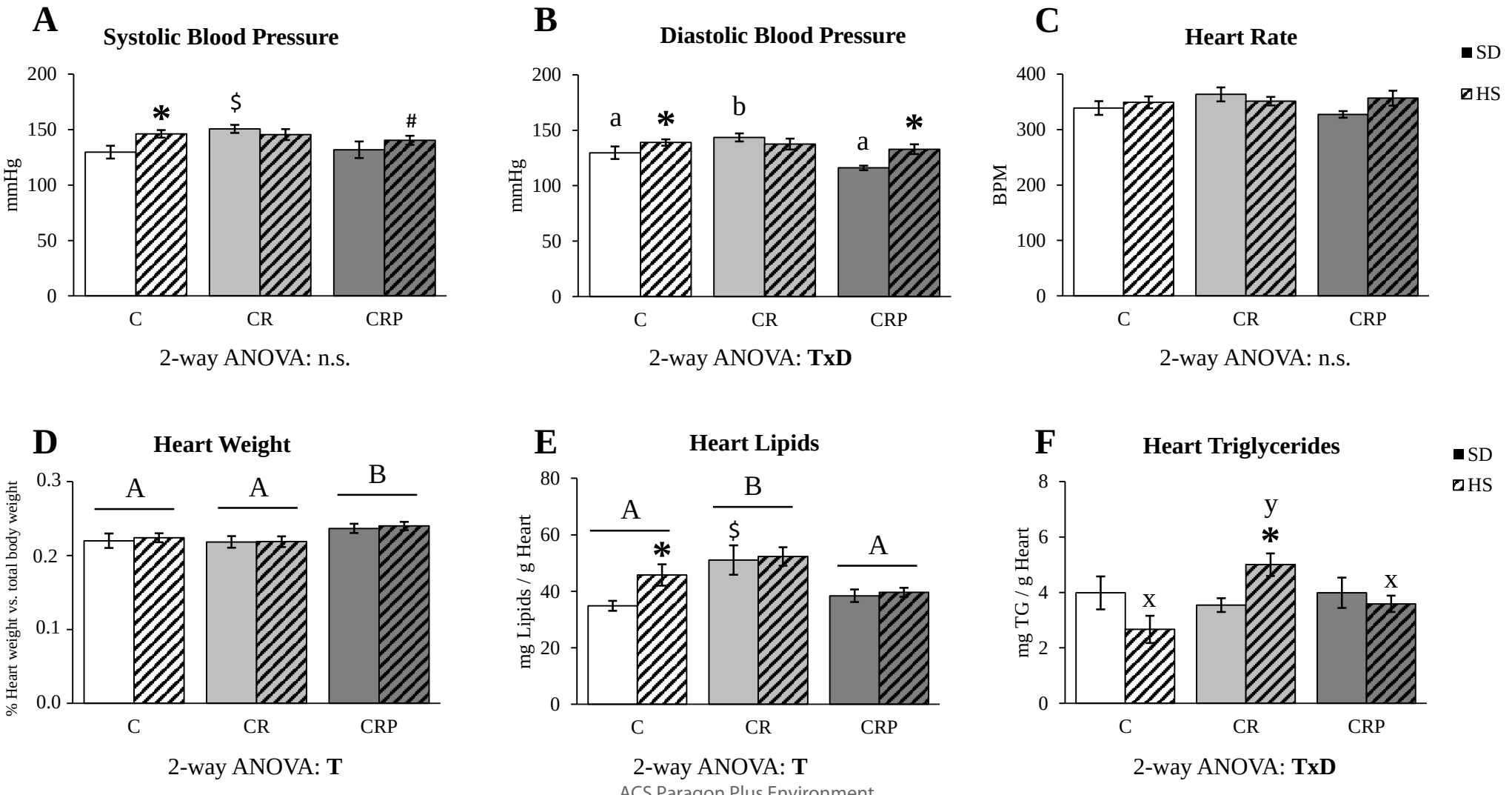
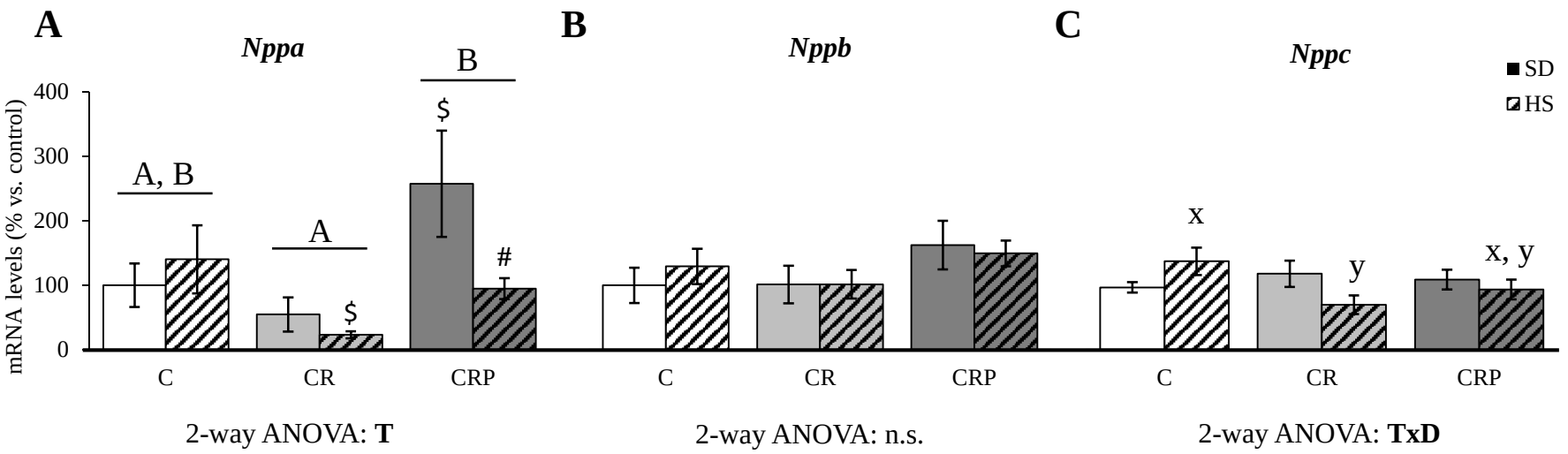
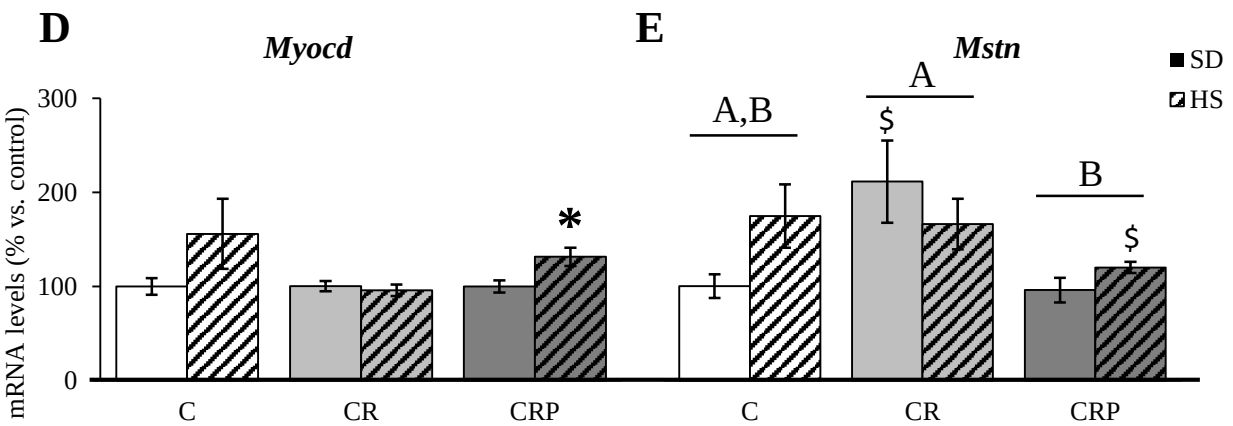

A
Systolic Blood Pressure
mmHg
200
150
100
50
0
*
$
#
C
CR
CRP
2-way ANOVA: n.s.
B
Diastolic Blood Pressure
mmHg
a
*
b
a
*
2-way ANOVA: TxD
C
Heart Rate
BPM
400
300
200
100
0
SD
HS
2-way ANOVA: n.s.
D
Heart Weight
% Heart weight vs. total body weight
0.3
0.2
0.1
0.0
A
A
B
2-way ANOVA: T
E
Heart Lipids
mg Lipids / g Heart
80
60
40
20
0
A
B
A
*
$
2-way ANOVA: T
F
Heart Triglycerides
mg TG / g Heart
8
6
4
2
0
x
y
*
x
SD
HS
2-way ANOVA: TxD

Figure 2

Heart





PBMC

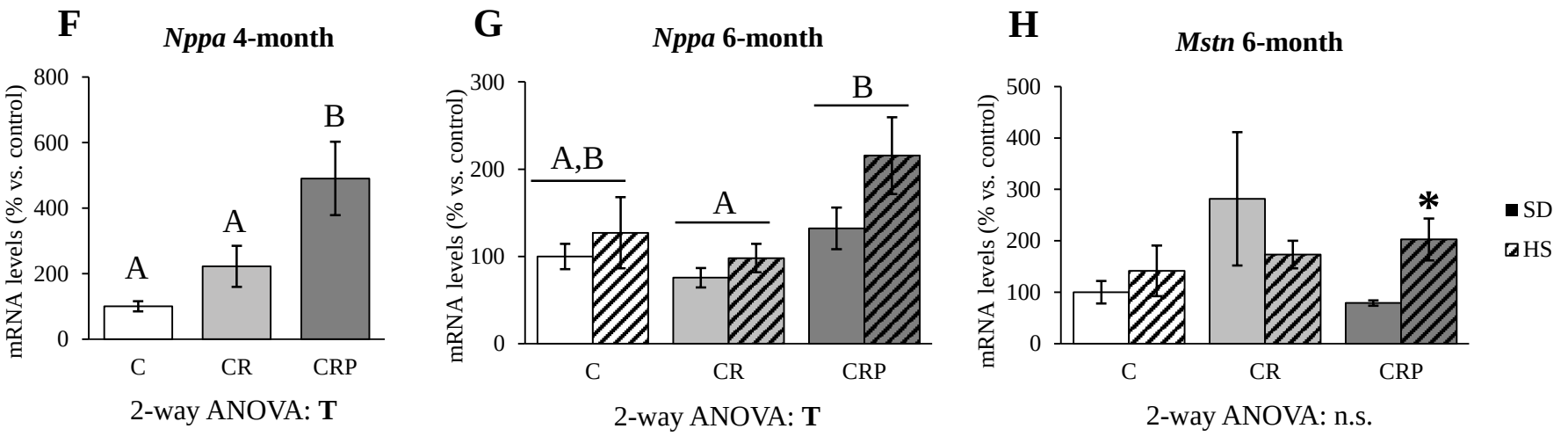
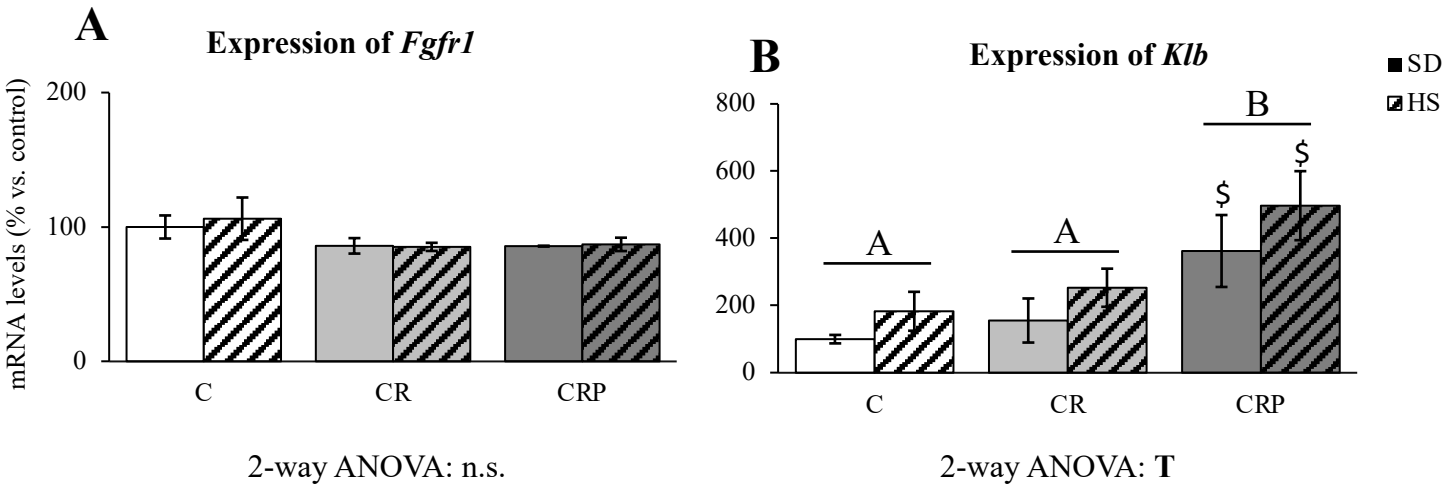


Figure 4

Heart mRNA

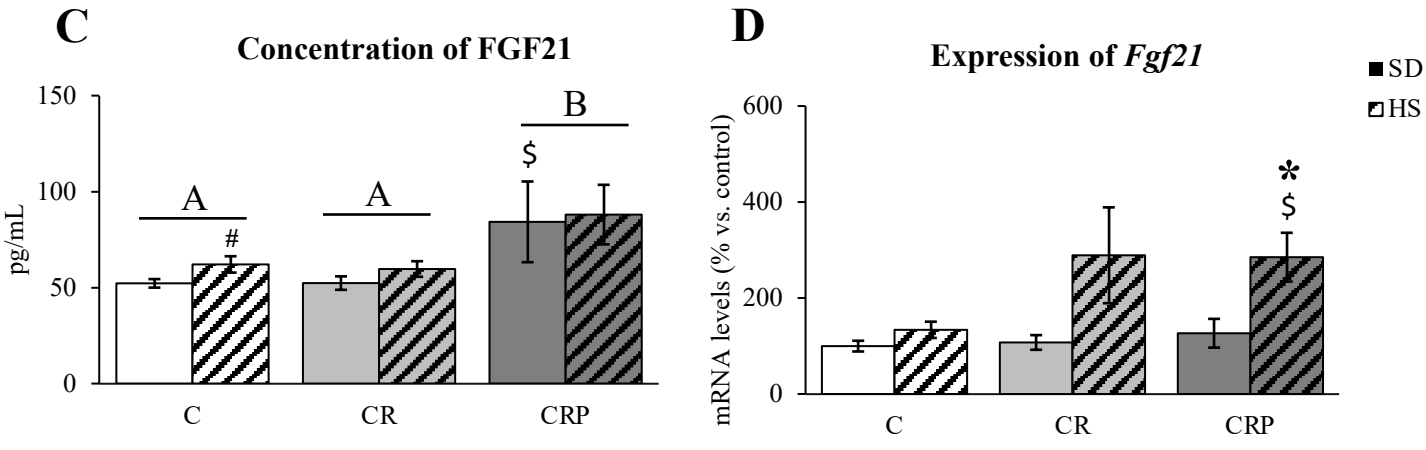


2-way ANOVA: n.s.

2-way ANOVA: **T**

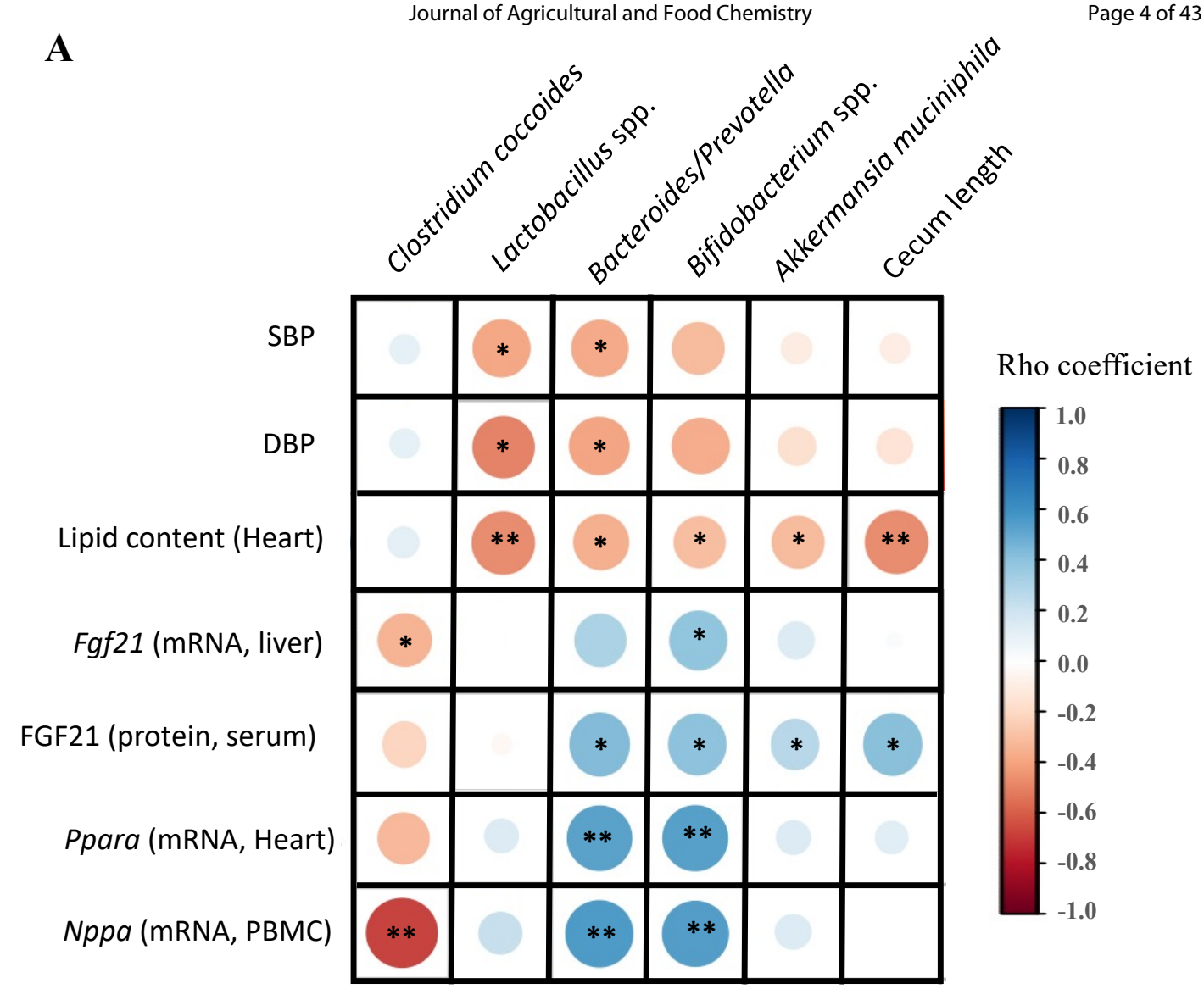
Serum

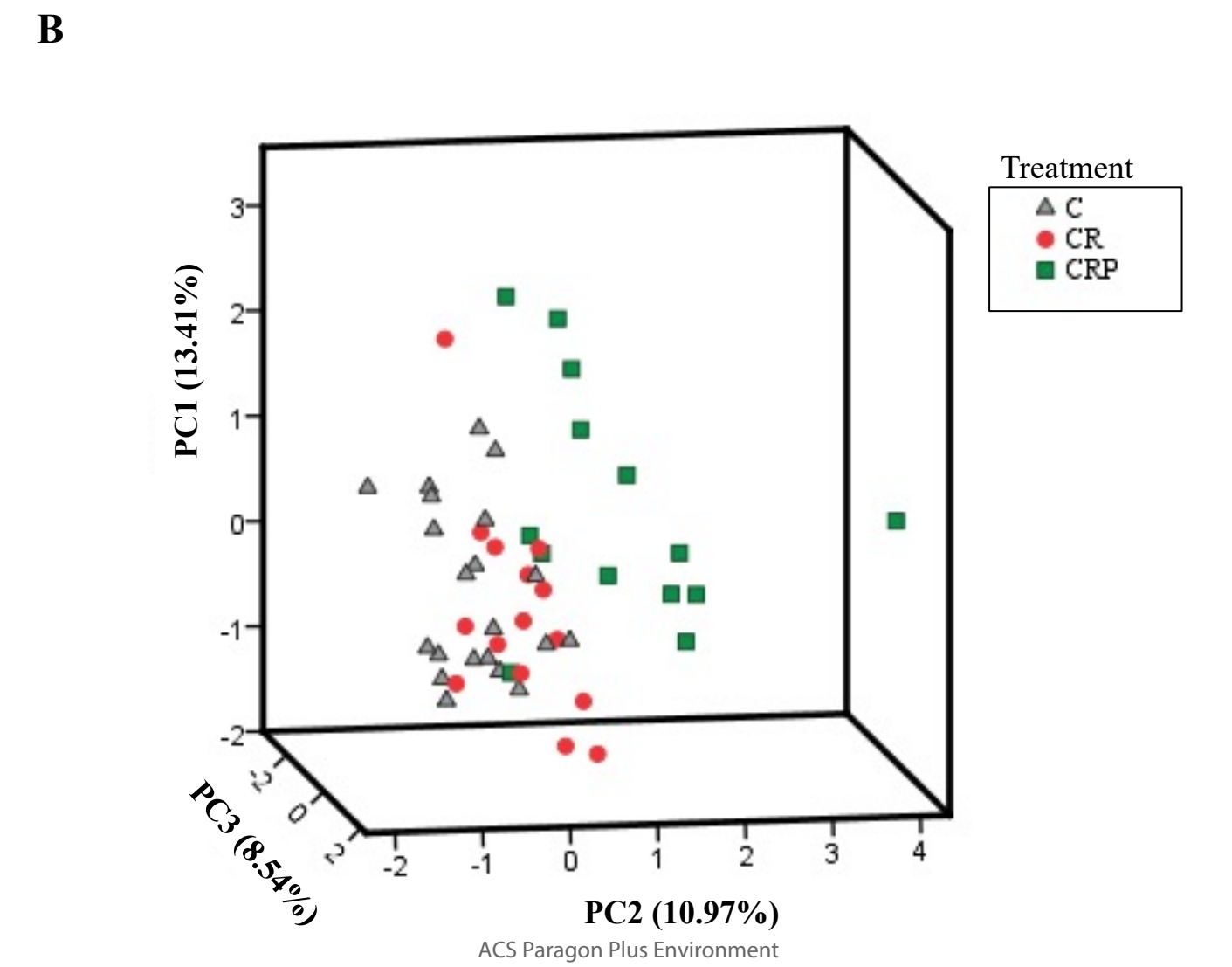
Liver

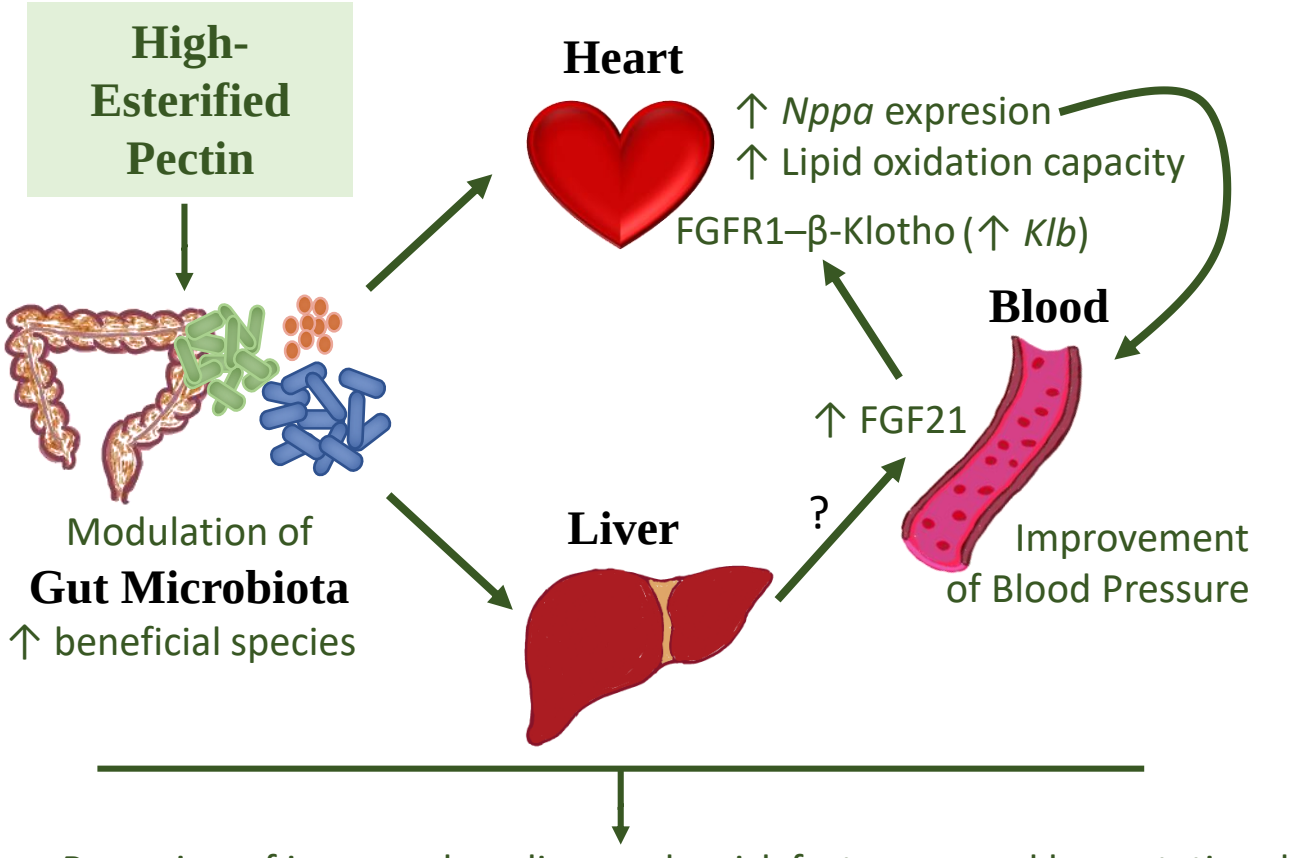


2-way ANOVA: **T**

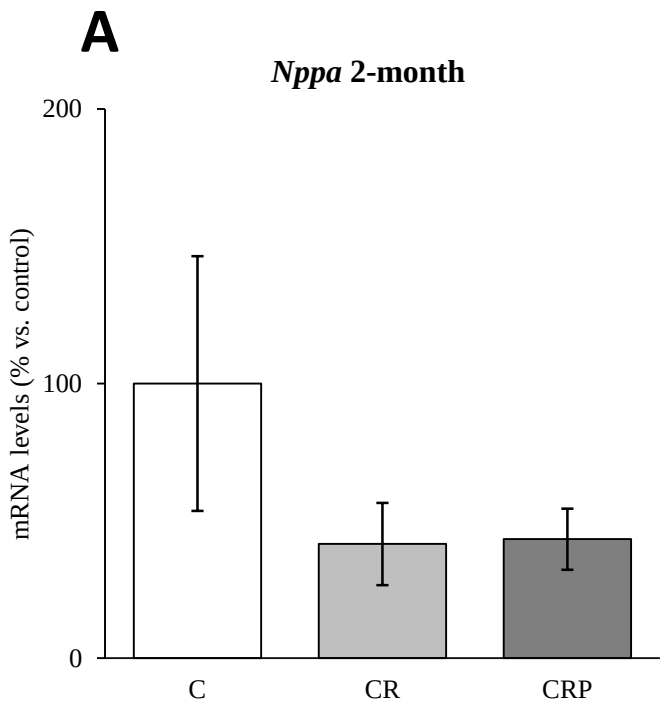
2-way ANOVA: **D**


A
Clostridium coccoides
Lactobacillus spp.
Bacteroides/Prevotella
Bifidobacterium spp.
Akkermansia muciniphila
Cecum length
SBP
DBP
Lipid content (Heart)
Fgf21 (mRNA, liver)
FGF21 (protein, serum)
Ppara (mRNA, Heart)
Nppa (mRNA, PBMC)
Rho coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0

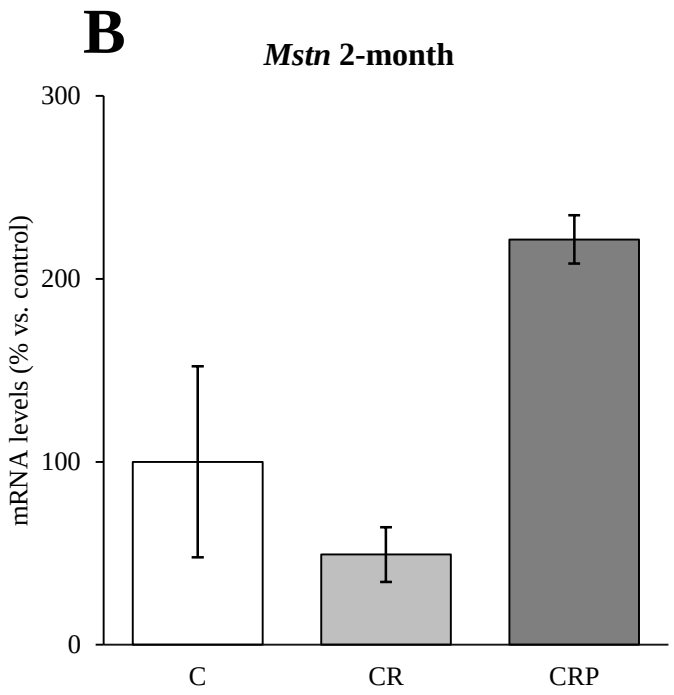

B
Treatment
C
CR
CRP
PC1 (13.41%)
PC2 (10.97%)
PC3 (8.54%)


High-Esterified Pectin
Heart
↑ Nppa expresion
↑ Lipid oxidation capacity
FGFR1–β-Klotho (↑ Klb)
Blood
↑ FGF21
?
Liver
Modulation of
Gut Microbiota
↑ beneficial species
Improvement of Blood Pressure
Reversion of increased cardiovascular risk factors caused by gestational calorie restriction and recovery/improvement of metabolic flexibility

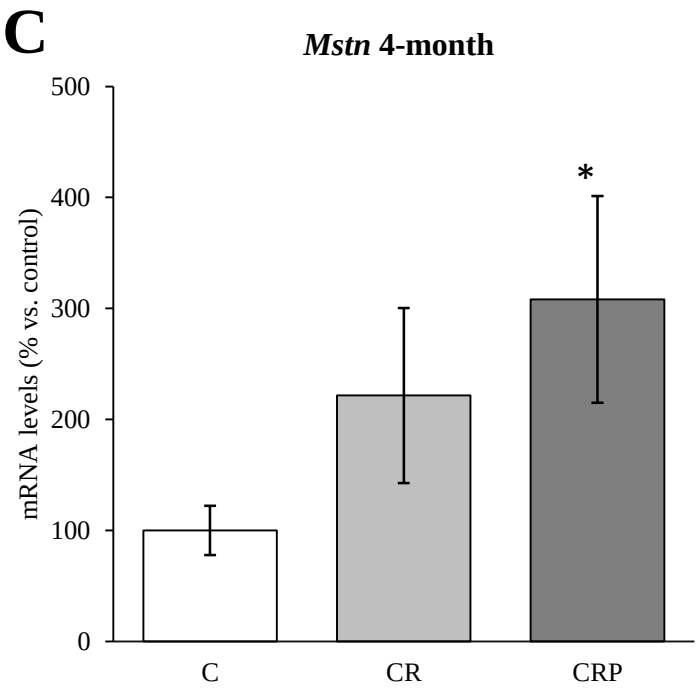
Supplementary Figure 1



2-way ANOVA: n.s.



2-way ANOVA: n.s.



2-way ANOVA: n.s.

Title and authorship

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Abstract and keywords

Abstract

Supplementation with the prebiotic pectin is associated with beneficial health effects. We aimed to characterise the cardioprotective actions of chronic high esterified pectin (HEP) supplementation (10%) in a model of metabolic malprogramming in rats, prone to obesity and associated disorders: the progeny of mild calorie-restricted dams during the first half of pregnancy. Results show that pectin supplementation reverses metabolic malprogramming associated with gestational undernutrition. In this sense, HEP supplementation improved blood pressure, reduced heart lipid content, and regulated cardiac gene expression of atrial natriuretic peptide and lipid metabolism-related genes. Moreover, it caused an elevation of circulating levels of fibroblast growth factor 21 and higher expression of its co-receptor β-klotho in the heart. Most effects are correlated with the gut levels of beneficial bacteria promoted by HEP. Therefore, chronic HEP supplementation shows cardioprotective actions and hence it is worth considering as a strategy to prevent programmed cardiometabolic alterations.

Keywords: High esterified pectin, Cardiovascular health, Microbiota, Perinatal malprogramming, Prebiotics

Introduction

Cardiovascular disease accounts for almost one-third of all deaths worldwide despite being, in most cases, preventable by addressing behavioural risk factors, such as unhealthy diet, obesity, or sedentarism (World Health Organization). There is growing evidence that gut microbiota participates in host metabolism, and dysbiosis (an imbalance in the gut microbiota) is associated with cardiovascular disease phenotypes [2]. Thus, new strategies to modify gut microbiota by favouring specific species with benefits in reducing cardiovascular disease risk are of interest. In this line, prebiotics have raised as candidates to reduce cardiovascular risk through microbiota modulation [3]. Among them, pectin has been associated with beneficial effects on metabolic health by reducing calorie intake, modulating chronic inflammation, reducing post-prandial glycaemic response and age-related insulin resistance [4–7]. Along these lines, we have previously demonstrated that physiological dietary supplementation with the prebiotic high esterified pectin (HEP) in rats improves adipostatic/adipokine sensitivity and regulates thermogenic capacity, preventing fat gain and deleterious effects associated with metabolic malprogramming and later exposure to an obesogenic diet [8,9].

Increased body weight and obesity have been consistently associated with increased cardiovascular risk factors, cardiovascular disease [10] and gut dysbiosis [11]. Besides genetic and environmental factors, conditions during the perinatal period are considered causal factors of increased obesity risk in adulthood [12]. In this regard, there is evidence from epidemiological studies and intervention studies in animal models showing that maternal calorie restriction during gestation may increase the propensity to develop obesity and related chronic diseases in adulthood, with different outcomes depending on the type and severity of restriction, as well as on the gender [13–16]. In humans, the emblematic study of the Dutch famine of 1944-45 has evidenced the adverse effects of severe gestational undernutrition, showing that men who were exposed to the famine during the first two trimesters of gestation had higher rates of obesity at the age of 19 years [17]. Notably, people exposed to the Dutch famine during early gestation also had a higher prevalence of coronary heart disease at 50 years of age [18]. In rats, we have shown

that maternal food restriction during gestation, even when it is mild/moderate, during the first 12 days of gestation programs the progeny to altered hypothalamic control of food intake and increased body weight and fat, among other disarrays, mainly in males [13,14]. Of interest, in the model mentioned above of adverse metabolic programming established in rats, chronic HEP supplementation in the offspring has been shown to prevent excess body weight/adiposity and various adverse metabolic disturbances, which may be related, in part, with an improved profile and sensitivity of the main adipostatic (leptin, insulin, and adiponectin) hormones, and the promotion of beneficial bacteria in the gut [8,9]. However, the effects of this maternal condition on the offspring's cardiovascular risk and the potential beneficial impact of HEP supplementation have not been assessed.

In this context and taking as a reference a non-infrequent condition in humans, here we aimed to study in rats the effects of mild calorie restriction (20%) during the first 12 days of gestation in the male progeny, and their performance under dietary stress (high-sucrose diet) in adulthood. Specifically, we meant to characterize the consequences of mild gestational calorie restriction on cardiovascular risk factors in terms of blood pressure (BP), heart rate, size, lipid content and gene expression, and circulating markers of cardiovascular risk, and whether chronic HEP supplementation in the offspring may have a reversal or protective effect against such potential detrimental outcomes.

Materials and methods

Animals and experimental design

The animal protocol was evaluated and approved by the Bioethical Committee of the University of Balearic Islands (Res. number 3513). The animals were from a cohort described in previous works [8,9]. In brief, pregnant Wistar dams were divided into two groups (6 rats per group): the control dams' group, fed *ad libitum* with standard diet (SD), containing 3.3 kcal/g, with 8% calories from fat and 4% (w/w) of cellulose (Panlab A08, Barcelona, Spain), and the calorie restriction dams' group, fed with the same diet but with 20% calorie restriction (compared to the

total intake of the control group) during 1-12 pregnancy days. The first day after parturition, the number of pups in each litter was adjusted to 10 per mother. From day 21 of life (weaning) to day 135, male offspring were divided into three groups, all fed ad libitum: Control (C) group included the progeny of control dams and were fed with SD, calorie restriction (CR) group was composed by the progeny of calorie-restricted dams and were fed with SD, and calorie restriction supplemented with pectin (CRP) group was the progeny of calorie-restricted dams and were fed with SD with 10% (w/w) of apple high-esterified pectin (HEP, with 70−75% degree of esterification, molecular weight 30-100 kDa, Sigma-Aldrich Chimie, Lyon, France, ref 76282). The intake in grams was measured every two days and the feeders were refilled with 100 grams of the powder SD hand-operated with pectin (w/w) (90 grams of SD+10 grams of pectin). From day 135 until day 180, half of the animals in each group were fed with the same diet but supplemented with 30% sucrose (HS—high-sucrose diet) (final n=6–10 animals/group) (C-HS, CR-HS, and CRP-HS, respectively), and the other half continued with SD (C-SD, CR-SD, and CRP-SD, respectively). The diet was in powder form to facilitate supplementations. In a previous study with the same cohort of animals [8], we accurately measured cumulative energy intake for a period of 48 h at 5 months of age. Considering this accurate measure of intake in the adult rats, and the percentage of HEP in the diet, we calculated the representative intake of HEP in the adult animals as a reference of daily HEP intake, which is 2.50±0.14 g in the CRP-SD group and 2.39±0.07 g in the CRP-HS group, without statistical differences between the two HEP-supplemented groups (p=0.631, Mann-Whitney U test). Finally, all animals were sacrificed at six months of age by decapitation for tissue recollection.

Blood pressure and heart rate measurements

Blood pressure (BP) −systolic (SBP) and diastolic (DBP)− and heart rate of the animals were measured at five months of age (n=6 per group) using a non-invasive method based on a rubber inflatable sphygmomanometer with a tail-cuff and a photoelectric sensor (NIPREM 546, Cibertec S.A, Madrid, Spain), without anaesthesia and after 30-minute acclimatisation to prevent animal stress hypertension. During this acclimatisation time, vasodilation was induced by warming the

rat with a red-light bulb. The Niprem V1.8 software was used to determine BP, and the rate values and the mean of at least five measurements per animal were used.

Peripheral blood mononuclear cell isolation

Blood samples were collected at 2, 4 and 6 months of age (6 animals per group), and Peripheral Blood Mononuclear Cells (PBMC) were isolated from total blood by density gradient separation using OptiPrep™ Density Gradient Medium (Axis-Shield, Dundee, UK) following the manufacturer guides.

RNA isolation, reverse transcription, and PCR

Total RNA from rats was isolated from liver, heart and PBMC at different times using two protocols, depending on the type of tissue or sample. For liver RNA extraction, TriPure Reagent (phenol-based, Roche Diagnostic GmbH, Mannheim, Germany) was used following the manufacturer's protocol. Total RNA was also extracted from the heart and PBMC (n=6 per group) by EZNA® TOTAL RNA kit I (Omega Bio-Tek, Norcross, GA, USA), as the manufacturer's protocol describes. Isolated RNA was quantified using the spectrophotometer NanoDrop ND-1000 (Nano-Drop Technologies, Wilmington, DE, USA), confirming its integrity by 1% agarose gel electrophoresis. Then, total isolated RNA was reverse transcribed into complementary DNA (cDNA) in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Madrid, Spain) and real-time quantitative polymerase chain reaction (RT-qPCR) with StepOne plus protocol (Applied Biosystems, Madrid, Spain) was performed to measure mRNA expression levels in heart, PBMC and liver, as described previously [19]. Regarding gene expression in the heart, we studied those genes coding for natriuretic peptides A (*Nppa*), B (*Nppb*) and C (*Nppc*), Myostatin (*Mstn*), Myocardin (*Myocd*), peroxisome proliferator-activated receptor α (*Ppara*), PPARγ-coactivator 1 α (*Ppargc1a*), 5'-AMP-activated protein kinase (AMPK) catalytic subunit alpha-2 (*Prkaa2*), carnitine palmitoyltransferase 1b (*Cpt1b*), fatty acid synthase (*Fasn*), FGF21 receptor (*Fgfr1*), and co-receptor β-Klotho (*Klb*). Furthermore, expression mRNA levels of *Nppa* and *Mstn* in PBMC and expression mRNA levels of *Fgf21* in the liver were analysed. GDP dissociation

inhibitor alpha (*Gdi1*) was used as a housekeeping gene for liver and heart, and proteasome subunit alpha type-6 (*Pmsa6*) for PBMC. All primers used were obtained from Sigma Genosys (Sigma-Aldrich Química SA, Madrid, Spain), and they are shown in **Table S1**.

Determination of total lipid and triacylglyceride content in heart

Total lipid determination was performed by mixing 100-150 mg of heart tissue with 1 mL of hexane/isopropanol (3:2, v/v), following the protocol established by Folch *et al*. [20]. Tubes with the samples were gassed with nitrogen before being closed to minimise lipid oxidation and then left overnight under orbital agitation at room temperature protected from light. The content of each tube was transferred into a new one, and 0.3 ml of Na_2SO_4 (0.47 M) was added and mixed for 5 min, left for 15 min in orbital agitation and, finally, centrifuged at 1000 x g for 10 min at 4º C. The upper phase containing lipids was dissolved in hexane and transferred to a clean, previously weighed glass tube. The hexane extract was then dried with nitrogen gas. Once the tube was dried, the percentage of lipids was determined as the weight difference between tubes with lipid extract and clean tubes, considering the initial amount of tissue present. Triglyceride (TG) content was determined from the lipid extracts dissolved in LPL buffer (28.75 mM Pipes, 57.41 mM $MgCl_2.6H_2O$, 0.569 mg/ml bovine serum albumin-fatty acid-free) with sodium dodecyl sulphate 0.1%, as described in the literature [21]). Samples were re-suspended in 3 ml of LPL buffer and were sonicated for 30 s. Tubes were left overnight in an orbital shaker and protected from light at room temperature. The following day, the tubes were coldly sonicated with three pulses of 30 s each. Their triglyceride levels were measured immediately using the Serum Triglyceride Determination Kit (Sigma-Aldrich, Saint Louis, MO, USA), following the manufacturer's instructions.

Western blot for heart proteins and circulating FGF21 measurement

Western blot was performed to determine cardiac protein levels of phosphorylated AMPK, the serine/threonine-protein kinase AKT (protein kinase B), adipose triglyceride lipase (ATGL), CPT1B and cytochrome c oxidase subunit 4 (COX4). A detailed Western blot protocol is

described elsewhere [9]. Briefly, total protein was extracted from the homogenised heart in radioimmunoprecipitation assay (RIPA) lysis buffer, and the protein content was determined by the Bradford method. For SDS-PAGE electrophoresis, 40 µg of total protein per sample was loaded. Electroblotting was carried out with the Trans-blot Turbo Transfer System (Bio-Rad). For labelling and detection, specific primary antibodies used appear in **Table S2.** Antibodies infrared (IR)-dyed 800 or IR-dyed 680LT (LI-COR Biosciences, Lincoln, NE, U.S.A.) were used as secondary antibodies. IR was detected by scanning in Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, U.S.A), and bands were quantified using the analysis software provided (Odyssey Software V.3.0). ACTB was used as the loading control. Serum FGF21 levels were analysed under fed conditions at five months of age using the ELISA kit Quantikine™ Mouse/Rat immunoassay (R&D Systems, MN, USA).

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Differences among C, CR and CRP groups, under SD or HS diet, were assessed by 2-Way ANOVA and LSD post-hoc analysis. When there was interaction in the two-way ANOVA, comparisons between groups (splitting by diet) were assessed by 1-way ANOVA and LSD post hoc analysis. The statistical assessment of differences between specific groups was carried out by Mann-Whitney *U* test test (this non-parametric test was selected as the most suitable since most groups had an $n \leq 10$). The significance threshold was set at $p < 0.05$, and *p*-values between 0.05-0.10 were considered non-significant tendencies. Analyses were performed with SPSS for Windows (SPSS version 27.0.0, Chicago, IL, USA). For correlation and integrative analysis of principal parameters with gut caecum bacteria relative content and short-chain fatty acid (SCFA) profile, previous data from García-Carrizo et al. [8] on the profile of bacteria/total bacteria for Firmicutes (*Clostridium coccoides*, *Clostridium leptum* and *Lactobacillus* spp.), Bacteroidetes (*Bacteroides/Prevotella*), Actinobacteria (*Bifidobacterium* spp.) and *Akkermansia muciniphila*, and acetate, propionate, butyrate (SCFA), and cecum length were included in the analyses of correlation and Principal Components Analysis (PCA). Spearman correlation assessment and PCA were carried out with

SPSS v27. Data were normalised to perform PCA. Correlation maps were performed using R Software Package corrplot, following the guidelines of Statistical Tools for High-throughput data analysis (STHDA).

Results

Pectin supplementation reverses adverse effects of maternal calorie restriction in blood pressure and heart lipid content of the progeny

The effects of moderate maternal calorie restriction and pectin supplementation, under standard or HS diet, on blood pressure (SBP and DBP), heart rate (at five months of age), and heart size, lipid and triglyceride content (at six months of age) in the C, CR and CRP rats are represented in **Fig. 1**. Concerning BP, C animals under the HS diet showed increased SBP than those fed with SD, and a tendency in this sense was also found in CRP animals (p=0.093). No differences between the animals fed with SD or HS diet were found in the CR group, but CR-SD rats showed increased SBP respect to C-SD (**Fig. 1A**). For DBP, there was a treatment per diet interaction (TxD): in the C and CRP groups, but not in the CR group, HS diet increased DBP. In addition, CR animals showed increased DBP under the SD, but this effect was reverted in the CRP group (**Fig. 1B**). Hence, the offspring of dams with gestational calorie restriction presented increased BP, not further increased by HS feeding, while HEP supplementation normalised the levels to the control situation. No significant differences were found between groups for heart rate (**Fig. 1C**). However, there was a significant effect of HEP treatment increasing heart size (in terms of % of body weight) (**Fig. 1D**), without effects of HS diet feeding.

Heart total lipid and triglyceride content in the different experimental groups are shown in **Fig. 1E-F**. Heart lipid content was increased in the CR group with respect to controls. Moreover, the HS diet caused an expected increase in heart lipid content in C animals, an effect lost in the CR animals, which already showed increased lipid content. This effect was reverted by pectin supplementation, which even prevented the HS diet-associated lipid increase (**Fig. 1E**). The amount of the main specific lipid type (triglycerides) was affected by both treatment and diet with

an interactive TxD effect: while under SD, there were no differences between treatments, under HS diet, the CR group showed increased heart TG content, an effect reverted, again, by HEP supplementation (**Fig. 1F**).

Gestational calorie restriction condition and pectin supplementation are associated with changes in gene expression of natriuretic peptides and myostatin

The mRNA expression levels of selected genes that may reflect cardiovascular risk status were analysed in the heart (**Fig. 2A-E**). We focused on heart key genes for cardiomyocyte function and the control of BP, such as those coding for natriuretic peptides [22], and for Myostatin (*Mstn*) and Myocardin (*Myocd*) (related to the control of cardiac muscle growth) [23,24]. Regarding mRNA expression levels of heart natriuretic peptides (*Nppa*, *Nppb* and *Nppc*, **Fig. 2A-C**), the CR group showed a tendency for decreased levels of *Nppa* mRNA versus controls (p=0.076). Still, HEP supplementation increased the levels of *Nppa* expression above those of the CR group, and the HS diet tended to decrease (p=0.071) its mRNA levels in the CRP animals (**Fig. 2A**). There were no significant changes in *Nppb* mRNA levels in CR animals compared to controls, but there was a tendency for upregulation in the HEP-supplemented rats (p=0.082) (**Fig. 2B**). Regarding the levels of *Nppc* mRNA (**Fig. 2C**), there was an interactive effect of treatment and diet, and when splitting the groups by diet, differences were found between groups under the HS diet, with CR animals showing decreased *Nppc* expression compared to the C group. This effect was partially reverted in the CRP group. Regarding the expression of *Myocd* and *Mstn*, *Myocd* mRNA levels were significantly increased under the HS diet only in the CRP group (**Fig. 2D**), while *Mstn* expression tended to be increased in the CR animals (the levels were significantly higher in CR animals with respect to C under SD), and was significantly reduced (respect to CR animals) in the CRP group (**Fig. 2E**).

Considering the changes found in the mRNA levels of *Nppa* and *Mstn* in the heart at six months of life, in response to gestational calorie restriction condition and/or to HEP supplementation, we considered of interest to analyse their expression levels in PBMC at different ages, to explore

their potential interest as biomarkers, to predict the adverse outcomes associated to CR or the protective role of pectin supplementation. No significant differences between groups were observed regarding the expression levels of *Nppa* (at two months) and *Mstn* (at 2 and 4 months) in PBMC (Supplementary Fig. 1). However, the treatment did already significantly affect the expression of *Nppa* at four months, when all animals were under SD (**Fig. 2F**), with a significant increase in the CRP group compared to C and CR groups. At six months, PBMC expression of *Nppa* (**Fig. 2G**) was also significantly affected by treatment, with the CRP group having the highest levels significantly different from the CR group. The overall profile of *Nppa* expression in PBMC at six months was partially comparable to the expression profile in the heart, particularly regarding the effects of gestational calorie restriction and pectin supplementation. Regarding *Mstn* expression, only a significant induction by HS with respect to SD feeding was observed in the CRP group (**Fig. 2H**).

Diet and pectin supplementation regulate gene expression and protein activity in the heart, potentially related to the observed changes in lipid content

Linked to changes in total lipid and TG profile content in the heart, expression (mRNA and protein) of selected genes related to lipid metabolism and/or the activation (phosphorylation) of key signalling molecules were also determined to ascertain potential molecular mechanisms involved. Therefore, *Prkaa2*, *Ppara*, *Pparagc1a*, *Cpt1b* and *Fasn* mRNA levels were analysed, and the results are shown in **Fig. 3A-E**. Generally, an effect of HS diet was found upregulating *Prkaa2*, *Ppara* and *Cpt1b* gene expression. For *Ppargc1a* and *Fasn*, expression was significantly increased only in the C and CR groups, respectively. The induction of these genes under an HS diet was expected, taking into account their involvement in lipid metabolism [25]. Regarding the effects of treatment, there was a significant increase of *Ppara* expression in the CRP group with respect to both C and CR groups, beyond the HS diet effects.

The activation of AMPKα and AKT was measured by their phosphorylation levels in Thr172 and Ser473, respectively. Interactive TxD effects were observed in both cases, and the groups were split by diet for one-way ANOVA analysis (**Fig. 3F-G**). Under SD, pAMPK levels were

significantly increased in the CR animals, an effect reverted by HEP supplementation. In the control and CRP animals, HS diet feeding tended ($p<0.1$) to increase phosphorylated AMPKα levels, while in CR animals, pAMPKα levels were significantly reduced in response to HS diet; therefore, under the HS diet, the highest pAMK levels were found in the CRP group. In the case of pAKT, CRP animals displayed decreased levels with respect to CR animals under SD, and CR and CRP animals showed higher levels with respect to C under the HS diet. In fact, HS diet feeding increased pAMPK levels in the CRP group. Concerning ATGL protein levels, both CR and CRP groups showed increased levels with respect to control animals (**Fig. 3H**). Regarding CPT1b and COX4 protein levels, there were TxD interactive effects. Therefore, when separating the groups by diet, the one-way ANOVA showed that, under SD, gestational calorie restriction condition triggered induction of CPT1b, an effect reverted by HEP supplementation (CRP group), but these differences were not observed among animals fed with HS diet (**Fig. 3I**), and there was a significant up-regulation of the protein levels in response to HS diet in the CRP animals (which was only a tendency, $p<0.1$, in the C group). For COX4 levels (**Fig. 3J**), significant differences between treatment groups were manifested in HS diet-fed animals, with CR animals showing lower levels than C, an effect reverted by HEP supplementation. This was mainly because the HS diet significantly upregulated COX4 levels in the C and CRP groups. On the contrary, it tended to reduce its levels in the CR animals, suggesting that the altered response to diet by the gestational calorie restriction condition was recovered by HEP supplementation.

Pectin supplementation increases FGF21 circulating levels and the cardiac expression of its specific co-receptor β-klotho

The expression (mRNA) levels of *Fgf21* in the liver, the circulating levels of the corresponding protein, and the expression (mRNA) of *Fgfr1* and *Klb* (genes for receptor and co-receptor of FGF21, respectively) in the heart are shown in **Fig. 4**. Despite there were neither treatment nor diet effects on heart mRNA levels of *Fgfr1* (**Fig. 4A**), there were clear effects of HEP supplementation on heart *Klb* gene expression (**Fig. 4B**) and on circulating FGF21 levels (**Fig. 4C**), where the CRP group showed higher levels of both parameters compared to the rest of the

283 groups. HS diet resulted in increased *Ffg21* liver expression, which was significant by Mann–
284 Whitney *U* test only in the CRP group (**Fig. 4D**).

285 *Correlation and Principal Component Analyses point out the relevance of gut microbiota*
286 *composition as a mediator of the pectin supplementation impact*

287 Analyses of correlation and Principal Component Analysis (PCA) were performed to assess
288 potential associations among the most outstanding cardiovascular health-related parameters
289 studied (by the results described above) and the profile of intestinal bacteria, cecum length, and
290 the main short-chain fatty acids produced by gut bacteria (acetate, propionate and butyrate). The
291 data of the levels of gut bacteria relative abundance and SCFA (acetate, propionate and butyrate)
292 concentration in peripheral blood was published in previous work [8].

Briefly, HEP supplementation was associated with increased levels of acetate in peripheral blood in comparison with CR animals. Furthermore, HEP supplementation was also associated with increased caecum abundance of specific beneficial bacteria (including *Bacteroides/Prevotella*, *Lactobacillus* spp., and especially *Bifidobacterium* spp.), decreased abundance of potentially detrimental bacteria (*C. coccoides*), and with the reversion of gestational calorie restriction effects on the levels of *A. muciniphila* (beneficial) [8].

Correlation analyses (**Fig. 5A**) revealed a significant inverse association of the relative gut abundance of *Lactobacillus* spp. and *Bacteroides*/*Prevotella* with SBP and DBP, and also an inverse association of the relative abundance of *Lactobacillus* spp., *Bacteroides*/*Prevotella* *Bifidobacterium* spp., *Akkermansia muciniphila* and cecum length with heart lipid content. The relative levels of *Clostridium coccoides* and *Bifidobacterium spp*. were negatively and positively correlated, respectively, with *Fgf21* liver mRNA levels. In addition, the relative gut abundances of *Bacteroides/Prevotella*, *Bifidobacterium* spp., *Akkermansia muciniphila* and the cecum length were positively associated with serum FGF21 levels. *Bacteroides/Prevotella* and *Bifidobacterium* spp. relative abundances were positively correlated with mRNA expression levels of heart *Ppara* and PBMC *Nppa*, while *Clostridium coccoides* was inversely associated with mRNA levels of *Nppa* in PBMC.

The PCA elaborated with three main components (PC) was able to explain 32.93% of the observed variability. Although the PCA does not explain a high percentage of variability, the representative plots show a separation of groups, especially in the case of the CRP animals, which more clearly move away from the control and CR animals (**Fig. 5B**). Component 2 (PC2, 10.97% explained variability) allowed the separation of the CRP group from the other groups, with the CRP animals showing the highest values for PC2, while C and CR groups were set by lower PC2 values. The highest (positive) contributors for PC2 were represented by the relative gut abundance of *Bifidobacterium* spp. (rotate component value: 0.773), *Bacteroides/Prevotella* (0.688), serum FGF21 (0.653), *Klb* heart mRNA expression (0.536), *Fgf21* liver mRNA expression (0.454), cecum length (0.448), acetate concentration in peripheral blood (0.413), PBMC *Nppa* mRNA

expression (0.403), gut *Lactobacillus* spp. (0.373), and COX4 protein levels in the heart (0.341). Otherwise, the most negative contribution for PCA2 was shaped by the gut relative abundance of *Clostridium leptum* (-0.653) *and C. coccoides* (-0.535), *Fas* (-0.462) and *Ffgr1* (-0.286) mRNA heart expression, heart lipid content (-0.234), DBP (-0.223), SBP (-0.187), *Myocd* (-0.148) and *Nppc* heart mRNA expression (-0.132), and TG content in the heart (-0.116).

All in all, the results derived from the correlation and PCA analyses suggest that changes in cardiovascular health-related parameters associated with HEP supplementation could be intimately related to positive changes in gut bacterial composition.

Discussion

As shown in previous works [7–9], chronic HEP supplementation can ameliorate metabolic disturbances produced by perinatal malprogramming, associated with specific gut microbiota selection, modulating the beneficial/detrimental gut bacterial species balance. These changes have implications in leptin and insulin sensitivity, energy metabolism, and thermogenic capacity [8,9]. Along these lines, we aimed to study the potential beneficial effects of HEP supplementation in cardiovascular protection, studying the progeny of calorie-restricted (20%) dams during the first half of pregnancy (CR animals), using the same animal cohort as in previous works [8,9]. The results suggest that HEP supplementation can reduce or counteract cardiovascular risk factors associated with metabolic malprogramming. The potential cardiovascular protective effects of pectin supplementation may be achieved in different ways, as discussed below.

We show here that mild gestational calorie restriction caused a significant raise of BP (both SBP and DBP) in the adult offspring, evidenced under SD, which was accompanied by a significant increase in total heart lipid content and a misbalance in triglyceride management under an HS diet, factors that can be associated with cardiac dysfunction and increased cardiovascular risk [26,27]. However, chronic HEP supplementation clearly counteracted the impairments mentioned above and may even show a further protective role against the damages of the obesogenic HS diet. The reversion of increased BP by HEP supplementation was more evident

346 for DBP since the CRP group under SD displayed significantly lower values than the CR group.
347 It can be noted that the repercussion of SBP and DBP in cardiovascular disease development may
348 be different; e.g., variability in DBP has been recently suggested as a more important predictor of
349 cardiovascular adverse events than SBP in certain patients (with stroke), and DBP and isolated
350 diastolic hypertension seem to be more related to the drive of coronary risk in younger subjects
351 [27,28]. Moreover, the pectin-supplemented animals showed a higher percentage of heart weight than
352 both C and CR animals. Although cardiac hypertrophy is usually considered a risk factor, the
353 surrounding observed physiological conditions suggest that such increase in the relative heart
354 weight might be associated with a favourable cardiovascular profile, as may happen, for instance,
355 in trained athletes [29].

356 To better characterise the cardio benefits of pectin supplementation at a molecular level, the
357 expression levels of genes encoding for natriuretic peptides or involved in heart size regulation
358 was analysed. On the one hand, natriuretic peptides play a central role in regulating blood pressure
359 and cardiovascular homeostasis, and dysregulation of these peptides could play a major role in
360 disorders such as hypertension, heart failure, and obesity [22]. Here, pectin supplementation
361 triggered significant increases in the expression of *Nppa* (which codes for natriuretic peptide A –
362 ANP) compared with the CR group, reverting the tendency to downregulation caused by the CR
363 condition, which might be related to the lower BP levels in the HEP supplemented (CRP) animals
364 compared to CR animals, considering the central role of ANP lowering blood pressure [30].
365 Moreover, it has also been described that the cardiac ventricular expression of ANP is decreased
366 in genetically obese or high-fat diet-fed mice, which also show increased cardiac triglyceride
367 content; the same authors reported that triglyceride accumulation in cultured atrial myocytes is
368 accompanied by downregulation of ANP mRNA [31]. Therefore, our results regarding the lipid
369 content profile in heart and *Nppa* expression are in line with such reports. The induction (respect
370 to CR animals) of *Nppa* expression in the pectin supplemented animals may be considered as
371 another of the beneficial effects reverting gestational calorie restriction malprogramming.
372 However, it was lost when the animals were exposed to an HS diet. On the other hand, *Myocd*

and *Mstn* encoded proteins (myocardin and myostatin) play a significant role in cardiac morphogenesis, contractility and heart energy homeostasis [23,24]. Myocardin is essential for heart development and cardiomyocyte differentiation, but it is also involved in cardiomyocyte hypertrophy [23]. During cardiac hypertrophy, a phenomenon of "foetal gene activation" is given, suggested as a protective physiological response against stress. The transcriptional co-activator myocardin has been proposed as fundamental in inducing the foetal gene program and cardiac hypertrophy [32]. Our results show that only CRP animals under the HS stimulus were able to significantly increase the levels of *Myocd* expression, suggesting that pectin supplementation might allow an improved molecular response to metabolic stress. Myostatin is a growth/differentiation factor that is a negative regulator of skeletal muscle mass. Its increased expression in the heart is involved in the pathogenesis of myopathy related to heart failure [24]. Here, the increase in *Mstn* mRNA levels in the heart due to metabolic programming effects of gestational calorie restriction condition (specially observed under SD) was reversed by pectin supplementation. Although the pectin supplemented animals showed lower *Mstn* mRNA levels with respect to CR animals, the differential profile of response to the experimental conditions with respect to relative heart weight points that would not be a key factor explaining the increased percentage of heart weight of the CRP animals. Altogether, the beneficial effects of pectin modulating BP may be related, at least in part, to the modulation of specific genes in the heart and especially to the induction of *Nppa* expression.

Due to the observed changes in both *Nppa* and *Mstn* mRNA levels in the heart, we considered of interest to study their expression at different ages in PBMC, trying to search for new biomarkers able to predict later disease outcomes in an accessible biological material (blood). Only *Nppa* mRNA levels in PBMC showed significant changes at a relatively early age (4 months of age), partially related to the later changes observed in cardiac expression and BP at six months. Therefore, considering both our results and the importance of *Nppa* expression in the heart regarding BP regulation and prevention of cardiometabolic diseases [22], *Nppa* expression in PBMC

399 may be of interest as a possible health biomarker that deserves more studies to confirm its
400 suitability and utility.

401 Excess of lipid accumulation in heart cells is associated with lipotoxicity and the development
402 of cardiac dysfunction and cardiomyopathies [33]. Due to the slight capacity of the heart to store
403 substrates, the control of energy uptake flux from food, together with energy production and
404 demand, is tightly controlled by mechanisms that induce genes encoding molecular regulators of
405 energy metabolism [34]. In this sense, the increased total lipid and TG (under HS diet) accumulation
406 in the heart of the CR animals suggests an impairment in heart lipid metabolism regulation due to
407 foetal malprogramming. The dysregulation of lipid content observed was accompanied by a series
408 of changes in the expression of key genes and in the activity of master signalling proteins. Still,
409 there were also interesting changes associated with pectin supplementation. In this way, the results
410 show that the HS diet upregulated the expression of *Prkaa2*, *Ppargc1a* and *Cpt1b* expression
411 significantly in the C animals while not in the other (CR, CRP) groups. On the contrary, *Fasn*
412 mRNA showed a significant upregulation in response to HS diet only in the CR group, prevented
413 in pectin-supplemented animals (CRP group). Considering the lipogenic role of *Fasn* encoded
414 protein (Fatty Acid Synthase), such response pattern may be partially responsible for the lower
415 heart lipid content in CRP animals with respect to CR, especially in those fed with HS diet. In
416 addition, the increase of *Ppara* (involved in the transcriptional regulation of fatty acid oxidation
417 [35]) mRNA levels driven by HEP supplementation may also make a significant contribution to
418 avoiding excess lipid accumulation in the CRP animals. Accordingly, this increase may be a factor
419 related to the changes observed in the levels of key proteins involved in lipid catabolism and
420 particularly associated with their capacity to respond to the HS diet. ATGL protein levels were
421 increased in CR animals compared to controls and even more increased with pectin
422 supplementation (in this case, when only considering the animals not exposed to HS diet). Given
423 the role of ATGL as the first enzyme in the process of TG lipolysis [36], this could be understood
424 as a physiological, metabolic adaptation (in CR animals) to increase lipid catabolism and therefore
425 avoid excess lipid accumulation in the heart, an event slightly potentiated by pectin

supplementation. The same argument could apply to the increase of CPT1B protein levels in CR animals since CPT1B is the main enzyme regulating the entry of long-chain fatty acids to the mitochondria for their oxidation [25]. However, in this case, the physiological capacity to increase lipid oxidation in response to metabolic stress (HS diet) seemed impaired in CR animals, which did not further increase CPT1B levels. This response was recovered/potentiated in the CRP animals, which showed similar CPT1B levels to control animals under SD, but significantly increased them in response to the obesogenic HS diet. A similar situation was given for the expression levels of the mitochondrial respiratory chain protein COX4 (used here as an indicator of oxidative capacity) since they tended to be downregulated in response to the HS stimulus in CR animals. In contrast, the opposite was observed in control animals; a response recovered in animals with pectin supplementation (CRP animals). Altogether, these results suggest that lipid oxidation control (and capacity to respond to metabolic stress) is altered by the malprogramming caused by the gestational calorie restriction condition. Still, pectin supplementation allows the recovery of the metabolic flexibility in the heart and may even increase it.

The activation (phosphorylation) state of the master metabolic regulator kinases AMPK and AKT also point to cardiometabolic protective effects of HEP supplementation. Phosphorylation of AMPKα (the catalytic subunit) was altered in CR animals. The control pAMPKα levels and response to HS diet were recovered in the pectin (CRP) supplemented animals. In this sense, the activation of AMPK in the heart might be suggested as a physiological response to the metabolic stress imposed by the HS diet since activated AMPK in the heart can favour processes such as glucose transport, glycolysis and fatty acid oxidation [37]. Our results suggest an impaired response to HS diet in CR animals but recovered in the pectin supplemented animals. In the case of pAKT, it showed increased levels in CR animals with respect to controls, but only in the HS groups, while the basal (under SD) levels were lower in the CRP animals respect to CR but significantly increased in response to HS diet, also suggesting a possible improved metabolic flexibility in the HEP supplemented group. FGF21 has been suggested to have multiple physiological functions, including protecting from cardiomyopathy by diminishing cardiac hypertrophy and oxidative

453 stress in the heart [38]. FGF21 is mainly produced by the liver and is released into the bloodstream
454 [39]. We report here that pectin supplementation increased *Fgf21* expression in the liver in response
455 to the HS diet, but only significantly in the pectin supplemented animals. Moreover, a significant
456 increase of FGF21 protein levels released into the bloodstream was observed in the CRP animals
457 with respect to C and CR groups. An effective response of FGF21 in heart tissue is determined
458 by the presence of specific receptors, especially when they form a complex with β-Klotho co-
459 receptor, which confers specific response capacity to FGF21 action [39]. In this sense, pectin
460 supplementation also stimulated the upregulation of the expression of *Klb* in the heart, without
461 changes in *Fgfr1* mRNA levels. Moreover, FGF21 cardio-protection is linked to the appropriate
462 function of AMPK and AKT activation in the heart [40]. As shown above, CR animals presented a
463 dysregulation of AKT and AMPK phosphorylation, which was corrected or even improved with
464 pectin supplementation. We suggest that, in our model, the increase of FGF21 levels in the blood,
465 accompanied by the increase in *Klb* expression, may be partly responsible for the described
466 protective effects of pectin supplementation, counteracting gestational calorie restriction
467 programmed cardiovascular risk.

468 Finally, the correlation and PCA analyses suggest that the HEP supplemented group of
469 animals tend to separate from the other two groups (C and CR) in its metabolic response, and how
470 the main beneficial outcomes of pectin supplementation described here in cardiovascular health-
471 related parameters were positively and negatively correlated with the relative abundance of
472 beneficial (*Lactobacillus spp.*, *Bacteroides/Prevotella*, *Bifidobacterium spp*. and *Akkermansia*
473 *muciniphila*) and detrimental (*Clostridium coccoides*) bacteria, respectively (e.g., BP levels were
474 inversely correlated with the relative gut abundance of *Lactobacillus* spp. and
475 *Bacteroides/Prevotella*; i.e., lower BP, a health positive effect, was associated with higher levels
476 of these beneficial bacteria). Therefore, it is suggested that the significant positive modulation of
477 gut microbiota caused by HEP supplementation could play a relevant role in the beneficial
478 cardiovascular effects described in our model.

In summary, the present study provides evidence that mild calorie restriction during the first half of pregnancy increases cardiovascular risk in the progeny in terms of BP, heart lipid content and gene expression biomarkers related to cardiac function. However, high esterified pectin supplementation can restore and even improve the basal control conditions, thus reducing the cardiovascular risk. The cardiac health improvement driven by pectin supplementation may be explained, at least in part, by modulation of the expression of natriuretic peptides and lipid oxidative capacity in the heart, which in turn may be partially explained by an increase in liver FGF21 production and its possible effects on the heart through its specific co-receptor β-Klotho. We also propose the role of specific microbiota selection by pectin supplementation as an underlying mechanism of the cardiovascular benefits observed (**Fig. 6**). All in all, the present work raises the possibility that high esterified pectin may become an interesting bioactive compound in the diet, able to provide protection against the increased cardiovascular risk associated with adverse metabolic programming. These results also support the interest in promoting the intake of fruits rich in pectins and in examining the possible interaction of these compounds with other bioactives present in fruits to make more targeted recommendations to prevent cardiovascular diseases, which represent one of the main causes of morbidity and mortality in humans.

Declaration of interest

The authors declare no conflict of interest.

Supplementary data

Table S1 contains the list of primers used for qPCR determinations. **Table S2** contains the list of primary antibodies used for Western Blot analysis**. List of Abbreviations**. **Supplementary Figure 1** shows PBMC expression levels of *Nppa* (at 2 months of age) and of *Mstn* (at two and four months of age).

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Figure Captions.

Figure 1. (A) Systolic Blood Pressure (SBP), (B) Diastolic Blood Pressure (DBP), (C) heart rate in beats per minute (BPM), (D) the percentage of heart weight with respect to total body weight, (E) total heart lipid content, and (F) heart triglyceride (TG) content. SBP, DBP and heart rate were measured at five months of age; heart weight, lipid content and TG were measured after sacrifice (at six months of age). Results are expressed as the mean ± SEM of 6 to 8 animals per group. C, offspring of control dams; CR, offspring of dams subjected to calorie restriction during first 12 days of pregnancy; CRP, CR rats supplemented with high-esterified pectin between days 21-180 of life. SD, standard diet; HS, high sucrose diet (supplemented between days 135-180 of life). Statistics: 2-way ANOVA was performed to analyse the effects of Treatment (T) and Diet (D), with LSD posthoc analysis (A≠B). In case of significant interaction (TxD), one-way ANOVA was performed, with LSD posthoc analysis, splitting individuals with standard diet (a≠b) and HS diet (x≠y). Specific differences between individual groups were assessed by Mann–Whitney *U* test ($p<0.05$): *HS versus SD, $^{\$}$CR or CRP group versus C group (same diet). n.s., non-significant.

Figure 2. Heart mRNA expression levels of genes coding for natriuretic peptides A (*Nppa*) (A), B (*Nppb*) (B) and C (*Nppc*) (C), Myocardin (*Myocd*) (D), and Myostatin (*Mstn*) (E) at six months of age. PBMC expression levels of *Nppa* at four (F) and six months (G) and of *Mstn* at six months of age (H). Results are expressed as a percentage of the mean value of the control group, mean ± SEM of 6 to 10 animals per group. C, offspring of control dams; CR, offspring of dams subjected to calorie restriction during first 12 days of pregnancy; CRP, CR rats supplemented with high-esterified pectin between days 21-180. SD, standard diet; HS, high sucrose diet (supplemented between days 135-180 of life). Statistics: ANOVA and post-hoc as explained in Figure 1 legend, A≠B, x≠y, Mann–Whitney *U* test ($p<0.05$): *HS versus SD, $^{\$}$CR or CRP group versus C group (same diet), $^{\#}$HS versus SD at the $p<0.1$ level. n.s., non-significant.

Figure 3. Heart mRNA and protein levels of genes related to lipid oxidation and its control at six months of age. (A-E) mRNA levels of the genes Prkaa2 (coding for AMP-activated protein kinase –AMPK– α subunit), Ppara (for peroxisome proliferator-activated receptor –PPAR– α), Ppargc1a (for PPARγ co-activator 1 α), Cpt1b (for carnitine palmitoyltransferase 1b –CPT1B) and Fasn (for fatty acid synthase). (F-J) Protein levels of phosphorylated AMPK, phosphorylated AKT, adipose triglyceride lipase (ATGL), CPT1B and cytochrome c oxidase subunit 4 (COX4). Below F-J graphs, representative western blot images of the corresponding bands are shown; pAMPK 63 kDa, pAKT 60-62 kDa, ATGL 54 kDa, CPT1B 75-85 kDa, COX4 19 kDa, and ACTB 42 kDa. Results are expressed as a percentage of the mean value of the control group, mean ± SEM of 6 to 10 animals per group. C, offspring of control dams; CR, offspring of dams subjected to calorie restriction during first 12 days of pregnancy; CRP, CR rats supplemented with high-esterified

pectin between days 21-180. SD, standard diet; HS, high sucrose diet (supplemented between days 135-180 of life). Statistics: ANOVA and post-hoc as explained in Figure 1 legend, A≠B, a≠b, x≠y. Mann–Whitney *U* test ($p<0.05$): *HS versus SD, $CR or CRP group versus C group (same diet), #HS versus SD at the $p<0.1$ level. n.s., non-significant.

Figure 4. FGF21 and its receptor and co-receptor expression. (A-B) levels of mRNA of Fgfr1 and Klb in the heart, (C) circulating FGF21 protein, and (D) Fgf21 mRNA in the liver (6 months of age), of C, CR and CRP groups under SD or HS diet. Results are expressed as a percentage of the mean value of the control group, mean ± SEM of 6 to 10 animals per group. C, offspring of control dams; CR, offspring of dams subjected to calorie restriction during first 12 days of pregnancy; CRP, CR rats supplemented with high-esterified pectin between days 21-180. SD, standard diet; HS, high sucrose diet (supplemented between days 135-180 of life). Statistics: ANOVA and post-hoc as explained in Figure 1 legend, A≠B. Mann–Whitney *U* test ($p<0.05$): *HS versus SD, $CR or CRP group versus C group (same diet), #HS versus SD at the $p<0.1$ level. n.s., non-significant.

Figure 5. Analyses of correlation and PCA (Principal Component Analysis). (A) Spearman correlation map between the relative abundance of selected health-relevant bacteria (see materials and methods section), cecum length and the cardiovascular health-related parameters studied in the present work. Positive correlations are indicated in blue and negative in red. *Spearman correlation *p*-value <0.05 **p-value <0.01. (B) PCA involves 39 different variables, including the health-related parameters of the present work, the relative abundance of selected health-relevant bacteria, cecum length, and peripheral blood concentration of SCFA (short-chain fatty acids: acetate, propionate and butyrate). Plots are coloured according to the received treatment. The highest positive and negative contributors for PC2 are detailed in the main text (see results section). All data were normalised for PCA performance. Variability explained of PC1, 2, and 3 are indicated next to each axis. Abbreviations: SBP -Systolic Blood Pressure, DBP -Diastolic Blood Pressure, *Fgf21 -Fibroblast growth factor 21*, *Ppara -Peroxisome Proliferator-Activated Receptor Alpha*, *Nppa -Natriuretic peptide A.*

Figure 6. Summary of suggested mechanisms involved in the cardiovascular improvement in gestational calorie-restricted animals, associated with High-Esterified Pectin (HEP) chronic supplementation. HEP supplementation promotes the modulation of gut microbiota by favouring the increase in the relative abundance of beneficial species. The changes may indirectly impact critical organs, such as the heart and the liver, modulating gene expression and increasing FGF21 circulating levels. Although the liver is the main productor of FGF21, from our results we cannot distinguish whether the elevated blood levels are caused by increased hepatic secretion or by other tissue/s. FGF21, via its specific receptor FGFR1 and co-receptor β-Klotho, might be partly responsible for the improvements observed in the heart, such as the increase in lipid oxidation capacity and in *Nppa* expression, which in turn would improve blood pressure. Overall, HEP supplementation reverses the increased cardiovascular risk factors caused by gestational calorie restriction and allows the recovery, or even improvement, of metabolic flexibility.

Supplementary Figure 1. PBMC expression levels of Nppa at two (A) and of Mstn at two (B) and four months (C) of age. Results are expressed as a percentage of the mean value of the control group, mean ± SEM of 6 to 10 animals per group. C, offspring of control dams; CR, offspring of dams subjected to calorie restriction during the first 12 days of pregnancy; CRP, CR rats supplemented with high-esterified pectin between days 21-180. Statistics: 2-way ANOVA (n.s., non-significant); Mann–Whitney U test ($p<0.05$): * significant differences vs. C group.

Figure graphics

Figure 1

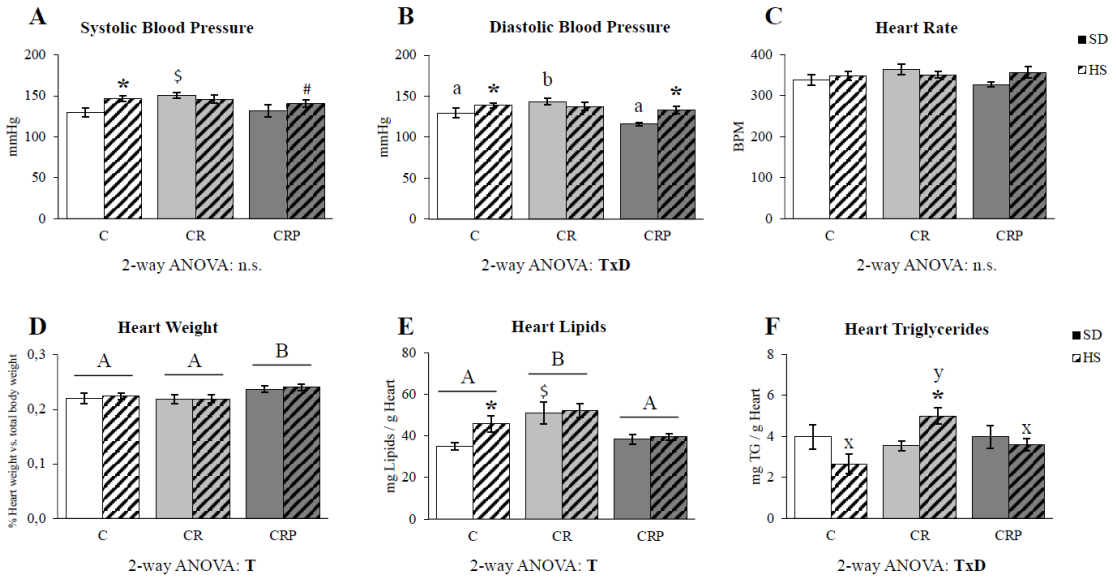


Figure 2

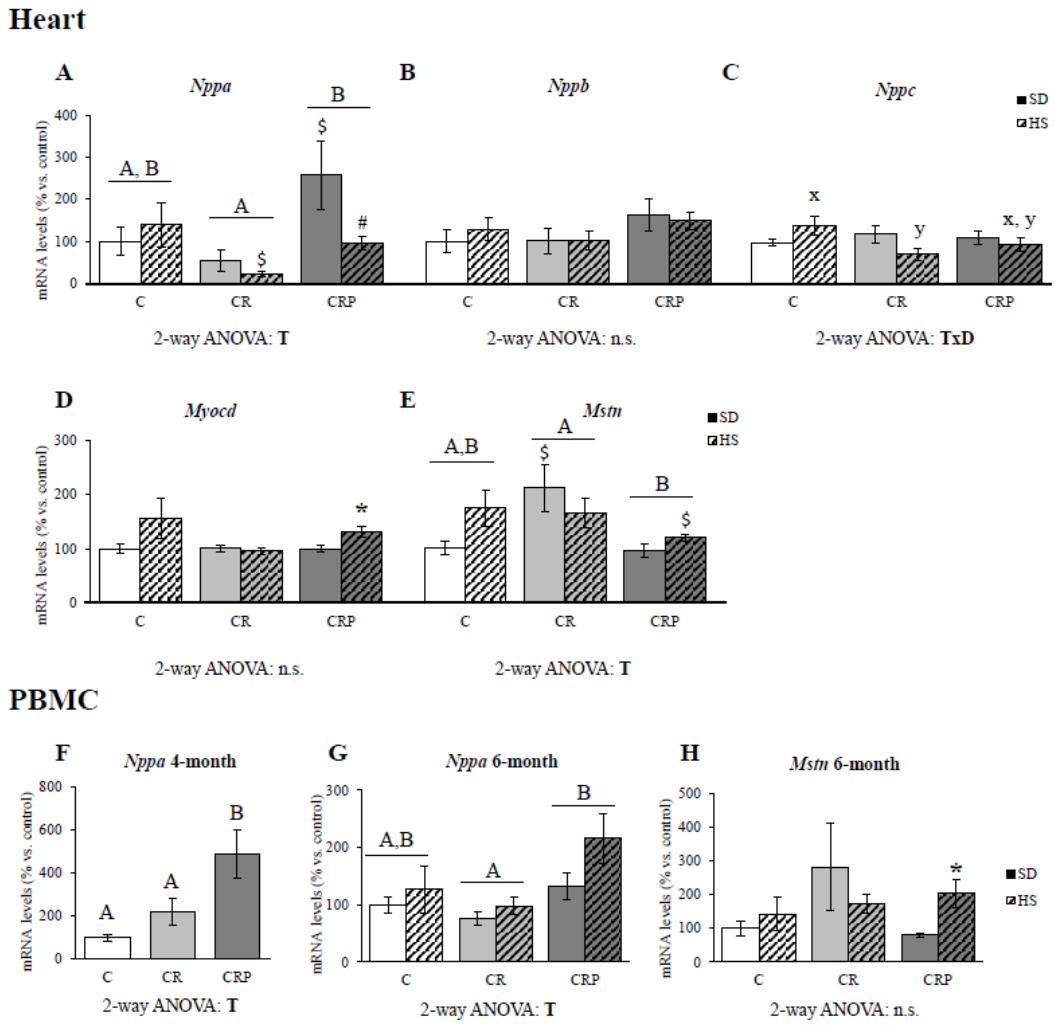

Heart
A
Nppa
B
Nppb
C
Nppc
SD
HS
mRNA levels (% vs. control)
A, B
A
B
$
$
#
x
y
x, y
C
CR
CRP
2-way ANOVA: T
2-way ANOVA: n.s.
2-way ANOVA: TxD
D
Myocd
E
Mstn
A,B
A
B
*
$
$
2-way ANOVA: n.s.
2-way ANOVA: T
PBMC
F
Nppa 4-month
G
Nppa 6-month
H
Mstn 6-month
A
A
B
A,B
A
B
*
2-way ANOVA: T
2-way ANOVA: T
2-way ANOVA: n.s.

Figure 3

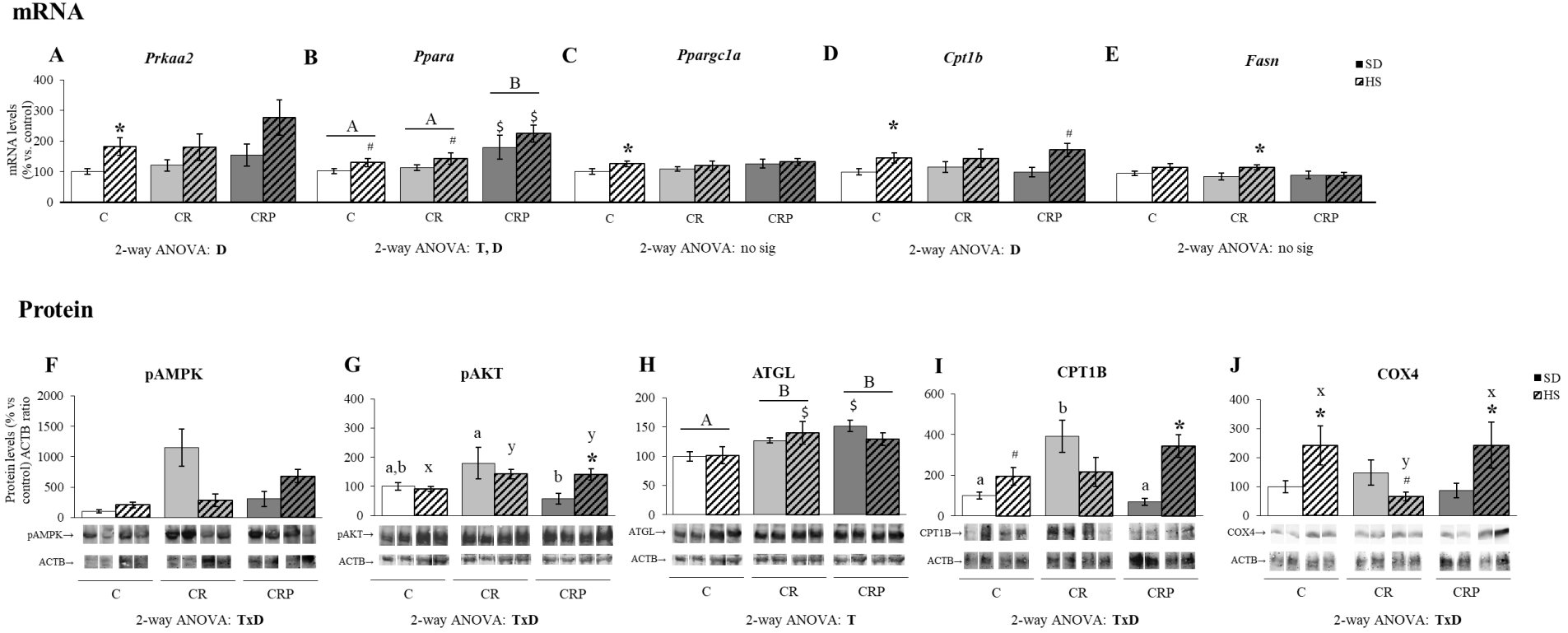

mRNA
A
Prkaa2
B
Ppara
C
Ppargc1a
D
Cpt1b
E
Fasn
SD
HS
mRNA levels (% vs. control)
C
CR
CRP
2-way ANOVA: D
2-way ANOVA: T, D
2-way ANOVA: no sig
2-way ANOVA: D
2-way ANOVA: no sig
Protein
F
pAMPK
G
pAKT
H
ATGL
I
CPT1B
J
COX4
Protein levels (% vs control) ACTB ratio
pAMPK→
pAKT→
ATGL→
CPT1B→
COX4→
ACTB→
2-way ANOVA: TxD
2-way ANOVA: TxD
2-way ANOVA: T
2-way ANOVA: TxD
2-way ANOVA: TxD

Figure 4

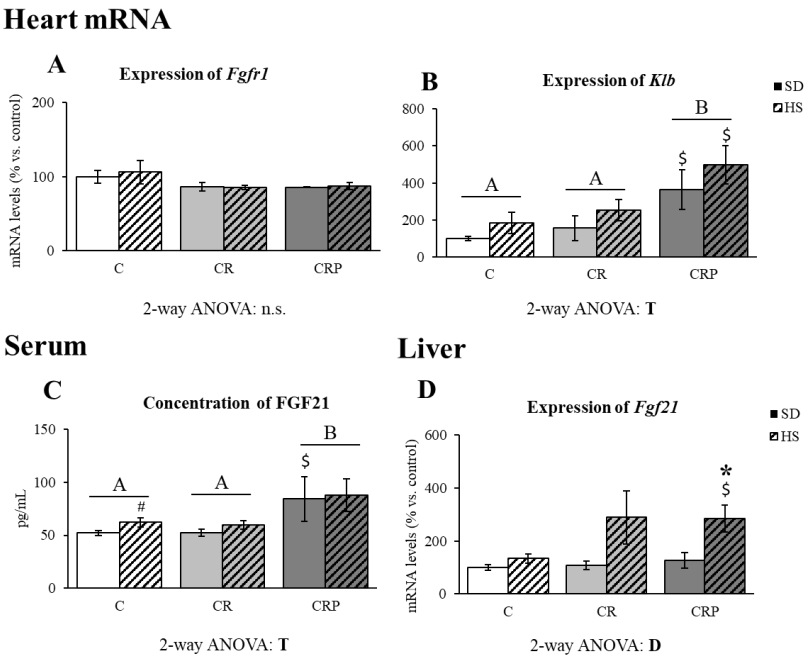

Heart mRNA
A
Expression of Fgfr1
mRNA levels (% vs. control)
200
100
0
C
CR
CRP
2-way ANOVA: n.s.
B
Expression of Klb
SD
HS
800
600
400
200
0
A
A
B
$
$
C
CR
CRP
2-way ANOVA: T
Serum
C
Concentration of FGF21
pg/mL
150
100
50
0
A
A
B
#
$
C
CR
CRP
2-way ANOVA: T
Liver
D
Expression of Fgf21
SD
HS
mRNA levels (% vs. control)
600
400
200
0
*
$
C
CR
CRP
2-way ANOVA: D

Figure 5

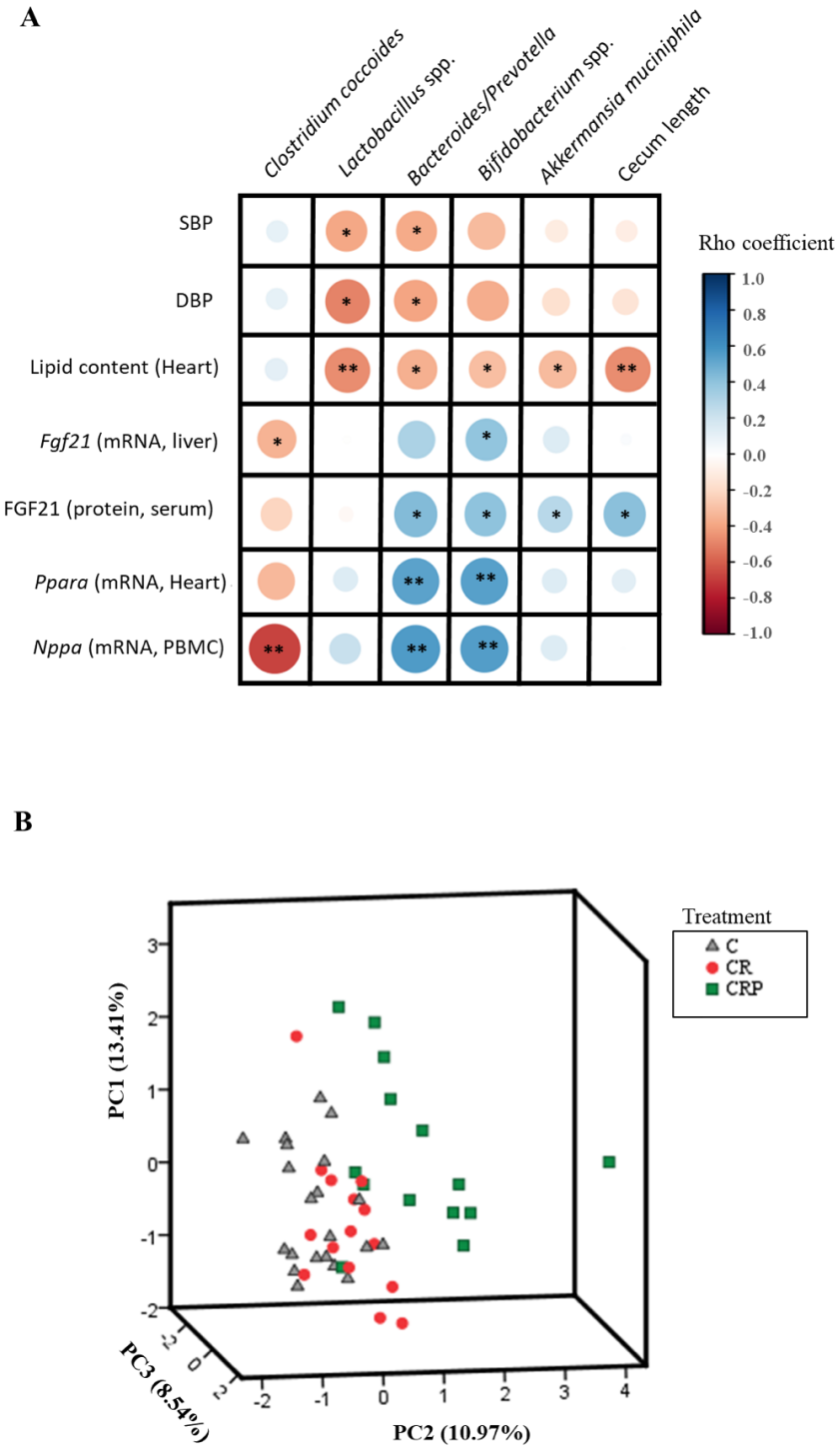
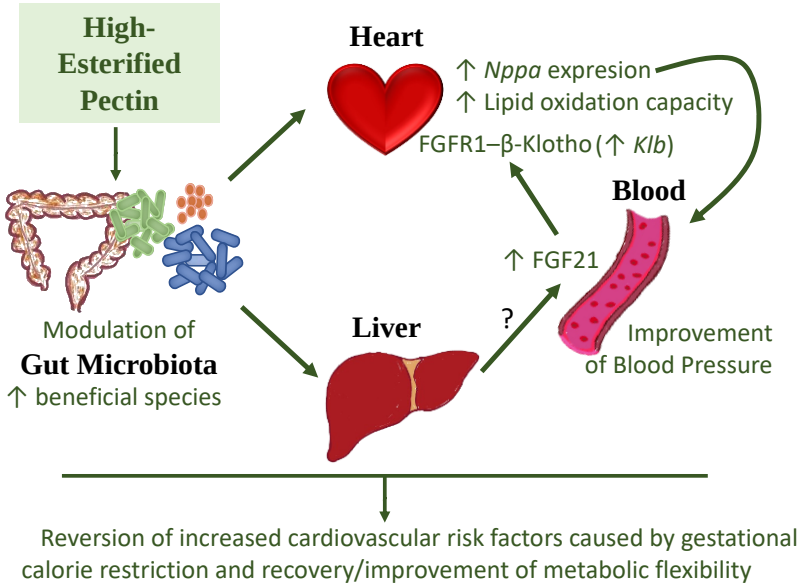
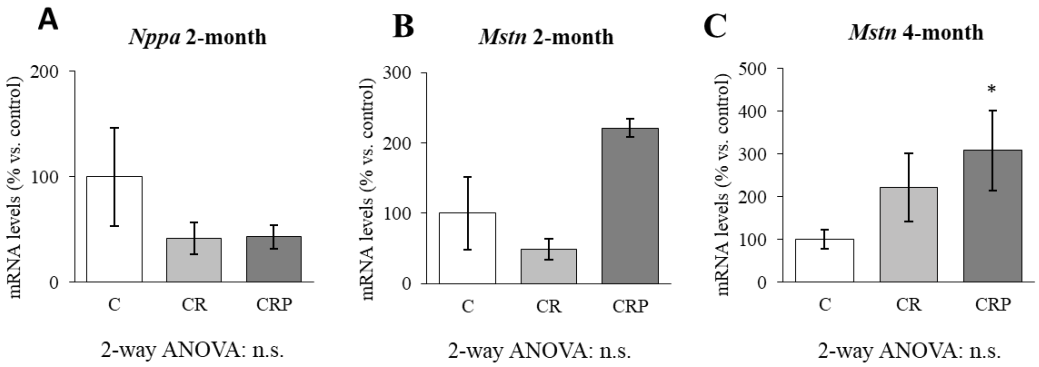

A
Clostridium coccoides
Lactobacillus spp.
Bacteroides/Prevotella
Bifidobacterium spp.
Akkermansia muciniphila
Cecum length
SBP
DBP
Lipid content (Heart)
Fgf21 (mRNA, liver)
FGF21 (protein, serum)
Ppara (mRNA, Heart)
Nppa (mRNA, PBMC)
Rho coefficient
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
B
Treatment
C
CR
CRP
PC1 (13.41%)
PC2 (10.97%)
PC3 (8.54%)

Figure 6


High-Esterified Pectin
Heart
↑ Nppa expresion
↑ Lipid oxidation capacity
FGFR1–β-Klotho (↑ Klb)
Blood
↑ FGF21
?
Liver
Improvement of Blood Pressure
Modulation of
Gut Microbiota
↑ beneficial species
Reversion of increased cardiovascular risk factors caused by gestational calorie restriction and recovery/improvement of metabolic flexibility

Supplementary Figure 1



Graphic for table of contents

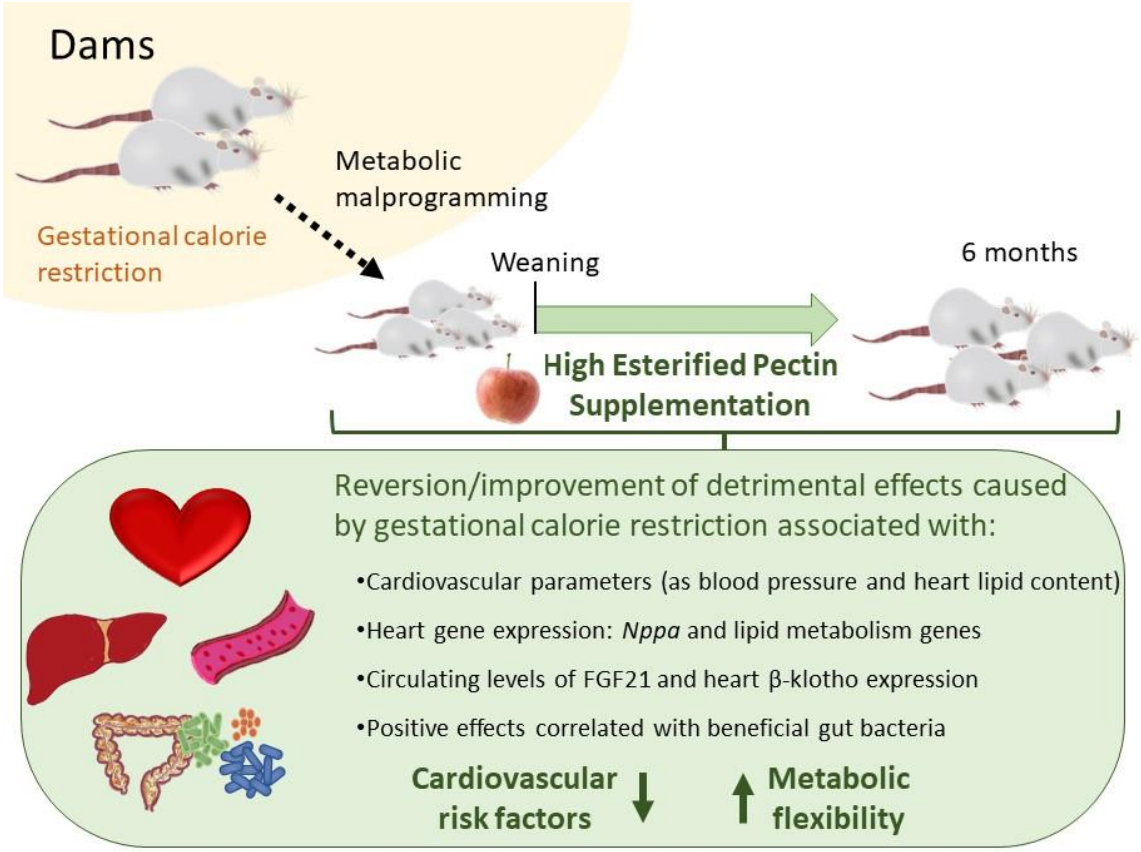
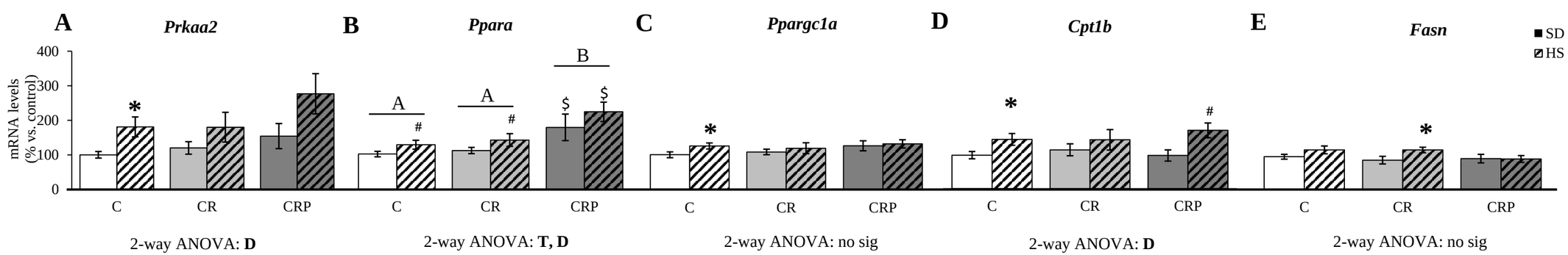
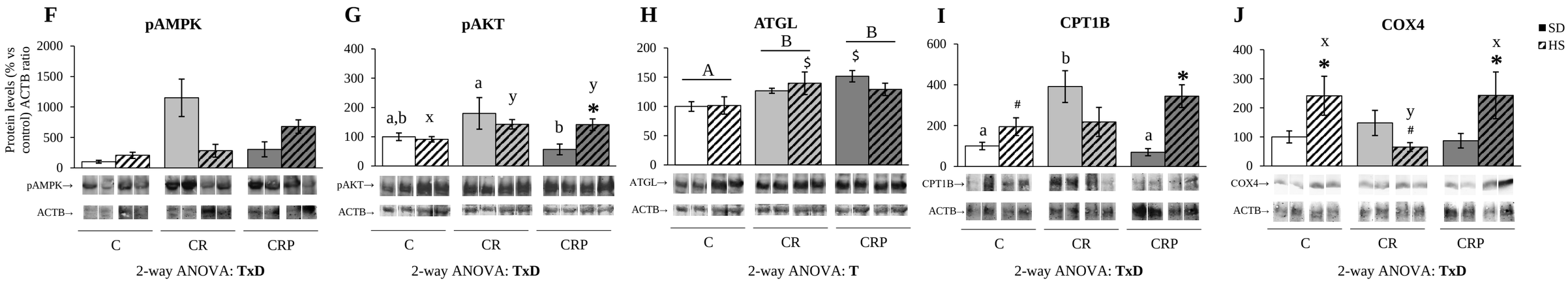


Figure 3


mRNA
A
Prkaa2
B
Ppara
C
Ppargc1a
D
Cpt1b
E
Fasn
SD
HS
mRNA levels (% vs. control)
400
300
200
100
0
C
CR
CRP
2-way ANOVA: D
2-way ANOVA: T, D
2-way ANOVA: no sig
2-way ANOVA: D
2-way ANOVA: no sig


Protein
F
pAMPK
G
pAKT
H
ATGL
I
CPT1B
J
COX4
SD
HS
Protein levels (% vs control) ACTB ratio
pAMPK→
pAKT→
ATGL→
CPT1B→
COX4→
ACTB→
C
CR
CRP
2-way ANOVA: TxD
2-way ANOVA: TxD
2-way ANOVA: T
2-way ANOVA: TxD
2-way ANOVA: TxD