RESEARCH

Hepatic inflammation precedes steatosis and is mediated by visceral fat accumulation

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Abstract

The negative aspects of unhealthy eating on obesity and hepatic health are well described. The axis between the adipose tissue and the liver participates in most of the damage caused to this tissue regarding obesogenic diets (OD). At the same time that the effects of consuming simple carbohydrates and saturated fatty acids are known, the effects of the cessation of its intake are scarce. Withdrawing from OD is thought to improve health; despite some studies had shown improvement in hepatic conditions in the long-term, short-term studies were not found. Therefore, we aimed to determine how OD intake and withdrawal would influence visceral and hepatic fat accumulation and inflammation. To this end, male 60-days-old Wistar rats received standard chow ($n = 16$) or a high-sugar/high-fat diet (HSHF) for 30 days ($n = 32$), a cohort of the HSHF-fed animals was then kept 48 h on standard chow ($n = 16$). In opposition to the generally reported, the results indicate that hepatic inflammation preceded hepatic steatosis. Additionally, inflammatory markers on the liver positively correlated visceral adipokines and visceral fat accumulation mediated them in a deposit-dependent manner. At the same time, a 48-h withdrawal was capable of reverting most of the risen inflammatory mediators, although MyD88 and TNF α persisted and serum non-HDL cholesterol was higher than control levels.

Key Words

- ▶ hepatic inflammation
- ▶ hepatic steatosis
- ▶ visceral adipose tissue
- ▶ obesogenic diet
- ▶ withdrawal

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Introduction

The negative aspects of obesogenic diets (OD) on adipose tissue accumulation, hepatic fat accumulation (steatosis), and inflammation are well described (Van Herck *et al.* 2017, Bortolin *et al.* 2018, Casagrande *et al.* 2019). Obesity and non-alcoholic fatty liver disease (NAFLD) are two parts of the same role, respectively, as causation and consequence of the metabolic syndrome (Kanwar & Kowdley 2016). It is believed that following the adipocyte-hepatocyte axis, an increase in adipose tissue deposits drives an increase in the hepatic fat content, mainly as triacylglycerol (TAG) (Smith & George 2009). NAFLD can progress to non-alcoholic steatohepatitis (NASH), with higher inflammation (Kanwar & Kowdley 2016).

The adipocyte-hepatocyte axis can promote an over-activation of the toll-like receptors pathway and the production of inflammatory cytokines. Likewise, simple carbohydrates and fatty acids from the diet produce this effect (Wagnerberger *et al.* 2012, Rocha *et al.* 2016, Totsch *et al.* 2017). This pathway can be mediated by myeloid differentiation factor 88 (MyD88) and lead to the phosphorylation and nuclear translocation of the nuclear factor kappa B (NF κ B) culminating in cytokine production (Lu *et al.* 2008).

The harms caused by unhealthy eating are widely known (Morris *et al.* 2015). Meanwhile, despite improving eating habits being recommended for populations

consuming ODs (Jensen *et al.* 2014), few studies describe the effects of stopping consuming an OD. In the long-term, stopping OD intake, that is, withdrawing from it, was reported to improve hepatic health (Das *et al.* 2017). At the same time, it decreased liver steatosis, but not pro-inflammatory macrophage activation (Zamarron *et al.* 2017). Conversely, we were not able to find studies applying a short-term withdrawal (WTD) and evaluating those parameters in the liver.

Considering that the harms associated with unhealthy eating patterns are proposed to decrease after dietary improvement and that some consequences may persist at short- and long-term withdrawal (Hazarika *et al.* 2016, Liu *et al.* 2016a, Das *et al.* 2017, Zamarron *et al.* 2017), our objective was to determine how visceral and hepatic fat contents and hepatic inflammation would respond to OD intake and a short-term withdrawal and, if so, whether they would be related or not.

Materials and methods

Ethics

The present study was approved by the ethics committee of the 'Federal University of Sao Paulo' (n° 4641210318). All procedures were carried out under the ethical principles to animal research, according to the 'National Board of Control for Animal Experimentation,' following national and international guidelines.

Experimental protocol

Thirty 60-day-old male young adult Wistar rats (*Rattus norvegicus*), 210–260 g, habituated to the bioterium for 7 days were randomly assigned into three groups. Animals were held in cages (40×35×15 cm) of 3–4 individuals during the experiment, in a 12 h light:12 h darkness cycle, at 22±2°C, receiving water and chow, the standard or an obesogenic diet (high-sugar and high-fat diet, HSHF) *ad libitum*. The first group (Control group, Ct, *n*=10) received the standard chow for 30 days; the second (HSHF-fed, Hd,

n=10) received the modified diet for 30 days; and the third (HSHF-fed+withdrawal, Hw, *n*=10) received the modified diet for 30 days and the standard chow for 2 days (Fig. 1). The standard chow used was Nuvilab CR (Quimtia, Colombo, Brazil). The modified diet (Table 1) consisted of grounded standard chow added with sweetened condensed milk (Nestle) and lard (Sadia, São Paulo, Brazil), to characterise an HSHF. In order to ensure adequate nutrition, it was added of casein (Labsynth, Diadema, Brazil), soybean oil (Bunge, Gaspar, Brazil), vitamins (RHOSTER, Araçoiaba da Serra, Brazil), and minerals (RHOSTER). At the end of the protocol, all animals were anaesthetised with isoflurane and killed. The ARRIVE checklist was used in the preparation of this manuscript (Kilkenny *et al.* 2010).

An additional cohort (Ct, *n*=6; Hd, *n*=6; Hw, *n*=6) was carried out with the same experimental design but was kied by perfusion with 0.9% sodium chloride in water (Labsynth) and subsequently with 4% paraformaldehyde in PB (phosphate buffer, 0.1 M, pH 7.4) (fixing solution) (Labsynth). The livers were kept in fixing solution for 48 h and transferred to a 70% alcohol solution.

Serum lipids

Total serum cholesterol (#76, Labtest, Lagoa Santa, Brazil), serum high-density lipoprotein cholesterol (HDL) (#13, Labtest), and serum triacylglycerol (#87, Labtest) were determined by an enzymatic method using a commercial colourimetric kit following manufacturer's guidelines. Non-HDL cholesterol was calculated subtracting HDL from total cholesterol.

Hepatic lipids

The content of hepatic triacylglycerol was determined by an adaptation of the method of total lipids extraction of Folch *et al.* (1957). A solution of chloroform-methanol-water (2:1:0.5) (Labsynth) was added to each sample, which was homogenised and centrifuged at 400 g for 10 min, twice. The precipitate was evaporated and resuspended with Triton-X 3% (Labsynth). The triacylglycerol (#87,

Figure 1

Experimental design. Duration of habituation and experimental protocol and chow provided during those phases. Ct, Control group; Hd, HSHF-fed; Hw, HSHF-fed + 48 h withdrawal; HSHF, high-sugar and high-fat diet.

Table 1 Composition and nutritional information of the diets.

	Standard chow	HSHF
Components (g/1000 g)		
Nuvelab CR1®	1000	334.96
Sweetened condensed milk	–	400
Lard	–	86.57
Casein	–	36.07
Sucrose	–	113.37
Soybean oil	–	12.63
Mineral mix	–	11.72
Vitamin mix	–	3.68
Nutrients (g/1000 g)		
Carbohydrates	600	511
Sucrose	0	33
Fibre	70	23
Protein	220	150
Lipids	40	132
Saturated fatty acids	0	55
Energy (%)		
Carbohydrates	63	53
Sucrose	0	35
Protein	26	16
Lipids	11	31
Saturated fatty acids	0	19
Energy (kcal/100 g)	3340	3833

Labtest) and cholesterol (#76, Labtest) content were determined by an enzymatic method using a commercial colourimetric kit. The percentage of hepatic cholesterol was calculated, dividing its value by the sum of hepatic cholesterol and triacylglycerol (hepatic cholesterol/(hepatic cholesterol+hepatic triacylglycerol)).

Protein content analysis

Tissue samples were individually homogenised in lysis buffer (100 mM Tris-HCl, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 0.04% bovine lung aprotinin, Sigma-Aldrich; 10 mM EDTA, 100 mM sodium fluoride, 1% Triton, Labsynth) and centrifuged at 20,000 *g* for 40 min at 4°C. The supernatant was collected, and the protein content was assessed with Bradford reagent. Interleukin 6, 10, 1 β , and TNF α protein contents were determined by the ELISA method (R&D Systems) following the manufacturer's instructions.

Western blotting, as previously described (Santamarina *et al.* 2018), was carried out to determine the protein content of phosphorylated nuclear factor kappa B p65 (pNF κ Bp65, p-p65) (1:1200, #3033 Cell Signaling Technology), myeloid differentiation factor 88 (MyD88) (1:5000, #4283S Cell Signaling Technology), and

β -actin (1:5000, #3700 Cell Signaling Technology), used as loading control. The respective secondary antibodies followed the primary antibodies, anti-mouse (1:20,000, #7076, Cell Signaling Technology) or anti-rabbit IgG, HRP-linked (1:20,000, #7074, Cell Signaling Technology). Images were obtained using chemiluminescence intensifier scanner UVITEC (Cambridge, UK), and quantification was performed with Scion Image software Beta 4.0.2 (NIH).

Hepatic histology

Posterior to fixation, the livers were dehydrated gradually in ethanol, diaphanized in xylol, and incorporated in paraffin. The paraffin blocks were cut in 3- μ m sections and stained with haematoxylin-eosin to histopathological evaluation. The criteria followed the proposed by Aguiar *et al.* (2011).

Statistical analysis

Sample size calculation was performed on G*Power v3.1.9.4, the *a priori* test accounted for one-way ANOVA, considering the effect size *f* as 0.6, α as 0.05, and power as 0.8; resulting in 30 individuals. Data was compiled with Microsoft Excel for Office 365 (16.0.12026.20312) and analysed with JASP v0.11.1. The data were assessed for normality and homoscedasticity, outliers were determined by ROUT test. For data with parametric sample distribution, we used one-way ANOVA and Tukey's post hoc in order to verify differences among and between groups, respectively; for data with non-parametric sample distribution, we used Kruskal–Wallis test and Dunn's post hoc. To determine effect size among groups, ANOVA's sum of squares was used in order to calculate Cohen's *f*, while for between groups, means, s.d., and sample size were used to determine Hedges' *g*, which is a small-sample-size correction for Cohen's *d*. Depending on the normality of data's distribution, Pearson's or Spearman's approach was used to determining correlations; the correlated data were then applied in mediation models and subsequently in structural equation modeling. For all analyses, the statistically significant limit was set at $P < 0.05$.

Results

High-sugar and high-fat diet intake and withdrawal

After consuming the HSHF diet, the Hd group presented greater body mass, serum TAG, mesenteric, epididymal and

retroperitoneal fat deposits, hepatic protein expression of p-p65 (Fig. 2A), Myd88 (Fig. 2B), IL6, IL10, IL1 β , and TNF α (Fig. 3 and Tables 2, 3). Withdrawing from HSHF promoted lower serum TAG, mesenteric fat accumulation, and protein content of hepatic p-p65 (Fig. 2A), IL6, IL10, IL1 β (Fig. 3), and partially lower body mass, and hepatic TNF α (Fig. 3). WTD resulted in higher serum cholesterol and serum non-HDL cholesterol, as well as the percentage of hepatic cholesterol. Still, neither HSHF intake nor WTD changed hepatic triacylglycerol content nor hepatic cholesterol content (Tables 2 and 3).

In the histopathological analysis, we observed no signs of steatosis nor focal inflammation under microscopical evaluation. All animals ($n=18$, Ct=6, Hd=6, Hw=6) rated '0' considering the criteria adopted (Aguilar *et al.* 2011). (Fig. 4A, B and C).

Figure 2

Graphical representation of hepatic protein levels assessed by Western blotting. (A) Hepatic protein content of phosphorylated nuclear factor kappa B p65 (pNFkBp65); (B) myeloid differentiation factor 88 (MyD88); (A.U.) Arbitrary units; values are presented as mean $\pm$ s.e.m. and are expressed relative to the Ct group mean. Ct, control group; Hd, HSHF-fed group; Hw, HSHF-fed + 48 h withdrawal group; Different letters indicate $P < 0.05$ between groups. The number of animals per group is described in Table 2.

Figure 3

Graphical representation of cytokine concentrations assessed by ELISA on the liver, mesenteric fat, epididymal fat, and retroperitoneal fat. Values are presented as mean $\pm$ s.e.m. in picograms per milligram of protein. Ct, control group; Hd, HSHF-fed group; Hw, HSHF-fed + 48h withdrawal group; Different letters indicate $P < 0.05$ between groups. The number of animals per group is described in Table 2.

Table 2 Summary of results.

Parameters	Ct group	Hd group	Hw group	ANOVA P-value
	Mean $\pm$ S.E.M.(n)	Mean $\pm$ S.E.M.(n)	Mean $\pm$ S.E.M.(n)	
Body mass	398.10 $\pm$ 6.98(10) ^a	436.20 $\pm$ 11.93(9) ^b	426.80 $\pm$ 9.62(10) ^{ab}	0.024
Serum cholesterol (mg/dL)	107.10 $\pm$ 8.70(10) ^a	125.00 $\pm$ 10.36(7) ^{ab}	141.80 $\pm$ 9.93(10) ^b	0.044
Serum HDLc (mg/dL)	39.09 $\pm$ 2.85(9) ^a	34.77 $\pm$ 2.63(7) ^a	44.48 $\pm$ 2.93(10) ^a	0.082
Serum n-HDLc (mg/dL)	64.39 $\pm$ 6.66(9) ^a	90.20 $\pm$ 8.84(7) ^{ab}	97.29 $\pm$ 9.18(10) ^b	0.021
Serum triacylglycerol (mg/dL)	108.00 $\pm$ 6.80(10) ^a	161.70 $\pm$ 13.67(7) ^b	101.7 $\pm$ 1.92(10) ^a	<0.001
Hepatic cholesterol (g/100 g)	20.64 $\pm$ 1.37(10) ^a	20.15 $\pm$ 0.99(7) ^a	23.66 $\pm$ 0.83(10) ^a	0.073
Hepatic triacylglycerol (g/100 g)	1.99 $\pm$ 0.19(10) ^a	1.67 $\pm$ 0.18(7) ^a	1.66 $\pm$ 0.11(10) ^a	0.257
Hepatic cholesterol (% of lipids)	51.57 $\pm$ 1.97(10) ^a	55.39 $\pm$ 2.65(7) ^a	59.10 $\pm$ 1.43(10) ^b	0.029
Mesenteric fat (g/100 g BM)	4.61 $\pm$ 0.42(10) ^a	8.37 $\pm$ 0.56(9) ^b	6.30 $\pm$ 0.54(8) ^a	<0.001
Epididymal fat (g/100 g BM)	7.57 $\pm$ 0.86(10) ^a	17.77 $\pm$ 1.81(9) ^b	16.86 $\pm$ 1.92(10) ^b	<0.001
Retroperitoneal fat (g/100 g BM)	7.25 $\pm$ 0.74(10) ^a	16.05 $\pm$ 1.21(9) ^b	14.62 $\pm$ 0.92(10) ^b	<0.001
Hepatic pNF κ Bp65 (A.U.)	100.00 $\pm$ 17.99(6) ^a	266.70 $\pm$ 58.39(6) ^b	119.30 $\pm$ 16.06(6) ^a	0.011
Hepatic MyD88 (A.U.)	100.00 $\pm$ 9.36(9) ^a	139.20 $\pm$ 13.65(7) ^b	140.00 $\pm$ 8.16(10) ^b	0.013
Liver				
IL6 (pg/mg)	704.60 $\pm$ 36.15(10) ^a	1219.00 $\pm$ 129.80(7) ^b	815.50 $\pm$ 54.92(10) ^a	<0.001
IL10 (pg/mg)	152.60 $\pm$ 11.12(10) ^a	255.20 $\pm$ 19.02(7) ^b	169.50 $\pm$ 11.75(10) ^a	<0.001
IL1 β (pg/mg)	185.10 $\pm$ 11.51(10) ^a	353.90 $\pm$ 51.43(7) ^b	223.10 $\pm$ 22.38(10) ^a	0.001
TNF α (pg/mg)	1245.00 $\pm$ 145.40(7) ^a	2157.00 $\pm$ 321.70(6) ^b	2023.00 $\pm$ 227.20(8) ^{ab}	0.029
Mesenteric fat				
IL6 (pg/mg)	47.73 $\pm$ 8.38(8) ^a	102.30 $\pm$ 11.08(6) ^b	94.28 $\pm$ 10.75(8) ^b	0.002
IL10 (pg/mg)	3.92 $\pm$ 0.45(8) ^a	6.53 $\pm$ 1.26(7) ^b	4.99 $\pm$ 0.54(7) ^{ab}	0.031
IL1 β (pg/mg)	2.65 $\pm$ 0.26(7) ^a	6.27 $\pm$ 0.68(6) ^b	3.84 $\pm$ 0.35(6) ^a	<0.001
TNF α (pg/mg)	45.63 $\pm$ 11.70(8) ^a	141.30 $\pm$ 31.95(6) ^b	53.23 $\pm$ 13.42(6) ^a	0.006
Epididymal fat				
IL6 (pg/mg)	5.8 $\pm$ 0.72(9) ^a	6.19 $\pm$ 1.49(7) ^a	6.28 $\pm$ 0.79(9) ^a	0.931
IL10 (pg/mg)	2.47 $\pm$ 0.18(9) ^a	3.07 $\pm$ 0.43(7) ^a	3.38 $\pm$ 0.28(9) ^a	0.088
IL1 β (pg/mg)	2.58 $\pm$ 0.22(10) ^a	2.67 $\pm$ 0.65(7) ^a	3.19 $\pm$ 0.58(10) ^a	0.622
TNF α (pg/mg)	1.34 $\pm$ 0.08(10) ^a	1.55 $\pm$ 0.09(5) ^{ab}	1.66 $\pm$ 0.11(9) ^b	0.047
Retroperitoneal fat				
IL6 (pg/mg)	7.56 $\pm$ 0.68(9) ^a	5.3 $\pm$ 1.39(7) ^a	4.38 $\pm$ 0.73(8) ^a	0.056
IL10 (pg/mg)	3.94 $\pm$ 0.39(9) ^a	2.35 $\pm$ 0.41(7) ^b	2.16 $\pm$ 0.15(8) ^b	0.002
IL1 β (pg/mg)	2.26 $\pm$ 0.40(9) ^a	1.73 $\pm$ 0.44(5) ^a	1.79 $\pm$ 0.34(6) ^a	0.579
TNF α (pg/mg)	1.57 $\pm$ 0.07(9) ^a	1.3 $\pm$ 0.25(7) ^a	1.41 $\pm$ 0.10(8) ^a	0.437

Different letters indicate $P < 0.05$ between groups, for Tukey's post hoc and for one-way ANOVA.

A.U., arbitrary units; BM, body mass; Ct, Control group; Hd, HSHF-fed; Hw, HSHF-fed + withdrawal; IL6, interleukin 6; IL10, interleukin 10; IL1 β , interleukin 1 beta; MyD88, myeloid differentiation factor 88; pNF κ Bp65, phosphorylated nuclear factor kappa B p65; TNF α , tumor necrosis factor alpha.

Serum lipids and hepatic inflammation

Serum lipids positively correlated with hepatic inflammation markers. Total serum cholesterol ($r=0.45$, $r^2=0.20$, $P=0.04$) and non-HDL serum cholesterol ($r=0.53$, $r^2=0.28$, $P=0.02$) positively correlated with hepatic TNF α . Serum TAG positively correlated with hepatic IL10 ($r=0.48$, $r^2=0.23$, $P=0.01$). The percentage of hepatic cholesterol positively correlated with epididymal ($r=0.43$, $r^2=0.18$, $P=0.02$) and retroperitoneal fat ($r=0.48$, $r^2=0.23$, $P=0.01$) and with hepatic TNF α ($r=0.49$, $r^2=0.24$, $P=0.02$).

Visceral fat accumulation and hepatic inflammation

Visceral fat accumulation deposit-dependently positively correlated with hepatic inflammation. The mesenteric fat (g/100 g) was positively correlated with hepatic protein

content of p-p65, MyD88, IL6, IL10, and IL1 β ; the epididymal (g/100 g), with hepatic MyD88, IL10, IL1 β , and TNF α ; and the retroperitoneal (g/100 g), with hepatic MyD88, IL6, IL10, IL1 β , and TNF α (Table 4).

Visceral adipose tissue inflammation and hepatic inflammation

Considering the role played by visceral adipose tissue (VAT) in hepatic inflammation, we performed cytokine analysis in mesenteric, epididymal, and retroperitoneal fat tissues. HSHF intake caused mesenteric fat IL6, IL10, IL1 β , and TNF α to be higher and retroperitoneal fat IL10 to be lower than those of Ct group (Tables 2 and 3). After withdrawal, IL6 was kept elevated, IL10 was similar to both Ct's and Hd's, and IL1 β and TNF α returned to control-like

Table 3 Effect size among and between groups.

Parameters	Ct vs Hd Hedges' <i>g</i>	Ct vs Hw Hedges' <i>g</i>	Hd vs Hw Hedges' <i>g</i>	Among groups Cohen's <i>f</i>
Body mass	−1.24	−1.03	0.27	0.58
Serum cholesterol (mg/dL)	−1.81	−3.56	−1.58	0.55
Serum HDL (mg/dL)	1.48	−1.79	−3.27	0.49
Serum non-HDL (mg/dL)	−3.32	−3.93	−0.76	0.63
Serum triacylglycerol (mg/dL)	−5.03	1.20	6.49	1.17
Hepatic cholesterol (g/100 g)	0.38	−2.55	−3.69	0.49
Hepatic cholesterol (% of lipids)	−0.55	−1.32	−0.60	0.58
Hepatic triacylglycerol (g/100 g)	0.55	0.65	0.02	0.35
Mesenteric fat (g/100 g BM)	−2.31	−1.22	0.80	1.13
Epididymal fat (g/100g BM)	−2.31	−1.89	0.15	0.99
Retroperitoneal fat (g/100 g BM)	−2.78	−2.67	0.42	1.37
Hepatic NFκB (A.U.)	−1.60	−0.90	1.22	0.91
Hepatic MyD88 (A.U.)	−1.71	−1.42	−0.03	0.68
Liver				
IL6 (pg/mg)	−2.08	−0.72	1.50	1.03
IL10 (pg/mg)	−2.32	−0.45	1.90	1.10
IL1β (pg/mg)	−1.77	−0.65	1.22	0.86
TNFα (pg/mg)	−1.41	−1.36	0.18	0.69
Mesenteric fat				
IL6 (pg/mg)	−2.03	−1.62	0.26	0.96
IL10 (pg/mg)	−1.24	−1.18	0.56	0.66
IL1β (pg/mg)	−2.73	−1.45	1.68	1.45
TNFα (pg/mg)	−1.59	−0.22	1.35	0.91
Epididymal fat				
IL6 (pg/mg)	0.74	1.47	0.30	0.08
IL10 (pg/mg)	1.33	1.88	0.22	0.50
IL1β (pg/mg)	0.44	0.42	0.06	0.20
TNFα (pg/mg)	0.55	0.60	0.20	0.58
Retroperitoneal fat				
IL6 (pg/mg)	0.74	1.47	0.30	0.56
IL10 (pg/mg)	1.33	1.88	0.22	0.92
IL1β (pg/mg)	0.44	0.42	−0.06	0.26
TNFα (pg/mg)	0.55	0.60	−0.20	0.29

Number of rats per group is described in Table 2.

A.U., arbitrary units; BM, body mass; Ct, Control group; Hd, HSHF-fed; Hw, HSHF-fed + withdrawal; IL6, interleukin 6; IL10, interleukin 10; IL1β, interleukin 1 beta; MyD88, myeloid differentiation factor 88; pNFκBp65, phosphorylated nuclear factor kappa B p65; TNFα, tumor necrosis factor alpha.

levels in mesenteric fat; IL10 continued lower compared to Ct's; TNFα in epididymal fat was higher than Ct's.

Moreover, VAT cytokines correlated with most of the hepatic inflammatory parameters presented. Mesenteric fat IL6 positive correlated with hepatic IL1β and TNFα; IL10 with hepatic IL6, IL1β, and TNFα; IL1β with hepatic MyD88, IL6, 1β, and TNFα; and TNFα with hepatic MyD88 and IL1β (Table 5). Epididymal fat IL1β positively correlated with hepatic MyD88 ($r=0.46$, $r^2=0.21$, $P=0.022$) and its TNFα positively correlated with hepatic TNFα ($r=0.49$, $r^2=0.24$, $P=0.029$). Retroperitoneal fat IL10 negatively correlated with hepatic IL6 ($r=-0.48$, $r^2=0.23$, $P=0.019$).

Hepatic inflammation is mediated by visceral fat accumulation

To pursue a further relationship, we used mediation and structural equation modeling. Mediation models

can be seen as sets of linear regressions and show that the total effect observed from the intervention can be broken down into components (e.g. direct effect and indirect effect) displaying the contribution of the proposed mediator on the final result (Montoya & Hayes 2017). Structural equation modeling is a confirmatory factor analysis that allows questions involving multiple regression analyses to be answered, setting relationships between several variables of interest at the same time (Ullman & Bentler 2017).

Following the correlations found, we tested mediation models and structural equation modeling. Serum lipids and hepatic cholesterol (% of hepatic lipids) showed no mediation of intake's nor withdrawal's effect. On the structural equation modeling, we were able to see that mesenteric fat accumulation mediated the effect of HSHF intake on IL1β (Table 6 (1)) and p-p65 (Table 6 '2'), whereas p-p65 mediated the effect of mesenteric fat on

Figure 4

Hepatic tissue microphotographs (20 $\times$) of haematoxylin-eosin staining. (A) Control group; (B) Hd, HSHF-fed group; (C) Hw, HSHF-fed + 48 h withdrawal group. ($n = 6$ per group). A full color version of this figure is available at <https://doi.org/10.1530/JOE-19-0553>.

IL1 β (Table 6 (3)). Therefore, the effect followed the equation 'HSHF $\rightarrow$ Mesenteric Fat $\rightarrow$ pNF κ Bp65 $\rightarrow$ IL1 β ' (Table 6 (A)). Hence, the increment in IL1 β following HSHF intake was determined by mesenteric fat accumulation and higher hepatic p-p65.

In the same way, IL6 mediated both HSHF intake and withdrawal effect on IL1 β (Table 6 (41) and (51)), the HSHF intake effect on IL10 (Table 6 (42)), and partially the withdrawal effect on IL10 (Table 6 (52)), whereas, IL10 mediated IL6 effect on IL1 β (Table 6 (6)). Therefore, the effect followed the equation 'HSHF/WTd $\rightarrow$ IL6 $\rightarrow$ IL10 $\rightarrow$ IL1 β ' (Table 6 (B)). Hence, the increment in IL10 and IL1 β was determined or partially determined by higher hepatic IL6 (Fig. 5).

Additionally, in simple mediation models, epididymal fat accumulation mediated the effect of HSHF intake on MyD88 (Fig. 6A and Table 7 (1) and (2)). Also, the effect of HSHF intake on TNF α was mediated by retroperitoneal fat accumulation (Fig. 6B and Table 7 (3) and (4)). Hence, epididymal fat and retroperitoneal fat accumulation, respectively, determined higher hepatic MyD88 and TNF α .

Discussion

The consumption of an obesogenic diet is known to increase body mass, VAT, and to promote hepatic inflammation and hepatic steatosis (Lozano *et al.* 2016, Casagrande *et al.* 2019). Still, there is not much information regarding these parameters when terminating the intake of OD. Our results indicate that a 48-h dietary improvement was efficient in returning most of the analysed parameters to control-like levels. Still, body fat accumulation, which decreases more slowly over time, persisted (Martire *et al.* 2014, Rinnankoski-Tuikka *et al.* 2014).

On the other hand, total serum cholesterol, mainly non-HDL cholesterol, composed majorly by LDL and VLDL, was higher. This rise could indicate higher lipid export in response to WTD, supported by a higher percentage of hepatic cholesterol. Interestingly, serum lipid levels were not correlated with intra-hepatic lipids nor VAT accumulation. As there was no similarity between fat accumulation and serum cholesterol, the observed greater hepatic lipid exportation might be the decisive factor.

In a broader perspective, higher non-HDL cholesterol is strongly linked with long-term risk for cardiovascular diseases (Brunner *et al.* 2019). This rise is of concern, pondering the frequent cycling of diet and weight in the population with obesity. Body mass fluctuations affecting

Table 4 Correlations between visceral fat accumulation and hepatic inflammation.

Hepatic parameters	Mesenteric fat			Epididymal fat			Retroperitoneal fat		
	<i>r</i>	<i>r</i> ²	<i>P</i> value	<i>r</i>	<i>r</i> ²	<i>P</i> value	<i>r</i>	<i>r</i> ²	<i>P</i> value
pNFκBp65	0.678	0.460	0.002	0.158	0.025	0.507	0.408	0.166	0.074
MyD88	0.426	0.181	0.038	0.647	0.419	<0.001	0.477	0.228	0.014
IL6	0.446	0.199	0.025	0.363	0.132	0.063	0.523	0.274	0.005
IL10	0.557	0.310	0.004	0.460	0.212	0.016	0.419	0.176	0.030
IL1β	0.616	0.379	0.001	0.446	0.199	0.020	0.511	0.261	0.006
TNFα	0.400	0.160	0.081	0.595	0.354	0.004	0.675	0.456	0.001

IL6, interleukin 6; IL10, interleukin 10; IL1β, interleukin 1 beta; MyD88, myeloid differentiation factor 88; pNFκBp65, phosphorylated nuclear factor kappa B p65; TNFα, tumor necrosis factor alfa.

cardiovascular health in the population with obesity are long known (Jeffery *et al.* 1992, Zeigler *et al.* 2018, Zou *et al.* 2019).

There is a relationship between VAT accumulation and hepatic tissue metabolism (Smith & George 2009, Barchetta *et al.* 2019, Cornide-Petronio *et al.* 2019, Franchitto *et al.* 2019, Ishtiaq *et al.* 2019). Adipocyte-hepatocyte lipid accumulation axis is proposed to be one of the most critical participants in NAFLD development (Smith & George 2009). Interestingly, adipose tissue inflammation, but not only fat accumulation, was linked to NAFLD (Asrih & Jornayvaz 2013). Our results lead us to believe that VAT inflammation links with hepatic inflammation and precedes NAFLD.

Hepatic inflammation occurred despite hepatic TAG accumulation following HSHF intake and is related to visceral fat accumulation and inflammation. We have recently shown that chronic OD intake, 13 weeks of a high-fat and high-fructose diet, can drive VAT accumulation and hepatic inflammation despite no significant hepatic TAG increase (Casagrande *et al.* 2019).

Combined fat and sugar seem to induce earlier inflammation and later TAG accumulation. Intrahepatic TAG accumulation was seen after 1 and 5 weeks of high-fat feeding (Shang *et al.* 2017, Santamarina *et al.* 2019); though, HSHF feeding increased hepatic TAG after 12 weeks, but not after 1 nor 4 weeks (Chiba *et al.* 2016).

Interestingly, free fatty acids (FFA) accumulation, but not TAG, was associated with NAFLD development (Liu *et al.* 2016b). Liu *et al.* (2016b) found that accumulation of TAG happened with 8 weeks of high-fat diet intake, whereas accumulation of FFA only after 16 weeks. Additionally, plasmatic FFA was suggested as an indicator for predicting advanced fibrotic states on NAFLD patients (Zhang *et al.* 2014). Concomitantly, time-dependent accumulation of FFA may reflect the progression of NAFLD (Konstantynowicz-Nowicka *et al.* 2019), and therefore, our protocol's length was perhaps not sufficient to induce those changes and thus NAFLD.

Fat tissue releases several factors – hormones, nutrients, cytokines – in the bloodstream, some of them are linked to hepatic inflammatory status (e.g. adiponectin, free fatty acids, and TNFα), which may have contributed to the results observed here (Musso *et al.* 2005, Asrih & Jornayvaz 2013, Berk & Verna 2016, Ishtiaq *et al.* 2019). Conversely, VAT cytokines positively correlated with hepatic inflammatory markers. Indeed, visceral fat accumulation and chronic low-grade inflammation play a leading role in the development and progression of metabolic syndrome following unhealthy diets (Zafar *et al.* 2018).

Interleukin 6 and TNFα are non-exclusive acute-phase cytokines (Feghali & Wright 1997). If their rise in the liver were solely dependant on the external stimuli (e.g. HSHF

Table 5 Correlations between mesenteric fat and hepatic inflammation.

Hepatic parameters	Mesenteric fat											
	IL6			IL10			IL1β			TNFα		
	<i>r</i>	<i>r</i> ²	<i>P</i> value	<i>r</i>	<i>r</i> ²	<i>P</i> value	<i>r</i>	<i>r</i> ²	<i>P</i> value	<i>r</i>	<i>r</i> ²	<i>P</i> value
pNFκBp65	0.172	0.030	0.575	0.130	0.017	0.660	0.337	0.114	0.284	0.342	0.117	0.276
MyD88	0.340	0.116	0.143	0.262	0.069	0.264	0.503	0.253	0.040	0.513	0.264	0.029
IL6	0.420	0.176	0.051	0.431	0.186	0.045	0.498	0.248	0.018	0.456	0.208	0.066
IL10	0.375	0.141	0.086	0.322	0.104	0.143	0.405	0.164	0.063	0.475	0.226	0.056
IL1β	0.637	0.406	0.003	0.790	0.624	<0.001	0.638	0.407	0.003	0.597	0.356	0.024
TNFα	0.497	0.247	0.026	0.732	0.536	<0.001	0.635	0.403	0.003	0.463	0.214	0.082

IL6, interleukin 6; IL10, interleukin 10; IL1β, interleukin 1 beta; MyD88, myeloid differentiation factor 88; pNFκBp65, phosphorylated nuclear factor kappa B p65; *r*, Pearson's correlation coefficient; TNFα, tumour necrosis factor alfa; *ρ*, Spearman's correlation coefficient.

Table 6 Structural equation modeling.

	a β (z)	b β (z)	c' β (z)	Mediated effect (a $\times$ b) β (z)	Total effect c (a $\times$ b + c') β (z)
(A) HSHF $\rightarrow$ Mesenteric Fat $\rightarrow$ pNF κ Bp65 $\rightarrow$ IL1 β					
(1) HSHF $\rightarrow$ Mesenteric Fat $\rightarrow$ IL1 β	0.62 (3.21) ^a	0.60 (2.79) ^a	0.16 (0.74)	0.37 (2.10) ^a	0.53 (2.57) ^a
(2) HSHF $\rightarrow$ Mesenteric Fat $\rightarrow$ pNF κ Bp65		0.54 (2.50) ^a	0.25 (1.16)	0.33 (1.97) ^a	0.58 (2.93) ^a
(3) Mesenteric Fat $\rightarrow$ pNF κ Bp65 $\rightarrow$ IL1 β	0.60 (2.79) ^a	0.69 (3.96) ^a	0.12 (0.66)	0.41 (2.28) ^a	0.54 (2.50) ^a
(B) HSHF/WTD $\rightarrow$ IL6 $\rightarrow$ IL10 $\rightarrow$ IL1 β					
(41) HSHF $\rightarrow$ IL6 $\rightarrow$ IL1 β	0.87 (4.75) ^a	0.54 (2.90) ^a	0.30 (1.28)	0.47 (2.48) ^a	0.77 (3.87) ^a
(42) HSHF $\rightarrow$ IL6 $\rightarrow$ IL10		0.73 (6.47) ^a	0.27 (1.88)	0.64 (3.83) ^a	0.91 (5.32) ^a
(51) WTD $\rightarrow$ IL6 $\rightarrow$ IL1 β	-0.65	0.54 (2.90) ^a	-0.24 (-1.13)	-0.35 (-2.24) ^a	-0.59 (-2.94) ^a
(52) WTD $\rightarrow$ IL6 $\rightarrow$ IL10	(-3.53) ^a	0.73 (6.47) ^a	-0.29 (-2.26) ^a	-0.47 (-3.10) ^a	-0.76 (-4.47) ^a
(6) IL6 $\rightarrow$ IL10 $\rightarrow$ IL1 β	0.73 (6.47) ^a	0.69 (2.36) ^a	0.03 (0.13)	0.50 (2.22) ^a	0.54 (2.90) ^a

Effect size (standardised β), z value, and significance of the models' coefficients.

^a indicate $P < 0.05$.

$\rightarrow$, orientation of effect; a, effect of predictor (X) on mediator (M); b, effect of mediator (M) on outcome (Y) controlling for predictor (X); c', direct effect of predictor (X) on outcome (Y); HSHF, high-sugar and high fat diet; IL6, interleukin 6; IL10, interleukin 10; IL1 β , interleukin 1 beta; MyD88, myeloid differentiation factor 88; pNF κ Bp65, phosphorylated nuclear factor kappa B p65; WTD, withdrawal.

diet), IL6 and TNF α levels would be both lower after the 48 h withdrawal, according to their peak concentration and half-life post-challenge (Sharma *et al.* 1992, de Vos *et al.* 1994). The reduction of IL6 and subsequently of IL10 and IL1 β and the maintenance of TNF α levels may indicate a persisting stimulus other than HSHF intake. The determinant could be retroperitoneal fat accumulation, as proposed by the mediation model (Fig. 6B), or epididymal fat TNF α and mesenteric fat IL6, both positively correlated with hepatic TNF α .

Based on the data from structural equation modeling, it is possible to propose an inflammatory cascade playing an essential role in the mechanisms of HSHF intake and VAT effect on hepatic inflammation. The production of IL1 β following p-p65 (Fig. 5) can be a consequence of the activation of 'nucleotide-binding oligomerisation domain and leucine-rich repeat-containing receptor' 3 (NLRP3) (Liu *et al.* 2017), whereas IL1 β production following IL10 increase might indicate an interplay of the mammalian

target of rapamycin complex 1 and NLRP3 (mTORC-NLRP3) pathway (Kabat & Pearce 2017).

In this sense, IL1 β would be one of the triggers and one of the results of the pathways' activation, since it promotes the cleavage of pro-IL1 β into its active form via NLRP3-caspase1 (Liu *et al.* 2017, Jin & Fu 2019). Associated with hypoxia, lipopolysaccharides (LPS), and TNF α increase in the adipose tissue, the NLRP3-IL1 β pathway plays an essential role in inflammation and obesity-associated comorbidities (Unamuno *et al.* 2019). Remarkably, mesenteric fat cytokines, consequently TNF α , were positively correlated with hepatic IL1 β , reinforcing this relationship.

Until the discovery of adipose tissue regulatory activity in energy metabolism, participating in the control of cerebral and peripheral mechanisms, it was acknowledged as an inert organ (Zhang *et al.* 1994, Kershaw & Flier 2004). Among the main effectors produced are leptin, adiponectin, adipokines, and regulatory peptides

Figure 5

Structural equation modeling for HSHF intake effect on hepatic pNF κ Bp65, IL1 β , and IL10. (1) and (2) display the models for mesenteric fat mediation of the intake effect on pNF κ Bp65 and IL1 β ; (3) displays the effect of mesenteric fat on IL1 β mediated by pNF κ Bp65; (41) and (51) display the models for IL6 mediation of intake effect on IL10 and IL1 β ; while (42) and (52) display the models for IL6 mediation of WTD on IL10 and IL1 β ; (6) displays the effect of IL6 on IL1 β mediated by IL10. (a) Effect of the predictor (X) on the mediator (M); (b) effect of the mediator (M) on the outcome (Y) controlling for the predictor (X); (c') direct effect of the predictor (X) on the outcome (Y); (continuous arrow) significant effect; (dashed arrow) non-significant effect.

Figure 6

Mediation models for HSHF intake effect on hepatic MyD88 and TNFα; (A) model for epididymal fat mediation of intake effect on MyD88; (B) model for retroperitoneal fat mediation of intake effect on TNFα; (a) effect of predictor (X) on mediator (M); (b) effect of mediator (M) on outcome (Y) controlling for predictor (X); (c) direct effect of predictor (X) on outcome (Y); (C) confounder; (continuous arrow) significant effect; (dashed arrow) non-significant effect.

(Kershaw & Flier 2004). A higher influx of fatty acids to this tissue, mainly from the diet sources, can promote enlargement of adipocytes, impairing its functions. The excessive size of adipocytes hardens the blood flow by restricting blood vessels diameters, leading to hypoxia. In low oxygen states, there is a rise in pro-inflammatory adipokines, as IL6, IL1β, and TNFα, besides macrophage migration and polarisation into M1 (Kihira *et al.* 2014, Lin & Yun 2015).

Nevertheless, withdrawal promoted lower hepatic IL6 and IL1β levels independently from VAT.

This decline suggests that WTD effect can also be linked to the absence of the negative stimuli from HSHF intake, possibly indicating a role for gut microbiota, which is being consolidated as a critical regulator for inflammatory diseases, endotoxemia, food intake, and obesity (Xiao & Zhao 2014, Guerville *et al.* 2017, van de Wouw *et al.* 2017, Mulders *et al.* 2018). At the same time, the mesenteric fat – mediator of HSHF intake's effect on p-p65 and IL1β – may be a direct target for inflammatory molecules passing intestinal barrier (e.g. LPS) in an obesogenic feeding condition (Konrad & Wuest 2014).

Altogether, our study reinforces the relationship between visceral adipose tissue and hepatic health status and postulates that an increase in hepatic inflammation may occur before and independently from hepatic steatosis. Our results showed that hepatic inflammation can be induced by unhealthy eating and can precede significant fat accumulation. Taking into account that the nutrients consumed have inflammatory potential and, that posterior to gut absorption, they enter the liver via the portal circulation, it is highly unlikely that hepatic fat accumulation and inflammation is a one-way road. The elevated levels of pro-inflammatory markers reported here indicate a response from the tissue, although, as seen post-withdrawal, not an irreversible one. The activation of the inflammatory pathways occurs despite the magnitude of the aggression. Although, a consistent and long-term activation with no resolution would promote damage and perhaps histopathological features.

Overall, a 48-h withdrawal was not entirely effective in decreasing hepatic inflammation and also increased non-HDL cholesterol. Since dietary improvement is the recommended nutritional approach to improve health status in an obesogenic-diet-consuming population and little information regarding the dietary modification *per se* is available, we see this as a promising research field craving for information.

Table 7 Mediation model.

	a β (z)	b β (z)	c' β (z)	Mediated effect (a × b) β (z)	Total effect c (a × b + c') β (z)
(1) HSHF → Epididymal Fat → MyD88	0.76 (4.60) ^a	0.53 (2.62) ^a	0.19 (0.80)	0.41 (2.28) ^a	0.59 (3.06) ^a
(2) WTD → Epididymal Fat → MyD88	−0.05 (−0.32)		0.04 (0.23)	−0.03 (−0.32)	0.01 (0.06)
(3) HSHF → Retroperitoneal Fat → TNFα	0.85 (5.23) ^a	0.59 (2.28) ^a	0.11 (0.39)	0.50 (2.09) ^a	0.62 (2.86) ^a
(4) WTD → Retroperitoneal Fat → TNFα	−0.11 (−0.70)		−0.03 (−0.13)	−0.07 (−0.67)	−0.09 (−0.43)

Effect size (standardised β), z value, and significance of the models' coefficients.

^a indicate $P < 0.05$.

→, orientation of effect; a, effect of predictor (X) on mediator (M); b, effect of mediator (M) on outcome (Y) controlling for predictor (X); c', direct effect of predictor (X) on outcome (Y); HSHF, high-sugar and high fat diet; MyD88, myeloid differentiation factor 88; WTD, withdrawal.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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