

# Cardiovascular disease is associated with high-fat-diet-induced liver damage and up-regulation of the hepatic expression of hypoxia-inducible factor 1 $\alpha$ in a rat model

Adriana L. BURGUENO\*, Tomas F. GIANOTTI\*, Noelia G. MANSILLA\*, Carlos J. PIROLA\*<sup>1</sup> and Silvia SOOKOIAN†<sup>1</sup>

\*Department of Molecular Genetics and Biology of Complex Diseases, Institute of Medical Research A Lanari-IDIM, University of Buenos Aires–National Council of Scientific and Technological Research (CONICET), Ciudad Autónoma de Buenos Aires, Argentina

†Department of Clinical and Molecular Hepatology, Institute of Medical Research A Lanari-IDIM, University of Buenos Aires–National Council of Scientific and Technological Research (CONICET), Ciudad Autónoma de Buenos Aires, Argentina

## Abstract

CVD (cardiovascular disease) is associated with abnormal liver enzymes, and NAFLD (non-alcoholic fatty liver disease) is independently associated with cardiovascular risk. To gain insights into the molecular events underlying the association between liver enzymes and CVD, we developed an HFD (high-fat diet)-induced NAFLD in the SHR (spontaneously hypertensive rat) and its control WKY (Wistar–Kyoto) rat strain. We hypothesized that hepatic induction of *Hif1a* (hypoxia-inducible factor 1 $\alpha$ ) might be the link between CVD and liver injury. Male SHRs ( $n = 13$ ) and WKY rats ( $n = 14$ ) at 16 weeks of age were divided into two experimental groups: standard chow diet and HFD (10 weeks). HFD-fed rats, irrespective of the strain, developed NAFLD; however, only HFD-SHRs had focus of lobular inflammation and high levels of hepatic TNF $\alpha$  (tumour necrosis factor  $\alpha$ ). SHRs had significantly higher liver weight and ALT (alanine aminotransferase) levels, irrespective of NAFLD. Liver abundance of *Hif1a* mRNA and Hif1 $\alpha$  protein were overexpressed in SHRs ( $P < 0.04$ ) and were significantly correlated with ALT levels ( $R = 0.50$ ,  $P < 0.006$ ). This effect was not reverted by a direct acting splanchnic vasodilator (hydralazine). Angiogenesis may be induced by the HFD, but the disease model showed significantly higher hepatic Vegf (vascular endothelial growth factor) levels ( $P < 0.025$ ) even in absence of dietary insult. *Hif1a* mRNA overexpression was not observed in other tissues. Liver mRNA of *Nr1d1* (nuclear receptor subfamily 1, group D, member 1;  $P < 0.04$ ), *Ppara* [Ppar (peroxisome-proliferator-activated receptor)  $\alpha$ ;  $P < 0.05$ ], *Pparg* (Ppar $\gamma$ ;  $P < 0.001$ ) and *Sirt1* (Sirtuin 1;  $P < 0.001$ ) were significantly up-regulated in SHRs, irrespective of NAFLD. *Sirt1* and *Hif1a* mRNAs were significantly correlated ( $R = 0.71$ ,  $P < 0.00002$ ). In conclusion, CVD is associated with *Hif1a*-related liver damage, hepatomegaly and reprogramming of liver metabolism, probably to compensate metabolic demands.

**Key words:** arterial hypertension, fatty liver, hypoxia, hypoxia inducible factor 1 $\alpha$ , liver damage, metabolic syndrome, non-alcoholic fatty liver disease

## INTRODUCTION

NAFLD (non-alcoholic fatty liver disease), a disease characterized by abnormal fat accumulation in the liver, has been recently regarded as the hepatic component of the metabolic syndrome [1]. The metabolic syndrome has received considerable attention

over the last decades, as the cluster of its associated diseases significantly increases the risk of CVD (cardiovascular disease) mortality [2]; in fact, it is also known as cardiometabolic syndrome. Although T2D (Type 2 diabetes), obesity and elevated plasma triacylglycerols are well-known contributors to cardiovascular morbimortality, NAFLD has been recently suspected as playing

**Abbreviations:** ALT, alanine aminotransferase; CVD, cardiovascular disease; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; HFD, high-fat diet; H&E, haematoxylin and eosin; Hif1 $\alpha$  (*Hif1a*), hypoxia-inducible factor 1 $\alpha$ ; *Hnf4a*, hepatocyte nuclear factor 4 $\alpha$ ; HOMA, homoeostasis model assessment; IL-6 (*Il6*), interleukin-6; IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; *Nr1d1*, nuclear receptor subfamily 1, group D, member 1; PAS, periodic acid–Schiff; *Pepck*, phosphoenolpyruvate carboxykinase 1; Ppar, peroxisome-proliferator-activated receptor; *Ppara*, Ppar $\alpha$ ; *Pparg*, Ppar $\gamma$ ; *Pgc1a*, Ppar $\gamma$  co-activator-1 $\alpha$ ; QTL, quantitative trait locus; RT, real-time; SBP, systolic blood pressure; SCD, standard chow diet; SHR, spontaneously hypertensive rat; *Sirt1* (*Sirt1*), sirtuin 1; *Tbp*, TATA box-binding protein; T2D, Type 2 diabetes; Tnf $\alpha$  (*Tnfa*), tumour necrosis factor  $\alpha$ ; VAT, visceral adipose tissue; Vegf (*Vegf*), vascular endothelial growth factor; WKY, Wistar–Kyoto.

<sup>1</sup>These authors contributed equally to this study.

**Correspondence:** Dr Silvia Sookoian (email sookoian.silvia@lanari.fmed.uba.ar) or Dr Carlos J. Pirola (email pirola.carlos@lanari.fmed.uba.ar)

a role in the development of CVD, independently of traditional risk factors [3]. For instance, a recent meta-analysis demonstrated that NAFLD patients have an increased risk of atherosclerosis, as they carry a 13% increase in carotid intima-media thickness [4]. In addition, we have observed that NAFLD is associated not only with increased circulating levels of molecular mediators of atherosclerosis, but also with their hepatic expression [5,6].

Interestingly, epidemiological evidence and clinical studies not only suggest that NAFLD might participate in the development and progression of the cardiovascular phenotype, but also that CVD is associated with abnormal levels of surrogate markers of liver injury, regardless of the presence of NAFLD. For example, several studies have consistently reported an association between abnormal liver enzymes, such as ALT (alanine aminotransferase), and increased incidence of cardiovascular events [7–10]. Moreover, abnormal liver function tests were associated with essential hypertension [11]. Hence CVD and liver injury seem to be strongly associated, but the mechanisms behind this relationship are unknown. Likewise, the biological mechanisms responsible for the association between NAFLD and CVD are still poorly understood. In addition, studies characterizing fatty liver in the absence of IR (insulin resistance) and cardiovascular risk factors are scarce because of methodological limitations in dissecting clinical phenotypes that usually co-exist.

To gain insights into the molecular events underlying the association between liver enzymes and CVD, we took advantage of a rat model of CVD and the metabolic syndrome, the SHR (spontaneously hypertensive rat). We developed an HFD (high-fat diet)-induced model of NAFLD in SHRs and their control normotensive insulin-sensitive WKY (Wistar–Kyoto) rat strain. The SHR strain has been studied and characterized for over 30 years; in fact, the SHR is the most widely used rodent model of CVD, showing all the metabolic traits related with the human disorder, including high blood pressure, dyslipidaemia and IR, and many other physiological and pathophysiological phenotypes [12,13]. Indeed, genetic analysis of the SHR strain showed QTLs (quantitative trait loci) for haemodynamic and metabolic traits on several chromosomes, including QTLs associated with phenotypes of lipid and carbohydrate metabolism [14]. In addition, the SHR strain has cardiac hypertrophy [15,16].

In the present study, we hypothesized that the liver is a target for CVD-associated organ damage, and up-regulation of liver *Hif1a* (hypoxia-inducible factor 1 $\alpha$ ) might be the link between CVD and liver injury. In addition, we explored whether CVD is associated with dysregulated patterns of liver gene transcription.

## MATERIALS AND METHODS

### Animals

SHRs ( $n = 13$ ) and WKY rats ( $n = 14$ ) (Charles River Laboratories) at 16 weeks of age were included in this experiment. The rats were housed under controlled conditions of temperature ( $23 \pm 1^\circ\text{C}$ ) and light (12 h light/12 h dark cycle). After acclimatization for 1 week, rats of both strains were randomly divided into two experimental groups. One group received SCD for 10 weeks

(control group, SCD;  $n = 7$  in both strains), in an amount restricted to that consumed at the beginning of the experiment. The other group was allowed *ad libitum* access to a high-fat solid diet {HFD, 40% (w/w) bovine and porcine fat added to the standard chow, as described previously [17]; SHR,  $n = 6$  and WKY,  $n = 7$ } for 10 weeks. In all of the animals, food intake and body weight were monitored daily for a 20-week period, and SBP (systolic blood pressure) was measured before and during the experiment weekly, as described previously [18].

At the completion of the study, food was withdrawn from 08:00 h to 16:00 h before the animals were anaesthetized with pentobarbital, and blood from individual rats was collected by cardiac puncture to determine plasma and serum levels of different parameters. Liver tissue was excised and weighed, and heart, intraperitoneal fat and retroperitoneal fat were weighed. Liver, heart and fat weights are expressed as liver/rat length (taken from nose to the tail origin) ratio (g/cm) to avoid the influence of body weight change. Liver was quickly snap-frozen and stored in  $-76^\circ\text{C}$  until gene expression analysis. A portion of each liver was fixed in 10% formalin for histological analysis. Serum and sodium EDTA/plasma was obtained by centrifugation and stored at  $-80^\circ\text{C}$  until needed. Fasting glucose, serum triacylglycerols, serum glutamic–pyruvate transaminase or ALT (alanine aminotransferase) and AP (alkaline phosphatase) levels were measured by an automatic biochemical analytical system (Architect; Abbott). Plasma insulin levels were determined using a commercial quantitative ultrasensitive rat ELISA kit (Crystal Chem), according to the manufacturer's instructions. IR was calculated by the HOMA (homoeostasis model assessment) index [fasting plasma insulin ( $\mu\text{-units/ml}$ )  $\times$  fasting plasma glucose (mmol/l)/22.5]. Leptin was measured by ELISA (Leptin ELISA Development Kit; PeproTech). All of these measurements were performed in a blinded manner.

Glucose level in liver tissue was measured using an enzymatic method (Accutrend; Roche Biodiagnostic); the amount is expressed per mg of liver protein after homogenizing the frozen liver tissue in 80% ethanol. The suspension was centrifuged at 15000 g for 10 min, the pellet was used for total protein determination using Bradford's reagent and the supernatant evaporated to dryness. The residue was resuspended in PBS.

All animals received humane care, and the studies were conducted according to the regulations for the use and care of experimental animals.

### Histological analysis of liver tissue

Light microscopy was used to assess steatosis and necroinflammation levels from sections of formalin-fixed paraffin-embedded samples that were stained with H&E (haematoxylin and eosin) and Masson's trichrome. The degree of steatosis was assessed irrespective of the experimental groups, and was graded from 0 to 4 according to the amount of fatty change [19]. The severity of necroinflammatory activity was expressed on a three-point scale as follows: grade 1 (mild), grade 2 (moderate) and grade 3 (severe), as described by Brunt et al. [19].

The amount of hepatocellular glycogen was assessed by PAS (periodic acid–Schiff) stain. Tissue glycogen content was

quantified using digital image analysis as described previously by our group [20].

### Measurement of liver triacylglycerol content

The liver triacylglycerol content was determined using an automatic biochemical analytical system (Architect; Abbott), and the results are expressed as  $\mu\text{g}$  of triacylglycerol/mg of liver tissue ( $\mu\text{g}/\text{mg}$  of liver).

### Evaluation of angiogenesis and inflammatory response

Tissue Vegf (vascular endothelial growth factor) and TNF $\alpha$  (tumour necrosis factor  $\alpha$ ) levels were measured in duplicate using an rat-specific ELISAs (Rat VEGF and Murine TNF $\alpha$  ELISA development kits; PeproTech). Plates were read at 405 nm using a microplate spectrophotometer. All samples were tested in a blinded manner. The results are normalized by mg of liver protein in the extract.

### RNA preparation and RT (real-time)-PCR for quantitative assessment of mRNA expression

Total RNA was prepared from rat livers using the phenol extraction step method, with an additional DNase digestion. The relative abundance of the target gene mRNA was normalized to the amount of a housekeeping gene [*Tbp* (TATA box-binding protein) or *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase)] to carry out comparisons between the groups. *Tbp* and *Gapdh* were found to be the most stable reference genes for testing liver and adipose tissue mRNA expression respectively, among other housekeeping genes tested before starting the experiment [cyclophilin A ( $\beta$ -actin, peptidylprolyl isomerase A) and GAPDH]. The levels of mRNA are expressed as the ratio of the estimated amount of the target gene relative to *Tbp* or *Gapdh* mRNA levels using fluorescence threshold cycle values ( $C_t$ ) calculated for each sample, and the estimated efficiency of the PCR for each product was expressed as the average of each sample efficiency value obtained.

The specificity of amplification and absence of primer dimers were confirmed using melting curve analysis and 1% agarose gel electrophoresis at the end of each run. The primer sequences and the resulting PCR product lengths are shown in Supplementary Table S1 (at <http://www.clinsci.org/cs/124/cs1240053add.htm>). Details of RT-PCR for the quantitative assessment of mRNA expression were as reported previously [18].

### Western blot analysis of Hif1 $\alpha$ protein

Nuclear proteins from liver tissue were denatured in the SDS sample buffer, separated by SDS/PAGE (8% gel) and electrotransferred on to Hybond-PVDF membranes (GE Healthcare;). After blocking with 5% non-fat dry milk in TBST (Tris-buffered saline with Tween) buffer [20 mmol/l Tris/HCl (pH 7.6), 137 mmol/l NaCl and 0.25% Tween 20], the membranes were probed with rabbit polyclonal anti-Hif1 $\alpha$  (1:1000 dilution; GeneTex), followed by incubation with HRP (horseradish peroxidase)-conjugated polyclonal anti-(rabbit IgG) secondary antibody (1:15000 dilution; GeneTex). Equal protein loading was confirmed by reblotting of the membranes with a goat polyclonal antibody against rabbit  $\beta$ -actin (1:500 dilution; GeneTex). Binding of the antibody was subsequently visualized with enhanced

chemiluminescence reagent (GE Healthcare), and the band images were detected and analysed using the Lab Works Analysis Software (Ultra-Violet Products).

### Per os treatment with hydralazine

Male SHR (at 14 and 16 weeks of age;  $n=6$  per group) were given either pellets containing the vasodilator hydralazine (10 mg/kg of body weight per day) or pellets without the drug for 28 consecutive days. Blood pressure was monitored weekly, and treatment with hydralazine significantly decreased SBP (see Supplementary Figure S1 at <http://www.clinsci.org/cs/124/cs1240053add.htm>). The animals were killed and liver tissue samples were obtained to measure the *Hif1 $\alpha$*  and *Sirt1* (sirtuin 1) mRNA levels, as described above.

### Statistical analysis

Quantitative data are expressed as means  $\pm$  S.E.M., unless otherwise indicated. The data were also adjusted for body length whenever applicable. Pairwise mean differences were evaluated using the non-parametric Mann-Whitney test, as most of the variables were ratios and not normally distributed, and non-homogeneous variances between the groups were evident. For the comparison of more than two groups, we used the Kruskal-Wallis test or two-way ANOVA after log-transformation of the dependent variable using strain and diet as categorical factors. To test the differences in steatosis gradation (as a categorical response variable), we used ANCOVA (analysis of co-variance) with ordinal multinomial distribution and probit as a link function and strain and diet as categorical factors adjusting for the indicated variables. Correlation between two variables was determined using the Spearman rank correlation test. A value of  $P < 0.05$  was considered to be statistically significant. We used the Statistica program package StatSoft to perform all the analyses.

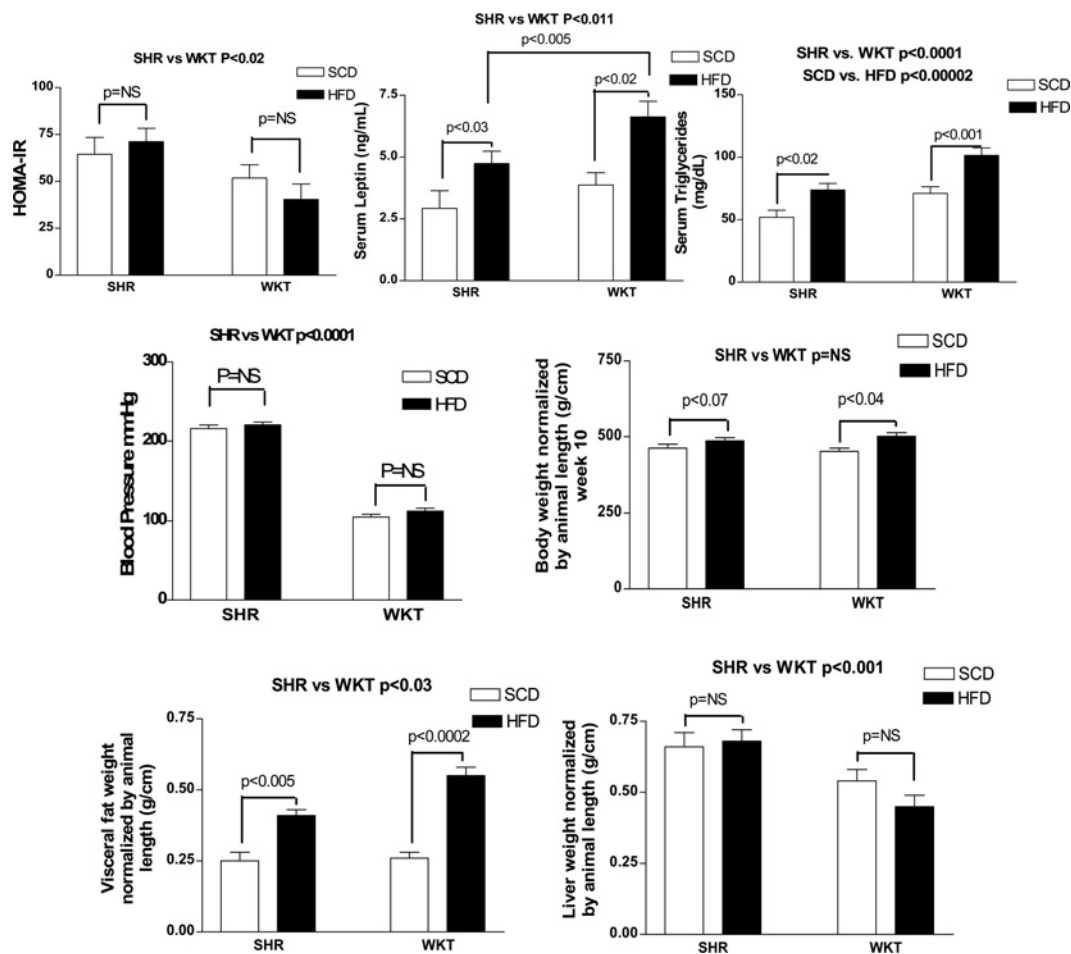
## RESULTS

### Phenotypic characterization of the disease model in comparison with the control strain after exposure to long-term HFD feeding

As described above, the SHR strain is an unquestionable rat model of CVD. In the present study, we hypothesized that CVD is associated with some degree of liver injury. In addition, we hypothesized that a phenotype characterized by the presence of CVD might be also clustered with phenotypic and genotypic changes in the liver, which render the organ more sensitive to exposure to nutritional insults such as an HFD, and thus more prone to develop a severe form of NAFLD. Hence we explored the phenotypic characteristics of SHRs in comparison with their controls, the WKY rats, after exposure to long-term HFD.

Overall, compared with the control strain, SHRs clustered some features of the metabolic syndrome, including elevated blood pressure and IR (Figure 1).

Exposure to HFD was associated with significant changes in both strains, including an increase in serum leptin levels and triacylglycerols (reduced levels of serum triacylglycerols in SHRs in comparison with normotensive WKY rats



**Figure 1** Phenotypic characteristics and biometric variables in the disease model (SHR) and the control strain (WKY) according to diet

SBP (mmHg), HOMA-IR, serum leptin levels (ng/ml), serum triacylglycerols (mg/dl), and body, liver and visceral fat weights (normalized by animal length) in SHR and WKY rats according to diet. Values are means  $\pm$  S.E.M. from SHRs ( $n = 13$ ) and WKY rats ( $n = 14$ ) divided into two experimental groups (SCD, SHR  $n = 7$  and WKY  $n = 7$ ; and HFD, SHR  $n = 6$  and WKY  $n = 7$ ). Statistical significance for SHR versus (vs) WKY represents the comparison between the strains independent of diet by two-way ANOVA. NS, not significant.

have similarly been reported [21]) and visceral fat deposition (Figure 1). In fact, in the whole experiment leptin levels were highly correlated with visceral fat deposition (Spearman  $R = 0.74$ ,  $P < 0.0001$ ).

Body weight was significantly increased only in the control strain (WKY) after exposure to the HFD; the disease model (SHR) showed a trend that did not reach statistical significance (Figure 1). The control strain did not show changes either in the HOMA index or blood pressure after the exposure to the HFD (Figure 1).

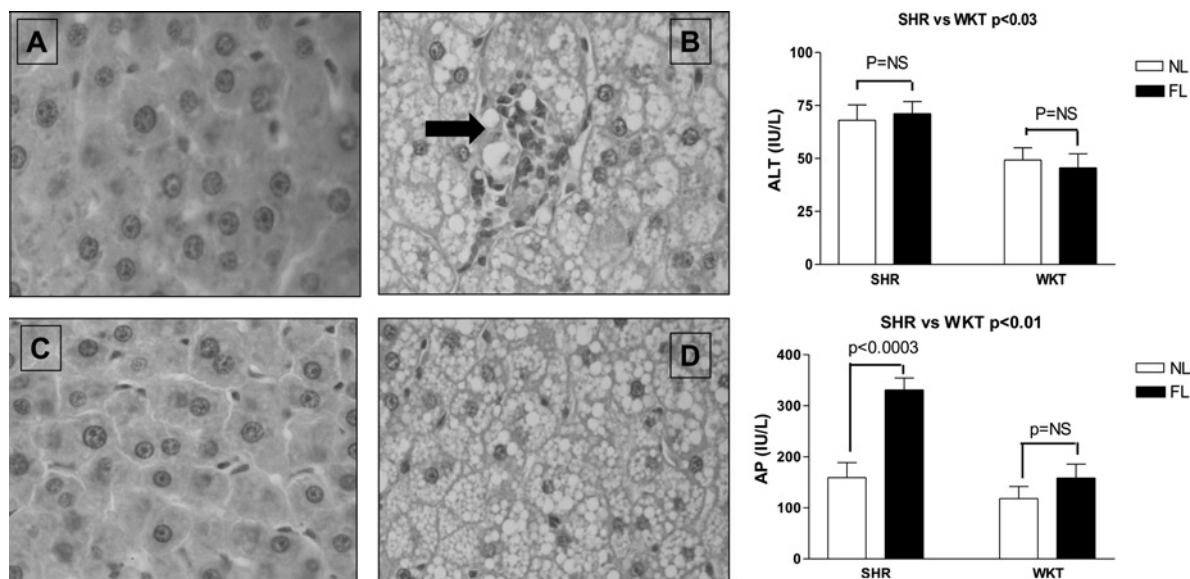
#### Analysis of liver-related characteristics in the disease model in comparison with the control strain after exposure to long-term HFD feeding

HFD-fed rats irrespective of strain developed severe hepatic microvesicular and macrovesicular steatosis (Figures 2B and 2D). In fact, at the end of the experiment, semi-quantitative evaluation

of the liver steatosis from H&E staining of liver sections using a steatosis score demonstrated significant differences between the SCD and the HFD groups, regardless of the rat strain (ANCOVA with steatosis gradation as a categorical response variable with ordinal multinomial distribution and probit as a link function, adjusted by the HOMA-IR, animal length and adipose tissue as continuous predictor variables) (SHR-SCD,  $0.17 \pm 0.30$ ; SHR-HFD,  $1.50 \pm 0.22$ ; WKT-SCD,  $0.01 \pm 0.28$ ; WKT-HFD,  $1.63 \pm 0.25$ ;  $P < 0.00001$ ,  $\chi = 24.5$ ).

Biochemical analysis of hepatic triacylglycerol content also showed that the amount of triacylglycerols was significantly ( $P < 0.00002$ ) increased in the HFD group (regardless of the rat strain, SHR,  $200 \pm 26.8 \mu\text{g/mg}$  of liver; and WKY,  $197.6 \pm 27.1 \mu\text{g/mg}$  of liver) in comparison with SCD rats (SHR,  $62.3 \pm 29.3 \mu\text{g/mg}$  of liver; and WKY,  $48.3 \pm 28.2 \mu\text{g/mg}$  of liver).

Interestingly, SHRs that developed NAFLD had significantly higher scores of histological necroinflammatory activity



**Figure 2** Liver histology and enzymes in the disease model (SHR) and the control strain (WKY) according to diet. Left-hand panel, liver histology of a representative animal from each experimental group. H&E staining of liver sections at the end of the experiment of a representative rat from each experimental group. The livers of rats fed on the SCD had normal histology and the absence of fat accumulation (A, SHR-SCD; C, WKY-SCD). The liver of SHRs fed on the HFD had severe panlobular microvesicular and macrovesicular steatosis and scattered focus of lobular inflammation (B, arrow). The livers of WKY rats fed on the HFD had severe panlobular microvesicular and macrovesicular steatosis without lobular inflammation (D). Original magnification,  $\times 400$ . Right-hand panel, ALT and AP levels in each experimental group. Statistical significance for SHR versus (vs) WKY represents the comparison between the strains independent of diet by two-way ANOVA. NS, not significant.

( $0.72 \pm 0.19$ ) in comparison with the WKY strain ( $0.33 \pm 0.12$ ;  $P < 0.04$ ,  $\chi = 4.3$ ). Figure 2(B) shows a scattered focus of inflammation in a representative SHR rat that developed fatty liver.

Regarding other liver-related features, we observed that SHRs as a group showed significantly higher liver weight compared with the control strain, regardless of HFD (Figure 1). In addition, the disease model showed significantly higher levels of AP and ALT, regardless the presence of fatty liver (Figure 2). In fact, AP was significantly higher in SHRs compared with WKY rats, even after adjusting by the HOMA index. A significant interaction between HFD and rat strain on AP was also observed ( $P < 0.01$ ). AP levels probably reflect liver isoenzyme because there was a significantly positive correlation between AP levels and liver weight (Pearson  $R = 0.65$ ,  $P < 0.0003$ ). Liver weight was significantly correlated with HOMA-IR ( $R = 0.5$ ,  $P < 1 \times 10^{-5}$ ).

Surprisingly, WKY rat liver weights (Figure 1) and liver enzymes (Figure 2) did not change with diet, even though fatty liver was present.

In conclusion, the disease model was associated with abnormal enlargement of liver tissue and increased levels of ALT and AP, even under basal conditions; after exposure to HFD the SHRs developed NAFLD with low degree of liver inflammation.

### Analysis of liver expression of Hif1 $\alpha$ in the disease model in comparison with the control strain

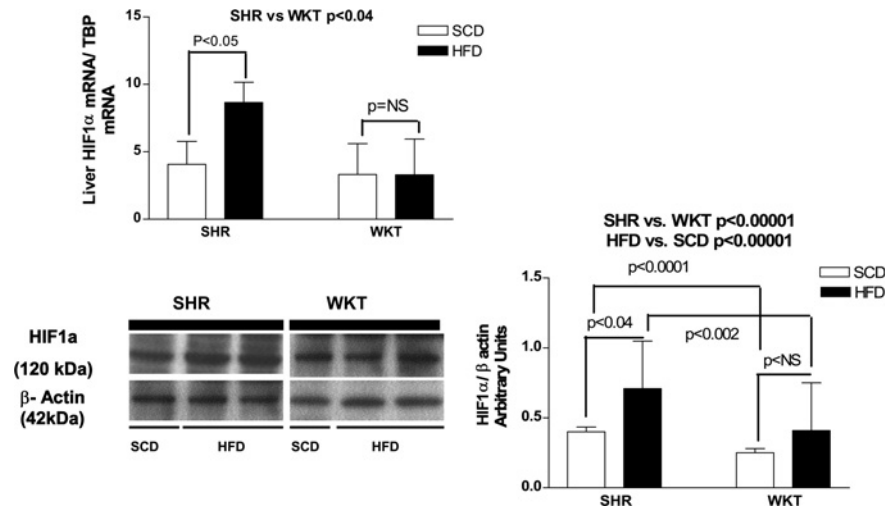
In a previous human study of NAFLD associated with the metabolic syndrome, we demonstrated that hypoxia-related genes were highly up-regulated in the liver tissue [6]. Evidence from animal studies also supports this finding [22,23].

In the present study, we hypothesized that CVD is associated with liver up-regulation of Hif1 $\alpha$ ; thus we explored the abundance of *Hif1a* mRNA in the liver tissue of SHRs and the control strain. We also aimed to investigate the hypothesis that elevated ALT levels observed in the SHR strain may be associated with overexpression of hepatic *Hif1a*. The rationale of this assumption is that, in many disease states, hypoxia and inflammation occur co-incidentally [24]. Interestingly, we observed that *Hif1a* mRNA was significantly up-regulated in the liver of SHRs as a group in comparison with the control WKY strain (Figure 3), irrespective of diet. In addition, exposure to the HFD was associated with a significant increase of the liver abundance of *Hif1a* mRNA in SHRs but not in the control strain (Figure 3).

Western blot analysis showed that the level of protein, in parallel with the liver *Hif1a* mRNA expression, was also significantly higher in the livers of SHRs (Figure 3).

Remarkably, global serum levels of ALT was correlated significantly with liver abundance of *Hif1a* mRNA ( $R = 0.50$ ,  $P < 0.006$ ). In conclusion, the disease model was associated with up-regulation of liver Hif1 $\alpha$  expression.

To test the possibility that Hif1 $\alpha$ -mediated liver damage is a consequence of systemic pathophysiological changes associated with CVD, we measured the local expression of *Hif1a* mRNA in another tissue, namely VAT (visceral adipose tissue). We chose VAT because previous evidence has shown that hypoperfusion and hypoxia in VAT was associated with metabolic syndrome phenotypes and inflammation [25]. Interestingly, CVD was not associated with local up-regulation of *Hif1a* mRNA in VAT, as there were no differences in



**Figure 3** Analysis of liver *Hif1α* mRNA and *Hif1α* protein expression in the disease model (SHR) and the control strain (WKY) according to diet

Upper panel: analysis of liver *Hif1α* mRNA abundance by quantitative RT-PCR in each experimental group. Values are the means  $\pm$  S.E.M. from SHR rats ( $n = 13$ ) and WKY rats ( $n = 14$ ) randomly divided into two experimental groups: SCD (SHR  $n = 7$  and WKY  $n = 7$ ) and *ad libitum* access to the HFD (SHR  $n = 6$  and WKY  $n = 7$ ) for 10 weeks. In each sample, the *Hif1α* expression was normalized to the expression of *Tbp*. Statistical significance for SHR versus (vs) WKY represents the comparison between the strains independent of diet by two-way ANOVA. Lower panel, analysis of liver *Hif1α* protein expression by Western blotting. Representative analysis of liver *Hif1α* protein levels in each experimental model (SHR or WKY) using  $\beta$ -actin as a control for loading, and densitometric quantification of liver *Hif1α* protein levels. Values are means  $\pm$  S.E.M. for 13 animals in SHR group and 14 in WKY).

its abundance between strains [Supplementary Figure S2 at <http://www.clinsci.org/cs/124/cs1240053add.htm>]. Nevertheless, HFD was associated with an increase in visceral fat *Hif1α* mRNA in the control strain ( $P < 0.03$ ; Supplementary Figure S2), probably reflecting an increased inflammatory response, as HFD-fed WKT rats had increased VAT levels of *Tnfα* (Supplementary Figure S2).

These findings demonstrate that the liver, but not VAT, seems to be a direct target of *Hif1α*-associated damage.

### Analysis of the inflammatory response in the disease model in comparison with the control strain: role of pro-inflammatory cytokines *Tnfα* and IL-6 (interleukin-6)

Because hypoxia and inflammation are strongly linked, as hypoxia can induce inflammation, and inflamed lesions often become severely hypoxic [26], we decided to explore whether the disease model was associated with liver induction of inflammatory factors, such as *Tnfα* and IL-6, because these cytokines are involved in the inflammatory response leading to a multiple inflammatory cascades.

As shown in Figure 4, hepatic abundance of *Tnfa* and *Il-6* mRNAs did not differ among groups and neither diet nor rat strain showed significant differences, despite a trend towards higher levels of *Tnfa* in HFD-fed SHR. Thus we further explored hepatic protein levels of *Tnfα* and observed that there was a significant increase induced by diet ( $P < 0.0008$ ; Figure 4), mostly observed in SHR because SHR fed on the HFD had significantly higher hepatic *Tnfα* levels in comparison with the control strain; these findings reinforce our observations about HFD in the

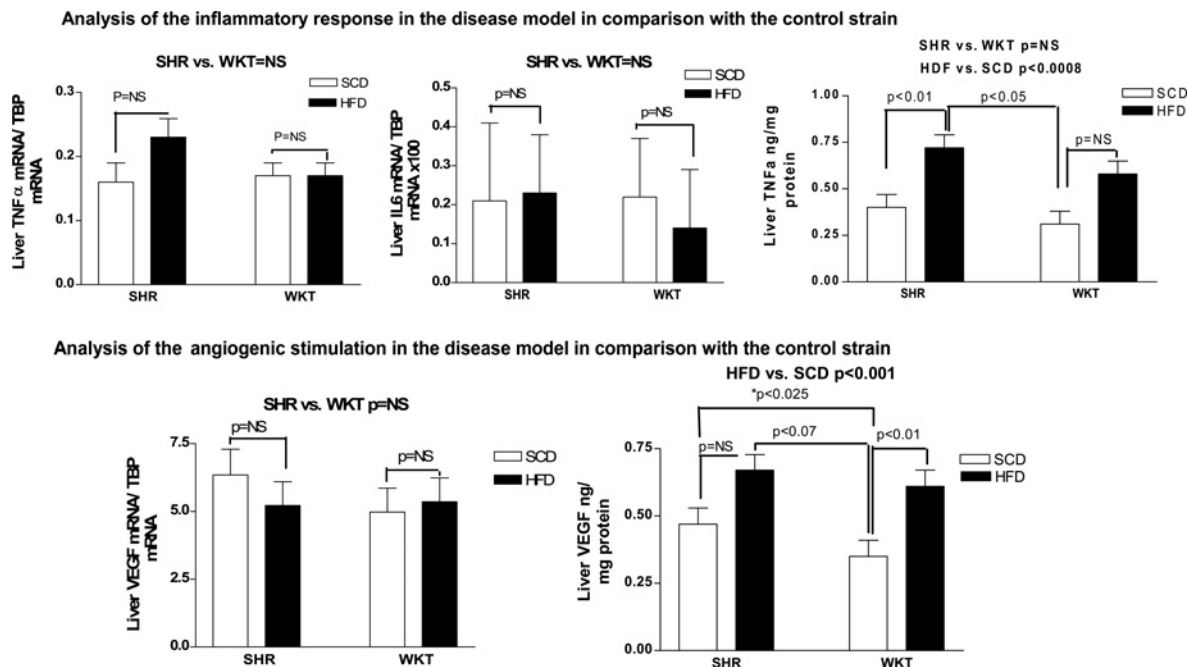
disease model being associated with higher foci of necroinflammatory activity in the liver histology (Figure 2).

### Interventional experiment: liver hypoxia was not reverted by the use of a direct acting splanchnic vasodilator, suggesting that hepatic *Hif1α* overexpression in the disease model is unrelated to systemic haemodynamic changes

It is well known that the major haemodynamic alteration in hypertension is an increased vascular resistance and vasoconstriction, which affects some vascular beds, particularly the splanchnic vasculature. To address the question of whether hepatic hypoxia is just a consequence of abdominal vasculature vasoconstriction associated with the cardiovascular phenotype, we decided to perform an additional interventional experiment in which we normalized the arterial blood pressure in the SHR strain by hydralazine, which does not appear to produce uniform vasodilation in all organs, but is a splanchnic vasodilator [27], directly dilating arteriolar smooth muscle of the liver [28]. Interestingly, there was no significant difference in the liver abundance of *Hif1α* and *Sirt1* mRNA between treated and untreated SHR, nor was there any significant difference in ALT and AP levels (Supplementary Figure S1).

### Analysis of putative factors associated with the liver induction of *Hif1α*: the role of angiogenic stimulation and modulation of metabolic response

The results of our previous experiment suggest that *Hif1α* overexpression in the liver of the SHR strain was not associated with splanchnic vasoconstriction; thus we investigated whether hypoxia occurs in the disease model as an adaptation for angiogenic



**Figure 4** Analysis of the inflammatory responses and angiogenic stimulation in the disease model (SHR) and the control strain (WKY) according to diet

Upper panel, analysis of the inflammatory response in the disease model in comparison with the control strain. Liver expression of *Tnfα* and *Il-6* mRNAs, and liver *Tnfα* protein levels were measured in the disease model in comparison with the control strain. Lower panel, analysis of the angiogenic stimulation in the disease model in comparison with the control strain. Liver expression of *Vegf* mRNA and *Vegf* protein levels were measured in the disease model in comparison with the control strain. Values are the means  $\pm$  S.E.M. from SHRs ( $n=13$ ) and WKY rats ( $n=14$ ) randomly divided into two experimental groups: SCD (SHR  $n=7$  and WKY  $n=7$ ) and *ad libitum* access to the HFD (SHR  $n=6$  and WKY  $n=7$ ) for 10 weeks. \**P* value represents the pairwise mean differences evaluated using the non-parametric Mann–Whitney test. NS, not significant.

stimulation. It is known that *Hif1α* binds to the promoter region of *Vegf* and thereby increases its expression under hypoxic conditions. Thus we evaluated *Vegf* mRNA and *Vegf* protein levels in the liver tissue of both strains. Although liver abundance of *Vegf* mRNA was not significantly associated with diet or rat strain, hepatic protein levels of *Vegf* were significantly up-regulated in HFD-fed rats (Figure 4). As shown before, HFD strongly modulates liver *Hif1α* expression and tissue inflammatory response, thus we investigated whether hepatic *Vegf* expression in the disease model was altered without a metabolic insult. For this purpose, hepatic *Vegf* levels were analysed among rats fed on the SCD. Interestingly, the disease model was significantly associated with liver *Vegf* overexpression in basal conditions ( $P < 0.025$ ; Figure 4), suggesting that the presence of CVD is associated with a hypoxic condition in the liver tissue.

In addition, physiological and pathological activation of *Hif1α* involves changes in the expression of genes that modulate metabolic control and energy metabolism [29]. Recent evidence has revealed a crucial cross-talk between *Hif1α* and *Sirt1*, a gene encoding a deacetylase [30]. Hence we speculated that *Sirt1* may also be dysregulated in the liver of this CVD model. Indeed, we observed significantly higher levels of hepatic *Sirt1* mRNA in the SHR strain in comparison with the control WKY rats (Figure 5), regardless of the exposure to long-term HFD feeding.

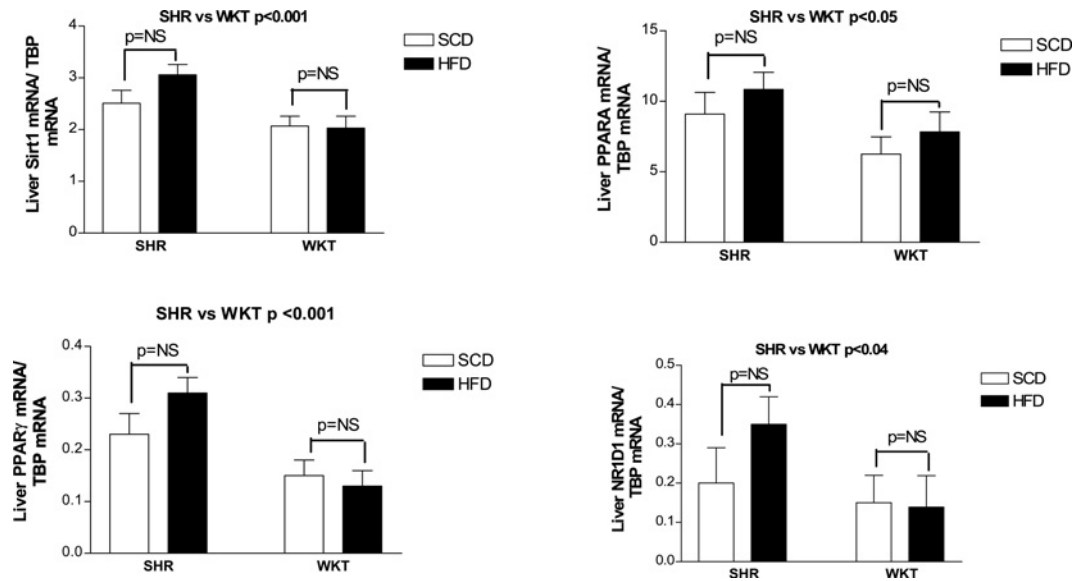
We evaluated further whether the altered pattern of liver gene expression described might explain the major phenotypic change

observed in the disease model, such as liver enlargement. Unexpectedly, we observed a positive and significant relationship between liver *Hif1α* mRNA abundance and liver weight (Spearman Rank order correlation  $R = 0.50$ ,  $P < 0.01$ ). In addition, we observed a positive and significant relationship between liver weight and liver mRNA expression of *Sirt1* (Spearman Rank order correlation  $R = 0.62$ ,  $P < 0.0005$ ). Liver expression of *Sirt1* and of *Hif1α* mRNA were also highly correlated ( $R = 0.71$ ;  $P < 0.00002$ ).

We then investigated whether the altered pattern of liver enzymes in the SHR strain was associated with the dysregulated pattern of liver *Sirt1* mRNA expression. We observed that serum levels of ALT ( $R = 0.60$ ,  $P < 0.002$ ) and AP ( $R = 0.50$ ,  $P < 0.004$ ) were correlated significantly with the liver abundance of *Sirt1* mRNA.

#### Glucose-related metabolism in the disease model in comparison with the control strain

Induction and stabilization of *Hif1α* is primarily, but not limited to, tissue hypoxia, as a growing number of factors can activate *Hif1α* even under non-hypoxic conditions, such as glucose and its metabolites [31]. To test whether intracellular metabolic signals are related to the observed phenotypic changes in the SHR strain, we first measured intrahepatic levels of glucose in all experimental groups. Remarkably, hepatic glucose levels were significantly decreased in the



**Figure 5** Liver expression analysis of *Sirt1*, *Ppara*, *Pparg* and *Nr1d1* mRNAs evaluated by quantitative RT-PCR in each experimental group

Values are means  $\pm$  S.E.M. from SHR rats ( $n=13$ ) and WKY rats ( $n=14$ ) randomly divided into two experimental groups: SCD (SHR  $n=7$  and WKY  $n=7$ ) and *ad libitum* access to the HFD (SHR  $n=6$  and WKY  $n=7$ ) for 10 weeks. In each sample, the gene expression was normalized to the expression of *Tbp*. Statistical significance for SHR versus (vs) WKY represents the comparison between the strains independent of diet by two-way ANOVA. NS, not significant.

SHR strain, regardless of HFD (Supplementary Figure S3 at <http://www.clinsci.org/cs/124/cs1240053add.htm>). In addition, hepatic glucose levels inversely correlated with either phenotypic characteristics, such as liver weight ( $R = -0.43$ ,  $P < 0.02$ ), ALT levels ( $R = -0.40$ ,  $P < 0.04$ ), AP levels ( $R = -0.40$ ,  $P < 0.04$ ) or liver transcripts such as *Sirt1* mRNA ( $R = -0.4$ ,  $P < 0.04$ ).

Accordingly, we investigated whether gluconeogenesis was deregulated in the liver tissue in the disease model. Thus we evaluated the liver transcriptional profile of genes highly involved in gluconeogenesis, such as *Hnf4a* [gene encoding Hnf4 $\alpha$  (hepatocyte nuclear factor 4 $\alpha$ )], *Pepck* (gene encoding phosphoenolpyruvate carboxykinase 1) and *Pgcl1a* [gene encoding Ppar (peroxisome-proliferator-activated receptor)  $\gamma$  co-activator 1 $\alpha$ ]. We observed that hepatic levels of *Hnf4a* were up-regulated in the disease model regardless of long-term HFD feeding (Supplementary Figure S3), suggesting the inefficiency of insulin to suppress hepatic glucose production, a pathological state associated with hepatic IR. No significant changes were observed in the liver expression of *Pepck* and *Pgcl1a* mRNAs, even though there was a trend to be higher in both strains after exposure to the HFD (Supplementary Figure S3).

Finally, we measured the amount of hepatocellular glycogen by PAS quantification, and observed that the SHR strain had lower liver glycogen levels, regardless of HFD feeding ( $P < 0.00001$ ; Supplementary Figure S3).

#### Analysis of liver pattern of gene expression of Hif1 $\alpha$ -related metabolic targets in the disease model in comparison with the control strain

We explored further whether the disease model was associated with dysregulated patterns of liver gene transcription. We targeted

specific transcripts in related pathways, such as nuclear receptors with a major role in metabolic homeostasis as Ppar, because previous findings have shown that Hif1 regulates Ppar signalling pathways [32]. Thus, in the present study, we evaluated the hepatic transcript levels of *Ppara* (encoding Ppar $\alpha$ ) and *Pparg* (encoding Ppar $\gamma$ ) mRNA in both strains. Remarkably, relative liver abundance of both mRNAs was significantly up-regulated in the liver of SHRs in comparison with WKY rats, regardless of the exposure to a nutritional insult such as long-term HFD feeding (Figure 5).

The transcriptional regulatory network integrated by the nuclear receptor super family co-ordinates not only metabolic homeostasis, but also cardiovascular physiology, blood pressure and circadian rhythm. Because the expression of *Nr1d1* [nuclear receptor subfamily 1, group D, member 1, also known as thyroid hormone receptor,  $\alpha$  like] is tightly involved in the regulation of circadian rhythm, and variations in the circadian rhythm strongly influence blood pressure and cardiovascular physiology [33], we explored whether the transcript level of liver *Nr1d1* mRNA was altered in the SHR strain. Interestingly, we observed significantly higher levels of *Nr1d1* mRNA in the liver of SHRs in comparison with the control strain, regardless of HFD feeding (Figure 5). In summary, the disease model is associated with dysregulated patterns of liver gene transcription of metabolic targets.

## DISCUSSION

In the present study, we addressed the hypothesis that CVD is associated with some degree of liver damage predisposing the organ to develop a more severe form of NAFLD after exposure



to a nutritional insult such as HFD. Our main hypothesis was centred on the potential role of the transcription factor Hif1 $\alpha$  as the link between liver injury and CVD. Thus, in this study we developed HFD-induced NAFLD in a rat model of essential hypertension, which has been extensively used to study CVD [34].

Surprisingly, we found that the SHR strain showed hepatomegaly and increased ALT levels compared with its control WKY, even in the absence of fatty liver and irrespective of the HFD. Liver weight in the SHR strain was significantly associated with plasma AP levels and HOMA-IR. SHRs fed with the HFD developed a model of metabolic syndrome-associated NAFLD with some degree of histological liver inflammation. By contrast, HFD intake in the control WKY strain resulted in a model of simple steatosis without liver inflammation and no change in either liver weight or enzymes in spite of a significant impact on serum leptin levels, body weight and visceral fat deposition.

Furthermore, the disease model-fed HFD was associated with increased hepatic levels of inflammatory cytokines, such as Tnf $\alpha$ , supporting the morphological findings about the SHR strain develops a more aggressive histological liver disease after exposure to a nutritional insult.

A remarkable finding of our present study is the observation that the disease model is associated with increased liver Hif1 $\alpha$  protein and *Hif1a* mRNA expression. Reinforcing this finding, we observed that the SHR strain showed significantly higher Vegf levels, irrespective of HFD.

Consequently, we may assume that Hif1 $\alpha$  is responsible for the enhanced susceptibility to liver injury in the disease model further aggravated after long-term HFD exposure.

In addition, we found that SHRs had increased levels of the liver *Sirt1* transcript. Surprisingly, liver weight was significantly associated with the liver transcript level of *Sirt1* and *Hif1a*, the levels of both mRNAs being highly correlated. These findings are biologically plausible, because recent evidence has shown a close Sirt1-Hif1 $\alpha$  interaction [30]. This pattern of gene transcription was not replicated in VAT, suggesting that up-regulation of the *Hif1a* transcript was restricted to the liver.

Interestingly, although we cannot completely rule out the possibility that increased levels of liver Hif1 $\alpha$  were directly related to hepatic hypoxia, our results using a direct vasodilator suggest that the splanchnic vasoconstriction associated with the systemic cardiovascular phenotype might not be the main cause. Thus, we wondered if some other local events might explain the abnormal phenotypic characteristics observed in the SHR strain, and we found that liver weight, ALT and AP levels, and *Sirt1* mRNA were significantly and inversely correlated with glucose levels in the liver tissue. These findings suggest that either the CVD phenotype causes high metabolic demands and elevated rates of hepatocyte glucose consumption, or hepatocytes abnormally regulate the cellular glucose levels due to the IR state. Our findings about up-regulation of *Hnf4a* and depleted glycogen stores in the disease model strongly suggest that SHRs have abnormalities in liver glucose metabolism that might reflect that the CV phenotype is associated with polygenic modulation at the hepatic level that significantly impact on systemic glucose metabolism as increased gluconeogenesis is the predominant mechanism responsible for

this increased glucose output after glycogen depletion. Moreover, adaptation to hypoxia is critically dependent on the reprogramming of glucose metabolism.

We evaluated further the liver transcriptional profile of highly metabolic genes and observed that SHRs significantly overexpressed *Ppara* and *Pparg* transcripts in comparison with the control strain, regardless of the exposure to HFD. It is well known that metabolic energetic demands directly impact on the regulation of body size [35]. In this study, we observed that the expression of *Ppara* and *Pparg*, two genes involved in the control of metabolic demands, was significantly up-regulated in the liver of SHRs. In fact, *Ppara* is highly expressed in tissues with high rates of fatty acid catabolism [36] and serves as a metabolic stress response factor by increasing the expression of enzymes involved in fatty acid utilization pathways, not only in response to normal physiological conditions but also in disease states [37]. Actually, evidence from animal models and human studies of patients with IR shows an abnormal activation of *Ppara* in the heart to compensate metabolic demands [38,39].

Furthermore, previous evidence has shown that SHRs overexpress the transcript of *Pparg* in aorta and mesenteric arteries [40], and this change has been attributed to pathophysiological adaptations to arterial hypertension. Hence, we might speculate that the observed changes in the transcription levels of the Ppars are pathophysiological adaptations to compensate for the metabolic needs associated with this disease model.

The liver of SHRs also showed significantly higher levels of *Nr1d1* in comparison with the control WKY strain. Although the meaning of this finding remains to be elucidated, it is reasonable to speculate that the CVD phenotype is associated with a dysregulation of the hepatic clock machinery, as *Nr1d1* is an integral part of the peripheral clock regulating metabolic pathways in concert with nuclear receptors [41]. Supporting our observations is the biological evidence showing that the up-regulated nuclear receptor transcripts observed in our study are highly related to each other [33]. In addition, a recent published study has reported that dysregulation of the cardiovascular diurnal rhythm in SHRs is associated with altered patterns of liver expression of core clock genes, including *Nr1d1* [42].

In conclusion, the results of the present study show that the liver seems to be a target for cardiovascular organ damage and CVD may predispose to an increased susceptibility of the liver to undergo pathological changes, including liver inflammation after exposure to a nutritional insult. The behaviour of liver transcripts in the disease model might suggest a singular programming of the liver metabolism associated with pathological stress such as CVD. More importantly, up-regulation of hepatic Hif1 $\alpha$  might be the link between CVD and liver injury.

Alternatively, it is worth noting that the hypothesis about fatty liver worsening CVD might also be valid, as HFD-fed rats showed a tendency to have higher levels of SBP, despite the fact that similar increments of serum triacylglycerols were observed in both strains (Figure 1). Nevertheless, more experimental data are needed to confirm whether the altered hepatic metabolism observed in the disease model is potentially able to worsening the cardiac or vascular function beyond traditional cardiovascular risk factors. In this regard, the evidence from human studies shed

light on this matter, as NAFLD was associated with abnormalities in left ventricular morphology and diastolic dysfunction [43–46], and also with carotid atherosclerosis [4,47].

Finally, in the analysis of the behaviour of the liver transcripts in the SHR strain altogether, a surprising pattern emerges, which is that CVD is associated with a shift of liver metabolism towards a stress metabolic response by activating transcriptional programmes similar to caloric restriction and fasting. Thus, not surprisingly, SHRs had increased levels of *Sirt1* mRNA, a stress-activated protein deacetylase that regulates different cell survival pathways, including inflammation and resistance to hypoxic and heat stress [48,49], and *Ppara* mRNA, which during fasting or in T2D contributes to increased hepatic glucose output [50]. Reinforcing this hypothesis, recent findings have shown that *Ppara* is located upstream from *Sirt1* and has a role in regulating *Sirt1* expression in fasting-induced anti-aging pathways [51]. Furthermore, the liver of SHRs showed partially depleted glycogen stores and enhanced gluconeogenesis.

## CLINICAL PERSPECTIVES

- Liver damage and CVD are clinically related and often co-existing entities, but the mechanisms behind this association are still unclear. We postulated that hypoxia might play a pathophysiological role linking CVD to liver injury.
- In the present study, we observed that SHRs with genetic CVD have hepatomegaly, increased ALT levels and an up-regulation of liver *Hif1 $\alpha$*  under baseline conditions. When rats were fed on an HFD, they developed fatty liver with some degree of inflammation, had increased hepatic levels of *Tnf $\alpha$*  and a more pronounced increase in *Hif1 $\alpha$* , which was associated with angiogenic stimulation and a shift in liver metabolism towards a stress metabolic response.
- The liver appears to be a target for organ damage in CVD, which may increase its susceptibility to undergo pathological changes, including inflammation, after exposure to a nutritional insult. A systematic assessment of liver damage may be an important addition to the risk assessment of patients with CVD and related hypoxic conditions, such as obstructive sleep apnoea.

## AUTHOR CONTRIBUTION

Adriana Burgueño and Tomas Gianotti performed the molecular experiments; Adriana Burgueño and Noelia Mansilla took charge of the experimental model. Carlos Pirola and Silvia Sookoian designed the study, analysed and interpreted the data, and prepared and wrote the paper.

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## SUPPLEMENTARY ONLINE DATA

# Cardiovascular disease is associated with high-fat-diet-induced liver damage and up-regulation of the hepatic expression of hypoxia-inducible factor 1 $\alpha$ in a rat model

Adriana L. BURGUEÑO\*, Tomas F. GIANOTTI\*, Noelia G. MANSILLA\*, Carlos J. PIROLA\*<sup>1</sup> and Silvia SOOKOIAN†<sup>1</sup>

\*Department of Molecular Genetics and Biology of Complex Diseases, Institute of Medical Research A Lanari-IDIM, University of Buenos Aires–National Council of Scientific and Technological Research (CONICET), Ciudad Autónoma de Buenos Aires, Argentina

†Department of Clinical and Molecular Hepatology, Institute of Medical Research A Lanari-IDIM, University of Buenos Aires–National Council of Scientific and Technological Research (CONICET), Ciudad Autónoma de Buenos Aires, Argentina

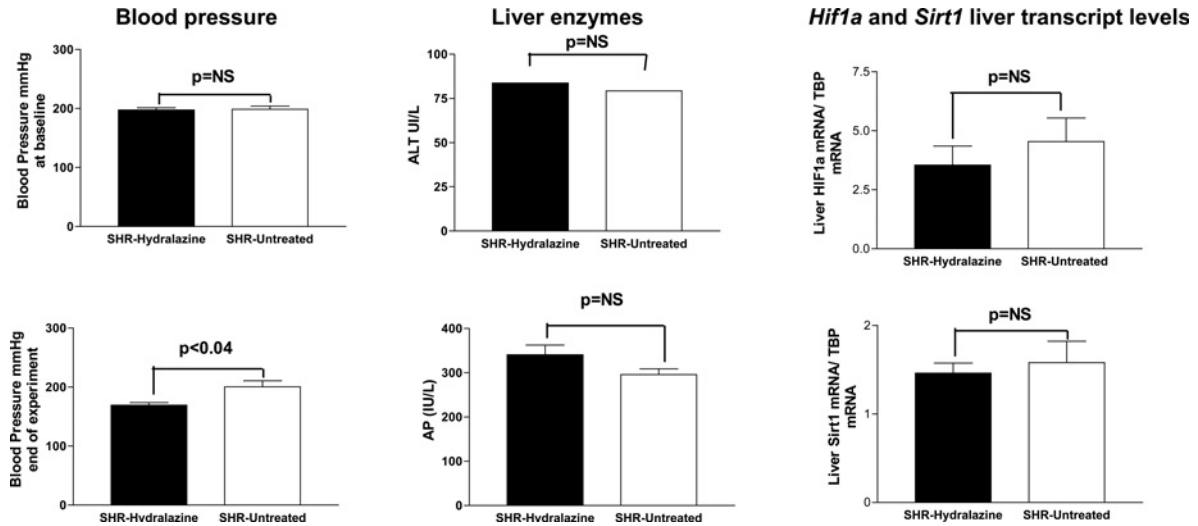
**Table S1 Primers used for the mRNA gene expression by RT-PCR**

Housekeeping genes for relative quantification of gene expression included *Actb* ( $\beta$ -actin), *Gapdh*, *Tbp* and *Ppia* [peptidylprolyl isomerase A (cyclophilin A)].

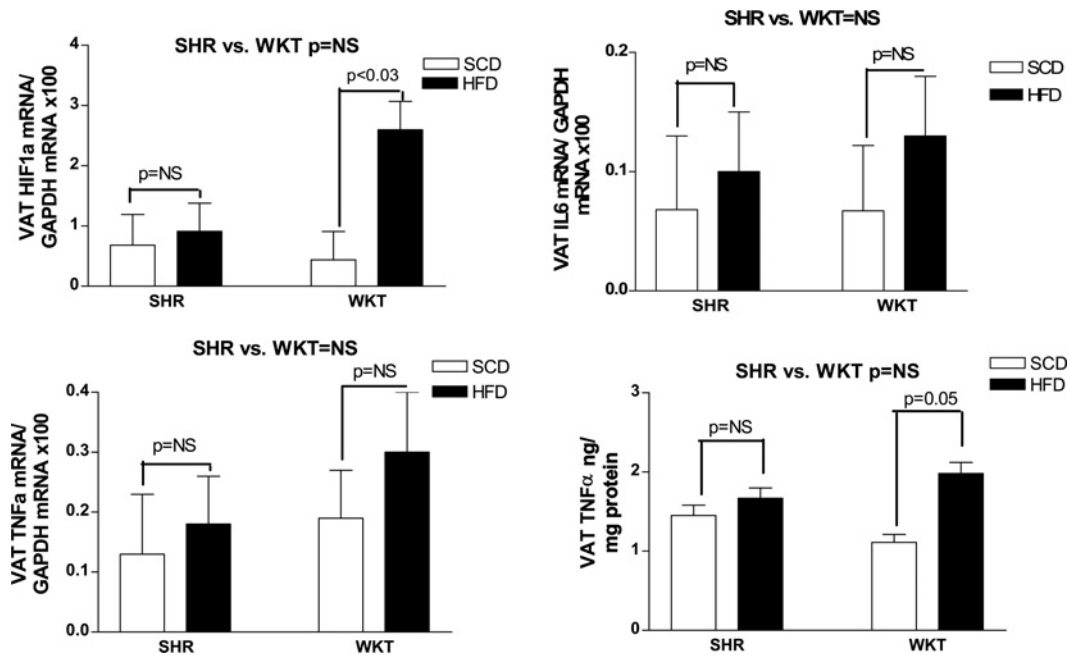
Gene	Primer sequence
<i>Sirt1</i>	Forward, 5'-CAACACCTCATGATTGGCAC-3'; reverse, 5'-GCTTTGGTGGTTCTGAAAGG-3'
<i>Hif1a</i>	Forward, 5'-TGTGTGTGTGAATTATGTTG-3'; reverse, 5'-GTCCTCAGATTCCACTTTAG-3'
<i>Tnfa</i>	Forward, 5'-CCACGCTCTTCTGTCTACTG-3'; reverse, 5'-GGCTACGGGCTTGCTCACTC-3'
<i>Il-6</i>	Forward, 5'-GGAAATGAGAAAAGAGTTGTG-3'; reverse, 5'-GCATCCATCATTCTTTGTAT-3'
<i>Vegf</i>	Forward, 5'-AATGATGAAGCCCTGGAGTG-3'; reverse, 5'-ATGCTGCAGGAAGCTCATCT-3'
<i>Pgc1a</i>	Forward, 5'-AAAAGCTTGACTGGCGTCAT-3'; reverse, 5'-TCAGGAAGATCTGGGCAAAG-3'
<i>Pepck</i>	Forward, 5'-TTGCCTGGATGAAGTTTGTG-3'; reverse, 5'-ATGGTGTCTTCTGGATGGTG-3'
<i>Hnf4a</i>	Forward, 5'-CCTGCCTCAAAGCCATCATC-3'; reverse, 5'-AACGACCCCGAGAGTCATAC-3'
<i>Ppara</i>	Forward, 5'-AAGGCCTCAGGATACCACTA-3' reverse, 5'-CTTGCAGCTTCGATCACACT-3'
<i>Pparg</i>	Forward, 5'-CTGAAGCTCCAAGAATACCA-3' reverse, 5'-TCCCCACAGACTCGGCACTC-3'
<i>Rev-Erba</i>	Forward, 5'-GCAAGGCAACACCAAGAATG-3'; reverse, 5'-ACCTGGTCGTGCTGAGAAAAG-3'
<i>Ppia</i>	Forward, 5'-AGCACTGGGGAGAAAGGATT-3'; reverse, 5'-CTTGCCACCAGTGCCATTAT-3'
<i>Actb</i>	Forward, 5'-TTCCTGGGTATGGAATCCTG-3'; reverse, 5'-CAGCAATGCCTGGGTACAT-3'
<i>Gapdh</i>	Forward, 5'-CTGACATGCCGCTGGAGAAAC-3'; reverse, 5'-CCAGCATCAAAGGTGGAAGAAT-3'
<i>Tbp</i>	Forward, 5'-TGGGATTGTACCACAGCTCCA-3'; reverse, 5'-CTCATGATGACTGCAGCAAACC-3'

<sup>1</sup>These authors contributed equally to this study.

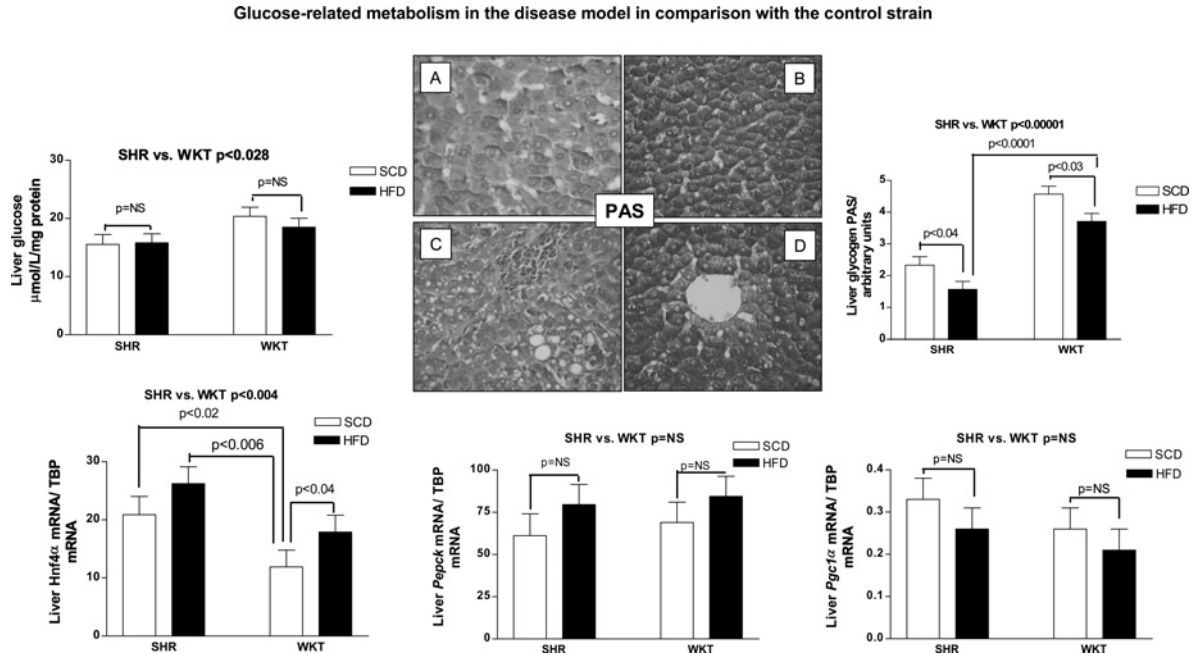
**Correspondence:** Dr Silvia Sookoian (email sookoian.silvia@lanari.fmed.uba.ar) or Dr Carlos J. Pirola (email pirola.carlos@lanari.fmed.uba.ar)



**Figure S1** **Interventional experiment: evaluation of the effect of the direct acting splanchnic vasodilator hydralazine on the hepatic expression of *Hif1a* and *Sirt1* mRNA and liver related-enzymes**  
 Values are the mean  $\pm$  S.D. for SHRs treated with hydralazine ( $n=6$ ) and untreated SHRs ( $n=6$ ). In each sample, the gene expression was normalized to the expression of *Tbp*. NS, not significant.



**Figure S2** **Analysis of VAT expression of *HIF1a*, *Tnfα* and *Il-6* mRNAs by quantitative RT-PCR in each experimental group**  
 Values are the means  $\pm$  S.E.M. from SHRs ( $n=13$ ) and WKY rats ( $n=14$ ) randomly divided into two experimental groups: SCD (SHR  $n=7$  and WKY  $n=7$ ) and *ad libitum* access to the HFD (SHR  $n=6$  and WKY  $n=7$ ) for 10 weeks. In each sample, the gene expression was normalized to the expression of *Gapdh*, as this housekeeping gene was the most stable gene in this experiment). Statistical significance for SHR versus (vs) WKY represents the comparison between the strains independent of diet by two-way ANOVA. NS, not significant.



**Figure S3** **Glucose-related metabolism in the disease model in comparison with the control strain**

Upper panels, liver tissue glucose levels in each experimental group and amount of liver glycogen assessed by PAS stain. PAS staining of liver tissue at the end of the experiment of a representative rat from each experimental group. Densitometric analysis of liver glycogen show that SHR rats fed with standard chow diet (A) and HFD (C) have lower levels in comparison with WKY rats fed on the SCD (B) and the HFD (D). Values are means  $\pm$  S.E.M. for 13 animals in the SHR group and 14 in WKY. Lower panels, liver expression analysis of *Hnf4a*, *Pepck* and *Pgc1a* mRNAs evaluated by quantitative RT-PCR in each experimental group. Values are the means  $\pm$  S.E.M. from SHRs ( $n=13$ ) and WKY rats ( $n=14$ ) randomly divided into two experimental groups: SCD (SHR  $n=7$  and WKY  $n=7$ ) and *ad libitum* access to the HFD (SHR  $n=6$  and WKY  $n=7$ ) for 10 weeks. Statistical significance for SHR vs WKY represents the comparison between the strains independent of diet by two-way ANOVA. NS, not significant.

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