The buckwheat iminosugar D-fagomine attenuates sucrose-induced steatosis and hypertension in rats

Sara Ramos-Romero[1,2,*], Mercè Hereu[1], Lidia Atienza[3], Susana Amézqueta[4], Josefina Casas[5,6], Silvia Muñoz[7], Isabel Medina[7], Bernat Miralles-Pérez[8], Marta Romeu[8], and Josep L. Torres[1]

[1] Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain

[2] Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

[3] Department of Pathology, Puerta del Mar University Hospital, Cádiz, Spain

[4] Departament d'Enginyeria Química i Química Analítica and Institut de Biomedicina (IBUB), Universitat de Barcelona, Barcelona, Spain

[5] Research Unit on BioactiveMolecules (RUBAM), Department of Biological Chemistry, Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain

[6] Center for Biomedical Research on Hepatic and Digestive Diseases (CIBEREHD), ISCIII, Madrid, Spain

[7] Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain

[8] Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, Reus, Spain

Keywords: diabetes, blood pressure; fructose; metabolic syndrome; D-fagomine.

* Dr. Sara Ramos-Romero

IQAC-CSIC, Jordi Girona 18-26,

08034 Barcelona, Spain

Phone: (+34) 934006100

Fax: (+34) 932045904

E-mail: sara.ramos@iqac.csic.es

Abbreviations

AT, Adipose Tissue
CVD, Cardiovascular Disease
DAGs, Diacylglycerols
DNL, De Novo Liposynthesis
F2-IsoPs, F_{2t} Isoprostanes
FG, D-fagomine
HF, High-Fat
HS, High-Sucrose
HS+FG, High-Sucrose with D-fagomine
IGT, Impaired Glucose Tolerance
IR, Insulin Resistance
MetS, Metabolic syndrome
MRM, Multiple Reaction Monitoring
NO, Nitric Oxide
OGTT, Oral Glucose Tolerance Test
OS, Oxidative Stress
qRT-PCR, Quantitative Real-Time PCR
RAAS, Renin-Angiotensin-Aldosterone System
SREBP-1c, Sterol Receptor Element Binding Protein–1c
STD, Standard
VAT, Visceral Adipose Tissue
VLDL, Very-Low-Density Lipoprotein
WKY, Wistar Kyoto

Abstract

Scope: This study examines the long-term functional effects of D-fagomine on sucrose-induced factors of metabolic dysfunctions and explores possible molecular mechanisms behind its action.

Methods & results: Wistar Kyoto (WKY) rats were fed a 35% sucrose solution with D-fagomine (or not, for comparison) or mineral water (controls) for 24 weeks. We recorded: body weight; energy intake; glucose tolerance; plasma leptin concentration and lipid profile; populations of Bacteroidetes, Firmicutes, bacteroidales, clostridiales, enterobacteriales, and *Escherichia coli* in feces; blood pressure; urine uric acid and F_{2t} isoprostanes (F_2-IsoPs); perigonadal fat deposition; and hepatic histology and diacylglycerols (DAGs) in liver and adipose tissue.

D-Fagomine reduced sucrose-induced hypertension, urine uric acid and F_2-IsoPs (markers of oxidative stress; OS), steatosis and liver DAGs, without significantly affecting perigonadal fat deposition and impaired glucose tolerance. It also promoted excretion of enterobacteriales generated by the dietary intervention.

Conclusion: D-fagomine counteracts sucrose-induced steatosis and hypertension, presumably by reducing the postprandial levels of fructose in the liver.

1. Introduction

s[1]Central fat accumulation and hypertension are risk factors for cardiovascular disease (CVD). Together with insulin resistance (IR), they are part of the cluster of factors known as metabolic syndrome (MetS) [1].

Dietary-fat-induced central adiposity results in ectopic fat deposition when subcutaneous adipocytes become insulin resistant and lose their capacity to store triacylglycerols, which are then deposited at undesirable sites such as the liver, the heart, the skeletal muscle or visceral adipose tissue (VAT) [2]. Then visceral adipocytes become resistant to the antilipolytic effect of insulin and free fatty acids drain directly into the liver through the portal vein, leading to impaired liver metabolism [3]. In contrast, sucrose (glucose/fructose)-induced adiposity develops through other pathways linked primarily to the excess of fructose. Unlike glucose, fructose is almost entirely catabolized, mainly in the liver, and escapes metabolic control by insulin [4]. Apart from contributing substrates for de novo liposynthesis (DNL), fructose may increase hepatic lipid levels by activating modulators of liposynthesis such as sterol receptor element binding protein–1c (SREBP-1c) [5]. Fructose-driven DNL also contributes to liver fat accumulation by inhibiting the hepatic oxidation of endogenous and exogenous fatty acids via increased levels of malonyl-coA [6]. Hepatic DNL triggers postprandial hypertriglyceridemia, which may promote lipid deposition as VAT rather than as subcutaneous adipose tissue [7].

As adipose tissue (AT) is a source of angiotensinogen, adiposity may lead to hypertension via activation of the renin-angiotensin-aldosterone system (RAAS) [8]. Free fatty acids from VAT may stimulate the RAAS via aldosterone production independently of renin [8], and they may also trigger hypertension via activation of the

sympathetic nervous system [9]. Increased levels of uric acid may contribute to hypertension through systemic and renal vasoconstriction by increasing intracellular oxidative stress (OS), activating the RAAS, and inhibiting the production of endothelial nitric oxide (NO) [10].

D-Fagomine (1,2-dideoxynojirimycin) is a polyhydroxylated nitrogen-containing ring structurally related to glucose and mannose. It is a minor component of buckwheat (*Fagopyrum esculentum* Moench, Polygonaceae) and traditional buckwheat-based foodstuffs such as noodles, pancakes, fried dough, beer, cookies and bread [11]. In rats, D-fagomine reduces elevated plasma insulin concentrations induced by a high-fat (HF) diet (Sprague-Dawley rats, 9 weeks) [12] and it counteracts fat-induced low-grade inflammation and impaired glucose tolerance (IGT) (Wistar Kyoto (WKY) rats, 13-21 weeks) via a mechanism that may involve modifications of gut microbiota [13]. As D-fagomine is poorly absorbed [14], it is likely to be largely in contact with the intestinal wall. There, D-fagomine also delays starch and sucrose digestion by inhibiting brush border glycosidases [15]. In previous work we have shown that an excess of fat or sucrose (glucose/fructose) triggers different risk factors of MetS at different times in WKY rats: an HF diet induced obesity and fast onset IR and IGT via low-grade inflammation; while a high-sucrose (HS) diet induced IGT later than the HF diet, through liver DNL from fructose. Meanwhile, only the HS diet triggered elevated blood pressure [16]. After reporting the long-term functional effect of D-fagomine on fat-induced MetS factors [13], we here examine its effects on sucrose-induced factors, namely VAT, fatty liver, IGT and hypertension.

2. Materials and Methods

2.1 Animals

A total of twenty-seven male WKY rats from Envigo (Indianapolis, IN, USA), aged 8-9 weeks were used. All animal manipulation was carried out in the morning to minimize the effects of circadian rhythms. All the procedures strictly adhered to the European Union guidelines for the care and management of laboratory animals and were licensed by the Catalan authorities (reference no. DAAM7921), as approved by the Spanish CSIC Subcommittee of Bioethical Issues.

2.2 Experimental Design and Sample Collection

The rats were kept under controlled conditions of humidity (60%), and temperature (22 ± 2 ºC) with a 12 h light-12 h dark cycle. They were randomly divided into 3 dietary groups (n = 9 per group), all fed a standard feed (2014 Teklad Global 14% Protein) from Envigo *ad libitum* with free access to water or sucrose solutions as follows: the standard (STD) group was given mineral water (Ribes, Girona, Spain); the HS group was given a 35% sucrose solution in mineral water as the only source of liquid intake; and the HS with D-fagomine (HS+FG) group was given a 35% sucrose solution in mineral water supplemented with D-fagomine (> 98% from Bioglane SLNE; Barcelona, Spain). The dosage of D-fagomine was the same used in the previous HF study (2 mg per g carbohydrate) [13].

Feed and drink consumptions were monitored daily and body weight was measured weekly throughout the experiment. Energy intake was estimated as metabolizable energy based on the Atwater factors:4 kcal per g protein, 9 kcal per g fat, and 4 kcal per g available carbohydrate. Fecal samples were collected by abdominal massage after week 20. The energy content of the feces was determined by differential scanning calorimetry (25-600 ºC in an O_2 atmosphere, 10 ºC min^{-1}) by means of a

92 thermogravimetric analyzer TGA/SDTA 851e (Mettler Toledo; Columbus, OH, USA)
93 with an integrated SDTA signal.
94 After week 23, the rats were placed in metabolic cages overnight for urine collection. At
95 the end of the experiment (week 24), the rats were anaesthetized intraperitoneally with
96 ketamine and xylazine (80 and 10 mg per kg body weight, respectively) after overnight
97 fasting. Blood was collected by cardiac puncture then plasma was immediately obtained
98 by centrifugation and stored at −80 ºC until analysis. Perigonadal AT, a type of VAT in
99 rats [17], and liver were removed, weighed and cut into small pieces. One part of the
100 liver was fixed in 10% formalin for histological analysis. The rest of the liver and
101 adipose tissue were washed with 0.9% NaCl solution and stored at −80 ºC for DAG
102 analysis.
103
104 **2.3 Oral Glucose Tolerance Test, Fasting Glucose and Plasma Insulin**
105 After weeks 13 and 21, an oral glucose tolerance test (OGTT) was performed on
106 overnight fasted animals. A solution of glucose (1 g per kg body weight) was
107 administered to the rats by oral gavage. Blood glucose concentration was measured by
108 the enzyme electrode method using an Ascensia ELITE XL blood glucose meter (Bayer
109 Consumer Care AG; Basel, Switzerland) before the experiment and 15, 30, 45, 60, 90
110 and 120 min after glucose intake. Fasting blood glucose was measured by the same
111 method after week 24 on animals fasted overnight. Plasma insulin was determined on
112 these samples using Milliplex xMAP multiplex technology on a Luminex xMAP
113 instrument (Millipore, Austin, TX). Milliplex Analyst 5.1 (Vigenetech, Carlisle, PA,
114 USA) software was used for data analysis. The standard curve was generated for the
115 range 69-50,000 pg mL^{-1} using a five-parameter logistic curve fit.
116

117

118 **2.4 Plasma Lipid Profile and Leptin Concentration**

119 Total plasma cholesterol, HDL and LDL cholesterol, and triglycerides were all
120 measured by spectrophotometric methods using the corresponding kits from Spinreact
121 (Girona, Spain) as described elsewhere [18]. Leptin levels were measured together with
122 insulin using the Milliplex xMAP multiplex technology (Millipore).

123

124 **2.5 Fecal Microbiota**

125 The levels of total bacteria and Bacteroidetes, Firmicutes, Enterobacteriales, and
126 *Escherichia coli* were estimated from fecal DNA by quantitative real-time PCR (qRT-
127 PCR). DNA was extracted from the feces using the QIAamp DNA StoolMini Kit from
128 Qiagen (Hilden, Germany) and quantified using a Nanodrop 8000 Spectrophotometer
129 (Thermo Scientific; Waltham, MA, USA). were qRT-PCR was carried out in triplicate
130 on diluted DNA samples (20 ng μL^{-1}), using a LightCycler 480 II (Roche; Basel,
131 Switzerland). Eachreaction mixture contained DNA solution (2 µL) and a master mix
132 (18 µL) made of 2XSYBR (10 µL), the corresponding forward and reverse primer (1 µL
133 each), and water (6 µL). Both Nontemplate controls (water) and positive controls were
134 included. The primers and annealing temperatures are detailed in Table S1, Supporting
135 Information. The PCR cycling conditions were: 10 s at 95 °C, then 45 cycles of 5 s at 95
136 °C, 30 s at the primer-specific annealing temperature (Table S1, Supporting
137 Information), and 30 s at 72 °C (extension). To determine the specificity of the qRT-
138 PCR after amplification, melting curve analysis was carried out: 2 s at 95 °C, 15 s at 65
139 °C, followed by atemperature gradient up to 95 °C at a rate of 0.11 °C s^{-1}, with five
140 fluorescence readings per °C. The relative DNA abundances for the different genes
141 were calculated from the second derivative maximum of their respective amplification

curves (Cp, calculated in triplicate) by considering Cp values to be proportional to the dual logarithm of the inverse of the specific DNA concentration, according to the equation: [DNAa]/[DNAb] = 2Cpb-Cpa [19]. Total bacteria was normalized as 16S rRNA gene copies per mg of wet feces (copies per mg).

2.6 Blood Pressure, and Urine and Plasma Uric Acid

Systolic and diastolic blood pressure was measured at time 0 and after weeks 4, 9, 15 and 22 by the tail-cuff method, using a non-invasive automatic blood pressure analyzer (Harvard Apparatus, Holliston, MA, USA).

Total urine and plasma uric acid were determined by a spectrophotometric method using a uricase/peroxidase kit from BioSystems (Barcelona, Spain) via measuring the absorbance at 520 nm on a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Creatinine levels in urine were determined by a colorimetric method using a commercial kit (C-cromatest Linear Chemicals, Montgat, Spain) via measuring absorbance at 510 nm.

2.7 Measurement of Isoprostanes

F_2-isoprostanes (F_2-IsoPs) were determined in urine samples by LC/ESI–MS/MS following a previously reported procedure [12] with some modifications. Samples (500 µL) were acidified and the mixtures were incubated for 2 h at 37 ºC in the presence of β-glucuronidase (90 U mL^{-1}) (Sigma, Saint Louis, MI, USA). , F_2-IsoPs were purified by SPE after adding [$^2H_4$]15-F_{2t}-IsoP (Cayman, Ann Arbor, MI, USA) (internal standard 100 µL, 10 µg L^{-1}) and analyzed using an Agilent 1260 chromatograph coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA,

166 USA) fitted with a Mediterranea Sea 18 column (10 cm x 2.1 mm i.d., 2.2 µm particle
167 size) (Teknokroma, Barcelona, Spain). The instrument was operated in the negative-ion
168 mode with a Turbo V source to obtain MS/MS data. The chromatography solvents used
169 were [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in acetonitrile, and the
170 solutes were separated with an increasing linear gradient (v/v) of [B]: time 0, 10% B; 7
171 min, 50% B; 7.1 min, 100% B; 8 min, 100% B; 8.1 min, 10% B; and 10 min, 10% B, at
172 a flow rate of 700 µL min^{-1} at 40 ºC. F_2-IsoPs were detected by multiple reaction
173 monitoring (MRM). Calibration curves were prepared using seven matrix-matched
174 standards covering the working concentration range. The LOQ was 0.4 µg L^{-1} for 15-
175 F_{2t}-IsoP and 2 µg L^{-1} for 5-F_{2t}-IsoP. The results were expressed as ng per mg creatinine,
176 to correct for urine dilution.

177

178 **2.8 Histology of the Liver**

179 Formalin fixed livers were dehydrated in alcohol and embedded in paraffin (Panreac
180 Quimica SLU; Barcelona, Spain), and then cut into 3 µm thick slices, using a steel knife
181 mounted in a microtome (HM 355S Rotary Microtome; Thermo Fisher Scientific,
182 Waltham, MA, USA). Sections were stained with hematoxylin (Hematoxylin solution
183 modified according to Gill III for microscopy; Merck KGaA, Darmstadt,
184 Germany)/eosin (Pharmacy Service of Puerta del Mar Hospital, Cádiz, Spain) then
185 viewed under a light microscope (NIKON Eclipse 80i; NIKON Corporation, Minato,
186 Japan). Three parameters were graded following the method described by *Taltavull et al*
187 [20]: steatosis, 0 (<5%), 1 (5%–33%), 2 (33%–66%), or 3 (>66%); steatosis
188 localization, 0 (absence), 1 (periportal), or 2 (non-zonal); and the presence of
189 lipogranuloma, 0 (absence) or 1 (presence).

190

2.9 Diacylglycerols in Liver and Adipose Tissue

Frozen samples were thawed and homogenized by sonication on a SFX150 Sonifier (Emerson Industrial Automation, St. Louis, MO, USA). DAGs were analyzed using the method described by Simbari *et al* [21] with some modifications. The internal standard was 1,3-17:0 D5 DG (Avanti Polar Lipids Inc., Alabaster, AL, USA; 200 pmol) and the incubation conditions were 12 h at 48 ºC. Then the samples were dried and suspended in methanol, centrifuged (9390 g, 3 min) and the supernatants (8 µL) loaded into an Acquity UPLC separation system connected to an LCT Premier orthogonal accelerated time-of-flight mass spectrometer (Waters; Milford, MA, USA), operated in positive ESI mode (LC-TOF-MS). DAG resolution was achieved using a C8 Acquity UPLC-bridged ethylene hybrid 100 x 2.1 mm i.d., 1.7 µm column (Waters) and a binary elution system consisting of [A] 0.2% (v/v) formic acid, 2 mM ammonium formate in water and [B] the same buffer in methanol, under linear gradient conditions: 0.0 min, 80% B; 3 min, 90% B; 6 min, 90% B; 15 min, 99% B; 18 min, 99% B; 20 min, 80% B; and 22 min, 80% B, at 30 ºC. The flow rate was 0.3 mL min^{-1}. Full-scan spectra from 50 to 1,500 Da were acquired, and individual spectra were summed to produce data points of 0.2 s each. To maintain mass accuracy and precision leucine enkephalin was used as an independent reference spray () via the LockSpray interface.. Quantification was carried out using the extracted ion chromatogram of each compound, with 50 mDa windows. The linear range was determined by injecting mixtures of internal standards. DAG content was calculated as DAG 16:0, 16:0 equivalents.

2.10 Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA). Quantitative data are expressed as mean values with their

standard errors (SEM). Normal distributions and heterogeneity of the data were evaluated and statistical significance was determined by two-way ANOVA for repeated measures (OGTT). One-way ANOVA, and Tukey's multiple-comparison test were used for mean comparison. Differences were considered significant when $P < 0.05$. The results from qualitative measurements of histological sections are expressed in frequencies (percentage of animals that present the value, or do not) and their statistical significance was determined using contingency tables and χ^2 statistics.

224 # 3. Results

225 ## 3.1. Feed and Drink Intakes, Energy Balance, and Body and Perigonadal Adipose
226 ## Tissue Weights

227 Feed intake was lower and drink intake was higher in both HS (HS and HS+FG) groups
228 than in the STD group (Table 1). The glucose/fructose intake was similar in the HS and
229 HS+FG groups. Energy intake was higher in both HS groups than in the STD group;
230 while energy in feces was similar in all three groups studied (Table 1).

231 Body weight was similar in all of the groups at the beginning (234.8 g, SEM 3.2), and
232 no differences were observed between the STD and either HS group throughout the
233 experiment (Table 1). Perigonadal AT weight was significantly higher in the HS and
234 HS+FG groups (P <0.001) and (P <0.01, respectively) (Table 1).

235

236 ## 3.2 Glycemic Status.

237 The OGTT test was run after weeks 13 and 21. There were no significant differences
238 between groups after week 13 (Figure 1A). After week 21, the levels of postprandial
239 glucose in animals that consumed an excess of sucrose (HS and HS+FG groups) were
240 higher (P < 0.01) than those in the STD group, 30 and 45 min after glucose
241 administration (Figure 1B). At 60 and 90 min after administration, the levels of plasma
242 glucose were higher (P < 0.01) in the HS than the STD group; while there was no
243 difference between the STD and HS+FG groups.

244 Fasting blood glucose was below 70 mg dL^{-1} in all three groups after week 21 and at the
245 end of the study (week 24) (Figure 1, Table 2). Fasting plasma insulin was significantly
246 higher in animals given either HS diet (HS and HS+FG groups) (Table 2).

247

248 **3.3 Plasma Leptin and Lipid Profile**

249 Plasma leptin was higher ($P < 0.001$) in the HS group than in the STD group (Table 2).
250 Total and LDL cholesterol in plasma were similar in all the groups (Table 2). HDL
251 cholesterol levels were higher ($P<0.05$) in the HS and HS+FG groups than in the STD
252 group. Plasma triglyceride levels were higher in both HS groups than in the STD group,
253 but this difference was only significant ($P<0.05$) between the HS+FG group and the
254 STD group.

255

256 **3.4 Fecal Microbiota**

257 The proportions of bacterial phyla, orders and *E. coli* in the gut microbiota were
258 evaluated after week 24 (Table 3). The Bacteroidetes, bacteroidales, enterobacteriales
259 and *E. coli* populations in feces were significantly higher ($P < 0.05$) in both HS groups.
260 The highest populations were recorded in the D-fagomine supplemented group.

261

262 **3.5 Blood Pressure and Uric Acid in Urine and Plasma**

263 After 23 weeks of sucrose intake, systolic (Figure 2A) and diastolic (Figure 2B) blood
264 pressures were significantly higher ($P < 0.05$) in animals fed the HS diet than in those
265 fed the STD or HS+FG diets. Animals in the two groups that consumed an excess of
266 sucrose (HS and HS+FG) presented a significantly ($P < 0.001$) higher concentration of
267 urine uric acid than those of the STD group after week 23 (Figure 2C). The group
268 supplemented with D-fagomine (HS+FG) presented significantly ($P < 0.05$) lower

concentration of urine uric acid than the HS group and the lowest concentration among groups in plasma (Figure 2C, D).

3.6 Urine Isoprostanes

The animals fed the HS diet presented increased concentrations of 5-F2t-IsoP and 15-F2t- IsoP ($P < 0.05$) compared with the STD group after 23 weeks of intervention (Figure 3A, B); while the animals supplemented with D-fagomine (HS+FG group) presented concentrations of IsoPs similar to those in the STD group (Figure 3A, B).

3.7 Liver Histology

An excess of sucrose induced significant ($P < 0.001$) and highly localized steatosis (Figure 4B, C, D) and lipogranuloma ($P < 0.001$; Figure 4E). D-Fagomine supplementation (HS+FG group) significantly ($P < 0.001$) reduced the grade of steatosis, from 2 (33%-66% steatosis) in the HS group to 1 (5%-33% steatosis), with no influence on its localization. Lipogranuloma was less ($P < 0.001$) present in animals in the HS+FG group than in those in the HS group (Figure 4E).

3.8 Liver and Adipose Tissue Diacylglycerols

The levels of DAGs 32:1, 32:2, 34:1 and 36:2 were higher in livers from rats in the HS group than from those in the STD and HS+FG groups (Figure 5 and Table S2, Supporting Information). There were no significant differences in any DAG content between the HS and HS+FG groups in AT (Table S3, Supporting Information).

4. Discussion

The present study explores the effects of D-fagomine on a rat model of sugar-induced metabolic alterations. From previous work we knew that WKY rats given a 35% sucrose (glucose/fructose) solution as the only source of liquid intake present moderate IGT, steatosis, deposition of perigonadal AT and raised blood pressure after 21-24 weeks of intervention, while remaining normoweight [16]. Here, we show that D-fagomine can counteract this induced steatosis and the elevation in blood pressure, while it had little effect on perigonadal fat (Table 1) and IGT (Figure 1). Gonadal AT is a type of VAT in rats [17] that has been associated with low-grade inflammation and metabolic complications, mainly owing to the production of free fatty acids and proinflammatory adipokines by adipocytes [2, 22]. Therefore, the increase in VAT may account, at least in part, for the IGT observed in the groups fed HS diets. Direct disruption of insulin signaling by lipid metabolites such as DAGs may also contribute to the sucrose-induced IGT [7, 16]. Fat accumulation and generation of DAGs in the liver would result from fructose-induced DNL [4]. Fructose-induced DNL is believed to trigger fat deposition in VAT by supplying triglycerides from the liver via upregulation of very-low-density lipoprotein (VLDL) production and secretion [7]. In contradistinction to this view, our observation that D-fagomine reduced steatosis (Figure 4) and not perigonadal fat deposition (Table 1) suggests that fructose-induced visceral fat accumulation may proceed independently of liver DNL. This hypothesis is supported by previous evidence that overconsumption of fructose may lead to rapid inflammation in subcutaneous adipocytes and an increase in intracellular cortisol that stimulates the flux of fatty acids into VAT [23] independently of any effect in the liver. The fact that D-fagomine only reduced fat deposition in the liver might be connected to its inhibitory activity on intestinal sucrase [15]. As fructose is mainly accumulated in the liver [4],

316 this organ may be more sensitive than AT to variations in fructose concentration.
317 Therefore, as D-fagomine consistently reduces postprandial blood glucose/fructose
318 levels by 25% at the dose supplied via the drink (2 mg per g sucrose) [15], it may be
319 reducing liver DNL through lowered activation of regulatory factors such as SREBP-1c
320 [5]. This reduction in liver DNL is consistent with the observation that D-fagomine
321 maintains the levels of liver DAGs similar to those in the STD group, while they are
322 significantly elevated ($P < 0.05$) in the HS group (Figure 5). In contrast, D-fagomine did
323 not modify DAG levels in perigonadal AT with respect to the HS group (Table S3,
324 Supporting Information), which confirms that a reduction in postprandial fructose
325 concentration would not significantly affect DNL in AT. The reduction of liver DAGs
326 may account, at least in part, for the slight effect of D-fagomine on sucrose-induced IGT
327 recorded at the end of the study (Figure 1, time 90).

328 D-Fagomine also affected the levels of fasting leptin: an anorectic hormone that helps to
329 regulate energy expenditure [24], produced by white AT and other organs [25]. In
330 agreement with studies on both rodents and humans [26, 27], fasting plasma leptin
331 levels where significantly higher ($P < 0.001$) in animals given an excess of sucrose than
332 in those given the STD diet (Tables 1 and 2). WKY rats supplement with D-fagomine
333 presented levels of fasting leptin similar to those in the STD group (Table 2). In a
334 similar experiment, Sprague-Dawley rats given free access to a 30% sucrose solution
335 developed insulin sensitivity-independent leptin resistance that was rapidly reverted
336 (days) after the excess of sucrose was eliminated from the diet [28]. This suggests that
337 adiposity as such may not be a major contributor to leptin resistance in that model. The
338 reduction of postprandial glucose/fructose concentration might be responsible for the
339 effect of D-fagomine on circulating leptin.

340 The animals consuming an excess of sucrose without D-fagomine supplementation (HS
341 group) showed elevated blood pressure (Figure 2) without increased body weight (Table
342 1) in agreement with other studies [16, 29-31]. The reduction of circulating fructose
343 levels may also explain the D-fagomine-mediated reduction in blood pressure (Figure
344 2). High levels of liver fructose triggers elevated blood pressure by generating an excess
345 of uric acid via overexpression of fructokinase C, increased ATP consumption and
346 nucleotide turnover [10, 16, 32]. Lower levels of postprandial fructose in the
347 supplemented group would be consistent with the reduction in the levels of urine and
348 plasma uric acid (Figure 2), which may explain, at least in part, the effect of D-fagomine
349 on lowering blood pressure. The levels of uric acid did not increase in plasma (Figure
350 2). This may be because urine concentration was measured in fasted animals while the
351 physiologically relevant high levels of plasma uric acid are likely to occur in the
352 postprandial period. In fasted animals, the levels of uric acid in urine are more likely to
353 reflect chronic effects of fructose consumption. Additionally to affecting uric acid, D-
354 fagomine may counteract the possible hypervolemic effect induced by a chronic high
355 intake of sucrose [29], also by lowering the circulating levels of glucose/fructose. D-
356 Fagomine also prevented the sucrose-induced systemic OS, which was monitored by
357 measuring urine F_2-IsoPs (Figure 3). This late (week 24) OS does not seem to be a
358 direct consequence of IR or IGT [16], while it may be related to elevated blood pressure
359 via generation of uric acid [33].

360 The role of intestinal microbiota in host homeostasis is increasingly being revealed [34].
361 At the phylum level, a reduced Bacteroidetes/Firmicutes ratio compared to the lean
362 phenotype has been associated with the obese phenotype in both humans and mice [35,
363 36]. In our experiment, the HS diet (HS and HS+FG groups) did not affect the
364 populations of the phylum Firmicutes in the gut microbiota of WKY rats while it

365 triggered a slight increase in Bacteroidetes that was more evident in the supplemented
366 group (Table 3). This is consistent with the absence of weight gain differences between
367 the STD, HS and HS+FG groups (Table 1). The populations of Enterobacteriales, and
368 particularly *E. coli*, were higher ($P < 0.05$) in the HS group than the STD group (Table
369 3). Previously, we have suggested elsewhere that this may be connected to an increase
370 in uric acid excreted via feces, which would foster the proliferation of Enterobacteriales
371 [16]. The inhibitory activity of D-fagomine on epithelial adhesion of *E. coli* [15] would
372 therefore explain the increase in excreted Enterobacteriales in the supplemented group
373 (Table 3).

374 In conclusion, D-fagomine counteracted sucrose-induced fatty liver and elevated blood
375 pressure in rats. The maintenance of DAG levels in the liver, though not in VAT,
376 together with the effect of lowering uric acid concentrations suggest that D-fagomine
377 selectively influences liver function, probably by reducing the levels of postprandial
378 fructose as a result of its inhibitory activity on intestinal sucrase. The selective action of
379 D-fagomine also suggests that fat deposition in VAT is not a direct consequence of liver
380 DNL in rats fed an excess of sucrose. As D-fagomine has been proven to lower
381 postprandial blood glucose/fructose levels in healthy volunteers (sucrose loading test,
382 clinical trial accessible at https://clinicaltrials.gov/ct2/show/NCT01811303), it is
383 reasonable to expect a protective action against in humans consuming an excess of
384 sucrose. The amount of D-fagomine consumed daily by the rats in this study (ca. 98 mg
385 kg^{-1} body weight) would translate to 15.5 mg kg^{-1} in humans by following the
386 conversion proposed by Reagan-Shaw et al. [37]. This dose is higher than the amount
387 provided by a diet rich in buckwheat-based foodstuffs [14] therefore, it should be
388 reached by dietary supplementation. The present results, together with the previous
389 report of the action on fat-induced low-grade inflammation, IR and IGT, are suggesting

that D-fagomine may be effective at preventing MetS by acting on different risk factors triggered by different components of unhealthy Westernized diets, such as excessive intake of saturated fat and refined sugar.

Acknowledgments: This work was supported by the Spanish Ministry of Economy, Industry and Competitiveness (grant numbers AGL2013-49079-C2-1,2-R and AGL2017-83599-R, and graduate fellowship BES2014-068592 to M.H.). We thank Eva Dalmau for the DAG analysis. English language revision by Christopher Evans is also appreciated.

Author contributions: S.R.-R., I.M., M.R. and J.L.T. conceived and designed the research; S.R.-R. and M.H. supervised and performed the animal intervention, the biometric determinations, evaluation of glycemic status and the qRT-PCR experiments; L.A. performed the histology; B.M-P. and M.R. evaluated lipid status; S.R.-R. and J.C. performed the DAG determinations; M.H. and S.A. performed the IsoP experiment; and S.R.-R. and J.L.T. analyzed the data and wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

5. References

[1] R. H. Eckel, K. G. M. M. Alberti, S. M. Grundy, P. Z. Zimmet, *Lancet,* **2010**, *375*, 181.
[2] J. P. Despres, I. Lemieux, *Nature,* **2006**, *444*, 881.
[3] S. D. Mittelman, G. W. Van Citters, E. L. Kirkman, R. N. Bergman, *Diabetes,* **2002**, *51*, 755.
[4] P. J. Havel, *Nut. Rev.,* **2005**, *63*, 133.
[5] T. Matsuzaka, H. Shimano, N. Yahagi, M. Amemiya-Kudo, H. Okazaki, Y. Tamura, Y. Iizuka, K. Ohashi, S. Tomita, M. Sekiya, A. Hasty, Y. Nakagawa, H. Sone, H. Toyoshima, S. Ishibashi, J. Osuga, N. Yamada, *Diabetes,* **2004**, *53*, 560.
[6] G. A. Cook, D. A. Otto, N. W. Cornell, *Biochem. J. ,* **1983**, *212*, 525.
[7] K. L. Stanhope, P. J. Havel, *Curr. Opin. Lipidol.,* **2008**, *19*, 16.
[8] B. Vogt, M. Bochud, M. Burnier, *Semin. Nephrol.,* **2007**, *27*, 529.
[9] R. J. Grekin, A. P. Vollmer, R. S. Sider, *Hypertension,* **1995**, *26*, 193.
[10] M. Mazzali, M. Kanbay, M. S. Segal, M. Shafiu, D. Jalal, D. I. Feig, R. J. Johnson, *Curr. Rheumatol. Rep.,* **2010**, *12*, 108.
[11] S. Amézqueta, E. Galán, I. Vila-Fernández, S. Pumarola, M. Carrascal, J. Abian, L. Ribas-Barba, L. Serra-Majem, J. L. Torres, *Food Chem.,* **2013**, *136*, 1316.
[12] E. Molinar-Toribio, J. Pérez-Jiménez, S. Ramos-Romero, L. Gómez, N. Taltavull, M. R. Nogués, A. Adeva, O. Jaúregui, J. Joglar, P. Clapés, J. L. Torres, *Food Funct.,* **2015**, *6*, 2614.
[13] S. Ramos-Romero, M. Hereu, L. Atienza, J. Casas, N. Taltavull, M. Romeu, S. Amézqueta, G. Dasilva, I. Medina, J. L. Torres, *Mol. Nutr. Food Res. ,* **2018**, *62*, e1800373.
[14] S. Amézqueta, S. Ramos-Romero, C. Martínez-Guimet, A. Moreno, M. Hereu, J. Lluis Torres*,* **2017**.
[15] L. Gómez, E. Molinar-Toribio, M. Á. Calvo-Torras, C. Adelantado, M. E. Juan, J. M. Planas, X. Cañas, C. Lozano, S. Pumarola, P. Clapés, J. L. Torres, *Br. J. Nutr.,* **2012**, *107*, 1739.
[16] S. Ramos-Romero, M. Hereu, L. Atienza, J. Casas, O. Jáuregui, S. Amézqueta, G. Dasilva, I. Medina, M. R. Nogués, M. Romeu, J. L. Torres, *Am. J. Physiol. Endocrinol. Metab.,* **2018**, *314*, E552.
[17] S. S. Choe, J. Y. Huh, I. J. Hwang, J. I. Kim, J. B. Kim, *Front. Endocrinol.,* **2016**, *7*, 30.
[18] L. Méndez, M. Pazos, J. M. Gallardo, J. L. Torres, J. Pérez-Jiménez, R. Nogués, M. Romeu, I. Medina, *Free Radic. Biol. Med.,* **2013**, *55*, 8.
[19] M. W. Pfaffl, *Nucleic Acids Res.,* **2001**, *29*, e45.
[20] N. Taltavull, M. Muñoz-Cortés, L. Lluís, M. Jové, À. Fortuño, E. Molinar-Toribio, J. L. Torres, M. Pazos, I. Medina, M. R. Nogués, *Lipids Health Dis.,* **2014**, *13*, 31.
[21] F. Simbari, J. McCaskill, G. Coakley, M. Millar, R. M. Maizels, G. Fabriás, J. Casas, A. H. Buck, *J. Extracell. Vesicles,* **2016**, *5*, 30741.
[22] E. Fabbrini, F. Magkos, B. S. Mohammed, T. Pietka, N. A. Abumrad, B. W. Patterson, A. Okunade, S. Klein, *Proc. Natl. Acad. Sci. U. S. A.,* **2009**, *106*, 15430.
[23] J. J. DiNicolantonio, V. Mehta, N. Onkaramurthy, J. H. O'Keefe, *Prog. Cardiovasc. Dis. ,* **2018**, *61*, 3.
[24] M. W. Schwartz, S. C. Woods, D. Porte, R. J. Seeley, D. G. Baskin, *Nature,* **2000**, *404*, 661.
[25] S. Blueher, C. S. Mantzoros, *Am. J. Clin. Nutr.,* **2009**, *89*, 991S.

[26] A. Lindqvist, C. D. de la Cour, A. Stegmark, R. Hakanson, C. Erlanson-Albertsson, *Regul. Pept.,* **2005**, *130*, 123.
[27] P. W. Franks, S. Brage, J. Luan, U. Ekelund, M. Rahman, I. S. Farooqi, I. Halsall, S. O'Rahilly, N. J. Wareham, *Obes. Res.,* **2005**, *13*, 1476.
[28] R. B. S. Harris, *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **2018**, *314*, R598.
[29] J. J. DiNicolantonio, J. H. O'Keefe, *Prog. Cardiovasc. Dis.,* **2016**, *59*, 219.
[30] C. Oudot, A. D. Lajoix, B. Jover, C. Rugale, *Kidney Int.,* **2013**, *83*, 674.
[31] L. T. Tran, V. G. Yuen, J. H. McNeill, *Moll. Cell Biochem.,* **2009**, *332*, 145.
[32] G. Van den Berghe, *Prog. Biochem. Pharmacol.,* **1986**, *21*, 1.
[33] F. Leyva, S. Anker, J. W. Swan, I. F. Godsland, C. S. Wingrove, T. P. Chua, J. C. Stevenson, A. J. S. Coats, *Eur. Heart J.,* **1997**, *18*, 858.
[34] A. F. Janssen, S. Kersten, *J. Physiol. ,* **2017**, *595*, 477.
[35] R. E. Ley, P. J. Turnbaugh, S. Klein, J. I. Gordon, *Nature,* **2006**, *444*, 1022.
[36] P. J. Turnbaugh, F. Baeckhed, L. Fulton, J. I. Gordon, *Cell Host Microbe,* **2008**, *3*, 213.
[37] S. Reagan-Shaw, M. Nihal, N. Ahmad, *FASEB J.,* **2008**, *22*, 659.

Tables

Table 1.- Feed, drink, and energy intake; and body and perigonadal AT weights of WKY rats fed the different diets for 24 weeks. Also, residual excreted energy in feces after 20 weeks of intervention.

	STD		HS		HS+FG	
	Mean	SEM	Mean	SEM	Mean	SEM
Feed intake (g per day per 100 g body weight)	5.4	0.2	2.8*	0.1	2.8*	0.1
Drink intake (mL per day per 100 g body weight)	7.4	0.2	10.5*	0.2	9.5*	0.2
Fructose intake (g per day per 100 g body weight)	–[a]		1.4	0.0	1.4	0.2
Total energy intake (kcal per day per 100 g body weight)	15.6	0.4	22.6*	0.5	21.5*	0.5
Energy in feces kcal °C g^{-1}[b]	310	20	290	20	260	20
Body weight (g)	380	10	370	10	390	10
Perigonadal adipose tissue weight (g)	8	0	13***	1	12**	1

Data are presented as means with their standard errors of the mean; n = 9 per group. Comparisons were performed using one-way ANOVA and Tukey's multiple-comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs* STD group

[a] Fructose intake as part of the 2014 Teklad Global feed is negligible compared to that in the HS diets.

[b] Integrated STDA signal (proportional to energy.

Table 2.- Fasting glucose and insulin, lipid profile and leptin in plasma of WKY rats fed different diets for 24 weeks.

	STD		HS		HS+FG	
	Mean	SEM	Mean	SEM	Mean	SEM
Fasting glucose (mg dL^{-1})	69	2	65	3	62*	1
Fasting insulin (ng mL^{-1})	0.4	0.1	0.8*	0.2	1.3*	0.5
Leptin (ng mL^{-1})	2.5	0.3	5.4***	0.6	3.9	0.4
Total cholesterol (mg dL^{-1})	135	4	132	3	132	2
HDL cholesterol (mg dL^{-1})	49	1	54*	2	53*	1
LDL cholesterol (mg dL^{-1})	23	2	21	1	22	1
Triglycerides (mg dL^{-1})	60	6	90	10	110*	10

Data are presented as means with their standard errors of the mean; n = 9 per group. Comparisons were performed using one-way ANOVA and Tukey's multiple-comparison test. * $P < 0.05$, and *** $P < 0.001$ *vs* STD group

Table 3.- Excreted intestinal bacteria (% copies per total bacteria) from rats fed different diets for 24 weeks.

	STD		HS		HS+FG	
	Mean	SEM	Mean	SEM	Mean	SEM
Bacteroidetes	30	3	80**	10	110***	10
Firmicutes	37	4	57	10	38	2
Bacteroidales	40	4	60*	6	73***	5
Clostridiales	12	2	15	2	14	2
Enterobacteriales	0.01	0.00	0.07*	0.03	0.14***$^{\delta}$	0.02
E. coli	0.004	0.003	0.040*	0.012	0.135***$^{\delta}$	0.032

Data are presented as means with their standard errors of the mean; n = 9 per group. Comparisons were performed using one-way ANOVA and Tukey's multiple-comparison test. * $P < 0.05$, and *** $P < 0.001$ *vs* STD group; δ $P < 0.05$ *vs* HS group

Figure 1. Time course of plasma glucose concentration after administration of a single dose of glucose (1 g per kg body weight) to WKY rats fed a standard (STD), high-sucrose (HS), or high-sucrose supplemented with D-fagomine (HS+FG) diet for 13 (A) and 21 (B) weeks.

Values are means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests or two-way ANOVA. ** $P < 0.01$ and *** $P < 0.001$ *vs* STD group.

Figure 2. Systolic (A) and diastolic (B) blood pressure, and uric acid in urine (C) and plasma (D) in rats fed a standard (STD, ○), high-sucrose (HS, □), or high-sucrose with D-fagomine (HS+FG, ■) diet for 24 weeks. Results are represented as means with their standard errors.

Comparisons were conducted using one-way ANOVA and Tukey's multiple-comparison test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ *vs* STD group; δ $P < 0.05$, δδ $P < 0.01$ *vs* HS group.

Figure 3. 5-F_{2t} isoprostane (A) and 15-F_{2t} isoprostane (B) in urine from WKY rats fed a standard (STD), high-sucrose (HS), or high-sucrose with D-fagomine (HS+FG) diet for 24 weeks of nutritional intervention.

Results are represented as means with their standard errors. Comparisons were conducted using one-way ANOVA and Tukey's multiple-comparison test. * $P < 0.05$ *vs* STD group

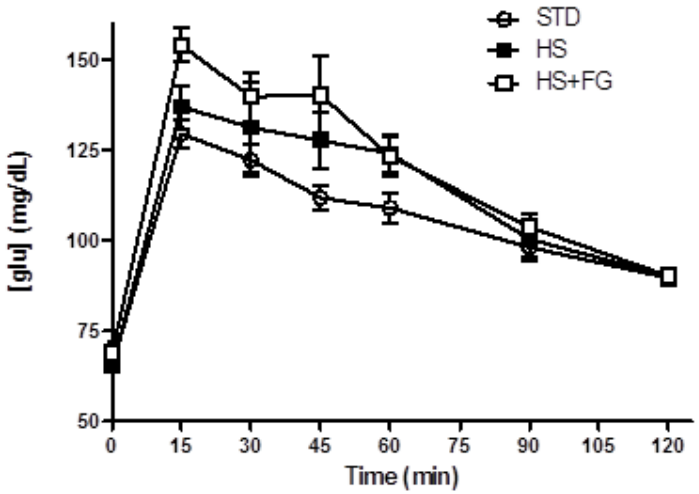
Figure 4. Histological sections (hematoxylin/eosin stained) from the livers of WKY rats fed a standard (STD) (A, 10x), high-sucrose (HS) (B, 10x), or high-sucrose supplemented with D-fagomine (HS+FG) (C, 10x) diet for 24 weeks and estimation of steatosis (D) and lipogranuloma (E).

Values are in frequencies (percentage of animals that present the value, or do not). Comparisons were performed using χ^2 statistics. ** $P < 0.01$ and *** $P < 0.001$ *vs* STD group; δδδ $P < 0.001$ *vs* HS group.

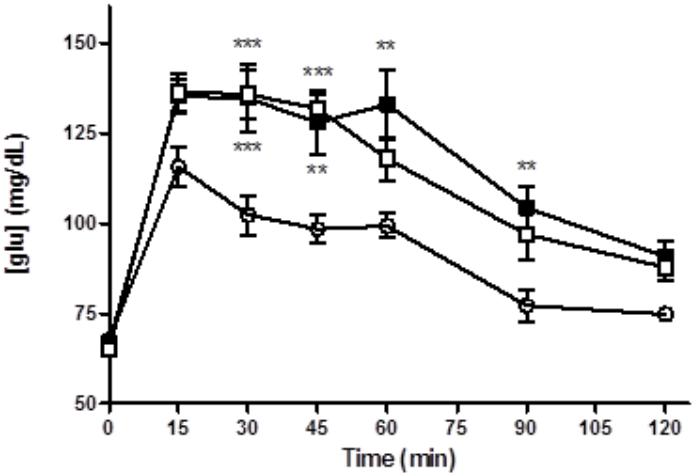
Figure 5. Hepatic levels of DAG 32:1 (A), DAG 32:2 (B), DAG 34:1 (C) and DAG 36:2 (D) in WKY rats fed a standard (STD), high-sucrose (HS), or high-sucrose supplemented with D-fagomine (HS+FG) diet for 24 weeks.

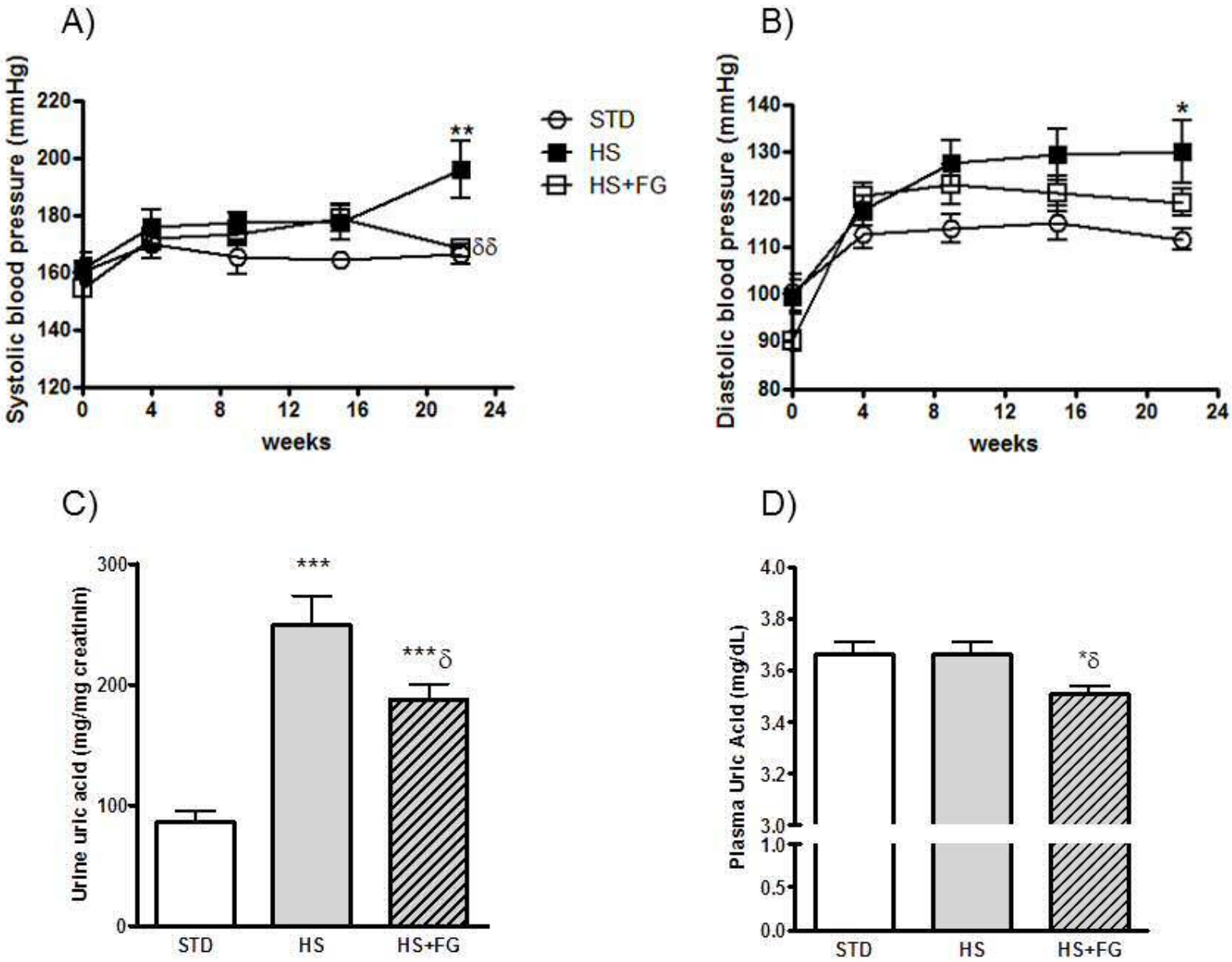
Values are means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests. * $P < 0.05$ *vs* STD group.

A) week 13

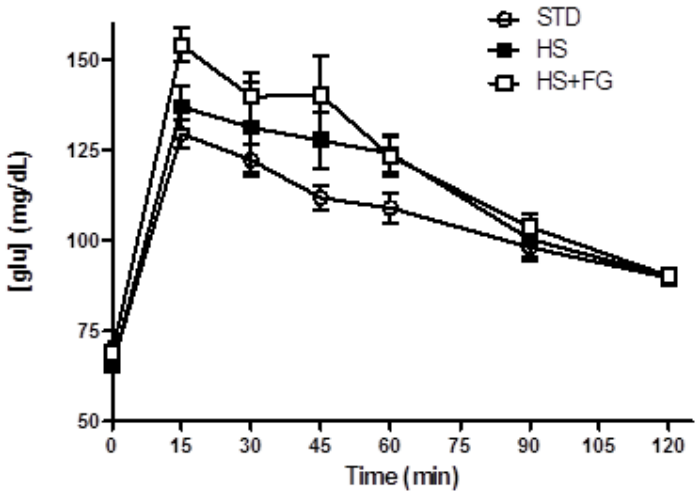


B) week 21

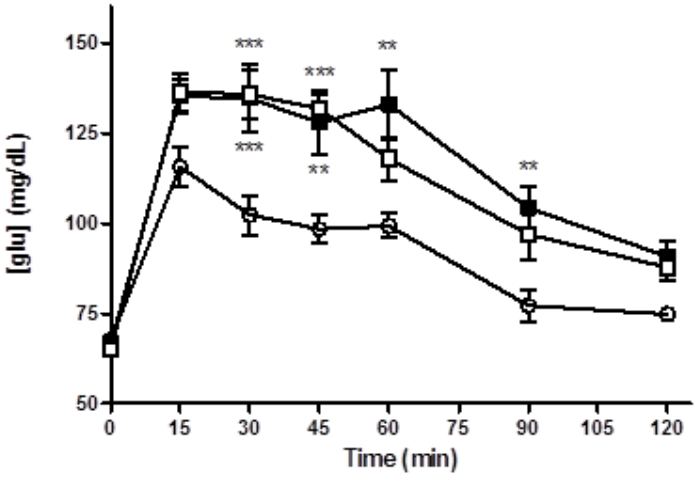


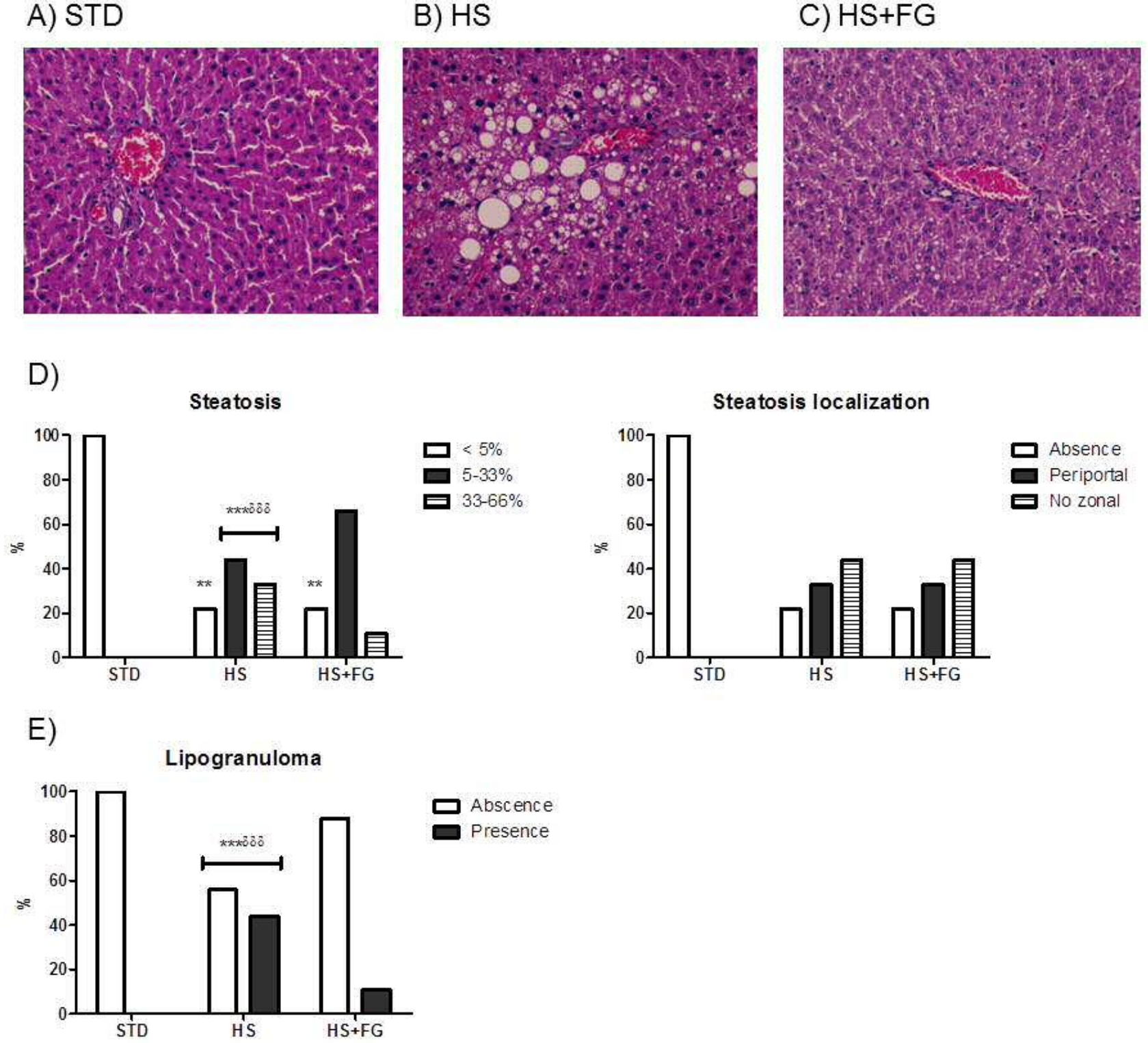

A)
Systolic blood pressure (mmHg)
220
200
180
160
140
120
0
4
8
12
16
20
24
weeks
**
δδ
STD
HS
HS+FG
B)
Diastolic blood pressure (mmHg)
140
130
120
110
100
90
80
0
4
8
12
16
20
24
weeks
*
C)
Urine uric acid (mg/mg creatinin)
300
200
100
0
***
***δ
STD
HS
HS+FG
D)
Plasma Uric Acid (mg/dL)
4.0
3.8
3.6
3.4
3.2
3.0
1.0
0.5
0.0
*δ
STD
HS
HS+FG

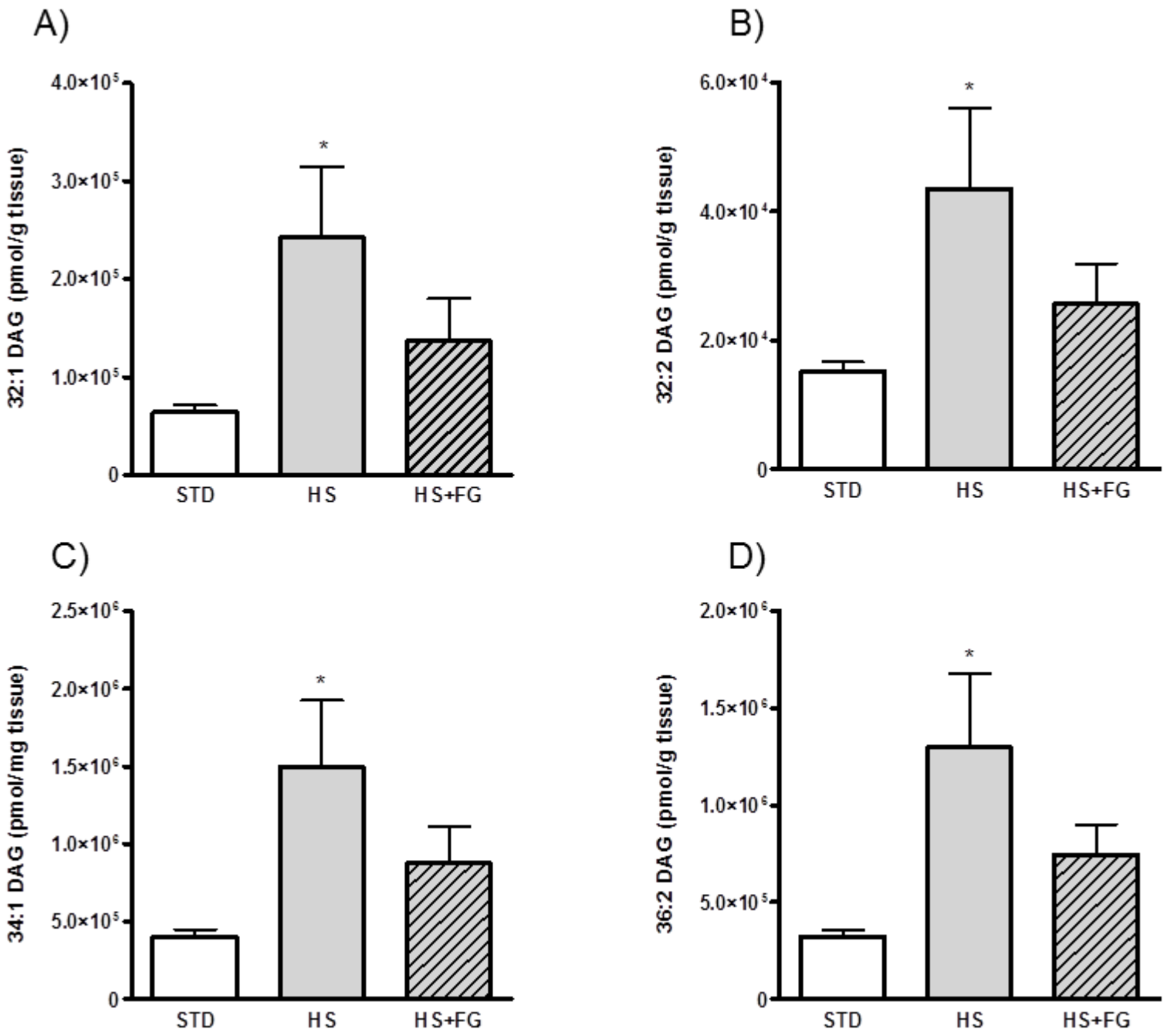
A) week 13



B) week 21




A) STD
B) HS
C) HS+FG
D)
Steatosis
100
80
60
40
20
0
%
***δδδ
**
**
STD
HS
HS+FG
< 5%
5-33%
33-66%
Steatosis localization
Absence
Periportal
No zonal
E)
Lipogranuloma
***δδδ
Abscence
Presence


A)
32:1 DAG (pmol/g tissue)
4.0×10⁵
3.0×10⁵
2.0×10⁵
1.0×10⁵
0
*
STD
HS
HS+FG
B)
32:2 DAG (pmol/g tissue)
6.0×10⁴
4.0×10⁴
2.0×10⁴
0
*
STD
HS
HS+FG
C)
34:1 DAG (pmol/mg tissue)
2.5×10⁶
2.0×10⁶
1.5×10⁶
1.0×10⁶
5.0×10⁵
0
*
STD
HS
HS+FG
D)
36:2 DAG (pmol/g tissue)
2.0×10⁶
1.5×10⁶
1.0×10⁶
5.0×10⁵
0
*
STD
HS
HS+FG