A high fructose diet worsens eccentric left ventricular hypertrophy in experimental volume overload.

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Running head: Fructose-fed rats and eccentric LVH

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Abstract

Aims: The development of left ventricular hypertrophy (LVH) can be affected by diet manipulation. Concentric LVH resulting from pressure overload can be worsened by feeding rats with a high-fructose diet. Eccentric LVH is a different type of hypertrophy and is associated with volume overload (VO) diseases. The impact of an abnormal diet on the development of eccentric LVH and on ventricular function in chronic volume overload is unknown. This study therefore examined the effects of a fructose-rich diet on left ventricular eccentric hypertrophy, ventricular function and myocardial metabolic enzymes in rats with chronic VO caused by severe aortic valve regurgitation (AR).

Methods: Wistar rats were divided in four groups: Sham-operated on control or fructose-rich diet (SC (n=13) and SF (n=12)) and severe aortic regurgitation fed with the same diets (ARC (n=16) and ARF (n=13)). Fructose-rich diet (F) was started one week before surgery and the animals were sacrificed 9 weeks later.

Results: SF and ARF had high circulating triglycerides. ARC and ARF developed significant LV eccentric hypertrophy after 8 weeks as expected. However ARF developed more LVH than ARC. LV ejection fraction was slightly lower in the ARF compared to ARC. The increased LVH and decreased ejection fraction could not be explained by differences in hemodynamic load. SF, ARC and ARF had lower phosphorylation levels of the AMP kinase compared to SC.

Conclusion: A fructose-rich diet worsened LV eccentric hypertrophy and decreased LV function in a model of chronic VO caused by AR in rats. Normal animals fed the same diet did not develop these abnormalities. Hypertriglyceridemia may play a central role in this phenomenon as well as AMP kinase activity.

42 Key words: cardiomyopathy, metabolic syndrome, heart hypertrophy, triglycerides,
43 glucose, fatty acids

Introduction

Chronic left ventricular volume overload (VO) causes severe left ventricular dilatation and eccentric hypertrophy. This type of left ventricular hypertrophy is encountered mainly in patients with valvular diseases such as chronic mitral (MR) or aortic valve regurgitation (AR). AR is associated with a long asymptomatic period during which the left ventricle (LV) progressively dilates and hypertrophies. In parallel with the LV dilatation, systolic function slowly decreases and symptoms eventually appear (4,5). Although it is not the most frequent valvular disease in Western countries, it has been estimated based on the Framingham study that 13% of the population suffer from AR of varying degrees of severity (40).

No drug treatment has been proven effective to decrease morbidity, mortality, or delay the evolution towards heart failure or valve replacement surgery in patients with chronic volume overload from valve disease (5). The search for an effective treatment is still ongoing. Patient lifestyle has a significant impact on the evolution of many cardiac diseases. Whereas good habits such as exercising and eating low fat/low sugar diets seem beneficial, a lack of physical activity and eating imbalanced diets may act in the opposite way. The impact of diet and exercise on the evolution of volume overload cardiomyopathy has received little attention. We have recently shown that exercise could improve survival, LV diastolic function, heart rate variability and reduce myocardial fibrosis in a rat model of severe AR (21; 22). A diet with a high glycemic load is strongly associated with an increased risk of coronary heart disease (9). It has been suggested that the current high prevalence of the metabolic syndrome in the population may be a consequence of the increasing use of high-fructose corn syrup and sucrose by the food

industry (41). Previous studies have reported that a fructose-rich diet fed to rats will eventually lead to the development of metabolic abnormalities sharing many similarities with the human metabolic syndrome (12, 27). This type of diet has also been shown to increase cardiac dysfunction and mortality in an animal model of LV pressure overload with concentric left ventricular hypertrophy (8; 27; 37; 38). The potential impact of a high-fructose diet on the progression of volume overload cardiomyopathy has never been explored. Therefore, this study was designed to assess the impact of a high-fructose diet on the development of eccentric left ventricular hypertrophy and its impact on ventricular function in rats with severe chronic left ventricular volume overload from severe aortic valve regurgitation.

Methods

Animals: Adult male Wistar rats were purchased from Charles River (Saint-Constant QC, Canada) and divided in 4 groups as follows: 1) Sham-operated animals on control diet (SC; n=13); 2) AR control diet (ARC; n=16), 3) Sham on High Fructose diet (60% Fructose Diet, Cat. No. TD.89247 Harlan Teklad Madison WI, (SF; n=12) and AR on High Fructose diet (ARF n=12). The animals were maintained either on the control diet (Purina Rat Chow #5075) containing 4.5% fat, 18.5% protein and 57.3% carbohydrate (41.2 g/kg from starch; 4.0 kCal/g) or the 60% fructose diet containing 5.2% fat, 18.3% protein and 60.4% carbohydrate (60 g/kg from fructose; 3.6 kCal/g). The high Fructose diet was started one week before the surgery in both SF and ARF groups and continued for 8 weeks until sacrifice. Food consumption was evaluated at mid-protocol by weighing consumed food pellets every day for a week and then averaged for a day. The protocol

was approved by the Université Laval's Animal Protection Committee and followed the recommendations of the Canadian Council for Laboratory Animal Care.

Aortic regurgitation: Severe AR was induced by retrograde puncture of the aortic valve leaflets as previously described (2; 30). A complete echocardiographic exam was performed two weeks after AR induction and the day before sacrifice 8 weeks later. At the end of the protocol, animals were sacrificed, hearts were quickly dissected and all cardiac chambers were weighed. LV was snap-frozen in liquid nitrogen and kept at -80^{o}C for further analysis. All sacrifices were scheduled at similar times of the day in the fed state to avoid circadian variations in metabolism. Lungs, liver and abdominal fat were rapidly collected and weighed. Blood samples were taken for the measurement of glucose, triglycerides, insulin, leptin and adiponectin levels in non-fasting animals.

Echocardiography

A complete M-mode, 2D, and Doppler echocardiogram was performed on the animals under 1.5% inhaled isoflurane anesthesia using a 12 MHz probe with a Sonos 5500 echograph (Philips Medical Imaging, Andover, Mass). LV dimensions, wall thickness, ejection fraction, fractional shortening, diastolic function, cardiac output (ejection volume in the LV outflow tract and heart rate) were evaluated as previously reported (2; 10; 31).

Hemodynamic Measurements

Aortic pressures, LV end-diastolic pressures and dP/dt (positive and negative) were measured invasively using a dedicated 2F impedance catheter (Millar Instruments,

Houston, TX) under 1.5% isoflurane anesthesia just before sacrifice as previously described (21; 22; 32,33).

Analysis of mRNA accumulation by quantitative RT-PCR

Tissues stored frozen in RNAlater® (Ambion, Austin, TX) were homogenized in Trizol (Invitrogen, Burlington, Ont, Canada) using a Polytron according to the standard Trizol procedure. Fifty ng of RNA was converted to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA), a procedure which includes a genomic DNA elimination step. The cDNA obtained was further diluted 10-fold with water prior to amplification (with the final concentration corresponding to 0.25 ng/µl of initial RNA). 1.25ng of diluted cDNA was amplified in duplicate (*technical* duplicates) by Q-PCR in a Rotor-Gene™ 6200 thermal cycler (Corbett Life Science, Sidney, Australia), using the QuantiTect® SYBR Green PCR kit and QuantiTect® Primer Assays (pre-optimized specific primer pairs from Qiagen). Each run included one tube with water only (no template control), one tube with a representative RNA sample (no RT control), and a series of 10-fold dilutions of a representative cDNA sample to confirm the efficiency of the amplification reaction.

The quantification of gene expression was based on the $-2^{\Delta\Delta Ct}$ method (24). Briefly, mean Ct values of technical duplicates for each gene of interest were subtracted from the mean Ct value (hence ΔCt) of the control "housekeeping" gene cyclophilin 1. The differences in the mean ΔCts between groups of rats ($\Delta\Delta Ct$) allow the calculation of relative levels of induction/repression of genes of interest.

Enzyme activity determinations

Left ventricle samples were kept at -80°C until assayed for maximal (V_{max}) enzyme activities. Small pieces of LV (20-30 mg) were homogenized in a glass-glass homogenizer with 39 volumes of ice-cold extracting medium pH7.4 (250 mM sucrose, 10mM Tris-Hcl, 1 mM EGTA). HADH (hydroxyacyl-Coenzyme A dehydrogenase) and CS (citrate synthase) enzyme activities were estimated by the reduction of NADP to NADPH in a spectrophotometer with wavelength set to 340 nm for the citric acid cycle, complex I for the respiratory chain and HADH for fatty acid β-oxidation (25). The method for measuring CK (creatine kinase) activity in cardiac tissues was adapted from a protocol provided by Sigma-Aldrich (details below), with the inclusion in the assay buffer of dithiothreitol for the reactivation of creatine kinase, and NaF for the inhibition of adenylate kinase (28). Immediately before assay, homogenates were diluted 1/80 in cold extraction buffer, and then 5 µl of those diluted samples were put into 195 µl assay buffer (glycylglycine 42 mM, pH 7.4; bovine serum albumin 0.017%; phosphocreatine 14 mM; adenosine diphosphate 1.4 mM; glucose 34 mM; beta-NADP 0.4 mM; magnesium acetate4.5 mM; dithiothreitol 20 mM; NaF 25 mM; hexokinase 10 U/ml; glucose-6-phosphate dehydrogenase 33 mU/ml). Absorbance at 340 nm was read at 30°C with readings every 15 sec for 15 minutes. The slope of the linear part of the absorbance curve was used to calculate enzyme activity, which was reported as mU OD per minute per ug protein. The activity of the complex I (NADH-ubiquinone oxidoreductase) was evaluated as described by Jarreta et al.(20) with some modifications. Small pieces of LV (20-30 mg) were homogenized in a glass-glass homogenizer with 39 volumes of ice-cold extracting medium. After centrifugation at 15000 x g, supernatant was used for

enzymatic assay. The activity was determined in the following reaction medium (1.1mL) (500mM potassium phosphate pH7.5, 50mg/ml BSA, 25mM decyubiquinone); 22.5µl of LV homogenate were added to reaction medium followed by a 5-min incubation at 37°C. The reaction was then initiated by adding NADH to a final concentration of 50µM. Enzyme activity was estimated by the reduction of NADH to NAD^+ in a spectrophotometer with wavelength set at 340nm. To measure the specific complex I activity, the same experiment was performed in presence of 2.5mM rotenone, an inhibitor of complex I. Subtraction of NADH oxidase activity measured with rotenone to the one measured without represent specific complex I activity. Succinate dehydrogenase (SDH) activity was measured on small pieces of LV (20-30 mg) homogenized in 10 volumes of a Tris-sucrose buffer (Tris 20mM pH 7.2, 0.8M sucrose, 2mM EGTA, 40 mM KCl and 1mg/ml BSA (bovine serum albumin). Four µl of the cleared homogenate were then added to 194.4µL of reaction buffer (50mM KH_2PO_4 solution (pH7.2), 10 mM succinate, 1mg/ml BSA, 140µM sodium 2, 6-dichloroindophenolate (DCIP), 0.2mM KCN (0.2mM), 8µM rotenone). A parallel reaction was also performed in presence of 10mM malonate, a SDH inhibitor. Reaction was incubated for 10 minutes at 37°C then decylubiquinone (100 µM in assay) was added to the mix. Rate of DCIP reduction was then measured on a spectrophotometer set at 600 nm for 5 min every 15 seconds. Rate of DCIP reduction was then calculated in presence or not of malonate in order to deduce SDH activity(16).

Immunoblotting

Crude LV homogenates were separated by SDS-PAGE. Volumes of samples loaded on gel were corrected for the amount of protein. Immunoblotting was performed as

described elsewhere (33). Membranes were hybridized with the indicated primary antibodies. All primary antibodies were used at a 1:1000 dilution and were purchased from Cell Signaling Technology (Beverly, MA). Bands were visualized and quantified with a ChemiImager system (Alpha Innotech Corporation).

Statistical analysis

Results are presented as mean ± SEM unless specified otherwise. Inter-group comparisons were done using two-way ANOVA and using Bonferroni post-test when indicated. Statistical significance was set at a $p<0.05$. Data and statistical analysis were performed using Graph Pad Prism version 5.02 for Windows, Graph Pad Software (San Diego CA).

Results

Clinical data and animal characteristics (Table 1):

All animals were alive at the end of the protocol. Fructose-fed rats (SF and ARF) had a slightly lower body weight compared to their respective controls (SC and ARC) at the end of the protocol although overall growth was similar as demonstrated by the comparable tibial lengths in all groups (Table 1). ARC had less retroperitoneal fat than the ARF animals. As illustrated in Figure 1a, fructose-fed animals (SF and ARF) had a lower caloric intake than animals on control diet.

As expected, heart weight was strongly increased in both AR groups compared to the sham-operated groups. The ARF had an increased total heart weight compared to ARC. This was due an increase in LV mass in the ARF group. Right ventricular, left atrial and

lung weights were also increased in ARC and ARF but the diets did not affect these measurements.

Plasma glucose levels (Fig. 1b) were similar between all groups with the exception of the ARF group which tended to have higher glucose levels (p=0.07) but this difference did not reach statistical significance. Triglyceride levels were strongly increased in both fructose-fed groups (SF and ARF) as expected with this diet composition (Fig. 1c). Insulin, leptin and adiponectin levels remained similar between groups (Figure 1d-e). There was a trend towards a diet-disease interaction for the insulin levels (p=0.06) but again the difference did not reach statistical significance.

Echocardiographic (table 2) and hemodynamic (table 3) data

As expected, severe aortic valve regurgitation led to enlarged end-diastolic and end-systolic dimensions in both groups (ARC and ARF) (Table 2). ARF had larger end-systolic diameters and slightly lower systolic ejection fraction than ARC. LV mass estimated by echo was significantly increased in ARF compared to ARC and therefore corroborated well with the direct measurement of heart weight at sacrifice.

AR severity was similar in both ARC and ARF groups (results not shown). Heart rate was slightly lower in the ARC and ARF groups. AR also resulted as expected in larger and similar forward stroke volumes and increased cardiac output in those groups (Table 3). End-diastolic LV pressure was significantly higher in ARC and ARF compared to their respective sham controls. There was however no clear diet effect on this parameter. There was no diet effect or diet-disease interaction for any of the measured hemodynamic parameters between the ARC and ARF groups.

Markers of LV remodeling

As illustrated in Figure 2, the gene expression of two markers of LV hypertrophy (atrial and brain natriuretic factors (ANP and BNP)) were increased in both AR groups. There was no diet effect or diet-disease interaction on these parameters.

Interstitial fibrosis is a late feature in our model (22; 32). Standard LV tissue staining for the quantification of fibrosis did not show any difference between groups (results not shown). The gene expression of pro-collagens type I, III and fibronectin were measured and are reported in figure 3. There was a clear disease effect towards an increase in the expression of pro-collagen I in the AR groups and a trend in the same direction for pro-collagen III ($p=0.06$) but post hoc testing was not significant. We did not find any diet effect on pro-collagen I gene expression. There was however a clear diet effect suggesting an increased expression of pro-collagen III in the fructose-fed animals but again this did not reach statistical significance after post hoc testing of the ANOVA results. Fibronectin expression was unaffected in all 4 groups without any measurable effect of the diet or the disease.

Myocardial metabolic enzymes

Data analysis suggested a disease effect on the level of LV enzymatic activity of HADH (hydroxyacyl-Coenzyme A dehydrogenase), the Complex 1 of the mitochondrial electron transport chain (ETC-1) and creatine kinase (CK) but not on citrate synthase (CS) or succinate dehydrogenase (SDH)/ETC-2 activities (Figure 4). Post hoc analysis did not reveal any significant differences however for these activity levels. There were no significant diet effects on the enzymatic activities reported in figure 4. However we did find a significant diet-disease interaction for the SDH/ETC-2 activity and this increase in

the ARF compared to the ARC was statistically significant. Total creatine kinase activity was lower in both AR groups (Fig. 4e) but no statistically significant diet effect or diet-disease interaction was found. Phosphofructokinase activity remained unchanged between all 4 groups (results not shown).

The increase in circulating triglycerides in fructose-fed animals (SF and ARF) was not accompanied by any changes in the mRNA levels of fatty acid transporters (FAT/CD 36 and carnitine palmitoyl transferases (Cpt)) although AR seemed to induce a slight decrease in Cpt2 gene expression which did not reach statistical significance (Fig. 5a-c). Glucose entry in the cardiac cell is mainly mediated by glucose transporters 1 and 4 (GLUT 1 and GLUT4). GLUT4 mRNA expression levels remained similar between SC and ARC animals. The fructose diet tended to increase this gene expression in SF and not in ARF group but this did not reach statistical significance (Fig. 5d-e). On the other hand, mRNA levels encoding for insulin-independent GLUT1 increased in both AR groups compared to SC rats. Again post hoc testing was not significant.
Peroxisome proliferator-activated receptor alpha (PPARα) is a main regulator of fatty acid metabolism. In our AR animals, LV mRNA levels of PPARα were slightly reduced but the diet had no significant effect on this parameter (Figure 5f).
The AKT/mTOR is a known prohypertrophic signaling pathway. We did not observe any modulation of this signaling pathway in the LVs of our animals as illustrated in Figure 6. Although the total protein content of AKT and 4EBP1 (a downstream effector of mTOR) were different in the SF group compared to controls, this did not translate to the content of their phosphorylated form (AKT (Ser473) and 4EBP1 (Ser65)). AR-induced LV

hypertrophy was accompanied by a significant decrease in the content of the phosphorylated form of AMPKα (Thr172). The fructose diet had a similar effect on this parameter. Activation of LKB1, an AMPK regulator, was not significantly affected by the diet or the disease (Figure 7).

Discussion

In this study, we show in that a relatively short exposition (8 weeks) to a fructose-rich diet increases eccentric LVH and slightly decreases LV ejection fraction in rats with severe volume overload from aortic valve regurgitation.

Rats can cope with this type of LV volume overload and tolerated severe LV dilation for a relatively long period with survival rates of more than 70% 6 months post AR-induction (22; 32). The present protocol was relatively short-termed and evaluated the compensated phase of the disease when LV dilation is almost maximal (30) but systolic function remains in the normal range. Despite this relatively short exposition to the high-fructose diet, we report a clear increase in LVH in the AR rats. We previously reported a clear link between the extent of LV hypertrophy and survival in our model (22). In the present study the AR animals fed with the high fructose diet for only 2 months had a larger heart and a lower ejection fraction than the AR animals fed with the control diet. This suggests that in the longer term the fructose-fed AR animals would probably have a poorer survival. This issue needs to be addressed in a longer study.

Cardiac disease in patients is often accompanied by metabolic abnormalities such as dyslipidemia, obesity, hypertension, insulin resistance or diabetes. The fructose-fed AR rat model provides an interesting glimpse at the impact of diet-induced metabolic

abnormalities in the context left ventricular hypertrophy but this type of diet has never been studied in a model of chronic LV volume overload with eccentric LVH (27). The ARF animals were not only hypertriglyceridemic but also had more retroperitoneal fat, a tendency for higher blood glucose levels and higher systolic blood pressure than those from the ARC group. The sham animals fed a high-fructose diet (SF) had similar metabolic abnormalities and hypertriglyceridemia than the ARF but they did not develop any LVH, LV dilatation or decrease in ejection fraction compared to the sham controls. It therefore seems that the AR animals coped less well with the metabolic challenge imposed by the high-fructose diet than healthy animals.

The reasons for this different behavior are not clear. We have previously reported that the sympathetic and renin-angiotensin systems are over-activated in our AR rats (27). The human metabolic syndrome has also been linked to an over-activation of the sympathetic and renin-angiotensin systems (11, 26). It is possible in our model that the chronic stress imposed by AR and the hyper-adrenergic state predisposed the ARF rats to have more difficulties in coping with the metabolic stress of the diet than the sham rats (17; 18).

The precise mechanisms responsible for the increased heart hypertrophy and decreased LV ejection fraction in AR rats fed with the high-fructose diet compared to those fed a normal chow remain to be elucidated. They are most probably related to a combination of multiple interacting factors involving several regulating pathways but based on our data they do not seem to be related to ANP or BNP activation or to differences in hemodynamic load. Increased LVH could be linked to increased insulin-triggered protein synthesis from the presence of high concentration of carbohydrates in this diet. However, we did not observe any increase in Akt activation in our animals.

We observed decreased levels of AMP kinase activation in SF and both AR groups. This observation may be important. The high fructose diet is associated with an increase in circulating triglycerides and fatty acid which may cause an overabundance of substrate for the myocardium (9) thus possibly reducing the need for the stimulating action of AMPK on fatty acid oxidation and glycolysis (14; 15). In the short term, the myocardium can probably cope with this situation as we did not observe any clear changes in the level of activity of metabolic LV enzymes except for succinate dehydrogenase/ECT-2 activity levels. It was shown in a model of ischemic cardiomyopathy that AMPK was activated but it is not clear if this is good or bad for the heart (14; 35). The same observation was made in a model of pressure overload (1). In our model the observation of AMPK inhibition may be related to the fact that we are still in the early stages of the disease. The findings may be different later in the evolution of the disease when animals start dying maybe due to a progressive incapacity of the myocardium to fulfill its need in energy production.

The accumulation of collagen is a late feature in our AR model and only occurs after 6-9 months (22; 31; 32). Therefore we did not expect to find any significant changes in collagen content in the myocardium of the animals after only 8 weeks. We observed a trend towards an increase in pro-collagen I expression and for an increase in pro-collagen III expression in the fructose groups compared to the ones fed with the standard diet. It is likely that this would translate into an increase in myocardial fibrosis after a longer follow-up and maybe an earlier deterioration of diastolic function in the ARF rats. This will have to be evaluated in longer protocols.

LVH and heart failure are usually associated with a shift from normal fatty acid to glucose as the preferred myocardial fuel (36). In our model, this shift was not clearly present after 8 weeks. Total creatine kinase, the complex 1 of the electron transport chain as well as succinate dehydrogenase (SDH)/ETC-2 enzymatic activities were reduced in AR rats suggesting a possible early alteration of mitochondrial function in these animals. Surprisingly, SDH activity seemed restored in AR animals on the fructose diet. The SDH/ETC-2 links the Krebs cycle to the electron transport chain (3). On one hand, this may be seen as a positive effect of the fructose diet by maintaining normal levels of SDH activity in the Krebs cycle. On the other hand, if the ETC function is impaired in the heart of AR animals, an increase in complex II activity by the fructose diet could be associated with an increase in reactive oxygen species production (19). This protocol unfortunately was not designed to test this hypothesis.

The impact of the fructose diet on some myocardial enzymatic activities seemed different in AR animals compared to sham controls. The shams on the fructose diet did not develop any hypertrophy or sign of LV dysfunction. How the dilated and hypertrophied left ventricle adapts to the high-fructose diet compared to a normal left ventricle and why it develops more hypertrophy remain a mystery. AR is associated with a decreased gene expression of PPARα which is known to stimulate fatty acid oxidation (34). The overabundance of circulating triglycerides combined with a lack of increase in fatty acid oxidation by the heart could possibly lead to myocardial lipotoxicity but this remains a hypothesis to be confirmed. The fructose diet slightly increased FAT/CD36 expression in both sham and AR animals. We previously observed that myocardial lipoprotein lipase activity remained unchanged after 6 months in AR animals (unpublished observation). We hypothesize that the myocardium placed in presence of

an excess of fatty acid substrate with a similar or reduced capacity for β-oxidation may develop lipotoxicity (6). This hypothesis will be tested in a specifically designed protocol.

Study limitations:

The results of this study have to be viewed in light of some limitations. Rodent heart metabolism may differ in some aspects from human heart metabolism. Substrate utilization was not directly assessed in vivo. The high fructose diet had a slightly higher fat content (5,2% vs. 4,5%) and lower caloric content (3,6 kCal/g vs 4,0 kCal/g) compared to the control diet. The impacts of a longer exposition to the abnormal diet have to be evaluated in longer protocols. Other signaling pathways potentially involved need to be investigated in more details

Conclusions:

The results of this study show that a short exposition (8 weeks) to a high fructose diet is sufficient to worsen LV eccentric hypertrophy and LV function in rats with volume overload due to severe aortic valve regurgitation. Exposing AR rats to this high fructose diet resulted in hypertriglyceridemia, a higher retroperitoneal fat content and a trend for higher glycemia and higher systolic blood pressure than those fed a normal diet. Put together, these results suggest that a high fructose diet has a clear, rapid and negative impact on the myocardium and on the metabolic profile of rats already suffering from a chronic stress such as volume overload. The exact mechanisms involved and consequences for the heart will need to be explored in longer studies. Our current findings in conjunction of those of other authors (37-39) working on LVH pressure-overload models strongly point toward a deleterious role of high fructose consumption in

395 subjects with concentric and eccentric LVH.

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Disclosures:

None to declare.

403 **Figure legends:**

404

405 **Figure 1**: Animal daily food intake (kCal/day) and plasma levels of glucose, triglycerides,
406 insulin, leptin and adiponectin at the time of sacrifice. Results are expressed as mean ±
407 standard error of the mean (SEM) (n=15/gr.). Two-way ANOVA analyses are displayed
408 on the right of each panel. *: $p<0.05$ versus corresponding control (ctrl) diet group by
409 Bonferroni post-test.

410

411 **Figure 2:** Evaluation by real-time quantitative RT-PCR of the LV mRNA levels of atrial
412 natriuretic peptide (ANP) and brain natriuretic peptide (BNP). Results are reported in
413 arbitrary units as mean ± SEM (n = 9–10 group). Sham (sham operated animals) group
414 on control diet mRNA levels were normalized to 1. Two-way ANOVA analyses are
415 displayed on the right of each panel.

416

417 **Figure 3:** Evaluation by real-time quantitative RT-PCR of the LV mRNA levels of pro-
418 collagen 1 (pro-col I), pro-collagen 3 (pro-col III) and fibronectin. Results are reported in
419 arbitrary units (AU) as mean ± SEM (n=9–10/group). Sham group on control diet mRNA
420 levels were normalized to 1. Two-way ANOVA analyses are displayed on the right of
421 each panel.

422

423 **Figure 4:** Activity of five enzymes implicated in myocardial energy metabolism. HADH
424 (hydroxyacyl-Coenzyme A dehydrogenase), CS (citrate synthase), ETC complex 1
425 (complex 1 from the electron transport chain (rotenone-sensitive activity)), CK (creatine
426 kinase) and SDH (succinate dehydrogenase) enzymatic activities were measured in LV

427 homogenates from at least 10 animals in each group as described in the Materials and
428 Methods. Results are reported as mean ± SEM (n=10-15/gr). Two-way ANOVA
429 analyses are displayed on the right of each panel.
430
431 **Figure 5**: Evaluation by real-time quantitative RT-PCR of the LV mRNA levels of several
432 genes related to cardiac metabolism. Evaluation by real-time quantitative RT-PCR of the
433 LV mRNA levels of two glucose transporters: GLUT 1 and 4, fatty acid transporters
434 (RAT/CD36, Cpt1b and Cpt2 (carnitine palmitoyl transferase) and PPARα: (peroxisome
435 proliferator activator receptor alpha). Results are reported in arbitrary units (AU) as
436 mean ± SEM (n=10-15/gr). Two-way ANOVA analyses are displayed on the right of
437 each panel. *: $p<0.05$ versus corresponding control (ctrl) diet group by Bonferroni post-
438 test.
439
440 **Figure 6:** Levels of activation of several members of the AKT/mTOR and the AMPK in
441 the LV of AR rats fed with a fructose-rich diet. Left panels: Quantification by
442 immunoblotting of phosphorylated forms of the indicated molecules. Right panels: Total
443 protein content. AKT (protein kinase B or serine/threonine protein kinase Akt), S6K
444 (RPS6-p70-protein kinase), 4EBP1 (eukaryotic translation initiation factor 4E binding
445 protein 1). Results are reported in arbitrary units (AU) relative to sham animals on
446 control diet (fixed to 1) as mean ± SEM (n=8-10/gr). Two-way ANOVA analyses are
447 displayed on the right of each panel. **: $p<0.01$ versus corresponding control (ctrl) diet
448 group by Bonferroni post-test.
449

450 **Figure 7:** Phosphorylated form and total protein content of LKB1 in the LV of AR rats fed
451 with a fructose-rich diet. Results are reported in arbitrary units (AU) relative to sham
452 animals on control diet (fixed to 1) as mean ± SEM (n=8-10/gr). Two-way ANOVA
453 analyses are displayed on the right of each panel.
454

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586

Table 1. Sacrifice data.

Parameters	SC (13)	SF (12)	ARC (15)	ARF (12)
Body Weight, g	578 ± 11.8	550 ± 6.9[a]	577 ± 11.3	561 ± 10.5[a]
Tibial length, mm	60.6 ± 0.35	61.0 ± 0.35	61.4 ± 0.43	60.9 ± 0.37
Heart, mg	1367 ± 40.8	1312 ± 33.0	1895 ± 37.9[c]	2181 ± 67.1[c,**]
LV, mg	929 ± 22.7	912 ± 25.9	1398 ± 23.8[c]	1568 ± 58.6[c,***]
iLV, mg/g	1.6 ± 0.03	1.7 ± 0.05	2.4 ± 0.05[c]	2.9 ± 0.09[c,**]
RV, mg	10.7 ± 0.26	11.7 ± 0.36	19.6 ± 0.79[c]	17.6 ± 0.92[c]
LA, mg	37.1 ± 3.59	39.8 ± 3.61	67.9 ± 5.51[c]	51.3 ± 4.40[c]
Lungs, g	2.4 ± 0.25	2.4 ± 0.19	3.3 ± 0.27[c]	3.4 ± 0.21[c]
Retroperitoneal fat, g	9.3 ± 0.90	10.3 ± 1.28	6.4 ± 0.43[b]	9.6 ± 0.97*

Values are expressed as mean ± SEM. The number of animals per group is indicated in parenthesis. LV: left ventricle. Two-way ANOVA analysis: [a]: $p<0.05$ vs. control diet groups, [b]: $p<0.01$ and [c]: $p<0.001$ vs. sham-operated animals. If interaction between AR and diet was found to have a P value below 0.05, a Bonferroni post-test was conducted: *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$ vs. control diet corresponding group.

Table 2 Echocardiography data.

Parameters	SC (13)	SF (12)	ARC (15)	ARF (12)
EDD, mm	8.5 ± 0.19	8.2 ± 0.22	10.6 ± 0.12[c]	10.9 ± 0.27[c]
ESD, mm	3.9 ± 0.18	3.6 ± 0.16	5.6 ± 0.17[c]	6.3 ± 0.24[c,*]
SW, mm	2.0 ± 0.10	1.8 ± 0.11	1.9 ± 0.08	2.0 ± 0.11
PW, mm	1.5 ± 0.05	1.6 ± 0.06	1.9 ± 0.04[a]	1.7 ± 0.09[a]
RWT	0.41 ± 0.018	0.42 ± 0.022	0.36 ± 0.011[c]	0.34 ± 0.012[c]
EF, %	79.4 ± 1.05	80.2 ± 1.53	72.0 ± 1.78[c]	66.5 ± 2.14[c,*]
FS (%)	54.6 ± 1.09	56.0 ± 1.76	47.5 ± 1.70[c]	42.4 ± 1.83 [c,*]
LV mass(echo), mg	1159 ± 43.9	1011 ± 42.9	1795 ± 45.1[c]	2039 ± 74.8[c,*]

Values are expressed as mean ± SEM. The number of animals per group is indicated in parenthesis. EDD: end-diastolic diameter, ESD: end-systolic diameter, SW: septal wall thickness, PW: posterior wall thickness, RWT: relative wall thickness ((SW + PW)/EDD) EF: ejection fraction. FS: fractional shortening, LV mass (echo): estimated LV mass by the method of Devereux (13). Two-way ANOVA analysis: [a]: $p<0.05$ and [c]: $p<0.0001$ vs. sham-operated animals. If interaction between AR and diet was found to have a P value below 0.05, a Bonferroni post-test was conducted: *: $p<0.05$ vs. control diet corresponding group.

Table 3. Hemodynamic values

Parameters	SC (13)	SF (12)	ARC (15)	ARF (12)
HR, bpm	411 ± 10.1	393 ± 6.0	389 ± 8.5[a]	380 ± 9.9[a]
SV, µl	286 ± 11.9	222 ± 12.9	445 ± 26.4[c]	461 ± 24.3[c]
CO, ml min^{-1}	118 ± 5.7	88 ± 5.8	178 ± 12.4[c]	170 ± 7.5[c]
dp/dt+	9145 ± 549.2	7772 ± 491.3	7285 ± 74.0	7597 ± 342.0
dp/dt-	7081 ± 543.3	8238 ± 1058.8	5734 ± 582.2	6785 ± 676.1
LVEDP, mmHg	11.6 ± 0.78	9.2 ± 1.51	17.3 ± 1.29[a]	15.4 ± 1.08[a]
Syst. BP mmHg	130 ± 6.3	133 ± 7.4	119 ± 2.9[a]	128 ± 2.6[a]
Diast. BP mmHg	98 ± 4.3	99 ± 4.7	59 ± 3.2[c]	63 ± 4.0[c]

Measurements obtained under inhaled 1.5% isoflurane anesthesia. HR: heart rate; SV: stroke volume in left ventricular outflow tract by pulsed Doppler; CO: cardiac output (SV X HR); dP/dt_{min}; minimal derivative of pressure/time; dP/dt_{max}: maximal derivative of pressure/time; LVEDP: left ventricular end-diastolic pressure; BP: blood pressure. Values are mean ± SEM of the indicated number of animals per group with the exception of for the dP/dt and LVEDP values (n=5). Two-way ANOVA analysis: [a]: $p<0.05$ and [c]: $p<0.0001$ vs. sham animals.

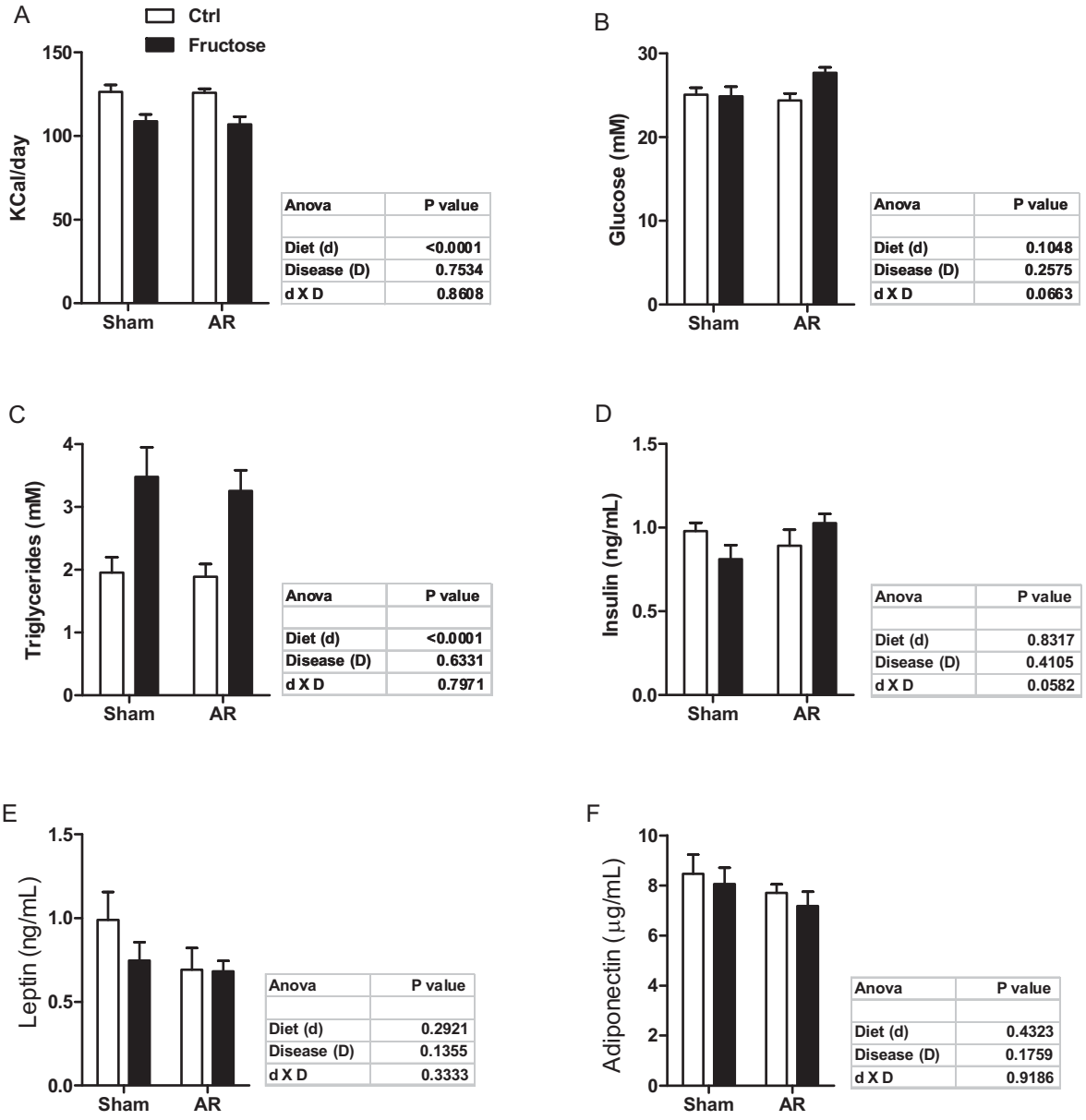

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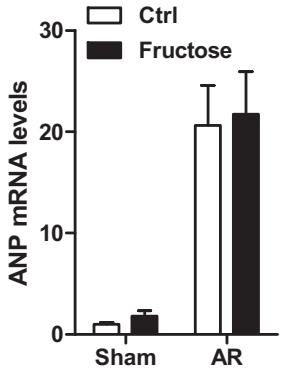
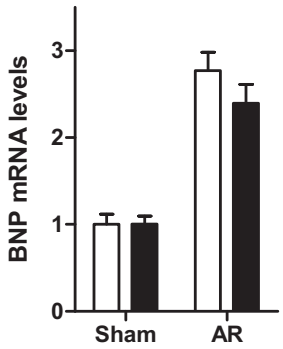
Figure 1



Anova	P value
Diet (d)	0.7449
Disease (D)	<0.0001
d X D	0.9598



Anova	P value
Diet (d)	0.2746
Disease (D)	<0.0001
d X D	0.2547

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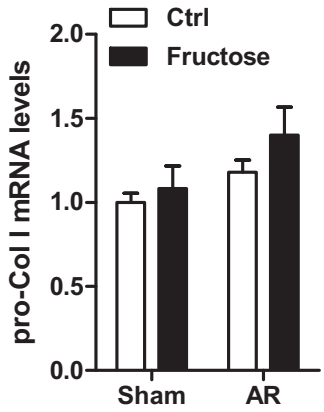
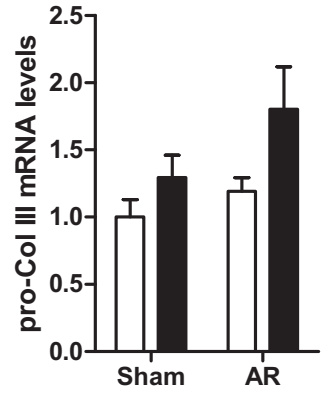
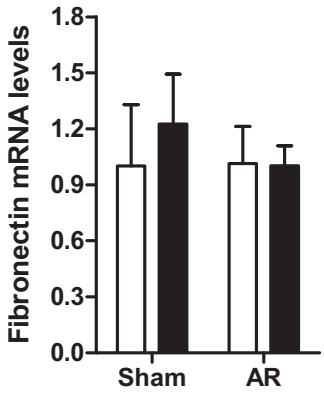
Figure 2



Anova	P value
Diet (d)	0.1973
Disease (D)	0.0381
d X D	0.5504



Anova	P value
Diet (d)	0.0176
Disease (D)	0.0623
d X D	0.3878



Anova	P value
Diet (d)	0.6197
Disease (D)	0.6250
d X D	0.5837

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Figure 3

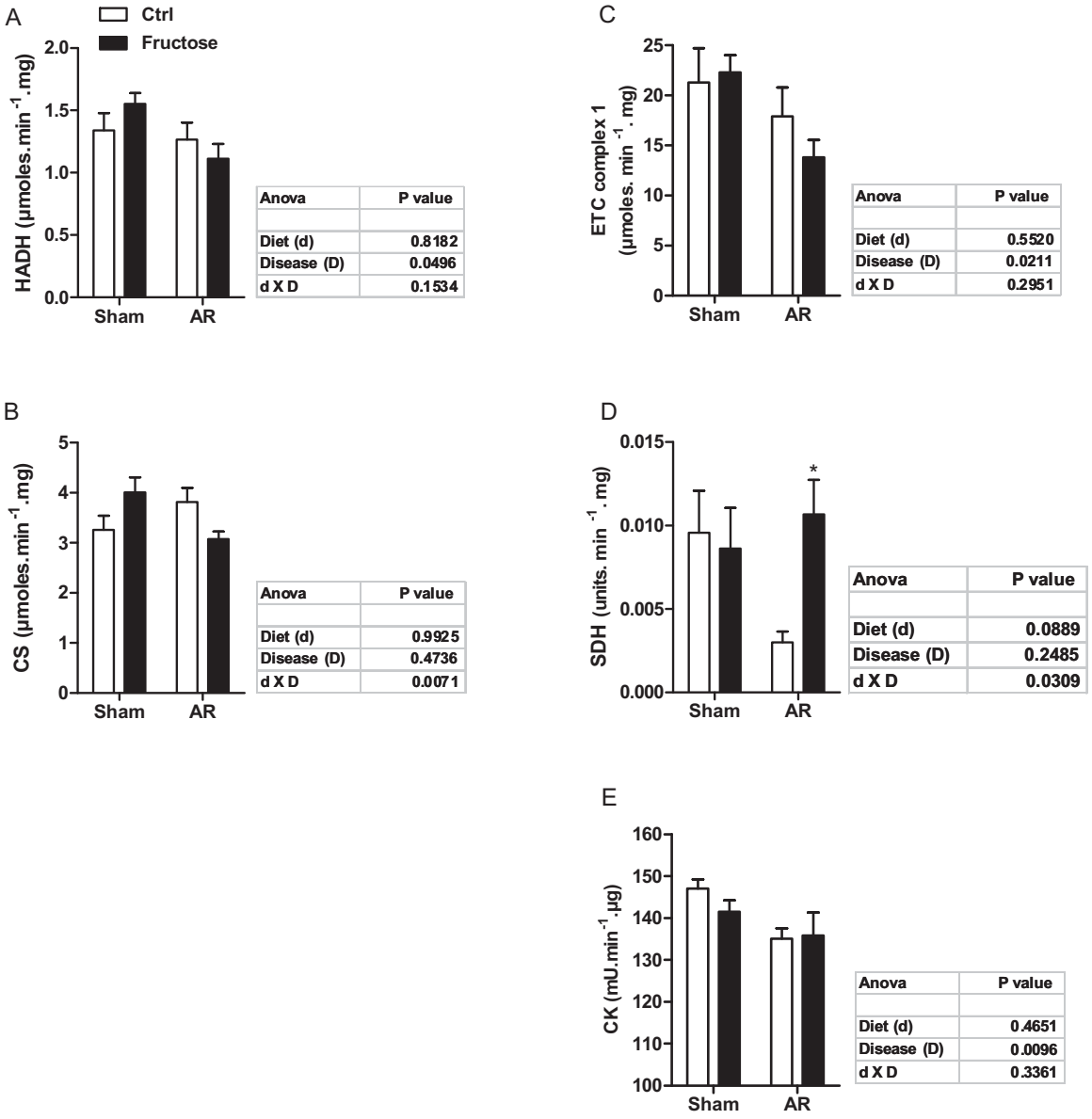

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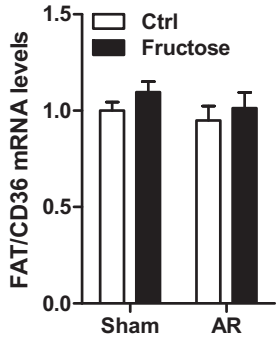
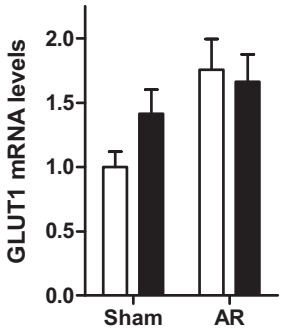
Figure 4

A



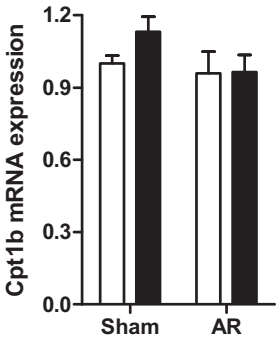
Anova	P value
Diet (d)	0.2271
Disease (D)	0.3114
d X D	0.8179

D



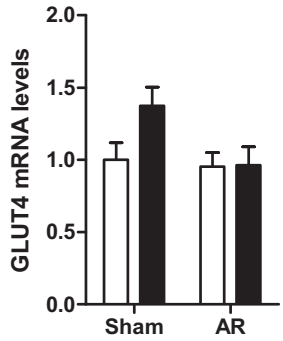
Anova	P value
Diet (d)	0.4029
Disease (D)	0.0124
d X D	0.1885

B



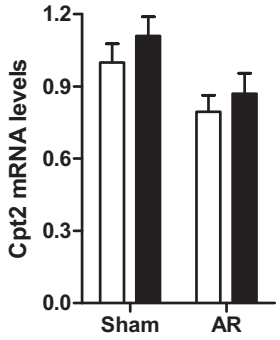
Anova	P value
Diet (d)	0.3126
Disease (D)	0.1299
d X D	0.3591

E



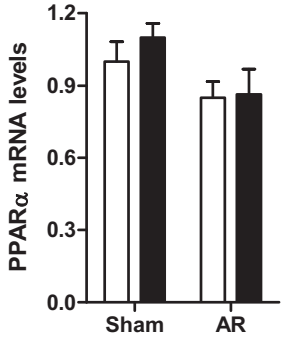
Anova	P value
Diet (d)	0.1173
Disease (D)	0.0637
d X D	0.1349

C



Anova	P value
Diet (d)	0.2453
Disease (D)	0.0075
d X D	0.8367

F



Anova	P value
Diet (d)	0.4803
Disease (D)	0.0201
d X D	0.5938

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Figure 5

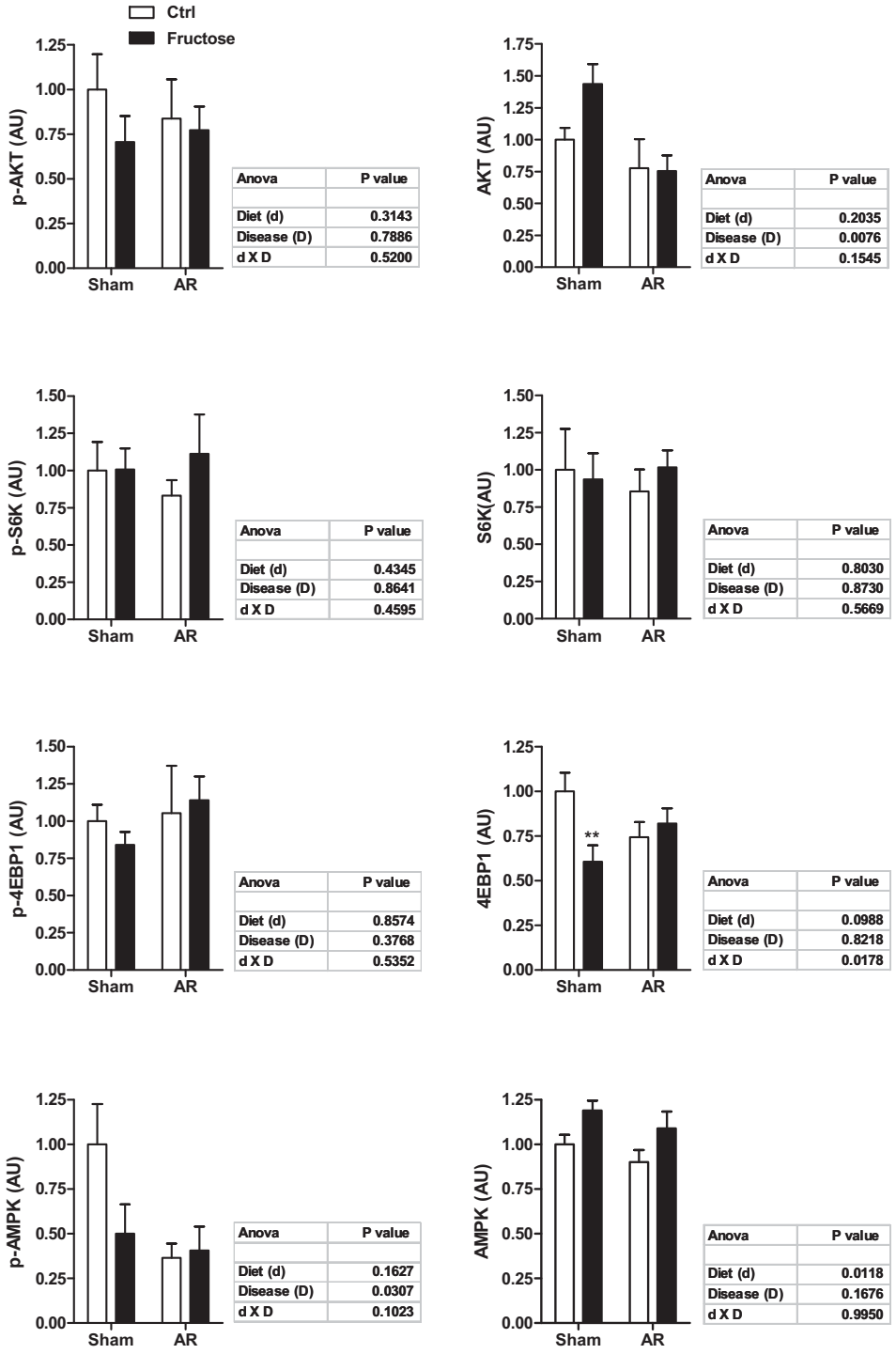

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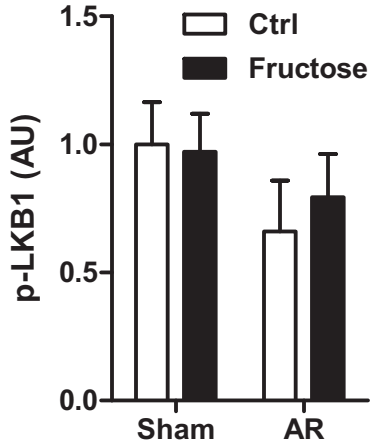
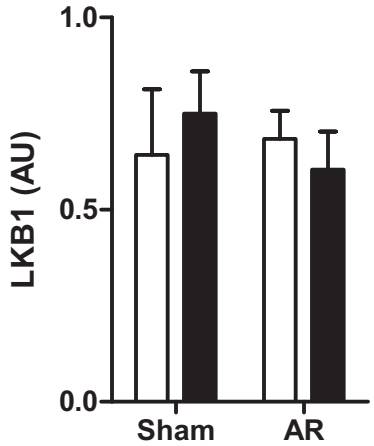
Figure 6



Anova	P value
Diet (d)	0.7634
Disease (D)	0.1426
d X D	0.6405



Anova	P value
Diet (d)	0.9095
Disease (D)	0.6726
d X D	0.4495

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Figure 7