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Characterization of liver changes in ZSF1 rats, an animal model of metabolic syndrome

Marta Borges-Canha[1], José Pedro Portela-Cidade[1], Glória Conceição[1], Cláudia Sousa-Mendes[1], Sara Leite[1], Dulce Fontoura[1], Daniel Moreira-Gonçalves[1], Inês Falcão-Pires[1], André Lourenço[1], Adelino Leite-Moreira[1,2] and Pedro Pimentel-Nunes[1,3,4]

[1]Department of Physiology and Cardiothoracic Surgery. Faculty of Medicine. University of Porto. Porto, Portugal. [2]Department of Cardiothoracic Surgery. Centro Hospitalar São João. Porto, Portugal. [3]Gastroenterology Department. Portuguese Oncology Institute. Porto, Portugal. [4]CIDES/CINTESIS. Faculty of Medicine. University of Porto. Porto, Portugal

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Correspondence: Marta Borges-Canha. Department of Physiology and Cardiothoracic Surgery. Faculty of Medicine of the University of Porto. Alameda Prof. Hernâni Monteiro. 4200-319 Porto, Portugal

e-mail: marta.canha@gmail.com

ABSTRACT

Background: The non-alcoholic fatty liver disease is the hepatic counterpart of the metabolic syndrome. ZSF1 rats are a metabolic syndrome animal model in which liver changes have not been described yet.

Aim: The characterization of liver histological and innate immunity changes in ZSF1 rats.

Methods: Five groups of rats were included (n = 7 each group): healthy Wistar-Kyoto control rats (Ctrl), hypertensive ZSF1 lean (Ln), ZSF1 obese rats with a normal diet (Ob), ZSF1 obese rates with a high-fat diet (Ob-HFD), and ZSF1 obese rats with low-intensity

exercise training (Ob-Ex). The animals were sacrificed at 20 weeks of age, their livers were collected for: a) measurements of the area of steatosis, fibrosis and inflammation (histomorphological analysis); and b) innate immunity (toll-like receptor [TLR] 2, TLR4, peroxisome proliferator-activated receptor γ [PPARγ], toll interacting protein [TOLLIP]) and inflammatory marker (tumor necrosis factor-alpha [TNFα], interleukin 1 [IL-1]) expression analysis by real-time PCR.

Results: Ob, Ob-HFD and Ob-Ex were significantly heavier than Ln and Ctrl animals. Ob, Ob-HFD and Ob-Ex animals had impaired glucose tolerance and insulin resistance. ZSF1 Ob, Ob-HFD and Ob-Ex presented a higher degree of steatosis (3,5x; $p < 0.05$) than Ctrl or ZSF1 Ln rats. Steatohepatitis and fibrosis were not observed in any of the groups. No differences in expression were observed between Ctrl, Ln and Ob animals (except for the significantly higher expression of TOLLIP observed in the Ob *vs* Ln comparison). Ob-HFD and Ob-Ex rats showed increased expression of PPARγ and TOLLIP as compared to other groups. However, both groups also showed increased expression of TLR2 and TLR4. Nevertheless, this did not translate into a differential expression of TNFα or IL-1 in any of the groups.

Conclusion: The ZSF1 model is associated with liver steatosis but not with steatohepatitis or a significantly increased expression of innate immunity or inflammation markers.

Key words: Fatty liver. Immunity. Innate. Liver. Metabolic syndrome X. Non-alcoholic fatty liver disease.

INTRODUCTION

Metabolic syndrome (MS) is a multi-component entity that represents a current major health concern in developed countries due to its impact on cardiovascular and metabolic mortality risk and associated decrease in lifetime survival (1,2). The prevalence of MS is increasing in several countries, with prevalence rates as high as 25% in the USA, 21% in China and greater than 15% in several European countries (3). The current definition of this syndrome includes central obesity, hypertension and

dyslipidaemia, and it is considered to be the major cause of the recent epidemic of diabetes, cardiovascular diseases and even cancer (1,2,4). MS is also a major risk factor for non-alcoholic fatty liver disease (NAFLD), which is now recognized as the hepatic component of the metabolic syndrome (5,6).

Recent studies show that MS and obesity are states of chronic low-grade inflammation with increased levels of several cytokines (such as TNF-α and IL-1) and increased activation of many pro-inflammatory pathways. The inflammation in this condition is thought to be the trigger of many pathologic alterations observed in the MS (7,8). It appears that many of these inflammatory effects in the MS are mediated, at least in part, by the activation of innate immunity receptors such as the toll-like receptors (TLRs), in particular TLR2 and 4 (9-12). TLR2 is essential for the innate immune response to Gram-positive bacteria, and TLR4 is the receptor of lipopolysaccharide of Gram-negative bacteria. However, TLR2 and TLR4 may also sense endogenous ligands such as free fatty acids (the levels of which are increased in MS) initiating inflammatory signals (12-15). TOLLIP and PPARγ block intracellular signalling pathways of TLR activation. In fact, studies have shown that a lower expression of these molecules, particularly of TOLLIP, may be associated with a higher activation of innate immunity and inflammation, particularly in colorectal carcinogenesis (16).

NAFLD is a metabolic liver disease characterized by an extensive continuum of liver injury, varying from non-alcoholic fatty liver to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis, which can ultimately give rise to hepatic carcinoma (17). The innate immune system may also be vital to its progression, and the activation of TLR4 appears to be crucial for the progression of the disease sequence of steatosis-steatohepatitis-fibrosis-cirrhosis and, finally, hepatocarcinoma, as we previously reviewed (18). It seems that TLR4 signaling is enhanced in chronic liver disease, such as NAFLD (19). Besides, in a human virus induced hepatic inflammation-fibrosis-cirrhosis sequence, we found an upregulation of TLR2 and TLR4 (20).

ZSF1 rats are considered to be a robust animal model for MS (21,22). These animals are generated by crossing non-hypertensive lean female Zucker diabetic fatty rats (ZDF, +/fa) with lean spontaneously hypertensive HF prone male rats (SHHF/Mcc,

+/facp), which share a common genetic background with Wistar Kyoto rats (used as control) and derive from spontaneously multifactorial hypertensive rats. Both lean and obese ZSF1 animals inherit a hypertensive gene from the spontaneously hypertensive rat strain and have high blood pressure (23-26). With regard to metabolism, ZSF1 animals develop obesity, abdominal adiposity, insulin resistance, oral glucose intolerance, hyperglycaemia and glycosuria, consistent with a type II diabetes mellitus phenotype (27). Our group previously described metabolic and cardiovascular changes in this animal model (22,28,29).

The present study investigates for the first time the metabolic, innate immunity and inflammatory liver changes in a ZSF1 model of MS, and demonstrates how strategies such as diet and exercise interfere with the inflammatory profile of these organs.

MATERIALS AND METHODS

All the procedures in this study followed the recommendation of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 2011). They have been certified by the Portuguese Veterinary Governmental Authorities, approved by the Portuguese Foundation for Science and Technology and approved by the faculty ethical committee. Trained researchers that are certified with a Laboratory Animal Science course according to the Federation of European Laboratory Animal Science Associations performed all animal handling.

Animal model

Nine-week-old male ZSF1 lean (Ln, n = 7), ZSF1 obese (Ob, n = 21) and Wistar-Kyoto rats (Ctrl, n = 7) were obtained from the Charles River Laboratories (Barcelona, Spain) and fed with a Purina Diet (#5008). All animals were allowed to acclimatize for seven days to laboratory conditions before baseline measurements. Animals were kept in individually ventilated chambers in a controlled environment with a 12 hours light/dark cycle at 22 °C and had unlimited access to food and water.

Experimental design

ZSF1 obese rats were randomly distributed between experimental groups, resulting in three different groups: a) Ob, ZSF1 with a sedentary lifestyle and normal diet (Purina 5008 diet with 0.28% salt, Research Diets Inc.); b) Ob-HFD, ZSF1 animals with a high-fat diet (HFD, Research Diet Inc. #D12468); and c) Ob-Ex, ZSF1 rats with a normal diet and low-intensity exercise training (treadmill ExT for five weeks, five days/week, 60 min/day at a speed of 20 m/min) from the 15th week onward. This procedure resulted in five experimental groups: Ctrl, Ln, Ob, Ob-HFD and Ob-Ex. The animals were sacrificed and liver samples were collected at week 20 (Fig. 1).

Samples collection and morphometric analysis

At 20 weeks of age the animals were sacrificed by exsanguination under sevoflurane-anesthesia. The livers were dissected and snap-frozen in liquid nitrogen, and stored at -80 °C. For histological analysis, samples were stored in buffered 10% formaldehyde. Organs were weighed and the tibia length (TL) was measured. Weights were normalized to TL due to body weight differences between the groups.

Quantitative histomorphometry

After fixation, histological samples were embedded in sectioned paraffin (3-µm-thick sections). Histomorphological analysis of liver samples was performed with picro-sirius red and hematoxylin and eosin. Hematoxylin and eosin staining was used to quantify steatosis, and picro-sirius red staining was used to quantify fibrosis. Sections were digitally photographed (Olympus XC30, Olympys). Digital images (magnification of X400) were analyzed using image analysis software (Image-Pro Plus version 6.0, Media Cybernetics Inc.). Seven randomly selected fields were analyzed per sample by two blinded observers. We used the algorithm described by Roullier V and Cavaro-Ménard C to quantify steatosis (30).

Quantitative RT-PCR

Expression of messenger RNA (mRNA) of TLR2, TLR4, IL-1, TNF-α, TOLLIP and PPARγ in the liver was quantified. Total mRNA was extracted using the RNeasy kit according to the manufacturer's instructions (Qiagen). Two-step RT-PCR was used for relative mRNA quantification (Step-OneTM, Appled Biosystems). Results are expressed as the ratio of the gene/glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which did not differ between groups. To calculate the ratio of inflammatory (TLR2xTLR4)/anti-inflammatory (PPARγxTOLLIP) genetic expression we used the values in arbitrary units set as the average value of the reference group after normalization with GAPDH. Specific PCR primer pairs for the studied genes were: rGAPDH - fw 5'- CCG CCT GCT TCA CCA CCT TCT -3' and rev 5'- TGG CCT TCC GTG TTC CTA CCC -3´; rTLR2 - fw 5´- AGGTCTCCAGGTCAAATCTCAG -3´ and rev 5´- CCTATCACAGCCATCAAGATCC-3´; TLR4- 5´- GAG AGG TCA TTT TTG TCT CCA -3´ and rev 5´- AGT GGC TGG ATT TAT CCA GGT GTG 3´; rIL-1 fw 5´- GTG GTT CAA GGC ATA ACA GGC- 3´ and rev 5´- AGG GAC AGT TGC CAT AGC TG -3´; rTNF-α fw 5´- GCT TGG TGG TTT GCT ACG ACG TGG -3´ and rev 5´- ACT TCG GGG TGA TCG GTC CCA A- 3´; rTOLLIP fw 5´- AGG TGT GGA CTC GTT CTA CC- 3´ and rev 5´- GAC TCG GGG ATA GTG ATG TG- 3´ and rPPARγ fw 5'- AAG ACA TCC CGT TCA CAA G -3' and rev 5'- CTA CTT TGA TCG CAC TTT GG -3'.

Statistical analysis

Values are given as means ± standard error of mean. Statistical significance was set at $p < 0.05$, and was obtained for multiple comparisons between groups by ANOVA followed by a Bonferroni test. Statistical analysis was performed using GraphPad (version 5).

RESULTS

Morphometrics and metabolic risk

The animals showed considerable body weight differences (Table 1). Therefore, the morphometric parameters measured were normalized to TL. The Ob, Ob-HFD and Ob-Ex groups had similar body weights and were significantly higher than the Ln and Ctrl animals. Ln body weights were higher than in Ctrl animals. With regard to body

composition differences, Ob animals showed a substantial increase in perigonadal and perirenal fat compared to the non-Ob groups. Ob, Ob-HFD and Ob-Ex animals had impaired glucose tolerance and insulin resistance, consistent with the presence of diabetes mellitus.

Liver analysis of steatosis and fibrosis

Many significant differences were found with regard to steatosis quantification assessed by hematoxylin and eosin staining of samples (Figs. 2 and 3). Both the Ctrl and Ln groups had significantly less steatosis than the Ob, Ob-HFD and Ob-Ex groups.

With regard to inflammation and fibrosis (Figs. 3 and 4), there was no histological evidence of steatohepatitis or fibrosis in the liver of these animals assessed by sirus red staining.

Liver evaluation of innate immunity and inflammatory markers

With regard to the expression of the innate immunity receptor TLR4, we noticed a tendency of higher levels of this receptor in the Ob groups compared to Ctrl (1.4 ± 0.03) animals, in particular in Ob-Ex rats (1.52 ± 0.01, $p < 0.05$). However, this tendency was not seen with TLR2 expression, which was only significantly increased in the Ob-Ex group (1.32 ± 0.01) when compared to Ctrl (1.2 ± 0.01, $p < 0.05$), Ln (1.17 ± 0.04, $p < 0.05$) and Ob (1.15 ± 0.04, $p < 0.05$) groups.

However, these increases of TLR2 and TLR4 were associated with higher levels of the antagonist molecules PPARγ and TOLLIP. In fact, Ob-HFD animals had higher levels of PPARγ (1.27 ± 0.01) compared to Ctrl, Ln and Ob animals (1.19 ± 0.01, 1.18 ± 0.03 and 1.20 ± 0.01, respectively, $p < 0.05$), and Ob-Ex had the highest levels among all the groups (1.38 ± 0.01, $p < 0.05$, *vs* all the other groups). With regard to TOLLIP, the Ob (1.13 ± 0.01) group had higher levels compared to Ln (1.07 ± 0.02, $p < 0.05$) rats. Likewise, Ob-HFD animals had a significantly increased expression (1.14 ± 0.01) compared to Ln ($p < 0.05$) and Ob ($p < 0.05$) animals. Finally, Ob-Ex rats demonstrated significantly increased TOLLIP expression levels (1.21 ± 0.01) compared to all the other groups. The ratio of increased TLRs/antagonists was 0.97 in Ob-HFD animals but only

0.93 in the Ob-Ex group, which may suggest a global anti-inflammatory effect triggered by exercise. No significant differences were found in the expression of inflammatory cytokines TNFα and IL-1. This information is summarized in figure 5.

DISCUSSION

In the present study we characterized for the first time liver histological and innate immunity changes in ZSF1 obese rats, an animal model of MS. Even though this is a validated model of MS and is associated with liver steatosis, we were not able to show steatohepatitis, fibrosis or increased expression of innate immunity or inflammation markers in ZSF1 rats. Therefore, this may not be a good model to evaluate the steatosis-inflammation-fibrosis pathway of liver pathology.

A potential limitation of this study may be the fact that animals were sacrificed too young, and this may explain why differences in hepatic histological architecture and in the inflammatory profile of the groups were not found. Histomorphological analysis of livers from 25-week old rats was also performed (data not shown), and the results were similar to those of the 20-week old animals (with no steatohepatitis). Thus, the genetic inflammatory profile in these older animals has not been analyzed. Moreover, Ishii et al. observed severe steatosis and inflammation at eight weeks of age and fibrosis started to occur at 32 weeks of age in spontaneously diabetic Torii fatty rats, a promising animal model of NAFLD (32). Furthermore, Agharpour et al. showed that steatohepatitis developed as early as 16 weeks of age and progressive fibrosis, from the 16th week onwards in B6/129 mice (33). Therefore, even though the 25-week old ZSF1 animals may be too young to exhibit inflammation or fibrosis in this particular model, we did not find any tendency for increased inflammation. Other models develop severe steatohepatitis and even fibrosis at an early stage, suggesting that ZSF1 rats are not a good model for studying the steatosis-steatohepatitis-cirrhosis-hepatocarcinoma pathway.

With regard to histological characteristics, the Ob group showed significantly more steatosis when compared to Ctrl and Ln groups. None of the groups presented either inflammation or fibrosis. Marques et al. reported a significant increase in liver steatosis

compared to control animals in a model of 16-week-old male C57BL/6J mice fed with very high-fat chow. However, in contrast to our results, these authors reported areas of inflammatory infiltrate, characteristic of steatohepatitis, which was not present in our histological analysis (31). Thus, significant liver steatosis can be achieved without a high-fat diet in the ZSF1 MS animal model, in contrast to other models.

With regard to the hepatic inflammatory profile, Ob animals had a slightly higher TOLLIP expression than their Ln counterparts, a finding that might represent the immune system response to the pro-inflammatory profile. Additionally, we reported a non-significant tendency of TLR4 levels to increase. Moreover, no significant differences were found in the expression of inflammatory cytokines TNFα and IL-1 between any of the groups. Overall, no large differences were found when comparing the Ob group to Ln and Ctrl animals.

With regard to the inflammatory profile, Li et al. analyzed regulatory microRNAs that lead to TLR2 expression in the liver in rats with NASH and MS induced by a high-fat diet. MicroRNA-144 regulates TLR2 signaling and its expression was negatively correlated with TLR2 expression in Kupffer cells, leading us to conclude that a decrease in microRNA-144 might contribute to TLR2 up-regulation and the progression of NASH (34). With regard to TLR4, Ye et al. investigated its role in the transition from simple steatosis to NASH, and concluded that it has a major role by inducing reactive oxygen species-dependent activation of XBP-1, a transcription factor involved in the unfolded protein responses (35). We may have not observed significant TLR4 alterations in our model as these animals had not developed steatohepatitis. In addition, Kawanishi et al. showed that a model of C57BL/6J mice fed with a high-fat diet and high-fructose water had greater TLR4 mRNA levels compared to control animals. The same authors stated that the groups fed with a high-fat diet and high-fructose water had higher TNFα levels, although no differences were observed in IL-1β mRNA levels (36). Cong et al. quantified PPARγ in a model of NASH using 23-week old C57BL/6 mice (*ad libitum* feeding with a high-fat diet, lower methinione and choline and higher fat content), and recorded significantly increased hepatic PPARγ mRNA expression (as well as suppressed hepatic PPARα mRNA expression) (37). No data on TOLLIP expression was

available. In contrast, ZSF1 Ob rats do not appear to show significant changes in the genetic inflammatory or innate immunity expression profile when compared to controls.

With regard to the *effect of exercise,* the degree of steatosis present in hepatic tissue of Ob-Ex was similar to Ob animals (and significantly higher than in the Ctrl and Ln groups). This contrasts with the effect of exercise in previous studies in other animal models. For example, Sakr et al. reported a beneficial effect of swimming exercise on hepatic histological architecture in 16-week old male Sprague-Dawley rats, a rat model of MS (38). In addition, Kapravelou et al. showed that an aerobic exercise protocol enhanced the degree of steatosis in Zucker rats. This was consistent with the study by Cameron et al. with male Wistar rats fed with a high-carbohydrate and high-fat diet as a model of the human metabolic syndrome (39,40). With regard to the inflammatory profile, the exercise program may have had a minor anti-inflammatory role due to the increase in the expression of TLR2 (compared to Ctrl, Ln and Ob animals) and the magnitude of the increase of TOLLIP and PPARγ expression (significant differences compared to all of the other groups). A previous study in Zucker rats reported an increase in PPARγ expression in the livers of animals submitted to an aerobic exercise-training program (39). Furthermore, we did not observe significant differences in TNFα and IL-1 expression (similar results between Ob and Ob-Ex rats). However, Kawanishi et al. reported decreased TNFα levels in animals submitted to an exercise programme, although no differences were seen in IL-1β mRNA levels. The same authors also reported a decrease in TLR4 mRNA levels in exercised mice (36).

The similarity between ZSF1 Ob-Ex and Ob animals with regard to the degree of liver steatosis and liver expression of TNFα and IL-1, as well as the potential but only minor anti-inflammatory effect of exercise recorded (ratio of increase TLRs/antagonists = 0.93), might suggest that the effect of exercise in the ZSF1 animal model may not be as powerful as in other scenarios. This is probably influenced by the intrinsic genetic profile of these rats.

When analyzing the *effect of a high-fat diet,* intriguingly, the degree of hepatic steatosis of Ob-HFD was similar to that of Ob animals (and significantly higher than Ctrl

and Ln groups). No inflammation or fibrosis was noticed. On the other hand, Carmiel-Haggai et al. reported a significantly higher steatosis grade (micro- and macrovesicular steatosis) and steatohepatitis in Zucker fatty rats fed with a high-saturated fat diet (41). With regard to the inflammatory profile, we were expecting a pro-inflammatory state as a non-significant tendency of TLR2 and TLR4 to increase in Ob-HFD animals was accompanied by a greater expression of PPARγ and TOOLIP (significant difference between Ob-HFD and Ob concerning PPARγ expression). Nevertheless, the role of a high-fat diet has not been well established in this model. We did not observe any increase in TNFα or IL-1 levels. However, we hypothesize that either this model has a different response to a high-fat diet (and exercise) compared to other MS models, or the mutation that these animals carry is a strong inducer of steatosis that makes these animals more insensitive to the influence of external factors.

In conclusion, this is a proof-of-principle study in a new animal model of MS. Indeed, 20-week-old ZFS1 rats have liver steatosis but not with steatohepatitis or fibrosis. In addition, these animals show some differences in the expression of innate immunity or inflammation markers in the liver but these do not match the expectations neither in quantity or quality. The effect of common liver steatosis interventions such as diet and exercise was lower than expected. Therefore, we believe that this is not a good model to study the steatosis-steatohepatitis-fibrosis pathway in MS rats.

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REFERENCES

1. Alberti KG, Zimmet P, Shaw J. The metabolic syndrome: A new worldwide definition. Lancet 2005;366(9491):1059-62. DOI: 10.1016/S0140-6736(05)67402-8

2. McNeill AM, Rosamond WD, Girman CJ, et al. The metabolic syndrome and 11-year risk of incident cardiovascular disease in the atherosclerosis risk in communities study. Diabetes Care 2005;28(2):385-90. DOI: 10.2337/diacare.28.2.385

3. McCullough AJ. Epidemiology of the metabolic syndrome in the USA. J Dig Dis 2011;12(5):333-40. DOI: 10.1111/j.1751-2980.2010.00469.x

4. Zimmet P, Magliano D, Matsuzawa Y, et al. The metabolic syndrome: A global public health problem and a new definition. J Atheroscler Thromb 2005;12(6):295-300. DOI: 10.5551/jat.12.295

5. Hamaguchi M, Kojima T, Takeda N, et al. The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. Ann Intern Med 2005;143(10):722-8. DOI: 10.7326/0003-4819-143-10-200511150-00009

6. Almeda-Valdes P, Cuevas-Ramos D, Aguilar-Salinas CA. Metabolic syndrome and non-alcoholic fatty liver disease. Ann Hepatol 2009;8(Suppl 1):S18-24.

7. Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006;444(7121):860-7. DOI: 10.1038/nature05485

8. Wree A, McGeough MD, Pena CA, et al. NLRP3 inflammasome activation is required for fibrosis development in NAFLD. J Mol Med (Berlin) 2014;92(10):1069-82. DOI: 10.1007/s00109-014-1170-1

9. Shi H, Kokoeva MV, Inouye K, et al. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest 2006;116(11):3015-25. DOI: 10.1172/JCI28898

10. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes 2007;56(8):1986-98. DOI: 10.2337/db06-1595

11. Kim F, Pham M, Luttrell I, et al. Toll-like receptor-4 mediates vascular inflammation and insulin resistance in diet-induced obesity. Circ Res 2007;100(11):1589-96. DOI: 10.1161/CIRCRESAHA.106.142851

12. Nguyen MT, Favelyukis S, Nguyen AK, et al. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. J Biol Chem 2007;282(48):35279-92. DOI: 10.1074/jbc.M706762200

13. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124(4):783-801. DOI: 10.1016/j.cell.2006.02.015

14. Beutler BA. TLRs and innate immunity. Blood 2009;113(7):1399-407. DOI: 10.1182/blood-2008-07-019307

15. Beutler B. Neo-ligands for innate immune receptors and the etiology of sterile inflammatory disease. Immunol Rev 2007;220:113-28. DOI: 10.1111/j.1600-065X.2007.00577.x

16. Pimentel-Nunes P, Goncalves N, Boal-Carvalho I, et al. Decreased Toll-interacting protein and peroxisome proliferator-activated receptor gamma are associated with increased expression of Toll-like receptors in colon carcinogenesis. J Clin Pathol 2012;65(4):302-8. DOI: 10.1136/jclinpath-2011-200567

17. Bugianesi E, Leone N, Vanni E, et al. Expanding the natural history of nonalcoholic steatohepatitis: From cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterol 2002;123(1):134-40. DOI: 10.1053/gast.2002.34168

18. Lopes JA, Borges-Canha M, Pimentel-Nunes P. Innate immunity and hepatocarcinoma: Can toll-like receptors open the door to oncogenesis? World J Hepatol 2016;8(3):162-82. DOI: 10.4254/wjh.v8.i3.162

19. Soares JB, Pimentel-Nunes P, Roncon-Albuquerque R, et al. The role of lipopolysaccharide/toll-like receptor 4 signaling in chronic liver diseases. Hepatol Int 2010;4(4):659-72. DOI: 10.1007/s12072-010-9219-x

20. Soares JB, Pimentel-Nunes P, Afonso L, et al. Increased hepatic expression of TLR2 and TLR4 in the hepatic inflammation-fibrosis-carcinoma sequence. Innate Immunity 2012;18(5):700-8. DOI: 10.1177/1753425912436762

21. Tofovic SP, Kusaka H, Jackson EK, et al. Renal and metabolic effects of caffeine in obese (fa/fa(cp)), diabetic, hypertensive ZSF1 rats. Renal Fail 2001;23(2):159-73. DOI: 10.1081/JDI-100103488

22. Hamdani N, Franssen C, Lourenco A, et al. Myocardial titin hypophosphorylation importantly contributes to heart failure with preserved ejection fraction in a rat metabolic risk model. Circ Heart Fail 2013;6(6):1239-49. DOI: 10.1161/CIRCHEARTFAILURE.113.000539

23. Tofovic SP, Kusaka H, Kost CK, et al. Renal function and structure in diabetic, hypertensive, obese ZDFxSHHF-hybrid rats. Renal Fail 2000;22(4):387-406. DOI:

10.1081/JDI-100100882

24. Tofovic SP, Dubey R, Salah EM, et al. 2-Hydroxyestradiol attenuates renal disease in chronic puromycin aminonucleoside nephropathy. J Am Soc Nephrol 2002;13(11):2737-47. DOI: 10.1097/01.ASN.0000031804.77546.F5

25. Tofovic SP, Kost CK Jr, Jackson EK, et al. Long-term caffeine consumption exacerbates renal failure in obese, diabetic, ZSF1 (fa-fa(cp)) rats. Kidney Int 2002;61(4):1433-44. DOI: 10.1046/j.1523-1755.2002.00278.x

26. Bilan VP, Salah EM, Bastacky S, et al. Diabetic nephropathy and long-term treatment effects of rosiglitazone and enalapril in obese ZSF1 rats. J Endocrinol 2011;210(3):293-308. DOI: 10.1530/JOE-11-0122

27. Zambad SP, Munshi S, Dubey A, et al. TRC150094 attenuates progression of nontraditional cardiovascular risk factors associated with obesity and type 2 diabetes in obese ZSF1 rats. Diabetes Metab Syndr Obes 2011;4:5-16. DOI: 10.2147/DMSO.S15323

28. Leite S, Rodrigues S, Tavares-Silva M, et al. Afterload-induced diastolic dysfunction contributes to high filling pressures in experimental heart failure with preserved ejection fraction. Am J Physiol Heart Circ Physiol 2015;309(10):H1648-54. DOI: 10.1152/ajpheart.00397.2015

29. Conceicao G, Heinonen I, Lourenco AP, et al. Animal models of heart failure with preserved ejection fraction. Neth Heart J 2016;24(4):275-86. DOI: 10.1007/s12471-016-0815-9

30. Roullier V, Cavaro-Menard C, Guillaume C, et al. Fuzzy algorithms to extract vacuoles of steatosis on liver histological color images. Conference proceedings: Annual International Conference of the IEEE Engineering in Medicine and Biology Society 2007;2007:5575-8.

31. Marques CM, Motta VF, Torres TS, et al. Beneficial effects of exercise training (treadmill) on insulin resistance and nonalcoholic fatty liver disease in high-fat fed C57BL/6 mice. Braz J Med Biol Res 2010;43(5):467-75. DOI: 10.1590/S0100-879X2010007500030

32. Ishii Y, Motohashi Y, Muramatsu M, et al. Female spontaneously diabetic Torii fatty rats develop nonalcoholic steatohepatitis-like hepatic lesions. World J Gastroenterol 2015;21(30):9067-78. DOI: 10.3748/wjg.v21.i30.9067

33. Asgharpour A, Cazanave SC, Pacana T, et al. A diet-induced animal model of non-alcoholic fatty liver disease and hepatocellular cancer. J Hepatol 2016;65(3):579-88. DOI: 10.1016/j.jhep.2016.05.005

34. Li D, Wang X, Lan X, et al. Down-regulation of miR-144 elicits proinflammatory cytokine production by targeting toll-like receptor 2 in nonalcoholic steatohepatitis of high-fat-diet-induced metabolic syndrome E3 rats. Mol Cell Endocrinol 2015;402:1-12. DOI: 10.1016/j.mce.2014.12.007

35. Ye D, Li FY, Lam KS, et al. Toll-like receptor-4 mediates obesity-induced non-alcoholic steatohepatitis through activation of X-box binding protein-1 in mice. Gut 2012;61(7):1058-67. DOI: 10.1136/gutjnl-2011-300269

36. Kawanishi N, Yano H, Mizokami T, et al. Exercise training attenuates hepatic inflammation, fibrosis and macrophage infiltration during diet induced-obesity in mice. Brain Behav Immun 2012;26(6):931-41. DOI: 10.1016/j.bbi.2012.04.006

37. Cong WN, Tao RY, Tian JY, et al. The establishment of a novel non-alcoholic steatohepatitis model accompanied with obesity and insulin resistance in mice. Life Sci 2008;82(19-20):983-90. DOI: 10.1016/j.lfs.2008.01.022

38. Sakr HF, Al-Hashem FH, El-Naby WM, et al. Preventive roles of swimming exercise and pioglitazone treatment on hepatic dysfunction in a rat model of metabolic syndrome. Can J Physiol Pharmacol 2014;92(2):162-70. DOI: 10.1139/cjpp-2013-0043

39. Kapravelou G, Martínez R, Andrade AM, et al. Aerobic interval exercise improves parameters of nonalcoholic fatty liver disease (NAFLD) and other alterations of metabolic syndrome in obese Zucker rats. Appl Physiol Nutr Metab 2015;40(12):1242-52. DOI: 10.1139/apnm-2015-0141

40. Cameron I, Alam MA, Wang J, et al. Endurance exercise in a rat model of metabolic syndrome. Can J Physiol Pharmacol 2012;90(11):1490-7. DOI: 10.1139/y2012-097

41. Carmiel-Haggai M, Cederbaum AI, Nieto N. A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats. FASEB J 2005;19(1):136-8.

Table 1. Morphometric and metabolic data

Parameters	*Ctrl*	*Ln*	*Ob*	*Ob-HFD*	*Ob-Ex*
Weights (g)	350.6 ± 9.4	421.7 ±	594.5 ± 9.5	557.8 ±	607.4 ±

		10.9$^{\alpha}$	$^{\alpha\beta}$	20.76$^{\alpha\beta}$	17.52$^{\alpha\beta}$
Liver (mg.mm^{-1})	290.9 ± 12.5	336.3 ± 18.8	981.0 ± 30.2 $^{\alpha\beta}$	714.9 ± 39.0 $^{\alpha\beta\chi}$	876.2 ± 54.6 $^{\alpha\beta\delta}$
Perirenal fat (mg.mm^{-1})	73.1 ± 6.5	50.6 ± 6.0	283.2 ± 34.0 $^{\alpha\beta}$	454.5 ± 29.8 $^{\alpha\beta\chi}$	294.5 ± 25.1 $^{\alpha\beta\delta}$
Perigonadal fat (mg.mm^{-1})	62.6 ± 7.1	55.5 ± 3.9	164.5 ± 21.3 $^{\alpha\beta}$	141.7 ± 11.0 $^{\alpha\beta}$	161.1 ± 2.3$^{\alpha\beta}$
Gastrocnemius (mg.mm^{-1})	54.39 ± 0.77	63.41 ± 1.32	54.20 ± 2.11	46.62 ± 0.79 $^{\beta}$	69.96 ± 7.91 $^{\alpha\beta\chi}$
TL (mm)	38.60 ± 1.12	41.01 ± 1.05	39.94 ± 0.47	37.73 ± 1.11	40.62 ± 0.48
IR AUC (mg.dl^{-1}.h^{-1})	50.0 ± 4.0	45.5 ± 7.5	136.2 ± 11.3 $^{\alpha\beta}$	158.3 ± 14.8 $^{\alpha\beta}$	127.2 ± 13.4 $^{\alpha\beta}$
OGT AUC (mg.dl^{-1}.h^{-1})	86.0 ± 0.0	70.0 ± 2.0	160.2 ± 12.6 $^{\alpha\beta}$	289.0 ± 12.9 $^{\alpha\beta\chi}$	141.2 ± 12.7 $^{\delta}$

TL: Tibia length; IR AUC: Insulin resistance area under the curve; OGT AUC: Oral glucose area under the curve. Values are expressed as mean ± SEM. $p < 0.05$: α *vs* Ctrl; β *vs* Ln; χ *vs* Ob; and δ *vs* Ob-HFD.

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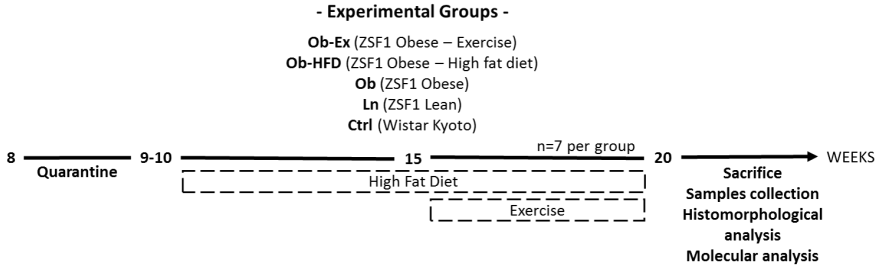

Fig. 1. Experimental design.

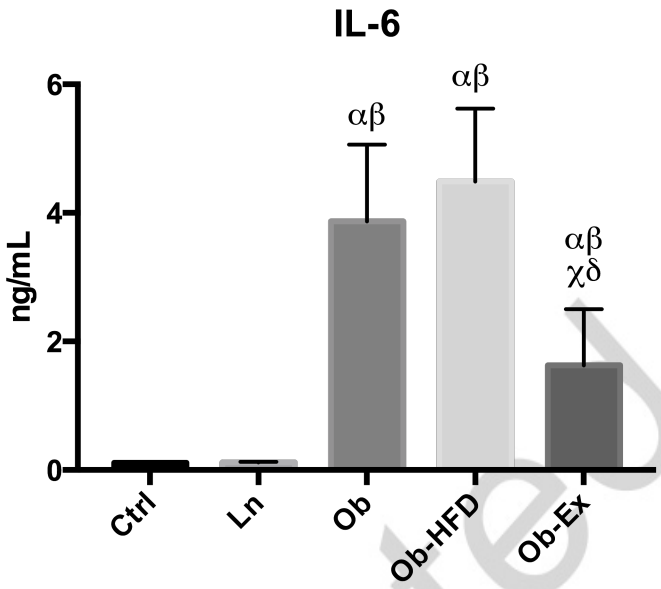

Fig. 2. Quantification of liver steatosis in all groups. Values are expressed as mean ± SEM, n = 7, each group. $p < 0.05$: α *vs* Ctrl; β *vs* Ln; χ *vs* Ob; and δ *vs* Ob-HFD.

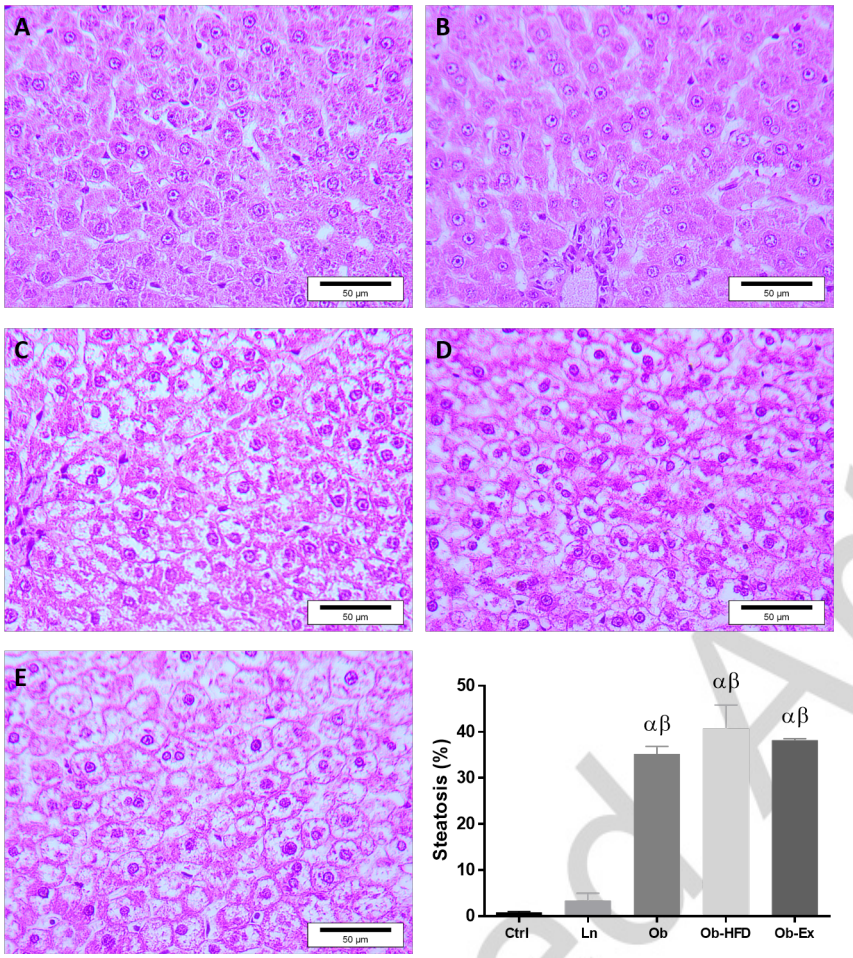

Fig. 3. Representative histological images of liver steatosis in all groups showing progressive fat infiltration (hematoxilin-eosin, 200x magnification). A. Ctrl. B. ZSF1 Ln. C. ZSF1 Ob. D. ZSF1 Ob-HFD. E. ZSF1 Ob-Ex.

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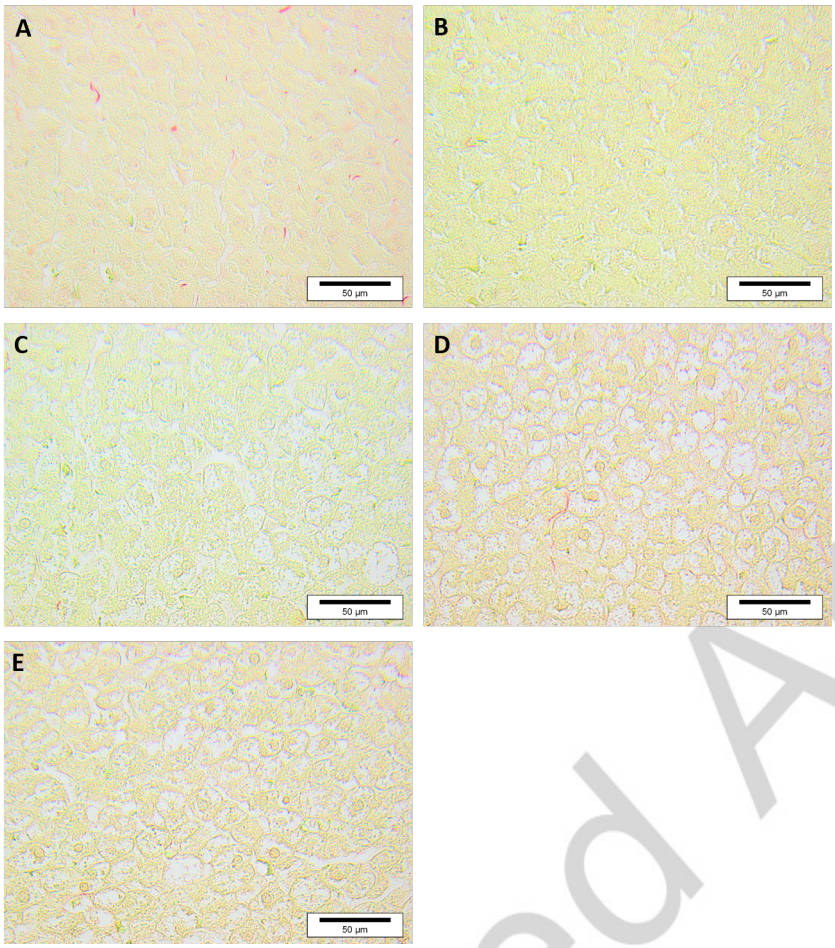

Fig. 4. Representative histological images of liver fibrosis (picro-sirius red, 200x magnification) in all groups. A: Ctrl; B: ZSF1 Ln; C: ZSF1 Ob; D: ZSF1 Ob-HFD; and E: ZSF1 Ob-Ex.

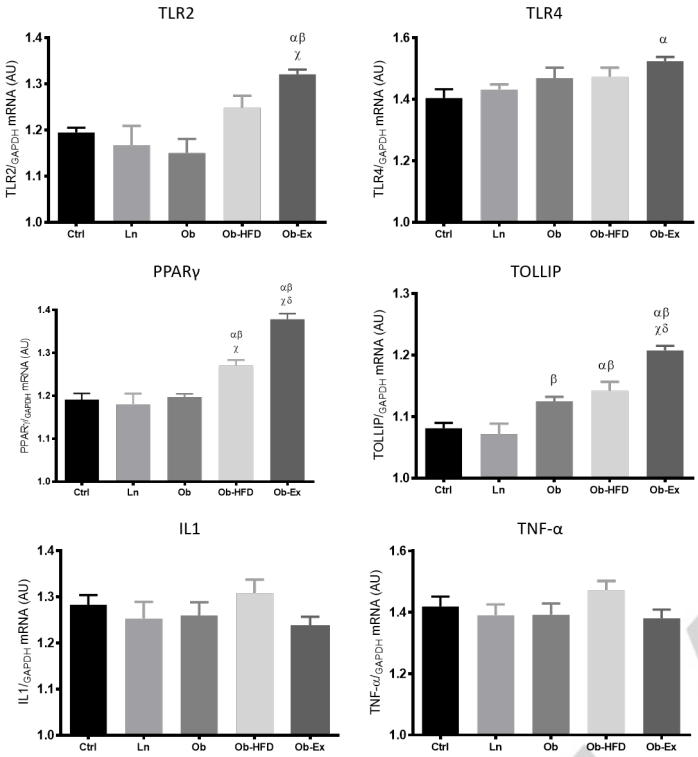

Fig. 5. mRNA quantification of PPARγ, TOLLIP, IL-1, TLR2, TLR4 and TNF-α. Values are expressed as mean ± SEM, n = 7, each group. $p < 0.05$: α *vs* Ctrl; β *vs* Ln; χ *vs* Ob; δ *vs* Ob-HFD. Results are normalized with GAPDH. IL-1: interleukin 1; PPARγ: Peroxisome proliferator-activated receptor γ; TLR2: Toll-like receptor 2; TLR4: Toll-like receptor 4; TOLLIP: Toll interacting protein; TNF-α: Tumor necrosis factor-alpha; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.