Journal of Developmental Origins of Health and Disease

cambridge.org/doh

Neonatal intake of oleanolic acid attenuates the subsequent development of high fructose diet-induced non-alcoholic fatty liver disease in rats

T. T. Nyakudya[1,2], E. Mukwevho[3], P. Nkomozepi[2] and K. H. Erlwanger[1]

[1]School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, [2]Department of Human Anatomy and Physiology, Faculty of Health Sciences, University of Johannesburg, Johannesburg, South Africa and [3]Department of Biology, Faculty of Agriculture, Science and Technology, North West University, Mmabatho, Mafikeng, South Africa

Original Article

Cite this article: Nyakudya TT, Mukwevho E, Nkomozepi P, Erlwanger KH. (2018) Neonatal intake of oleanolic acid attenuates the subsequent development of high fructose diet-induced non-alcoholic fatty liver disease in rats. *Journal of Developmental Origins of Health and Disease* **9**: 500–510. doi: 10.1017/S2040174418000259

Received: 18 October 2017
Revised: 21 February 2018
Accepted: 14 March 2018
First published online: 24 May 2018

Key words:
fructose; metabolic dysfunction; neonatal programming; non-alcoholic fatty liver disease; oleanolic acid

Address for correspondence:
T. T. Nyakudya, School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, 2193, Johannesburg, South Africa.
E-mail: trevorn@uj.ac.za

Abstract

Dietary manipulations during the early postnatal period are associated with the development of metabolic disorders including non-alcoholic fatty liver disease (NAFLD) or long-term protection against metabolic dysfunction. We investigated the potential hepatoprotective effects of neonatal administration of oleanolic acid (OA), a phytochemical, on the subsequent development in adulthood, of dietary fructose-induced NAFLD. Male and female suckling rats ($n = 112$) were gavaged with; distilled water (DW), OA (60 mg/kg), high fructose solution (HF; 20% w/v) and OA + HF (OAHF) for 7 days. The rats were weaned onto normal rat chow on day 21 up to day 55. From day 56, half of the rats in each treatment group were continued on plain water or HF as drinking fluid for 8 weeks. Hepatic lipid accumulation and hepatic histomorphometry were then determined. Fructose consumption in adulthood following neonatal fructose intake (HF + F) caused a 47–49% increase in hepatic lipid content of both male and female rats ($P < 0.05$). However, fructose administered in adulthood only, caused a significant increase ($P < 0.05$) in liver lipid content in females only. NAFLD activity scores for inflammation and steatosis were higher in the fructose-fed rats compared with other groups ($P < 0.05$). Steatosis, low-grade inflammation and fibrosis were observed in rats that received HF + F. NAFLD area fraction for fibrosis was three times higher in rats that received fructose neonatally and in adulthood compared with the rats in the negative control group ($P < 0.05$). Treatment with OA during a critical window of developmental plasticity in rats prevented the development of fructose-induced NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the accumulation of lipids in hepatocytes in the absence of alcohol abuse, steatogenic medication or underlying pathological conditions that cause steatosis.[1] The steatosis leads to hepatic fat build up and an increase in total liver mass of over 5%.[1,2] Hepatic lipid accumulation of between 5 and 10% is considered as fatty liver disease.[3] NAFLD is considered to be the 'hepatic manifestation' of metabolic syndrome (MetS) as its development is closely linked with health outcomes associated with MetS such as obesity, dyslipidemia, type 2 diabetes mellitus and hypertension.[4,5] NAFLD includes a wide range of clinicopathologies ranging from hepatic steatosis to non-alcoholic steatohepatitis (NASH) which can progress to hepatic fibrosis and eventually cirrhosis if left untreated.[6] NASH is regarded as the most severe form of NAFLD and is accompanied by lipid peroxidation and the generation of free radicals which in turn trigger an inflammatory response and activation of hepatic stellate cells resulting in fibrosis.[7,8]

NAFLD has a high global prevalence which is increasing.[9] According to Bellentani *et al.*,[10] the recent increase in the global prevalence of NAFLD ranges from 6.3–33% with an average of 20% of the population, depending on the diagnostic tool used. When other co-morbidities are considered, even higher prevalence is reported. The prevalence of NAFLD in obese diabetic individuals can be as high as 70–90%.[7] The main risk factors for the development of NAFLD include adoption of sedentary lifestyles, obesity, insulin resistance, genetics and altered dietary patterns such as excessive consumption of Western calorific diets that are high in fat and carbohydrate content, particularly fructose.[11,12]

Several mechanisms have been proposed in order to explain hepatic lipid accumulation and these include but are not limited to; (i) an increase in extrahepatic mobilization of fatty acids from visceral adipose tissue causing an influx of triglycerides (TGs) into the liver, (ii) *de novo* lipogenesis (DNL) and (iii) a decrease in hepatic fatty acid oxidation.[13]

© Cambridge University Press and the International Society for Developmental Origins of Health and Disease 2018.

CAMBRIDGE UNIVERSITY PRESS

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press

As proposed by Day and James[14] in their 'two hit hypothesis', the development and pathogenesis of NAFLD and NASH requires a 'double hit'. The 'first hit' results in hepatic lipid accumulation which is primarily due to insulin resistance. Insulin resistance triggers the secretion of adipokines from adipocytes, changes the rate of production and transport of TGs by hepatocytes and increases lipolysis in adipocytes which in turn releases free fatty acids thus exposing the liver to excess free fatty acids.[7] In the 'first hit' of NAFLD pathogenesis, there is also mitochondrial dysfunction and DNL which causes an increase in hepatic TG levels.[7] The 'second hit' is caused by reactive oxygen species and pro-inflammatory cytokines produced by adipocytes and reactive oxygen species which in turn activates hepatic stellate cells to increase hepatic fibrosis and lipid peroxidation.[15,16]

The development of metabolic disorders such as NAFLD in adulthood has been known to be programmable in the different phases of ontogeny such as gestation and in the neonatal phase of development which are characterized by phenotypic plasticity.[17,18] Studies have shown that increased consumption of fructose during the critical periods of developmental plasticity predisposes offspring to the development of metabolic disorders later on in adult life.[19–21]

Rats are an altricial species and several of the developmental changes occurring during the early neonatal phase correspond to those occurring in the third trimester of precocious species including humans.[22] Consequently, the neonatal rat represents an experimental model for studying developmental changes that are associated with the human third trimester of pregnancy.[22] Thus for our study we chose to use neonatal rats as an experimental model for developmental metabolic plasticity.

Current therapeutic options for MetS and NAFLD include lifestyle changes such as a reduction in dietary calorific intake, and an increase in physical activity.[23] Traditional pharmacological agents that are currently being used to treat and manage metabolic disorders and NAFLD include insulin-sensitizing drugs like metformin, antioxidants such as vitamin E and lipid-lowering drugs like fenofibrate.[23,24] This treatment approach often administered to adults, amongst other mechanisms is directed at weight loss and increasing energy expenditure so that less energy is stored in the adipose tissue.[25] These conventional synthetic medicines are often associated with a number of side effects including lactic acidosis, liver toxicity and hypoglycemia.[23] There is need to develop alternative treatments or prophylactic interventions, preferably using affordable natural products that form part of our normal diets, having fewer side effects, and safer than conventional medicines. Ideally the alternative pharmacological interventions should be administered during the critical phases of development to provide long-term health benefits.

Oleanolic acid (OA) is a naturally occurring triterpenoid[26] derived from plants including olives (*Olea europaea*), Mistletoe (*Viscum album* L.) and Chinese Weldelia (*Wedelia chinensis*).[27] OA was selected for this study due to its several pharmacological benefits.[28,29] The reported pharmacological bioactivities of OA include protection against chemically induced liver injury,[30] anti-diabetic properties,[31,32] anti-inflammatory[33] and anti-oxidant properties.[34,35] Previous studies on murine models have shown that OA treatment in adulthood significantly reduces diet-induced hepatic lipid accumulation and confers hepatic protection through the modulation of hepatic SREB-1c-mediated expression of genes responsible for DNL.[36] Most studies on the beneficial pharmacological effects of OA have been performed in adult animals but none have been performed in the neonatal phase.

The neonatal period selected for the experimental treatments in the current study is a critical window of developmental phenotypic plasticity in which development and programming of several phenotypes such as good health profiles and metabolic disorders that are observed later in adulthood occur.[37,38]

The current study was designed to investigate the potential hepatoprotective effect of OA administered in the neonatal phase to attenuate the development of fructose-induced hepatic manifestations of metabolic dysfunction in a murine experimental model.

Materials and methods

Ethical clearance for the study

All animal experimental procedures were performed according to the protocols approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC ethics clearance number: 2014/47/D).

Housing and animal husbandry

Sprague–Dawley (*Rattus norvegicus*) dams, each with between 8 and 12 neonatal rats, were used in this study. The rats were supplied by the Central Animal Services, University of the Witwatersrand and housed in a conventional animal housing facility. Each dam and its respective litter were housed in Perspex cages in which wood shavings was used as bedding. The bedding was changed once a week. The room temperature was maintained at 25 ± 2°C and a 12-h light and dark cycle followed (with lights on at 7 am). Adequate positive pressure ventilation of the room was maintained at all times. The dams were supplied with standard commercially sourced rat chow (SRC) (Epol®, Johannesburg, South Africa) and water *ad libitum* throughout the suckling period. The dams were allowed to freely nurse (including during the 7-day neonatal experimental period) until weaning of the rat pups on postnatal day (PD) 21. The dams were returned to stock after weaning of their pups. The weaned rats were then housed individually in cages as described above.

Study design and experimental dietary treatments

This was an interventional comparative study in which 112 male and female neonatal rats from 10 nursing dams were used. The study was divided into two experimental interventional phases. The first phase of the study was between PD7 and PD13. During the first phase, the first nutritional intervention was introduced in order to induce neonatal programming of liver metabolism. The litter from each of the 10 nursing dams were randomly assigned to the four neonatal treatment groups resulting in each treatment group consisting of 28 male and female neonatal rats. This random allocation of littermates to each of the different treatment groups was done to avoid dam-effect bias. For easy identification, a colour-coded numbering system was used. The neonatal rats were marked on their tails with different coloured non-toxic ink based permanent marker pens.

The neonatal rats were weighed daily to monitor the health of the animals and adjust treatment amounts to maintain a constant dosage per body mass and they received the following treatments: *Group* 1: distilled water (DW) – DW with dimethyl sulphoxide (0.5% v/v) which was used as a vehicle control to dissolve the OA; *Group* 2: OA – OA (60 mg/kg body mass) reconstituted in 0.5% dimethyl sulphoxide; *Group* 3: high fructose solution (HF) – 20% fructose solution (w/v) made up in 0.5% dimethyl sulphoxide; *Group* 4: oleanolic acid and high fructose solution (OAHF) – OA (60 mg/kg body mass) and 20% fructose solution (w/v) constituted in dimethyl sulphoxide.

All treatments were administered once daily in the morning (between 9 and 11 am) for 7 days (PD7 to PD13), at a volume of

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press

10 ml/kg body mass via orogastric gavage using a 20G orogastric tube attached to a 1 ml syringe. After administration of treatments, all the neonatal rats were observed for 20 min for unusual behavioural changes. Following the treatments between PD7 and PD13, the rats were allowed continue to suckle until they were weaned on PD21 after which they had *ad libitum* access to SRC and plain drinking tap water until PD55 which is generally recognized as post-pubertal and the commencement of adulthood in rats. In the second experimental interventional phase, a second dietary intervention was introduced PD56 up to PD112 (adult phase). During this adulthood period, PD56 to PD112, all rats received *ad libitum* access to SRC, however, half ($n=56$) the number of male and female rats in each group received plain drinking water whilst the other half ($n=56$) received 20% fructose (w/v) as drinking fluid. Each treatment group thus had 6–8 male or female pups. The fructose was given in order to induce health outcomes associated with metabolic dysfunction.[39] In the rats that did not receive the early 'hit' with fructose in the neonatal phase, the fructose in adulthood would represent a single first hit in adulthood. For the rats which received an early hit as neonates, the fructose intervention in adulthood represented a late second hit. Thus the latter group of rats had a double hit of fructose in their lifespan. Consequently in terms of fructose intake, the rats either had only an early (neonatal) hit with fructose or a late (in adulthood) hit with fructose or a double hit (neonatally and then later as adults).

Terminal procedures

The rats were then euthanized on PD112 by an overdose of intraperitoneally injected sodium pentobarbital (200 mg/kg body mass; Eutha-Naze®, Bayer Corporation, Johannesburg, South Africa). Blood samples were collected into serum separator vacutainers via cardiac puncture, and centrifuged to collect serum. The abdominal cavity was opened via a midline incision and the liver was carefully dissected out. The liver samples were weighed (Precisa 310M®, Precision Instruments, Switzerland) and masses recorded. Thereafter a section of each liver sample was dissected from the right medial lobe and preserved in 10% phosphate buffered formalin for histological analyses. The remainder of the liver sample was stored at −20°C for determination of liver lipid content.

The left tibia was dissected from the hindlimb of each rat, defleshed, dried in an oven at 50°C for 5 days and then the length measured and used in the computation for the relative mass of liver.

Measurement of non-specific tissue alkaline phosphatase (ALP) and alanine aminotransferase (ALT) as a surrogate marker of liver function

Using the serum samples collected at termination, tissue non-specific ALP and ALT were measured using a calibrated automatic biochemical analyzer (IDEXX VetTest™, Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA). The results from the measurement of enzyme markers were reported as units per litre (U/l). The assays were carried out according to the manufacturer's instructions.

Preparation and processing of liver tissue for histological examination

Following fixation, the liver tissue samples were processed using an automatic tissue processor (Microm STP 120, ThermoScientific, MA, USA), embedded in paraffin wax, and sectioned at 3 μm thickness using a rotary microtome. The tissue sections were then either stained with haematoxylin and eosin (HE) to assess hepatocellular changes or Masson's trichrome (MT) stain to assess fibrosis according to standard protocols as described by Bancroft and Gamble.[40] Photomicrographs of the stained sections were acquired using a Leica ICC50 HD video camera linked to a Leica DM 500 microscope. Composite images were prepared with CorelDraw X3 Software (Version 13, Corel Corporation, Ottawa, Canada). No pixelation adjustments of the captured photomicrographs were undertaken except for adjustment of contrast and brightness.

Histomorphometry and histological examination of the liver samples

The HE-stained sections of the liver were semi-quantitatively scored for inflammation according to Kleiner *et al.*[41] Steatosis was determined by analyzing hepatocellular vesicular steatosis, based on the total area affected, grading was done as follows: Grade 0 = <5% steatosis; Grade 1 = 5–33% steatosis; Grade 2 = 33–66% steatosis; Grade 3 = >66% steatosis per camera field of the parenchyma. Inflammation was scored by counting the number of inflammatory cell aggregates in the liver parenchyma and graded as follows: Grade 0 = none or no foci of inflammation per camera field; Grade 1 = fewer than 2 foci per camera field; Grade 2 = 2–4 foci per camera field; Grade 3 = >4 foci per camera field at ×20.

Fibrosis and steatosis was quantitatively assessed from photomicrographs of the portal areas of MT-stained sections at ×40 using ImageJ.[42] In brief, the images were converted to 8-bit scale, a threshold set manually and area fraction covered by connective tissue was quantified.

To avoid sampling errors, liver samples were obtained from the right medial lobe and all the histological features were semi-quantitatively and quantitatively assessed by a histologist (PN) who was blinded to the animal treatments.

Determination of hepatic lipid content

Liver samples from each of the four different treatment groups for male and female rats were pooled together for the determination of intrahepatic lipid content. In brief, the liver samples were dried (lyophilized) in a freeze dryer (Model BK-FD12, SP Scientific, New York, USA) and ground into a fine powder. The ground sample (2.5 g) was placed in an extraction thimble and the oil extracted using 60 ml of petroleum ether in a Soxhlet extractor (Tecator Soxtec System HT 1043, Gemini BV Laboratories, Apeldoorn, Nederland). The total lipid was then determined gravimetrically. Liver lipids for each experimental group were determined in triplicate.

Statistical analyses

Results were presented as mean ± standard deviation (S.D.) and analyzed using GraphPad Prism for Windows Version 7.0 (GraphPad Software Inc., San Diego, USA). For parametric data, one-way ANOVA followed by Bonferroni's *post-hoc* test were used. The Kruskal–Wallis test was used to analyze NAFLD scores for steatosis and inflammation followed by Dunn's *post-hoc* test. $P \leqslant 0.05$ was considered significant.

Results

The effect of neonatal OA administration on body mass in fructose-fed adult male and female rats

Body masses of male and female rats from all experimental treatment groups increased significantly from induction (P7)

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press

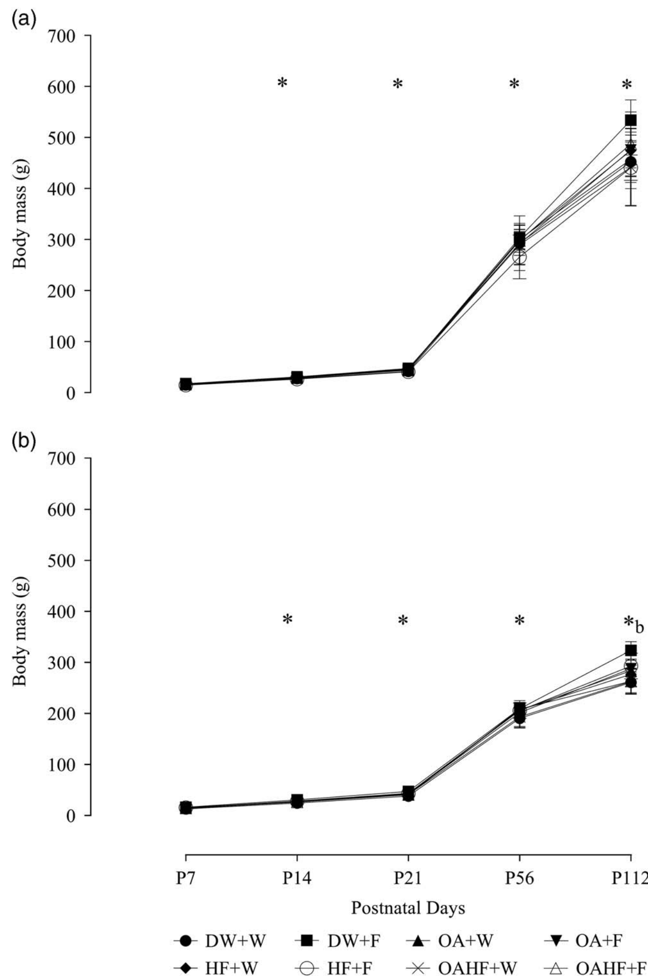

Fig. 1. The effect of neonatal oral administration of oleanolic acid or fructose on body masses of male rats (*a*) and female (*b*) fed a high fructose diet, as neonates and, or in adulthood. All data presented as mean ± S.D. *Significant increase in body mass from induction to weaning and from weaning to termination ($P < 0.005$). [b]Significant increase in terminal body mass for female rats receiving a double hit of fructose (HF + F) compared with all of the other treatment groups ($P < 0.005$). DW + W, distilled water neonatally + plain drinking water post-weaning and throughout adulthood (M = 7; F = 7); DW + F, distilled water neonatally + high fructose solution as drinking fluid in adulthood (M = 6; F = 6); OA + W, oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (M = 8; F = 8); OA + F, oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (M = 7; F = 8); HF + W, high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (M = 6; F = 7); HF + F, high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (M = 7; F = 8); OAHF + W, combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (M = 7; F = 6); OAHF + F, combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (M = 7; F = 7); M, male; F, female, P, postnatal day.

to termination (P112) ($P < 0.05$; Fig. 1a and 1b). There were no significant differences in terminal body masses for male rats from all experimental treatment groups ($P > 0.05$; Fig. 1a).

In female rats, a double hit of fructose, first early in the neonatal period and then later in adulthood (HF + F) resulted in a significant increase in terminal body mass (P112) compared with other treatment groups ($P < 0.05$; Fig. 1b). Rats neonatally administered OA and subjected to the double hit of fructose (OAHF + HF) did not show the significant increase in terminal body mass observed in rats receiving the double hit (HF + F) of fructose ($P < 0.05$; Fig. 1b). Thus OA prevented the increase in terminal body mass due to a double hit with fructose.

The effect of neonatal OA administration on surrogate markers of liver function, liver masses (absolute and relative to tibia length) and hepatic lipid content in fructose-fed adult male and female rats

In male rats, consumption of fructose either late in adulthood (DW + F) or as a double hit early in the neonatal period and late in adulthood (HF + F) resulted in up to 19% increase in liver masses compared with other treatment groups ($P < 0.05$; Table 1). Oral administration of OA in the neonatal period prevented the late single hit (OA + F) and double hit (OAHF + F) effects of fructose on gain in liver mass ($P < 0.05$). There were no significant differences in liver masses between neonatal administration of OA and the control group which did not receive any fructose throughout its lifespan (DW + W) ($P > 0.05$).

Although the absolute liver mass was significantly higher ($P < 0.05$; Table 2) for female rats that received a double hit of fructose (HF + F), when adjusted to relative tibial length, the relative liver mass was not significantly different compared with other treatment groups ($P > 0.05$; Table 2).

In male rats, only the double hit (HF + F) of fructose (early in the neonatal period and late in adulthood) resulted in a significant accumulation (47% increase) in hepatic lipid content compared with other treatment groups ($P < 0.05$; Fig. 2). Oral administration of OA in the neonatal period prevented the increased hepatic lipid content observed as a result of the double hit with fructose (OAHF + HF *v.* HF + F; $P < 0.001$; Fig. 2).

In females, hepatic lipid accumulation was not restricted to the double hit with fructose. The liver lipid content for female rats that received a late fructose hit (DW + F) and those that received a double hit neonatally and in adulthood (HF + F) was up to 49% higher than rats from the other treatment groups ($P < 0.05$; Fig. 3). Oral administration of OA in the neonatal period prevented the increased hepatic lipid content observed as a result of the late single hit and double hit with fructose (OA + F *v.* DW + F and OAHF + F *v.* HF + F; $P > 0.001$; Fig. 3).

The effect of neonatal oral administration of OA on hepatic histomorphometry of fructose-fed adult male and female rats

In male rats, steatosis scores were greater in the rats that received a double hit of fructose neonatally and in adulthood (HF + F) compared with all the other experimental groups (Kruskal–Wallis; Table 1). In addition, both macro and microvesicular steatosis that was periportally distributed was observed in the HF + F group (Fig. 4f). However, there were no significant differences in the scores for hepatic inflammation across all treatment groups ($P > 0.05$). Fibrosis area fraction was up to three times higher in the rats that only received a late fructose hit (DW + F) and a double fructose hit (neonatally and in adulthood; HF + F) compared with other treatment groups ($P < 0.05$; Table 1). Fibrosis in these two groups (DW + F and HF + F) was mainly periportal (Fig. 5b and 5f). Neonatal administration of OA prevented the development of steatosis and fibrosis induced by a late (DW + F) and double hit of fructose (HF + F).

In female rats, steatosis was higher in the rats that received a single late hit (DW + F) and double hit of fructose neonatally and

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press

Table 1. The effect of neonatal oral administration of oleanolic acid and fructose on the absolute (g) and relative (g/cm tibia) liver masses, steatosis, fibrosis and inflammation scores, a surrogate marker of liver function (alkaline aminotransferase) and non-tissue specific alkaline phosphatase levels in adult male rats fed a high fructose diet in adulthood

Parameter	DW + W	DW + F	OA + W	OA + F	HF + W	HF + F	OAHF + W	OAHF + F
Liver (g)	11.7 ± 1.8	13.5 ± 1.2	11.0 ± 0.7	12.5 ± 1.4	13.5 ± 1.4	17.2 ± 1.8[b]	12.1 ± 1.3	12.8 ± 2.1
Liver (g/cm tibia)	2.8 ± 0.4	3.6 ± 0.3[a]	2.5 ± 0.2	2.9 ± 0.4	3.1 ± 0.3	3.7 ± 1.6[b]	2.4 ± 1.1	3.0 ± 0.6
Steatosis	0.3 ± 0.6	1.7 ± 0.6	0 ± 0	0.7 ± 0.6	0.7 ± 0.6	2.3 ± 0.6	0.3 ± 0.6	1 ± 0
Inflammation	1.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	1.7 ± 0.6	0.3 ± 0.6	0.7 ± 0.6	1.3 ± 0.6
Fibrosis (area fraction)	1.2 ± 1.2	10.4 ± 1.8[a]	0.9 ± 0.9	3.9 ± 0.4	2.6 ± 0.8	12.2 ± 3.1[b]	1.1 ± 0.9	3.6 ± 1.0
ALT (U/l)	81.9 ± 10.6	82.7 ± 27.8	93.1 ± 39.7	82.1 ± 34.5	76 ± 10.1	88.0 ± 40.3	66.4 ± 28.9	78.0 ± 15.3
ALP (U/l)	87.3 ± 5.7	108.5 ± 12.5	102.1 ± 17.7	111.4 ± 12.0	96.9 ± 29.8	140.7 ± 21.8	80.6 ± 9.5	86.14 ± 15.3

DW + W, distilled water neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); DW + F, distilled water neonatally + high fructose solution as drinking fluid in adulthood ($n = 6$); OA + W, oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood ($n = 8$); OA + F, oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$); HF + W, high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 6$); HF + F, high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$); OAHF + W, combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); OAHF + F, combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$); ALT, alanine aminotransferase; ALP, alkaline phosphatase.
All data presented as mean ± S.D.
[a,b]Significant increase in absolute and relative liver masses and hepatic fibrosis area fraction in rats that received DW + F and HF + F compared with rats from the other experimental treatment groups ($P < 0.05$), compared with the other groups, respectively.

Table 2. The effect of neonatal oral administration of oleanolic acid and fructose on the absolute (g) and relative (g/cm tibia) liver masses, steatosis, fibrosis and inflammation scores, a surrogate marker of liver function (alkaline aminotransferase) and tissue non-specific alkaline phosphatase levels in of adult female rats fed a high fructose diet in adulthood

Parameter	DW + W	DW + F	OA + W	OA + F	HF + W	HF + F	OAHF + W	OAHF + F
Liver (g)	6.1 ± 0.5	7.8 ± 0.4	6.2 ± 0.6	6.6 ± 0.8	8.4 ± 0.5	9.2 ± 0.7[a]	6.2 ± 0.6	6.7 ± 0.6
Liver (g/cm)	1.6 ± 0.1	2.0 ± 0.1	1.6 ± 0.1	1.7 ± 0.2	2.2 ± 0.1	2.1 ± 0.7	1.6 ± 0.2	1.6 ± 0.5
Steatosis	0.0 ± 0.0	2.7 ± 0.6	0.3 ± 0.6	0.7 ± 0.6	0.7 ± 0.8	3.0 ± 0.0	0.3 ± 0.6	1.0 ± 0.0
Inflammation	0 ± 0	1.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	0.3 ± 0.6	1.7 ± 0.6	0.7 ± 0.6	0.3 ± 0.6
Fibrosis (Area fraction)	1.6 ± 0.4	12.5 ± 0.7[a]	1.6 ± 0.7	4.2 ± 0.3	2.5 ± 0.5	12.5 ± 2.3[b]	1.8 ± 0.8	4.3 ± 0.9
ALT (U/l)	67.8 ± 2.3	81.6 ± 9.7	72.3 ± 8.7	78.1 ± 10.4	77.3 ± 11.0	79.6 ± 7.4	63.5 ± 9.8	67.9 ± 8.8
ALP (U/l)	64.3 ± 3.8	67.1 ± 14.7	68.9 ± 13.9	62.6 ± 12.8	63.5 ± 15.8	69.7 ± 14.3	65.0 ± 24.6	63.7 ± 13.1

DW + W, distilled water neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); DW + F, distilled water neonatally + high fructose solution as drinking fluid in adulthood ($n = 6$); OA + W, oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood ($n = 8$); OA + F, oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood ($n = 8$); HF + W, high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); HF + F, high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 8$); OAHF + W, combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 6$); OAHF + F, combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$); ALT, alanine aminotransferase; ALP, alkaline phosphatase.
All data presented as mean ± S.D.
[a,b]Significant increase in absolute liver masses (HF + F) and higher hepatic fibrotic area fraction in rats that received a single late hit (DW + F) and double hit of fructose neonatally and in adulthood (HF + F) than other experimental treatment groups ($P < 0.05$).

in adulthood (HF + F) compared with all the other experimental groups (Kruskal–Wallis; $P < 0.05$; Table 2). In addition, periportally distributed macrovesicular steatosis was observed in the rats that received a single late hit (DW + F) and those that received a double hit of fructose neonatally and in adulthood (HF + F) (Fig. 6b and 6f). Scores for hepatic inflammation were also higher in the rats that received a single late hit (DW + F) and double hit of fructose neonatally and in adulthood (HF + F) compared with all the other experimental groups (Table 2). Furthermore, fibrosis was three times higher in the in rats that received DW + F and HF + F compared with other treatment groups ($P < 0.05$; Table 2). Fibrosis in these two groups was mainly periportal (Fig. 7b and 7f). Neonatal oral administration of OA prevented the development of steatosis and fibrosis induced by a late (DW + F) and double hit of fructose (HF + F).

The effect of neonatal OA administration on a surrogate marker of liver function and tissue non-specific ALP in fructose-fed adult male and female rats

The serum levels of liver enzymes were similar in all experimental treatment groups for both male and female rats ($P > 0.05$; Tables 1 and 2).

Discussion

In the current study, the potential protective effects of neonatal oral intake of OA on the development of fructose-induced NAFLD were investigated in male and female rats. As confirmed by the terminal liver masses, total hepatic lipid content (more than 5% of mass as fat) and histological findings, a double

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press

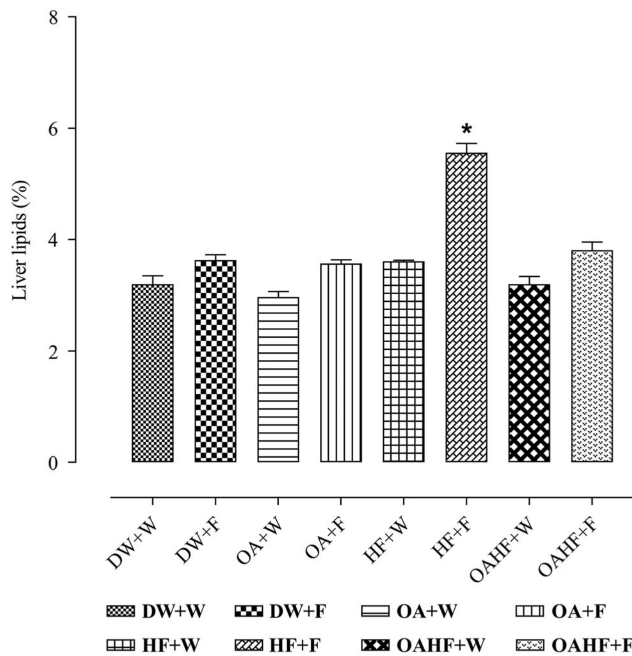

Fig. 2. The effect of neonatal oral administration of oleanolic acid on hepatic lipid storage in male rats fed a high fructose diet as neonates and, or in adulthood. All data presented as mean ± S.D. *Significant increase in hepatic lipid content in rats that received a double hit of fructose neonatally and in adulthood (HF + F) than the treatment groups ($P < 0.05$). DW + W, distilled water neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); DW + F, distilled water neonatally + high fructose solution as drinking fluid in adulthood ($n = 6$); OA + W, oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood ($n = 8$); OA + F, oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$); HF + W, high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 6$); HF + F, high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$); OAHF + W, combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); OAHF + F, combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$).

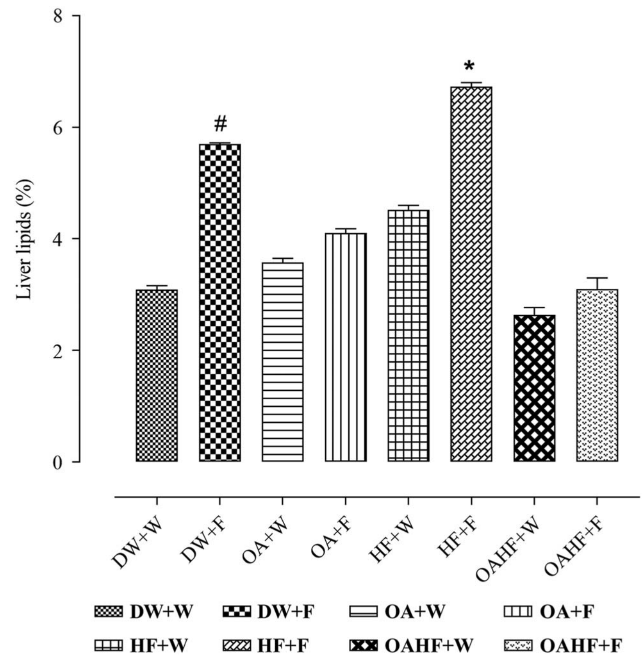

Fig. 3. The effect of neonatal oral administration of oleanolic acid on hepatic lipid storage in adult female rats fed a high fructose diet as neonates and, or in adulthood. All data presented as mean ± S.D. #, *Significant increase in hepatic lipid content in rats that received only fructose late (DW + F) and those that received fructose as neonates and as adults (HF + F) than the control rats that did not receive any fructose throughout the study period (DW + W) ($P < 0.05$). DW + W, distilled water neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); DW + F, distilled water neonatally + high fructose solution as drinking fluid in adulthood ($n = 6$); OA + W, oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood ($n = 8$); OA + F, oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood ($n = 8$); HF + W, high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); HF + F, high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 8$); OAHF + W, combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 6$); OAHF + F, combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$).

hit of fructose (neonatally with the subsequent administration in adulthood) induced the development of NAFLD in both male and female rats. Moreover, the late fructose hit alone also induced NAFLD in female, but not in male, rats. Neonatal oral administration of OA was effective in attenuating the development of the fructose-induced NAFLD in both male and female rats.

The results of this current study showed an increase in body masses of the rats across treatment groups from weaning to termination in male and female rats. There were no differences in body masses of male rats throughout the study period. However, in female rats, administration of a double hit of fructose neonatally and as a secondary dietary insult in adulthood caused an increase in terminal body mass which was not observed in male rats and was prevented by neonatal administration of OA. Due to the obesogenic nature of fructose, the neonatal treatment with fructose and its consumption in adulthood as a secondary dietary insult could be the main culprit in the observed increase in terminal body mass in female rats that received a double hit. The sex differences in response to the dietary interventions are discussed later. Neonatal oral administration of OA prevented the fructose-induced increase in terminal body mass observed in female rats, suggesting that neonatal interventions with OA do not negatively affect growth. The specific molecular mechanism(s) through which OA prevents fructose-induced body mass increases requires further investigation.

It is notable that the timing of fructose intake had an impact on the liver. When administered early in the neonatal phase only, both male and female rats did not develop fatty liver disease. When administered in adulthood only, only the females developed the fatty livers. When the rats were administered with fructose as neonates and then later as adults both male and females rats had a significant increase in liver lipid accumulation. In addition in females the intake of excessive fructose early and late (double hit) resulted in a greater increase in fatty liver compared with the rats that only had fructose late. Thus whilst our results confirm the findings from other studies that have shown that exposure to dietary insults during critical windows of cellular plasticity results in increased risks of developing NAFLD later in life,[18,43] the sex differences in response to the high fructose diets emphasize the need for studies to be designed so as to account for possible differences between sexes and to avoid extrapolating findings from one sex to another without scientific verification. The importance of sex-specific medicine is indeed taking centre stage these days.[44]

The liver masses for male and female rats that received a double hit of fructose, initially as neonates and later in adulthood (HF + F) were up to 19% higher than other experimental groups.

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press

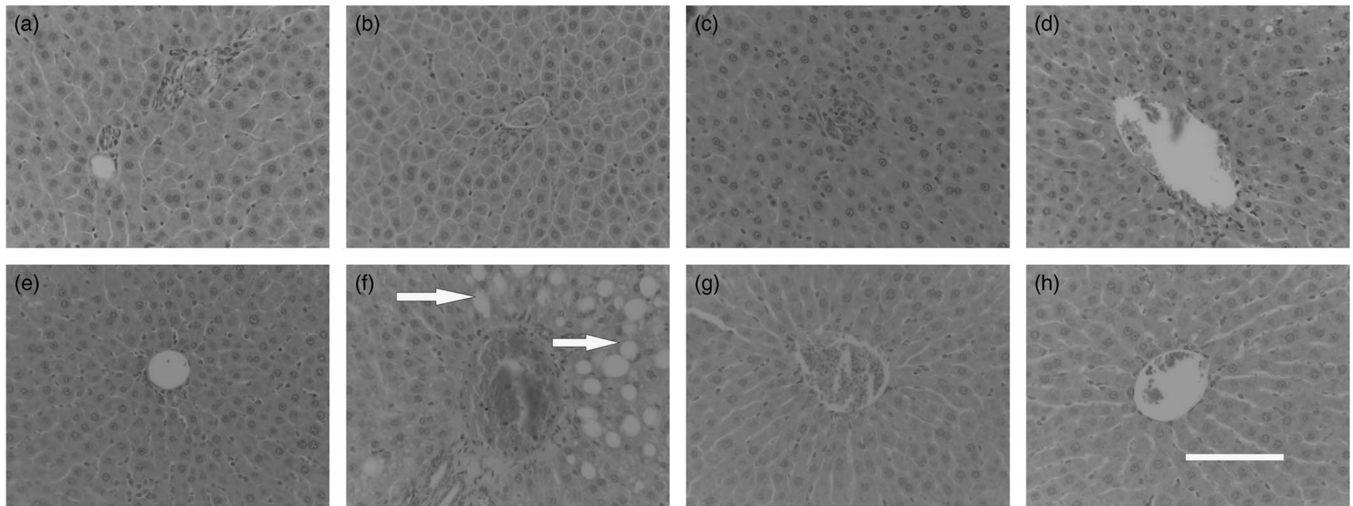

Fig. 4. Photomicrographs showing histopathological features after haematoxylin and eosin (HE) staining of liver cross-sections from a representative male rat from each experimental treatment group (×40). (*a*) Distilled water neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); (*b*) distilled water neonatally + high fructose solution as drinking fluid in adulthood ($n = 6$); (*c*) oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood ($n = 8$); (*d*) oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$); (*e*) high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 6$); (*f*) high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$); (*g*) combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); (*h*) combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$). The open arrows indicate steatosis. Scale bar = 30 μm in the HE stain sections.

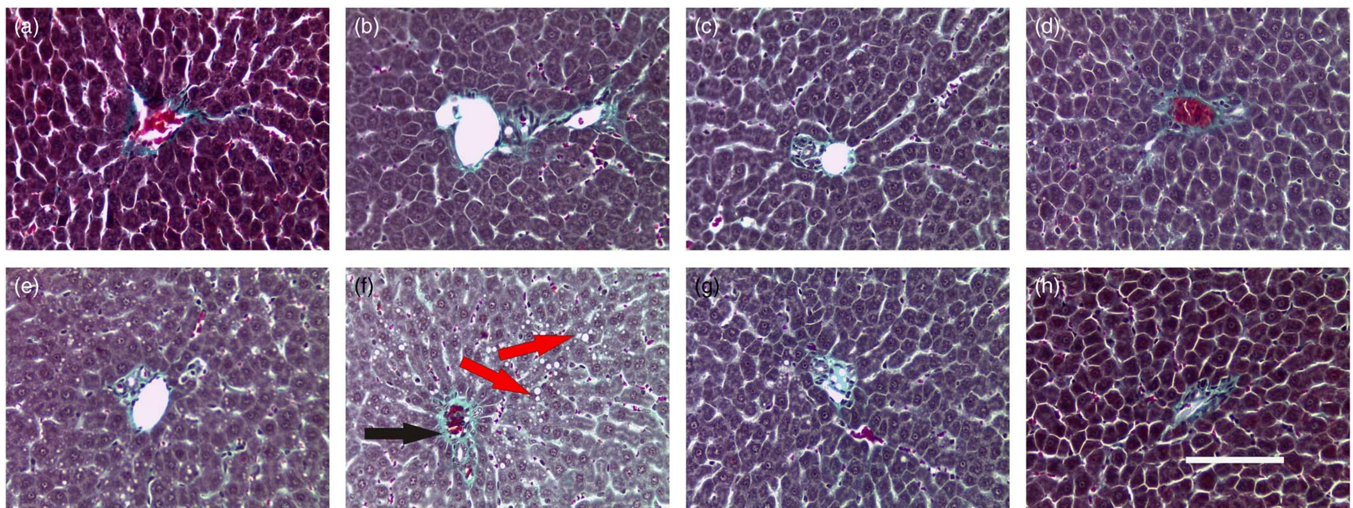

Fig. 5. Photomicrographs showing histopathological features after Masson's trichrome staining of liver cross-sections from a representative male rat from each experimental treatment group (×40). (*a*) distilled water neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); (*b*) distilled water neonatally + high fructose solution as drinking fluid in adulthood ($n = 6$); (*c*) oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood ($n = 8$); (*d*) oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$); (*e*) high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 6$); (*f*) high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$); (*g*) combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); (*h*) combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$). The open arrows indicate steatosis. Scale bar = 30 μm in the haematoxylin and eosin stain sections. Red arrows indicate steatosis and black arrows indicate fibrosis. Scale bar = 30 μm in the Masson's trichrome stain sections.

These findings are consistent with those of Bruggeman *et al.*[45] who also reported that dietary fructose in female rats increases the relative liver masses. Studies have shown that the increase in liver masses in fructose-fed rats can be attributed to the hepatic lipid storage from DNL and glycogen accumulation from hepatic metabolism of fructose.[46] We speculate that the increase in terminal liver masses observed in this study could most likely be due to lipid accumulation rather than glycogen since we fasted the rats for several hours before termination, as such glycogen would not have been present in significant amounts. Fasting rats overnight (8–12 h) would have initiated a catabolic state and resulted in the depletion of glycogen stores.[47] The fructose-induced increase in liver masses was prevented by neonatal oral administration of OA, suggesting that OA potentially programs against the increase in liver mass possibly through modulating hepatic lipogenic enzymes.[36] Other studies performed in adult mice have shown that short-term administration of OA protected against the increase in body and liver masses.[48] An exciting finding in our study is the fact that the OA administered in the neonatal period showed long-lasting effects, about 14 weeks, after the last intake of OA.

Total liver lipid content followed a similar trend as recorded for liver masses, with the double hit of fructose resulting in an

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press

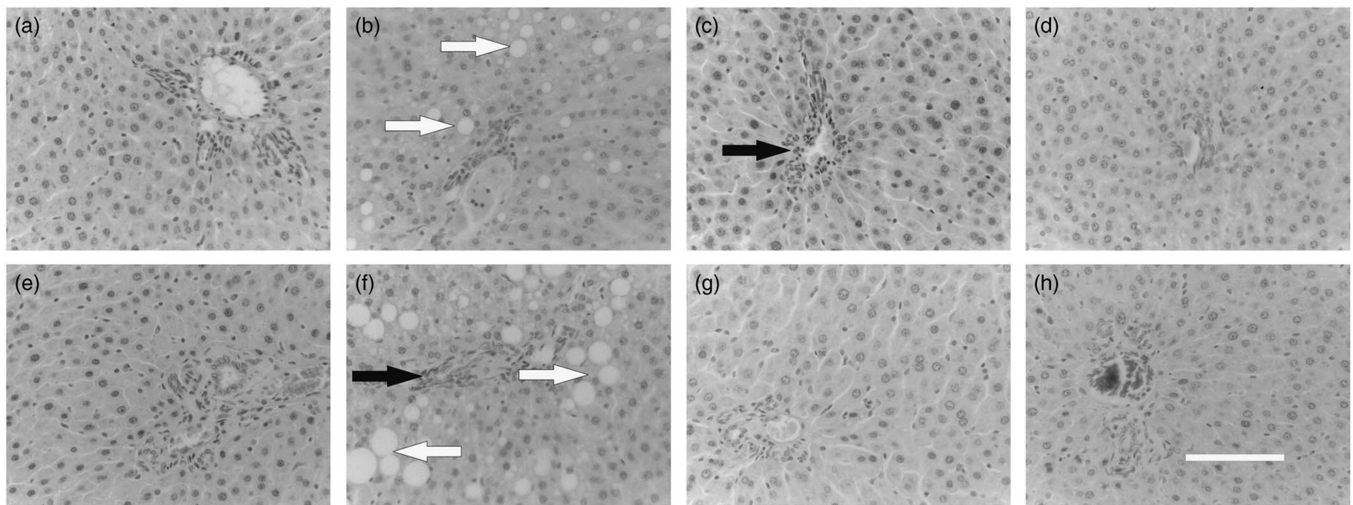

Fig. 6. Photomicrographs showing histopathological features after heamatoxylin and eosin (HE) staining of liver cross-sections from a representative female rat of each group (×40). (*a*) Distilled water neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); (*b*) distilled water neonatally + high fructose solution as drinking fluid in adulthood ($n = 6$); (*c*) oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood ($n = 8$); (*d*) oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood ($n = 8$); (*e*) high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); (*f*) high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 8$); (*g*) combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 6$); (*h*) combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$). Solid black arrows indicate clusters of inflammatory cells. The open arrows indicate steatosis. Scale bar = 30 µm in the HE stain sections.

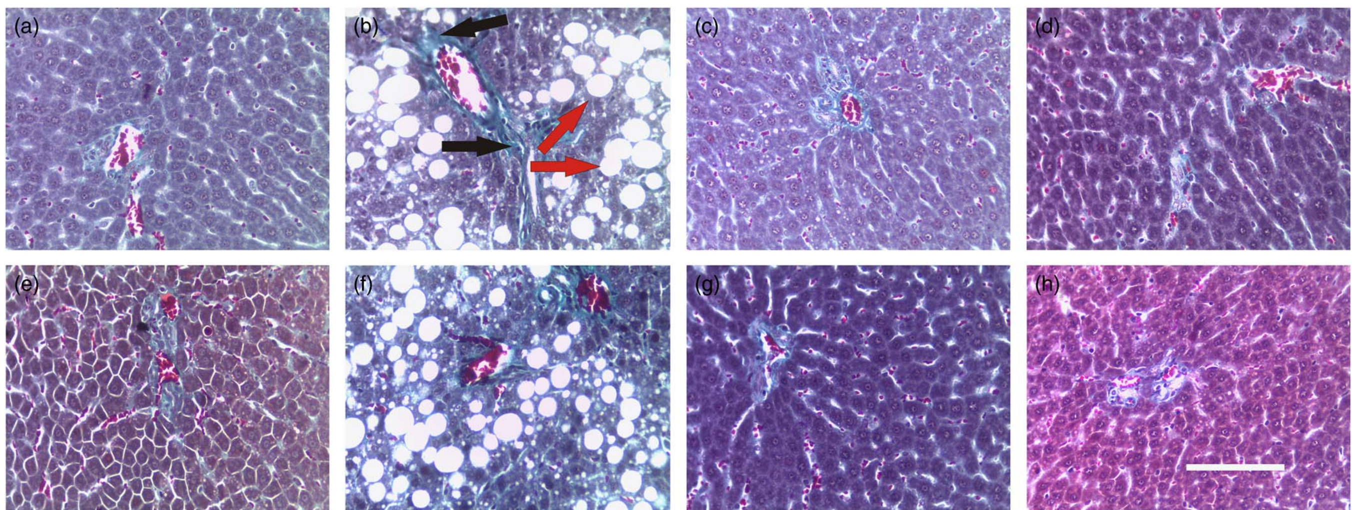

Fig. 7. Photomicrographs showing histopathological features (fibrosis and steatosis) after Masson's trichrome (MT) staining of liver cross-sections from a representative female rat from each experimental treatment group (×40). (*a*) Distilled water neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); (*b*) distilled water neonatally + high fructose solution as drinking fluid in adulthood ($n = 6$); (*c*) oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood ($n = 8$); (*d*) oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood ($n = 8$); (*e*) high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 7$); (*f*) high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 8$); (*g*) combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood ($n = 6$); (*h*) combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood ($n = 7$). Red arrows indicate steatosis and black arrows indicate fibrosis. Scale bar = 30 µm in the MT-stained sections.

increase in hepatic lipid accumulation in both male and female rats. The accumulation and storage of hepatic lipids in male and female rats reported in this study confirm previous findings that demonstrated the role of fructose in the pathogenesis of hepatic steatosis and NAFLD.[49,50] The increase in the liver lipid content observed in the current study could be attributed to the fructose-induced accumulation of lipids through its stimulation of hepatic DNL.[51] The fructose-induced increase in hepatic liver lipid content was attenuated by the neonatal administration of OA. Studies have shown that OA, in addition to protecting against liver and body mass gain, also protects against fructose-induced hepatic TG accumulation and hepatic morphological changes associated with NAFLD.[36]

NAFLD is a common feature of MetS that is characterized by hepatic steatosis, inflammation, hepatocellular ballooning and fibrosis.[52,53] Histomorphological examination of the liver confirmed the presence of sex-specific steatosis in rats from different treatment groups. The present study reports the presence of periportal microvesicular steatosis and periportal macrovesicular steatosis in male and female rats that received fructose neonatally and in adulthood. However steatosis was only observed in females that received a late hit of fructose in adulthood (DW + F), but not

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press

in male rats that received the same treatment. Female rats that received fructose had a more severe form of steatosis (macrosteatosis) and appeared to be more vulnerable to the development of NAFLD compared with males given the same fructose treatments. The sex differences in liver masses and hepatic steatosis that we observed could possibly be explained by alterations in hepatic gene expression in the neonatal rats due to fructose administration which results in sex-dependent changes in lipid metabolism of the adult rats as previously reported.[54] It is also possible that the sex differences observed in liver masses and hepatic steatosis could be due to differential sex-specific neonatal programming of metabolic dysfunction as previously reported.[54,55]

Our findings on the vulnerability of female rats to develop NAFLD are in contradiction with several human epidemiological studies which showed that male subjects had a higher prevalence of NAFLD than females.[56–58] These observed sex differences were considered to be a reflection the role of sex steroid hormones and genetic vulnerability in the pathogenesis of NAFLD.[59] In addition, in the human studies, differences in lifestyle of males and females were also suggested as playing a role wherein it was observed that the weekly consumption of non-diet soda by males was significantly greater than by females.[57] This would translate to an increased consumption of fructose by the males, the implications of which were discussed earlier. In our study the male and female rats had *ad libitum* access to the fructose solutions and similar housing conditions resulting in the females being more susceptible to developing NAFLD. There is thus need to investigate in human populations where males and females have the same lifestyles whether the susceptibility to develop NAFLD is reversed to show a similar trend to that of rats. Future studies are recommended to establish the mechanisms responsible for our observed findings.

We nevertheless propose that neonatal OA administration may have prevented fructose-induced hepatic metabolic dysfunction through neonatal programming of hepatocellular protective mechanisms. The possible mechanism through which OA could have prevented hepatic lipid storage may be the neonatal programming of mechanisms regulating lipogenic pathways such as antagonizing the action of fructokinase activity which in turn alters hepatic conversion of fructose to fructose-1-phosphate.

Histomorphological analyses of the livers showed the presence of inflammatory cells in female rats that received a double hit of fructose neonatally and in adulthood, but not in males receiving the same treatment. Excess fructose in the liver causes fructosylation of proteins and superoxide formation which contributes to hepatic inflammation.[60] Accumulation of visceral white adipose tissue in fructose-consuming obese individuals is an important source of pro-inflammatory cytokines in the development of NAFLD.[61,62] Hepatic inflammation is promoted by infiltration of macrophages[63] and the chemokine monocyte chemoattractant protein-1 and its receptor C-C chemokine receptor-2 which play an important role in the recruitment of macrophages to the sites of hepatic inflammation.[62] Other studies have shown that malondialdehyde generated during hepatic metabolism of fructose causes inflammation by activating NF-κB, a transcription factor regulating the expression of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 8.[64] Excessive fructose intake has also been reported to cause hepatic inflammation by enhancing production of TNF-α and activation of the c-Jun amino-terminal kinase which are pro-inflammatory cytokines (Shimatmoto and Nobuyuki, 2006).

After 8 weeks of fructose feeding in adulthood following neonatal administration of fructose, male and female rats presented with fibrotic septa and there was evidence of accumulation of collagen fibres, as shown by a tripling in fibrosis area fraction in rats receiving a late hit and a double fructose hit. Excessive fructose consumption causes hepatic steatosis and accumulation of hepatic lipids which eventually causes lipid peroxidation. Aldehyde products of lipid peroxidation such as 4-hydroxynonenal and MDA activate hepatic stellate cells,[14,65] the main collagen producing cells in the liver, resulting in fibrosis.[66] The dual effect of increased inflammation and oxidative cellular damage often result in accumulation of connective tissue and possibly contributes to the progression of NAFLD to NASH.[67]

Our study did not reveal any significant differences in the blood levels of the enzymes, ALT and non-tissue specific ALP. Similarly, Zarghani *et al.*[68] reported no significant changes in liver enzymes following diet-induced NAFLD. Human studies have shown that histologically confirmed NAFLD and NASH may exist without elevation of liver function enzymes.[69] In this study we have confirmed the presence of NAFLD in fructose-fed rats through histological analyses, liver masses and hepatic lipid storage, although the enzymes serving as surrogate markers of liver function were normal. The lack of significant increase in liver enzymes in rats that were administered with OA means that OA did not cause any hepatotoxicity and is safe for use as a phytochemical that potentially programs against the development of NAFLD. Although liver enzymes are useful indicators of hepatocellular damage, they may not be used as conclusive diagnostic tools of liver damage as such there is need to make use of other confirmatory diagnostic test panels such as non-invasive molecular biomarkers such as hyaluronic acid, tissue inhibitor of metalloproteinase 1 and amino-terminal propeptide of type III collagen to confirm liver damage.[70] Histology is considered a gold standard method for the diagnosis of NAFLD but is invasive and requires biopsies, as such the use of newer techniques is advisable.

Conclusion

We demonstrated that a high fructose diet can induce fatty liver disease, however the timing of the fructose intake in the life stage of rats has an impact on the phenotype. Sex-specific differences were also noted in response to the high fructose diets. It is thus important to note that studies should not just focus on a single sex but should be comparative between the sexes. We have also demonstrated, for the first time, that neonatal administration of OA attenuates the development of fructose-induced NAFLD by reducing hepatic lipid storage, terminal liver masses and hepatic histomorphological changes associated with NAFLD. We conclude that dietary supplementation with OA in the neonatal phase of development potentially programs for hepatoprotection against the development of NAFLD in adult life. This opens the potential for further exploration of the use of prophylatic intereventions during periods of developmental plasticity for long-term health benefits.

Acknowledgements. The authors thank the Central Animal Services staff for the care and husbandry of the animals.

Financial Support. The study was funded by the National Research Foundation, the South African Department of Higher Education and Training and the Medical Research Endowment Fund.

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press

References

1. Basaranoglu M, Basaranoglu G, Bugianesi E. Carbohydrate intake and nonalcoholic fatty liver disease: fructose as a weapon of mass destruction. *Hepatobiliary Surg Nutr*. 2014; 4, 109–116.
2. Li Y-C, Hsieh C-C. Lactoferrin dampens high-fructose corn syrup-induced hepatic manifestations of the metabolic syndrome in a murine model. *PLoS One*. 2014; 9, e97341.
3. Younossi ZM, Stepanova M, Rafiq N, *et al*. Pathologic criteria for nonalcoholic steatohepatitis: interprotocol agreement and ability to predict liver-related mortality. *Hepatology*. 2011; 53, 1874–1882.
4. Lonardo A, Ballestri S, Marchesini G, Angulo P, Loria P. Nonalcoholic fatty liver disease: a precursor of the metabolic syndrome. *Dig Liver Dis*. 2015; 47, 181–190.
5. Asrih M, Jornayvaz FR. Metabolic syndrome and nonalcoholic fatty liver disease: is insulin resistance the link? *Mol Cell Endocrinol*. 2015; 418, 55–65.
6. Liang W, Menke AL, Driessen A, *et al*. Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology. *PLoS One*. 2014; 9, e115922.
7. Castro GS, Cardoso JF, Vannucchi H, Zucoloto S, Jordão AA. Fructose and NAFLD: metabolic implications and models of induction in rats. *Acta Cir Bras*. 2011; 26, 45–50.
8. Dietrich P, Hellerbrand C. Non-alcoholic fatty liver disease, obesity and the metabolic syndrome. *Best Pract Res Clin Gastroenterol*. 2014; 28, 637–653.
9. Al Rifai M, Silverman MG, Nasir K, *et al*. The association of nonalcoholic fatty liver disease, obesity, and metabolic syndrome, with systemic inflammation and subclinical atherosclerosis: the Multi-Ethnic Study of Atherosclerosis (MESA). *Atherosclerosis*. 2015; 239, 629–633.
10. Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. *Dig Dis*. 2010; 28, 155–161.
11. Tessari P, Coracina A, Cosma A, Tiengo A. Hepatic lipid metabolism and non-alcoholic fatty liver disease. *Nutr Metabol Cardiovasc Dis*. 2009; 19, 291–302.
12. Khitan Z, Kim DH. Fructose: a key factor in the development of metabolic syndrome and hypertension. *J Nutr Metabol*. 2013; 2013, 1–12.
13. Musso G, Gambino R, Cassader M. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Prog Lipid Res*. 2009; 48, 1–26.
14. Day CP, James OF. *Steatohepatitis: A Tale of Two "Hits"?* 1998; *Gastroentorol*. 1998; 114, 842–845.
15. James O, Day C. Non-alcoholic steatohepatitis: another disease of affluence. *Lancet*. 1999; 353, 1634–1636.
16. Rector RS, Thyfault JP, Wei Y, Ibdah JA. Non-alcoholic fatty liver disease and the metabolic syndrome: an update. *World J Gastroenterol*. 2008; 14, 185.
17. Vickers MH. Developmental programming of the metabolic syndrome-critical windows for intervention. *World J Diabetes*. 2011; 2, 137–148.
18. Li M, Reynolds CM, Segovia SA, Gray C, Vickers MH. Developmental programming of nonalcoholic fatty liver disease: the effect of early life nutrition on susceptibility and disease severity in later life. *BioMed Res Int*. 2015; 2015, 1–12.
19. Mortensen OH, Larsen LH, Ørstrup LK, Hansen LH, Grunnet N, Quistorff B. Developmental programming by high fructose decreases phosphorylation efficiency in aging offspring brain mitochondria, correlating with enhanced UCP5 expression. *J Cereb Blood Flow Metab*. 2014; 34, 1205–1211.
20. Tain Y-L, Wu KL, Lee W-C, Leu S, Chan JY. Maternal fructose-intake-induced renal programming in adult male offspring. *J Nutr Biochem*. 2015; 26, 642–650.
21. Rodríguez L, Panadero MI, Roglans N, *et al*. Fructose only in pregnancy provokes hyperinsulinemia, hypoadiponectinemia, and impaired insulin signaling in adult male, but not female, progeny. *Eur J Nutr*. 2016; 55, 665–674.
22. Clancy B, Darlington R, Finlay B. Translating developmental time across mammalian species. *Neuroscience*. 2001; 105, 7–17.
23. Adams L, Angulo P. Treatment of non-alcoholic fatty liver disease. *Postgrad Med J*. 2006; 82, 315–322.
24. Harrison SA. New treatments for nonalcoholic fatty liver disease. *Cur Gastroenterol Rep*. 2006; 8, 21–29.
25. Tolman KG, Dalpiaz AS. Treatment of non-alcoholic fatty liver disease. *Ther Clin Risk Manag*. 2007; 3, 1153–1163.
26. Castellano JM, Guinda A, Delgado T, Rada M, Cayuela JA. Biochemical basis of the antidiabetic activity of oleanolic acid and related pentacyclic tritepenes. *J Diab*. 2013; 62, 1791–1799.
27. Liu J. Pharmacology of oleanolic acid and ursolic acid. *J Ethnopharmacol*. 1995; 49, 57–68.
28. Camer D, Yu Y, Szabo A, Huang XF. The molecular mechanisms underpinning the therapeutic properties of oleanolic acid, its isomer and derivatives for type 2 diabetes and associated complications. *Mol Nutr Food Res*. 2014; 58, 1750–1759.
29. Lin C, Wen X, Sun H. Oleanolic acid derivatives for pharmaceutical use: a patent review. *Expert Opin Ther Pat*. 2016; 26, 643–655.
30. Liu J, Liu Y, Klaassen CD. Protective effect of oleanolic acid against chemical-induced acute necrotic liver injury in mice. *Zhongguo yao li xue bao = Acta pharmacol Sin*. 1995; 16, 97–102.
31. Musabayane C, Tufts M, Mapanga R. Synergistic antihyperglycemic effects between plant-derived oleanolic acid and insulin in streptozotocin-induced diabetic rats. *Ren Fail*. 2010; 32, 832–839.
32. Zeng X-Y, Wang Y-P, Cantley J, *et al*. Oleanolic acid reduces hyperglycemia beyond treatment period with Akt/FoxO1-induced suppression of hepatic gluconeogenesis in type-2 diabetic mice. *PLoS One*. 2012; 7, e42115.
33. Nkeh-Chungag BN, Oyedeji OO, Oyedeji AO, Ndebia EJ. Anti-inflammatory and membrane-stabilizing properties of two semisynthetic derivatives of oleanolic acid. *Inflammation*. 2015; 38, 61–69.
34. Tsao S-M, Yin M-C. Antioxidative and antiinflammatory activities of asiatic acid, glycyrrhizic acid, and oleanolic acid in human bronchial epithelial cells. *J Agric Food Chem*. 2015; 63, 3196–3204.
35. Yin M-C, Chan K-C. Nonenzymatic antioxidative and antiglycative effects of oleanolic acid and ursolic acid. *J Agric Food Chem*. 2007; 55, 7177–7181.
36. Liu C, Li Y, Zuo G, *et al*. Oleanolic acid diminishes liquid fructose-induced fatty liver in rats: role of modulation of hepatic sterol regulatory element-binding protein-1c-mediated expression of genes responsible for de novo fatty acid synthesis. *Evid Based Complement Alternat Med*. 2013; 2013, 1–11.
37. Wang J, Wu Z, Li D, *et al*. Nutrition, epigenetics, and metabolic syndrome. *Antioxid Redox Signal*. 2012; 17, 282–301.
38. Wang X-M. Early life programming and metabolic syndrome. *World J Pediatr*. 2013; 9, 5–8.
39. Mock K, Lateef S, Benedito VA, Tou JC. High-fructose corn syrup-55 consumption alters hepatic lipid metabolism and promotes triglyceride accumulation. *J Nutr Biochem*. 2017; 39, 32–39.
40. Bancroft JD, Gamble M. *Theory and Practice of Histological Techniques*. 2008. Elsevier Churchill Livingstone: London.
41. Kleiner DE, Brunt EM, Van Natta M, *et al*. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005; 41, 1313–1321.
42. Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. *Nature Methods*. 2012; 9, 671.
43. Stewart MS, Heerwagen MJ, Friedman JE. Developmental programming of pediatric non-alcoholic fatty liver disease: redefining the 'first-hit'. *Clin Obstet Gynecol*. 2013; 56, 577.
44. Taqueti VR, Bairey Merz CN. *Sex-Specific Precision Medicine: Targeting CRT-D and Other Cardiovascular Interventions to Those Most Likely to Benefit*. 2017. Oxford University Press: Oxford.
45. Bruggeman EC, Li C, Ross AP, *et al*. A high fructose diet does not affect amphetamine self-administration or spatial water maze learning and memory in female rats. *Pharmacol Biochem Behav*. 2011; 99, 356–364.
46. Rippe JM, Angelopoulos TJ. Sucrose, high-fructose corn syrup, and fructose, their metabolism and potential health effects: what do we really know? *Adv Nutr Int Rev J*. 2013; 4, 236–245.
47. Ayala JE, Samuel VT, Morton GJ, *et al*. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Dis Model Mech*. 2010; 3, 525–534.

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press

48. Wang X, Liu R, Zhang W, *et al.* Oleanolic acid improves hepatic insulin resistance via antioxidant, hypolipidemic and anti-inflammatory effects. *Mol Cell Endocrinol*. 2013; 376, 70–80.
49. Lê K-A, Ith M, Kreis R, *et al.* Fructose overconsumption causes dyslipidemia and ectopic lipid deposition in healthy subjects with and without a family history of type 2 diabetes. *Am J Clin Nutr*. 2009; 89, 1760–1765.
50. Lim JS, Mietus-Snyder M, Valente A, Schwarz J-M, Lustig RH. The role of fructose in the pathogenesis of NAFLD and the metabolic syndrome. *Nat Rev Gastroenterol Hepatol*. 2010; 7, 251–264.
51. Softic S, Cohen DE, Kahn CR. Role of dietary fructose and hepatic de novo lipogenesis in fatty liver disease. *Dig Dis Sci*. 2016; 61, 1282–1293.
52. Honda Y, Yoneda M, Kessoku T, *et al.* The characteristics of non-obese NAFLD: effect of genetic and environmental factors. *Hepatol Res*. 2016; 46, 1011–1018.
53. Leitão HS, Doblas S, Garteiser P, *et al.* Hepatic fibrosis, inflammation, and steatosis: influence on the MR viscoelastic and diffusion parameters in patients with chronic liver disease. *Radiology*. 2016; 151570.
54. Clayton ZE, Vickers MH, Bernal A, Yap C, Sloboda DM. Early life exposure to fructose alters maternal, fetal and neonatal hepatic gene expression and leads to sex-dependent changes in lipid metabolism in rat offspring. *PLoS One*. 2015; 10, e0141962.
55. Rodríguez-Ortiz D, Reyes-Pérez A, León P, *et al.* Assessment of two different diagnostic guidelines criteria (National Cholesterol Education Adult Treatment Panel III [ATP III] and International Diabetes Federation [IDF]) for the evaluation of metabolic syndrome remission in a longitudinal cohort of patients undergoing Roux-en-Y gastric bypass. *Surgery*. 2016; 159, 1121–1128.
56. Weston SR, Leyden W, Murphy R, *et al.* Racial and ethnic distribution of nonalcoholic fatty liver in persons with newly diagnosed chronic liver disease. *Hepatology*. 2005; 41, 372–379.
57. Williams CD, Stengel J, Asike MI, *et al.* Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology*. 2011; 140, 124–131.
58. Caballería L, Pera G, Auladell MA, *et al.* Prevalence and factors associated with the presence of nonalcoholic fatty liver disease in an adult population in Spain. *Eur J Gastroenterol Hepatol*. 2010; 22, 24–32.
59. Torres DM, Williams CD, Harrison SA. Features, diagnosis, and treatment of nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol*. 2012; 10, 837–858.
60. Cichoż-Lach H, Michalak A. Oxidative stress as a crucial factor in liver diseases. *World J Gastroenterol*. 2014; 20, 8082.
61. Mulder P. The contribution of metabolic and adipose tissue inflammation to non-alcoholic fatty liver disease. 2017, Doctoral thesis.
62. Mulder P, Morrison M, Wielinga P, Van Duyvenvoorde W, Kooistra T, Kleemann R. Surgical removal of inflamed epididymal white adipose tissue attenuates the development of non-alcoholic steatohepatitis in obesity. *Int J Obes*. 2016; 40, 675–684.
63. Seki E, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. *Hepatology*. 2015; 61, 1066–1079.
64. Jaeschke H, Wang Y, Essani N. Reactive oxygen species activate the transcription factor NF-KB in the liver by induction of lipid peroxidation. In *Hepatology*. 1996; pp. 445–445. WB Saunders Co Independence Square West Curtis Center: Philadelphia, PA.
65. Lettéron P, Fromenty B, Benoît T, Degott C, Pessayre D. Acute and chronic hepatic steatosis lead to in vivo lipid peroxidation in mice. *J Hepatol*. 1996; 24, 200–208.
66. Reeves HL, Friedman SL. Activation of hepatic stellate cells—a key issue in liver fibrosis. *Front Biosci*. 2002; 7, 808–826.
67. Miller A, Adeli K. Dietary fructose and the metabolic syndrome. *Cur Opin Gastroenterol*. 2008; 24, 204–209.
68. Zarghani SS, Soraya H, Zarei L, Alizadeh M. Comparison of three different diet-induced non alcoholic fatty liver disease protocols in rats: a pilot study. *Energy (Kcal/g)*. 2016; 3, 3.18.
69. Calvaruso V, Craxì A. Implication of normal liver enzymes in liver disease. *J Viral Hepat*. 2009; 16, 529–536.
70. Pearce SG, Thosani NC, Pan J-J. Noninvasive biomarkers for the diagnosis of steatohepatitis and advanced fibrosis in NAFLD. *Biomark Res*. 2013; 1, 7.

https://doi.org/10.1017/S2040174418000259 Published online by Cambridge University Press