Accepted Manuscript

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PII: S0955-2863(16)30333-3
DOI: doi: [10.1016/j.jnutbio.2017.04.008](https://doi.org/10.1016/j.jnutbio.2017.04.008)
Reference: JNB 7769

To appear in: *The Journal of Nutritional Biochemistry*

Received date: 2 August 2016
Revised date: 7 January 2017
Accepted date: 11 April 2017

Please cite this article as: Díaz-Rúa Rubén, Keijer Jaap, Palou Andreu, van Schothorst Evert M., Oliver Paula, Long-term intake of a high protein diet increases liver triacylglycerol deposition pathways and hepatic signs of injury in rats, *The Journal of Nutritional Biochemistry* (2017), doi: [10.1016/j.jnutbio.2017.04.008](https://doi.org/10.1016/j.jnutbio.2017.04.008)

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Long-term intake of a high protein diet increases liver triacylglycerol deposition pathways and hepatic signs of injury in rats

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Running title: High protein diet and liver fat deposition

Financial support: This work was supported by the Spanish Government (Ministerio de Economía y Competitividad, INTERBIOBES AGL2015-67019-P) and by the EU FP7 project (BIOCLAIMS -FP7-244995-). RD-R was recipient of a fellowship from the Spanish Government. CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative of the ISCIII.

Conflicts of interest: None.

Abstract

Intake of high protein (HP) diets has increased over the last years, mainly due to their popularity for body weight control. Liver is the main organ handling ingested macronutrients and it is associated with the beginning of different pathologies. We aimed to deepen our knowledge on molecular pathways affected by long-term intake of a HP diet. We performed a transcriptome analysis on liver of rats chronically fed with a casein-rich HP diet and analysed molecular parameters related to liver injury. Chronic increase in the dietary protein/carbohydrate ratio up-regulated processes related with amino acid uptake/metabolism and lipid synthesis, promoting a molecular environment indicative of hepatic triacylglycerol (TG) deposition. Moreover, changes in expression of genes involved in acid-base maintenance and oxidative stress indicate alterations in the pH balance due to the high acid load of the diet, which has been linked to liver/health damage. Up-regulation of immune-related genes was also observed. In concordance with changes at gene expression level, we observed increased liver TG content, and increased serum markers of hepatic injury/inflammation (aspartate transaminase, C-reactive protein and TNF- α). Moreover, the HP diet strongly increased hepatic mRNA and protein levels of HSP90, a marker of liver injury. Thus, we show for the first time that long-term consumption of a HP diet, resulting in a high acid load, results in a hepatic transcriptome signature reflecting increased TG deposition and increased signs of health risk (increased inflammation, alterations in the acid-base equilibrium and oxidative stress). Persistence of this altered metabolic status could have unhealthy consequences.

Key words: liver steatosis, liver disease, hyperproteic diet, acid load, microarray analysis

1. Introduction

Obesity and related comorbidities, such as metabolic syndrome and cardiovascular diseases, are a major public health concern in Western countries. Intake of diets with a high percentage of protein replacing carbohydrates is one of the main dietary strategies for weight loss purposes and also to maintain normal body weight [1, 2]. In fact, intake of high protein (HP) diets as daily dietary practice and/or as part of a life style pattern is increasing [3]. HP diets have been associated with body weight loss and with positive metabolic effects, such as control and normalization of some risk factors related to metabolic syndrome, like hyperglycaemia, hypercholesterolemia and hypertriglyceridemia, in obese people [2]. However, there is a scientific controversy on the effects of long term-consumption of these diets, and it has recently been reported that high protein intake is related with a higher risk of weight gain and with increased risk of fatal and non-fatal outcomes [4]. Although there are many human and animal studies focused on the physiological, biochemical and/or pathological effects induced by specific nutrients and dietary factors, there have been relatively few studies investigating safety and potential adverse effects of HP diets. Potential deleterious effects of these diets may be associated with the nonspecific effects of individual amino acids and/or generated metabolites in the different steps of amino acid metabolism [5], as well as to alterations in the acid-base balance due to increased dietary acid load [6]. There is evidence indicating that HP diets could be related to impairment of kidney and liver function [7, 8], but diets with a high protein content are generally considered safe and healthy in subjects without any pathology [9].

Liver is a key tissue involved in dietary nutrient handling. Metabolic alterations in this organ resulting from the intake of unbalanced diets constitute a starting point for numerous

pathologies [10]. Thus, molecular studies in liver are very useful for understanding metabolic effects generated by the intake of diets with an altered macronutrient composition. Furthermore, transcriptome analysis using whole genome microarrays can provide an unbiased overview at the molecular level of alterations that can occur [11]. For this reason, we assessed the global effects of HP diets on liver gene expression in healthy adult male Wistar rats to better understand the long term effects of such diets on health.

2. Materials and methods

2.1. Animals. All animal experimental procedures followed in this study were reviewed and approved by the Bioethical Committee of the University of the Balearic Islands, and guidelines for the use and care of laboratory animals of the University were followed. 6-month-old male Wistar rats (Charles River Laboratories España, SA, Barcelona, Spain) single-housed at 22° C with a period of light/dark of 12 hours (light on at 8:00 pm) were examined. At the age of two months, the animals were stratified on body weight into two groups: a control group (n=7) and a pair-fed high-protein group (HP, n=6), as part of a larger animal study [12]. Sample size was selected based on similar experimental designs, which show that this size is adequate to detect statistical differences. Control animals were fed a normolipidic diet (D12450B, Research Diets) containing 70% of energy (kcal) from carbohydrates, 10% from fats and 20% from proteins. HP animals were fed a high protein diet (Research Diets) containing 45% of energy from carbohydrates, 10% from fats and 45% from proteins (mainly casein). Diets (detailed composition in Table 1) were purchased from Brogaarden (Gentofte, Denmark) and were given isocalorically for 4 months. Food intake was recorded daily to calculate the daily and cumulative caloric intake; body weight was recorded three times a week. Our HP-fed animals ingested 2.3 times higher amount of protein than controls; this overconsumption is similar to the extent of overconsumption observed for humans following high-protein weight loss diets [13]. Animals were sacrificed in the fed state at the beginning of the light cycle by decapitation and truncal blood was collected from the neck to collect serum. Liver was rapidly removed, weighed, snap-frozen in liquid nitrogen, and stored at -70°C until further analysis. One week prior to sacrifice animals were submitted to nocturnal 14-h fasting to collect serum in fasted conditions to analyse the HOMA-IR score using the formula of Matthews *et al.* [14].

2.2. Estimation of dietary acid load. The estimation of cumulative dietary acid load was calculated based on the diet composition as shown in Table 1, using two different approaches: estimated net endogenous acid production (NEAP) from the potassium and protein content of the diet according to the formula of Frassetto *et al.* [NEAP (mEq/d) = (54.5 x protein (g/d) / potassium (mEq/d)) - 10.2] [15, 16], and the estimated diet-induced potential renal acid load (PRAL) directly calculated from dietary intakes applying the formula reported by Remer *et al.* [PRAL (mEq/d) = 0.49 x protein (g/d) + 0.037 phosphorus (mg/d) - 0.021 potassium (mg/d) - 0.026 magnesium (mg/d) - 0.013 calcium (mg/d)] [17].

2.3. Quantification of circulating parameters. Circulating glucose was measured using an Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). ELISA kits were used to measure serum insulin (DRG Instruments, Marburg, Germany), TNF-alpha (R&D Systems Europe, Minneapolis, MN, USA), and C-reactive protein levels (eBioscience, Affymetrix, San Diego, CA, USA). Other serum parameters were measured using the following enzymatic kits: non-esterified free fatty acids (NEFA) (Wako Chemicals, Neuss, Germany), triacylglycerols (TG) (Sigma Diagnostics, Madrid, Spain), total circulating cholesterol and urea (BioSystems, Barcelona, Spain), and aspartate transaminase (AST) (Bioo Scientific, Austin, TX, USA).

2.4. Determination of lipids and glycogen in liver. Lipid extracts were obtained from liver as previously described [18] and used to measure triacylglycerol content using the Serum Triglyceride Determination Kit (Sigma Diagnostics), lipid content using the Folch method [19] and glycogen content using an anthrone method [20].

2.5. Liver histological analysis and oil red O staining. Liver tissue samples were embedded in paraffin blocks for light microscopy and 5 µm thick sections were mounted on slides and

counterstained with haematoxylin/eosin. To determine the presence of hepatic steatosis we analysed the entire area ($\sim 2 \text{ cm}^2$) of histological stained liver sections from animals of the different conditions, and searched for the presence of fatty vesicles. For lipid highlight, 15 μm cryostat sections were fixed 1 hour in 4% paraformaldehyde, washed in distilled water and stained with Oil Red O Stain Kit (Abcam, UK) following manufacturer's instructions. Briefly, sections were incubated with oil red O during 6 min, washed in distilled water, counterstained with Mayer hematoxylin and mounted in aqueous medium (Mount Quick aqueous, Bio-Optica, Italy). All groups were examined in a blind manner.

2.6. Total RNA isolation. Total RNA from liver samples was extracted using Tripure Reagent (Roche Diagnostic, Barcelona, Spain) and purified with Qiagen RNeasy Mini Kit spin columns (Izasa SA, Barcelona, Spain). RNA yield was quantified on a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies Wilmington, Delaware, USA) and its integrity was measured on an Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, United Kingdom).

2.7. Microarray processing. For the microarray analysis, a total of 13 individual liver RNA samples from the control (n=7) and HP (n=6) groups were used, which were part of a larger set of dietary groups that were simultaneously processed. Samples were randomized before hybridization and all arrays were hybridized simultaneously. Labelling and microarray processing (hybridisation, washing, and scanning) were performed as previously described [21]. Briefly, one μg RNA of each sample was reverse transcribed, half was labelled with Cy5, the other half with Cy3. After purification and quantification, Cy3 labelled cRNA samples were pooled on an equimolar basis. Each individual Cy5-labelled sample was hybridized with Cy3 pool on 4x44K G4131F rat whole genome Agilent microarrays (Agilent Technologies).

2.8. Microarray data analysis. Quantification and quality control of the array data was performed as previously described [22]. In total, about 54% of all probes were considered to be expressed (22,765 out of 41,012 probes). The data were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE57858. Statistical differences between the HP group vs the control group was assessed by Student's-*t* test in GeneMaths XT. Fold change equals HP/control ratio in the case of increase or equals -1/ratio in the case of decrease. For pathways analysis using Metacore (Thomson Reuters, St. Joseph, Michigan, USA), a threshold of $p < 0.001$ was selected. Subsequently, a statistically generated list of genes was manually analysed in regard to their biological information, obtained with the use of available databases (Rat Genome Database (RGD), Genecards, KEGG, NCBI, Reactome, UniProt, USCN, WikiPathways, and PubMed) based on key biological domains, such as molecular function and biological process. Some of these processes overlapped, thus they were collected, renamed and all the unique genes were assigned into several biological processes according to their function. We manually supplemented the significantly enriched biological processes with non-annotated genes from the selected gene-set using biological databases (Biocarta, SOURCE, GenMAPP, and KEGG) and scientific literature. As processes overlap, we bundled some processes and renamed them. In some cases we annotated probes manually in order to have the best annotated list of probes.

2.9. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. In order to validate the microarray data analysis, real-time RT-PCR was used to measure mRNA expression levels in liver samples of animals of the control and HP groups. Genes analysed were: *Cps1*, *Gnpat*, *Got1*, *Gpt*, *Gstm4*, *Hsp90*, *Kcnma1*, *Slc4a2*, *Slc43a1*, *Slc25a21* and *Tpi1* using conditions as previously described [23]. Primers were obtained from Sigma

Genosys (Sigma Aldrich Química S.A., Madrid, Spain); sequences are provided as Supplementary Table. Data were normalized against *β-actin*, a well-known reference gene and *Gdi1* which we have previously identified as a good constitutive gene based on microarray studies; both reference genes showed equal and high expression in all microarrays of the two experimental groups.

2.10. Western blot analysis. Two selected proteins, HSP90 and GPT/ALT were analysed by Western blot in liver using 30 µg of total protein per lane in a 4–20% Criterion TGX Precast Gel (Bio-Rad, Madrid, Spain). Membranes were incubated during one hour with a rabbit monoclonal anti-HSP90 antibody (#4877, Cell Signaling Technology, Danvers, MA, USA) or overnight with a rabbit polyclonal anti-GPT antibody (HPA031059, Sigma-Aldrich, Madrid, Spain). Antibodies were diluted 1:1,000 and 1:250 respectively in 0.02 M Tris-buffered saline and 0.1% Tween-20. HRSP12 antibody (Catalog number: HPA022856, Sigma-Aldrich) was used as transfer and loading control; this protein was selected based on the results of gene expression in our microarray analysis. Infrared-dyed secondary anti-IgG antibodies (LI-COR Biosciences, Lincoln, NE, USA) were used, membranes were scanned in Odyssey Imager (LI-COR Biosciences) and bands were quantified using the software Odyssey V3.0 (LI-COR Biosciences).

2.11. Immunohistochemistry analysis of HSP90 in liver. Five-micrometers sections of formalin-fixed, paraffin-embedded liver samples from the control and HP groups were pretreated in a microwave oven at 700W with 0.01 M citrate buffer (pH 6) for 20 min, incubated with 5% H₂O₂ in water for 10 min to block endogenous peroxidase, and then immunostained by means of the avidin-biotin technique [24], as previously described [23]. The HSP90 antibody (#4877, Cell Signaling Technology) was diluted 1:200 in

SignalStain® Antibody Diluent (Cell Signaling Technology); incubation was overnight at 4°C. Murine epididymis was used as positive control (according to [25]).

2.12. Statistical analysis. Data of body weight, adiposity, serum parameters and the confirmatory results of the microarray data are expressed as the mean $\pm$ SEM. Differences between groups were analysed using Student's-*t* test. The test used for each comparison is specified in the footnote of the specific figures and tables. Data followed normal distribution. All the analyses were performed with SPSS for windows (version 20, SPSS, Chicago, IL, USA). Threshold of significance was defined at $p < 0.05$ or indicated when different. The statistical analysis of the microarray data is in the microarray data analysis section.

3. Results

3.1. Estimated dietary acid load

Diet is a major determinant of the acid load that must be excreted by the kidney to maintain acid-base balance [26]. The estimated dietary acid load is an indirect measure of the contribution of diet to endogenous acid production [6], which was calculated based on the cumulative intake of the two studied diets over the whole experimental period. The two parameters of estimated dietary acid load, NEAP and PRAL, were around 2 times higher in HP-fed animals than in animals fed the control diet (Table 2A).

3.2. Body weight, adiposity and serum parameters

Data of body weight, adiposity, and serum parameters of the experimental model used in this study have been published previously [12], and are summarized in Table 2A. In brief, compared to rats on a control diet, HP-fed animals showed a lower cumulative food intake and lower body weight in comparison to controls and, although the size of subcutaneous adipocytes was decreased (data not shown), this lower body weight was not related to decreased adiposity. In addition, HP-fed animals presented lower serum cholesterol levels, and were apparently healthy according to parameters related to metabolic syndrome: no differences were found in circulating NEFA or TG in comparison to controls. Remarkably, HP-fed animals displayed increased circulating insulin, but this was not related to insulin resistance as evidenced by HOMA-IR index or to alterations in glycaemia. Serum TNF- α levels, measured as a marker of inflammation, were increased in HP group, as were serum urea levels, in accordance to the higher protein content of the diet. In addition to these previously reported serum parameters, two additional serum markers were analysed as indicators of hepatic dysfunction (Table 2A): serum C-reactive protein and AST; both of them were increased in the HP group.

3.3. Liver composition

As shown in Table 2B, animals of the HP group presented 75% higher TG levels ($p=0.06$, Student's-*t* test) in liver. However, total lipid content was not increased and no clear sign of macrovesicular lipid accumulation was yet evident at morphological level when analysing paraffin-embedded haematoxylin/eosin-stained tissue sections, although disperse intracellular fat microvesicles were observed in oil red O stained sections (data not shown). Although liver weight was not affected, increased liver weight was observed when expressed as percentage of total body weight. Hepatic glycogen content was not affected by the dietary treatment.

3.4. Hepatic transcriptomics analysis

We used transcriptomics of liver to elucidate potential metabolic changes induced by the HP diet. Our microarray analysis showed that hepatic gene expression was affected by chronic intake of a HP diet. A total of 148 probes, representing 133 unique genes (110 known and 23 unknown) were differentially expressed between the HP and the control group (considering $p < 0.001$, Student's *t*-test). Of the known genes, the major part, 84%, were up-regulated, and only 16% were down-regulated.

As could be expected, regulation of amino acid metabolism by HP diet appeared as the most affected pathway using MetaCore analysis (Table 3). MetaCore allows the same gene to be included in different metabolic pathways. To gain specificity, we subsequently manually classified the 110 unique known genes affected by the HP diet, with each individual gene being included in one metabolic process only. This classification resulted in 10 different processes: again amino acid metabolism was the most affected process (18 up-regulated genes), followed by signal transduction, gene expression, transport, cell cycle, lipid synthesis, immune response, oxidative stress/detoxification, cell communication, and

carbohydrate metabolism (Figure 1). A small subset of genes could not be classified as they were involved in other biological processes (n=23) or had an unknown role (n=23).

The most relevant (i.e. informative) genes that were affected by the intake of a HP diet are detailed in Table 4. HP diet affected mainly the expression of genes related to amino acid metabolism; all of them were up-regulated. These genes encoded mainly for transport proteins involved in importing amino acids into the cell, deamination and transamination of amino acids, and urea cycle. Moreover, five of the ten most regulated genes belong to these processes (data not shown). Genes related to lipid synthesis were mainly involved in TG metabolism (included TG synthesis), while carbohydrate metabolism genes were related to gluconeogenesis/glyceroneogenesis. The results also showed substantial changes in genes coding to protein transport proteins involved in dicarboxylic acids transport, acid-base equilibrium and potassium homeostasis. Other relevant processes affected by HP diet were oxidative stress, detoxification, and inflammation. All these processes will be discussed in detail. In addition, the interconnection of the HP-regulated genes involved in amino acid, glycerol, and lipid metabolism are shown schematically in Figure 2.

3.5. Confirmation of array results by real-time RT-PCR and Western blot analysis

Real-time RT-PCR analysis was performed to substantiate the microarray data focusing on the most prominent processes. A total of 10 genes were verified (Figure 3A and 3B): 3 genes related to amino acid metabolism, one related to glyceroneogenesis, one related to TG biosynthesis, one to oxidative stress and 3 genes related to transport processes. These genes followed the same pattern as observed in the microarray analysis. Glutamic-pyruvate transaminase (GPT), also known as alanine aminotransferase (ALT), has a key role in amino acid metabolism and was selected for confirmation of microarray data at the protein

level. Indeed, Western blot analysis showed higher levels of GPT/ALT in the HP group (Figure 3C).

3.6. Western blot and immunohistochemical analysis of HSP90 in liver

When considering all the genes that were differentially expressed in liver between the HP and control groups, one of the up-regulated genes in this tissue was *Hsp90* (Figure 4A). HSP90 is a heat shock protein that has been related to liver steatosis induced by chronic alcoholic injury and to hepatocellular carcinoma [27-29]. The increased *Hsp90* mRNA expression was confirmed by real-time RT-PCR (Figure 4B). Moreover, Western blot analysis revealed that HSP90 protein levels were dramatically increased in liver of the HP-fed animals (Figure 4C). In concordance to these data, immunohistochemistry of hepatic tissue showed an intense HSP90 positive staining in the HP group in comparison to the control group (Figure 4D).

4. Discussion

The consumption of HP diets is common in Western societies, particularly in association with weight reduction, weight loss maintenance, or physical training. However, there is a lack of information on the health effects of chronic intake of these diets. Thus, we considered it important to analyse the effects of prolonged exposure to a HP diet using healthy animals. We used a HP diet rich in casein. Casein has a high nutritional value compared to other protein sources due to a relatively high content of essential amino acids and good digestibility [30], and it is very extensively used in human and animal nutrition. The increase in protein intake in our study is associated with a concomitant decrease in dietary carbohydrates; which makes it impossible to specifically ascribe the observed metabolic effects to the protein content alone. However, sucrose content and other components of the diet, including fat, vitamins, minerals, and fibre were maintained in the same proportion to avoid interferences from these components in our study.

Previous results in the same cohort of animals fed the HP diet showed an alteration in the composition of different key homeostatic tissues such as liver, adipose tissue, muscle and kidney [12]. One of the most interesting and unexpected altered parameters was a 75 % increase in the hepatic TG content ($p=0.06$) in the HP group relative to the control group, even if HP-fed animals had a lower cumulative caloric intake, while the fat content of the HP diet and the control diet was identical. This is of relevance as lipid deposition, mainly in the form of TG, is considered a prerequisite for the development of nonalcoholic fatty liver disease (NAFLD) [31]. Even if we consider that HP-fed animals were in an initial stage of fatty liver disease, as we did not observe clear signs of macrovesicular fat deposition, the increase in circulating AST and C-reactive protein levels, serum markers of hepatic injury, support hepatic cellular damage in the HP-fed animals. Moreover, in liver of the HP group,

we found increased levels mRNA and protein levels of HSP90, a heat shock protein that has been linked to hepatic steatosis induced by chronic alcoholic injury as well as hepatocellular carcinoma ([27-29]).

To better understand how HP diets affect the liver, a key metabolic tissue with a crucial role in dietary protein handling, we performed whole genome gene expression analysis, which revealed a strong upregulation of genes involved in amino acid metabolism; this result is in agreement with other authors [32]. The chronic increase in protein intake (4 months of HP diet) up-regulated hepatic amino acid metabolism at three different levels: (i) amino acid import into the cell; (ii) deamination; and (iii) urea cycle. This is in agreement with the well-known effect of an increased flow of amino acids into the liver, and a subsequent increase in urea production and serum urea, which constitute a biochemical adaptation associated with increased amino acid deamination in liver to excrete nitrogen to maintain nitrogen balance [33].

It is known that an increase in protein intake is associated with marked increase in amino acid catabolism to obtain energy and glucose [34]. This agrees with the observed increase in the expression of genes involved in the tricarboxylic acid cycle (TCA) and in electron transport chain in the HP-fed animals (see also Figure 2). However, for a major part of the dietary amino acid carbon skeletons derived from amino acid deamination under long-term exposure to HP diet, the exact metabolic fate is unclear. There is no evidence that all deaminized carbon skeletons are oxidized [35]. Fromentin *et al.* [36] concluded that after HP diet adaptation in rats only half of the deaminated amino acids were immediately oxidized during the postprandial phase because of limitations in hepatic energy requirements. We suggest that the other half is temporary stored as TG. During amino acid oxidation, a severe non-volatile acid load is generated in liver mitochondria, which has

been suggested to be alleviated mainly by the respiratory proton chain pump in the form of uncoupled respiration to maintain the acid-base equilibrium in the mitochondria to avoid organelle damage [34]. This overload of the electron transport chain could slow down the oxidation of carbon skeletons generated by deamination of the dietary amino acids, and could generate a temporary pool of intermediate metabolites from amino acid metabolism, being dicarboxylic acids [34]. Interestingly, in the HP group we observed an increased expression of the gene encoding SLC25A21, which transports C5-C7 oxodicarboxylic acids, produced by amino acid metabolism, across the mitochondrial membranes [37]. Thus, the excess of dicarboxylic acids resulting from increased dietary amino acid degradation could be directed out of the mitochondria through SLC25A21 mediated transport. The high expression of this transport protein suggests a mechanism to ameliorate the acid load in the mitochondria through the extrusion of the dicarboxylic acids derived from amino acids as indicated by Aoi and Marunaka for monocarboxylic acids [38]. These dicarboxylic acids can then be used for TG formation [34]. In agreement, we observed an increase in the expression of genes involved in TG synthesis, which was confirmed at the functional level by increased hepatic TG accumulation. Our microarray data further indicate that glycerol, necessary for TG formation, could be synthesized “*de novo*”, as we observed an up-regulation of genes involved in gluconeogenesis pathway only up to the trioses (glyceroneogenesis pathway). Fatty acids could be derived from increased lipolysis in the subcutaneous adipose tissue, as we previously reported that our HP-fed animals presented increased levels of the key lipolytic protein ATGL correlating with a lower size of subcutaneous adipocytes [12], and liver is responsible for the up-take of about one-third of the free fatty acids (FFA) removed from the blood stream [39]. Alternatively, the carbon skeletons of some amino acids can be converted into fatty acids [34]. This molecular

scenario is supported by the transcriptome data of the HP group, that showed increased expression of key hepatic genes involved in TG synthesis: *Gnpat* that catalyses the first link between dihydroxyacetone phosphate produced in the glyceroneogenesis with FFA; *Agpat6* that converts lysophosphatidic acid in phosphatidic acid; *Mogat2* that catalyses the formation of diacylglycerol (DAG) from monoacylglycerol; and *Dgat1*, coding for an enzyme involved in the last step of TG formation from DAG. We also observed up-regulation of the novel gene *Lpin2*, which contains a domain coding for phosphatidic acid phosphatase activity [40]. Interestingly, recent experiments have demonstrated that overexpression of LIPIN2 promotes hepatic insulin resistance, and increases hepatic DAG levels and hepatic TG production in diet-induced obese mice, which is associated with its phosphatidate phosphatase activity [41], as well as and chronic endoplasmatic reticulum stress caused by chronic inflammation and nutrient overload [42]. In accordance with these data, our HP-fed rats presented not only higher TG content, but also higher levels of serum inflammatory markers, such as TNF-alpha and C-reactive protein as well as up-regulation of the immune response pathway in the array data.

Briefly, according to our results, summarized in Figure 2, the glycerol and dihydroxyacetone produced “*de novo*” in the liver by using the excess of dicarboxylic acids would be re-esterified with the FFA resulting from increased lipolysis in adipose tissue, thus increasing hepatic TG content. This mechanism of energy storage in the liver could help to dissipate the acid load generated in the hepatic mitochondria as result of amino acid metabolism. A large fraction of the FFA is trapped into TG, which are either stored in the hepatocytes or released from the liver into the bloodstream as VLDL. In our case, data show a down-regulation in the *Apoa4* gene, coding for an apolipoprotein that promotes very low density lipoprotein particle expansion and which enhances TG secretion and reduces

hepatic lipid content when over-expressed in transgenic mice [43]. An increased TG synthesis capacity in liver with a concomitant reduction of hepatic VLDL-related TG secretion could help to explain the higher hepatic TG content observed in animals fed with HP diet. It has been previously reported that rats supplemented with branched-chain amino acids under vitamin B6 deficiency showed TG deposition in liver partly explained by impaired secretion of VLDL [44]; this could be relevant as casein, our dietary protein source, is rich in branched-chain amino acids. Intriguingly, other authors have reported reduced liver fat accumulation in rodents with long term increase of the protein content of the diet [45-48]. Differences with our results could be explained by differences in diet composition, mainly the sucrose content. Sucrose is known to importantly induce liver steatosis [49]. However, the HP diets administered in the experiments of Schwarz *et al.* [45] and Lacroix *et al.* [46] provided half of the amount of sucrose than their control diets, which could affect to the lower hepatic liver accumulation observed in their HP-fed animals, especially in long-term studies. On the contrary, the content of sucrose was equal in the control and high protein diets used in our experiment. Finally, the different dietary protein source, casein in our study, or whey or soy protein used by other authors [47, 48], could have influenced the results.

The composition of diets is a major determinant of the acid load that must be excreted by the kidney to maintain acid-base balance [26]. Protein-rich diets are considered acidogenic due to the excess of sulphuric anions produced by catabolism of sulphur amino acids and an imbalance in the protein-potassium ratio [15]. One of the most up-regulated genes in our study was *Kcnma1*, coding for a potassium channel. This suggests disequilibrium in the potassium flow that could also alter the cellular acid-base and potassium homeostasis. In accordance with alterations in the cellular pH, our results have shown up-regulation of gene

expression of *Slc9a6* and *Slc4a2*, coding for integral membrane ion transporters that mediate the exchange of H^+ and HCO_3^- , respectively, and involved in the regulation of cytoplasmic pH [50, 51]. Disturbance in cellular acid-base equilibrium has been related to several metabolic repercussions in metabolic health and disease [38, 52]. For example, low levels of interstitial fluid pH are linked with dysfunction of insulin signalling through alteration of insulin binding to its membrane receptor and subsequent downstream phosphorylation [52]. Such a dysfunction in insulin signalling could be counteracted by increased insulin release by the pancreas, in agreement with the observed higher circulating insulin levels in the fed state. The excess of dietary sulphur containing amino acids could explain increased gene expression of *Tpmt* and *Nnmt*, coding for enzymes which catalyse metabolism of the sulphur containing amino acids through methylation reactions [53]. Moreover, the production of free radicals associated with these metabolic pathways could be responsible for the increased expression levels of *Gstm7*, *Nqo1*, and *Gstm4*. These genes code for enzymes involved in the detoxification of, among others, endogenous electrophilic compounds to prevent cytotoxicity and cellular damage [54-56]. Oxidative stress and the consequent organelle damage may play a critical role in the progression of hepatic injury. The maintenance of this metabolic status could generate tissue damage and inflammation as revealed by the high levels of circulating AST and C-reactive protein compared with control animals. Although diet-induced “low grade” metabolic acidosis has minimal adverse effects in the short-term, a prolonged diet-induced high acid load may lead to a mild form of chronic metabolic acidosis which is considered having significant clinical relevance. In fact, and in accordance with our results, different studies suggest that there may be an association between diet-induced acid load and NAFLD [57] and dietary acid

load and mild metabolic acidosis with hyperinsulinemia and insulin resistance in humans [58].

We performed our study using males to avoid potential interferences of hormonal fluctuations during the menstrual cycle which occurs in females. However, possible gender differences should be taken into account in future studies. Regarding the age of the animals, adult rats were fed with the different diets during 4 months, which corresponds to approximately 10 years in humans, as we wanted to analyse the effect of chronic intake of a HP diet. As our control animals had the same age as the experimental animals fed the HP diet, we can discard an effect of age on the differential response observed and in the increased health risk, which was only observed in the HP-fed animals.

Conclusion

Long-term intake of a casein-rich high protein diet had an impact on metabolic pathways in the liver that indicate higher TG deposition, inflammation, and activation of processes in response to increased pH unbalance and oxidative stress. Thus, in general, care has to be taken with the intake of HP diets over longer periods of time to control body weight. In particular, subjects with other pathologies such as diabetes, liver or kidney disease, can be expected to be more susceptible to the potential adverse effects of such diets. Although a moderate increase in the dietary ratio of proteins/carbohydrates could be a valid and scientifically accepted strategy to lose weight, our gene expression data indicate that a drastic and prolonged rise in protein intake induces homeostatic imbalances with clear functional signs of unhealthy effects such as increased serum levels of AST, inflammation markers, liver TG content and liver weight. Moreover, we describe for the first time that chronic intake of a HP diet produces a dramatic increase of hepatic HSP90, a marker of liver injury.

Acknowledgements. We thank Enzo Ceresi and Paula Núñez for technical assistance in the immunohistochemistry and Western blot analysis, respectively.

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Figure legends

Figure 1. Regulated pathways. Manual classification into biological pathways of the genes affected by the intake of HP diet in comparison to animals fed a control diet (Student's *t*-test, $p < 0.001$).

Figure 2. Schematic representation of the interconnection of the HP-regulated genes involved in amino acid, glycerol, and lipid metabolism. Microarray data showed an up-regulation of genes related with different metabolic process, cumulatively integrating the fate of excess amino acids. The excess of dietary amino acids increased the expression of amino acids importers. This excess of amino acids in the hepatocytes increased expression of genes involved in carbon skeleton transformation and deamination/transamination of the different dietary amino acids, and the urea cycle to remove excess ammonia. Also, genes of the tricarboxylic acid cycle (TCA) and electron transport chain increased. The excess of carbon skeleton generated as result of amino acid catabolism up-regulated the expression of *Slc25a21* coding form a protein implicated in the transport of dicarboxylic acids. Amino acid carbon skeleton could be used for cytoplasmic glyceroneogenesis as result of up-regulation of key genes. This increased glyceroneogenic capacity, together with increased free fatty acids (FFA) levels released by lipolysis in white adipose tissue, and increased expression of key genes involved in TG synthesis, likely underlies the increased hepatic TG levels. Also, we observed a decreased expression of lipoprotein *Apoa4* related to particle size of VLDL. The increase in TG synthesis and the decrease of the flow of the hepatic TG could be related to the TG deposition in liver of the HP animals in respect to the control animals.

Underlined genes were part of the set of genes confirmed by real-time RT-PCR analysis. The arrows (up/down) show the direction of the gene expression regulation under

conditions of HP diet. Solid arrows show a p-value (Student's *t*-test) lower than 0.001, while empty arrows show a p-value (Student's *t*-test) of 0.001-0.05.

Figure 3. Microarray data confirmation. A) Selected microarray data to be confirmed consisting of genes of prominent pathways differentially expressed in liver between animals fed a high protein (HP) or a control (C) diet. Fold change indicates hepatic expression levels in HP diet over control diet group. B) Microarray confirmation by real-time RT-PCR. Results represent means $\pm$ SEM (n=6-7) of ratios of specific mRNA levels relative to *β -actin* and *Gdi1*, and are expressed as a percentage of the value of control group that was set to 100%. * indicates values significantly different vs control animals (Student's *t* test, $p < 0.05$). C) Confirmation of a selected protein, GPT/ALT, by Western blot analysis. Results represent means $\pm$ SEM (n=6-7) of specific protein levels. Data of the control group was set to 100%. * vs control animals (Student's *t*-test, $p < 0.05$). Representative bands obtained in the Western blot are shown: 30 μ g of protein was loaded per lane. HRSP12 was used as transfer and loading control. The represented bands correspond to samples resolved together in the same gel.

Figure 4. HSP90 expression in liver. A) *Hsp90* differential gene expression by microarray analysis between animals fed a high protein (HP) or a control (C) diet. B) *Hsp90* mRNA levels measured by real-time RT-PCR in control and HP animals. Results represent means $\pm$ SEM (n=6-7) of ratios of specific mRNA levels relative to *β -actin* and *Gdi1*, and expressed as a percentage of the value of control group that was set to 100%. * indicates values significantly different vs control animals (Student's *t* test, $p < 0.05$). C) HSP90 protein levels measured by Western blot. Results represent means $\pm$ SEM (n=6-7) of specific protein levels. Data of the control group was set to 100%. * vs control animals (Student's *t*-test, $p < 0.05$). Representative bands obtained in the Western blot are shown: 30

µg of protein was loaded per lane. HRSP12 was used as transfer and loading control. The represented bands correspond to samples resolved together in the same gel. D) Immunohistochemistry of HSP90 in control and HP animals. Positive staining: brown colour. V: central vein.

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Figure 1

Figure 2

Figure 3

A) Selected microarray data

	Gene name	Gene symbol	Sequence ID	Microarray	
				Fold change	p-value
Amino acid metabolism	Carbamoyl-phosphate synthetase 1	<i>Cps1</i>	NM_017072	1.66	2.12E-04
	Glutamic-oxaloacetic transaminase 1, soluble	<i>Got1</i>	NM_012571	2.10	1.30E-04
	Glutamic-pyruvate transaminase	<i>Gpt</i>	NM_031039	1.72	7.76E-05
	Solute carrier family 43 (amino acid system L transporter), member 1	<i>Slc43a1</i>	NM_001107742	1.53	4.58E-04
Glyceroneogenesis	Similar to triosephosphate isomerase 1	<i>Tpi1</i>	XR_007561	1.29	2.88E-04
Lipid synthesis	Glyceronephosphate O-acyltransferase	<i>Gnpat</i>	NM_053410	1.21	2.23E-04
Oxidative stress	Glutathione S-transferase mu 4	<i>Gstm4</i>	NM_001024304	2.14	1.04E-06
Transport	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	<i>Kcnma1</i>	NM_031828.1	4.31	1.02E-04
	Anion exchange protein 2	<i>Slc4a2</i>	NM_017048.2	1.25	9.95E-04
	Mitochondrial oxodicarboxylate carrier	<i>Slc25a21</i>	NM_001171170.1	1.90	1.22E-04

B) Confirmation of selected microarray data by real-time RT-PCR

C) Confirmation of a selected protein by Western blot: GPT/ALT protein levels

Figure 4

A) Microarray results of HSP90 mRNA expression

	Gene symbol	Sequence ID	Microarray	
			Fold change	p-value
Similar to Heat shock protein HSP 90-beta (HSP 84)	<i>Hsp90</i>	RGD1562936	1.18	0.0273

B) HSP90 mRNA levels

C) HSP90 protein levels

D) Immunohistochemistry of HSP90

Table 1. Diet composition

	Control diet		HP diet	
	g%	kcal%	g%	kcal%
Protein	19.2	20	43.2	45
Carbohydrate	67.3	70	43.3	45
Fat	4.3	10	4.3	10
Energy (kcal/g)	3.85		3.85	
Ingredients				
Casein	200	800	450	1800
L-Cystine	3	12	6	24
Corn starch	315	1260	62	248
Maltodextrin	35	140	35	140
Sucrose	350	1400	350	1400
Soy bean oil	25	225	25	225
Lard	20	180	20	180
Cellulose	50	0	50	0
Mineral Mix, S10026 \$	10	0	10	0
Dicalcium Phosphate \$	13	0	13	0
Calcium Carbonate \$	5.5	0	5.5	0
Potassium Citrate \$	16.5	0	16.5	0
Vitamin Mix, V10001	10	40	10	40
Choline Bitartrate	2	0	2	0

Control and high protein (HP) diets were from Research Diets and were purchased from Brogaarden (Gentofte, Denmark) and served as dry pellets. The macronutrient percentage is given in g (gram) and in kcal. The energy content is given in kcal/g of food. Diet ingredients composition is also given in g/kg and in kcal/kg. The control diet was rich in corn starch, while the HP diet was rich in casein; both diets had identical amounts of sucrose, fat, cellulose, vitamins, and minerals, coming from the same sources. \$ 1 kg of diet contains 2.96 g phosphorus, 6.3 g potassium; 0.5 g magnesium and 0.2 g calcium (both diets).

Table 2**A) Body weight, adiposity, dietary acid load and circulating parameters of the control and HP groups**

	Control group	HP group
Body weight (g)	500 ± 13	455 ± 12#
Adiposity index (% of fat)	8.43 ± 0.70	8.31 ± 1.07
Body fat content (% of fat)	17.8 ± 1.6	18.3 ± 1.7
Cumulative caloric intake (kcal)	10,227 ± 182	8,971 ± 495 #
Daily food intake (Kcal)	73.2 ± 3.4	69.0 ± 3.4
Cumulative NEAP (mEq)	53624 ± 0	120668 ± 0 #
Cumulative PRAL (mEq)	260 ± 5	514 ± 28 #
Circulating parameters in fed state:		
Glucose (mg/dl)	97.1 ± 4.4	91.5 ± 3.0
Insulin (µg/l)	1.23 ± 0.22	2.12 ± 0.27 #
HOMA-IR index	2.17 ± 0.49	1.85 ± 0.65
NEFA (µM)	0.62 ± 0.07	0.52 ± 0.05
TG (mg/ml)	7.08 ± 0.63	6.93 ± 1.02
Cholesterol (mg/dl)	200 ± 27	103 ± 27 #
TNF-alpha (pg/ml)	2.64 ± 0.55	13.3 ± 5.7 #
C-reactive protein (ng/ml)	53.1 ± 0.9	76.6 ± 7.0 #
Aspartate transaminase (U/l)	416 ± 29	544 ± 58 # (p=0.07)
Urea (mg/dl)	5.08 ± 0.26	8.10 ± 0.68 #

B) Liver composition in animals of the control and HP groups

	Control group	HP group
Liver weight (g)	13.9 ± 0.4	13.9 ± 0.3
Liver/body weight ratio (%)	2.83 ± 0.05	3.16 ± 0.10 #
Liver glycogen content (mg glycogen/ g tissue)	25.3 ± 1.5	22.3 ± 3.9
Liver lipid content (mg lipid/g tissue)	42.6 ± 3.3	38.8 ± 1.3
Liver TG content (mg TG/ g tissue)	22.7 ± 3.1	39.9 ± 6.8 # (p=0.06)

A) The adiposity index was computed as the sum of epididymal, inguinal, mesenteric and retroperitoneal white adipose tissue depot weights and expressed as a percentage of total body weight. Body fat content was measured using an EchoMRI-700TM (Echo Medical Systems, LLC, TX, USA). The cumulative net endogenous acid production (NEAP) and the potential renal acid load (PRAL) were calculated using the formulas of Frassetto and Remer, respectively, and expressed as milliequivalents (mEq). All circulating metabolites were measured in the fed state as described in the methods section. B) Hepatic glycogen, total lipids and triacylglycerol (TG) content were measured as described in the methods section. Results represent mean $\pm$ SEM (n=7 in control and n=6 in HP group). # vs. control group (Student's *t*-test, $p < 0.05$ or indicated when different).

Table 3. MetaCore pathway analysis of genes affected by HP diet feeding in liver

	Cell process	Pathway	Objects		p-value
			Number of genes (p < 0.001)	All genes annotated (MetaCore)	
1	(L)-Alanine, (L)-cysteine, and (L)-methionine metabolism	Amino acid metabolism	6	30	5.39 E-05
2	Urea cycle	Amino acid metabolism	4	30	1.23 E-04
3	Aspartate and asparagine metabolism	Amino acid metabolism	5	30	1.53 E-04
4	Glycine, serine, cysteine and threonine metabolism	Amino acid metabolism	2	16	1.05 E-03
5	Phenylalanine metabolism	Amino acid metabolism	6	31	1.99 E-03
6	(L)-Arginine metabolism	Amino acid metabolism	4	3	2.75 E-03
7	Huntingtin-depended transcription deregulation in Huntington's Disease	Cell cycle	2	24	3.62 E-03
8	Transcription regulation of amino acid metabolism	Amino acid metabolism	4	21	3.92 E-03
9	Regulation of G1/S transition (part 2)	Cell cycle	2	33	4.24 E-03
10	Transcription factors in segregation of hepatocytic lineage	Cell cycle	2	30	5.62 E-03

MetaCore analysis was performed for 133 genes differentially regulated by HP diet feeding (p<0.001). The top ten pathways obtained are sorted based on significance. For each pathway its p-value (as calculated by MetaCore software) and the number of regulated genes and total number of genes are shown.

Table 4. Most relevant genes affected by HP diet feeding in liver

Metabolic pathway	Gene name	Gene symbol	Sequence ID	Fold change	p-value	Function
Amino acid metabolism	<u>Amino acid transport</u>					
	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	<i>Slc7a2</i>	NM_022619	1.67	9.15E-04	Cellular uptake of arginine, lysine and ornithine
	Solute carrier family 38, member 9	<i>Slc38a9</i>	NM_001035251	1.21	2.69E-04	Putative sodium-dependent amino acid/proton antiporter
	Solute carrier family 43 (amino acid system L transporter), member 1	<i>Slc43a1</i>	NM_001107742	1.53	4.58E-04	Transports branched-chain amino acids and phenylalanine
	<u>Deamination and carbon skeleton process</u>					
	Alanine-glyoxylate aminotransferase	<i>Agxt</i>	NM_030656	1.52	4.76E-04	Alanine-glyoxylate aminotransferase
	Cystathionine beta synthase	<i>Cbs</i>	NM_012522	1.66	2.98E-05	Conversion of homocysteine to cystathionine
	Glutamic-oxaloacetic transaminase 1, soluble	<i>Got1</i>	NM_012571	2.10	1.30E-04	Biosynthesis of L-glutamate from L-aspartate or L-cysteine
	Glutamic-pyruvate transaminase	<i>Gpt</i>	NM_031039	1.72	7.76E-05	Transamination between alanine and 2-oxoglutarate to form pyruvate and glutamate
	4-hydroxyphenylpyruvate dioxygenase	<i>Hpd</i>	NM_017233	1.90	1.22E-04	Degradation of tyrosine
	Pyrroline-5-carboxylate reductase-like	<i>Pycrl</i>	NM_001011993	1.64	9.25E-05	Metabolism of amino groups and proline biosynthesis II
	Serine dehydratase	<i>Sds</i>	NM_053962	2.85	4.72E-04	Converts L-serine to pyruvate and ammonia
	<u>Urea cycle</u>					
	Argininosuccinate lyase	<i>Asl</i>	NM_021577	2.10	2.31E-04	Hydrolytic cleavage of argininosuccinate into arginine and fumarate
Inflammation/ Oxidative stress/ SAM activity	Argininosuccinate synthase 1	<i>Ass1</i>	NM_133614	1.82	6.46E-04	Breakdown of argininosuccinate producing arginine and fumarate
	Carbamoyl-phosphate synthetase 1	<i>Cps1</i>	NM_017072	1.66	2.12E-04	Synthesis of carbamoyl phosphate from ammonia and bicarbonate
	C Protein Reactive	<i>Crp</i>	NM_017096	1.56	1.29E-04	Several functions associated with host defense
	Glutathione S-transferase mu 4	<i>Gstm4</i>	NM_001024304	2.14	1.04E-06	Conjugation of reduced glutathione
	Glutathione S-transferase, mu 7	<i>Gstm7</i>	NM_031154	1.73	3.58E-04	Conjugation of reduced glutathione
	Nicotinamide N- methyltransferase	<i>Nnmt</i>	NM_001106819	1.81	4.92E-04	Catalyzes the N-methylation of nicotinamide
Transport protein	NAD(P)H dehydrogenase, quinone 1	<i>Nqo1</i>	NM_017000	1.96	3.99E-04	Reduction of quinones that results in the production of radical species
	Thiopurine S-methyltransferase	<i>Tpmt</i>	NM_001079531	1.35	6.13E-05	Catalyzes the S-methylation of thiopurine drugs
	Anion exchange protein 2	<i>Slc4a2</i>	NM_017048.2	1.25	9.95E-04	Plasma membrane anion exchange protein
	Sodium/hydrogen exchanger 6	<i>Slc9a6</i>	XM_006257691.1	1.17	8.45E-04	Electroneutral exchange of protons for Na(+) and K(+)
	Mitochondrial oxodicarboxylate	<i>Slc25a21</i>	NM_001171170.1	1.90	1.22E-04	Transport of dicarboxylic

	carrier Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	<i>Kcnma1</i>	NM_031828.1	4.31	1.02E-04	acids Protein that exhibits calcium-activated potassium channel activity
Carbohydrate metabolism	Phosphoglycerate mutase 2	<i>Pgam2</i>	XM_340952	1.33	7.25E-04	Catalyzes the interconversion of 2-phospho-D-glycerate and 3-phospho-D-glycerate
	Similar to triosephosphate isomerase 1	<i>Tpi1</i>	XR_007561	1.29	2.88E-04	Isomerization of glyceraldehyde 3-phosphate and dihydroxy-acetone phosphate
Lipid metabolism	<u>Triacylglycerol synthesis</u> Diacylglycerol O-acyltransferase 1	<i>Dgat1</i>	NM_053437	1.30	1.00E-03	Conversion of diacylglycerol and fatty acyl CoA to triacylglycerol
	Glyceronephosphate O-acyltransferase	<i>Gnpat</i>	NM_053410	1.21	2.23E-04	Synthesis of acyldihydroxyacetone from dihydroacetone phosphate and fatty acyl CoA
	Lipin2	<i>Lpin2</i>	TC610999	1.33	4.95E-05	Involve in the synthesis of diacylglycerol from monoacylglycerol
	<u>Desaturation of fatty acids</u> Stearoyl-Coenzyme A desaturase 2	<i>Scd2</i>	NM_031841	1.38	1.81E-04	Insertion of a double bond into a spectrum of fatty acyl-CoA
	<u>Others</u> Phosphatidylinositol glycan anchor biosynthesis, class O	<i>Pigo</i>	ENSRNOT00000013213	1.27	1.32E-04	Involved in glycosylphosphatidylinositol-anchor biosynthesis

Categorization and expression profiles of the most relevant regulatory and functional genes affected by HP diet in liver ($p < 0.001$). Genes are listed based on alphabetic order of the gene symbol. Fold change indicates hepatic expression levels in HP diet over control.

Highlights:

- Chronic high protein diet intake affects liver transcriptome promoting TG deposition.
- Chronic high protein diet results in molecular changes indicative of hepatic injury.
- Long-term increase of protein diet content could have unhealthy consequences.