

## Food restriction by intermittent fasting induces diabetes and obesity and aggravates spontaneous atherosclerosis development in hypercholesterolaemic mice

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### Abstract

Different regimens of food restriction have been associated with protection against obesity, diabetes and CVD. In the present study, we hypothesised that food restriction would bring benefits to atherosclerosis- and diabetes-prone hypercholesterolaemic LDL-receptor knockout mice. For this purpose, 2-month-old mice were submitted to an intermittent fasting (IF) regimen (fasting every other day) over a 3-month period, which resulted in an overall 20% reduction in food intake. Contrary to our expectation, epididymal and carcass fat depots and adipocyte size were significantly enlarged by 15, 72 and 68%, respectively, in the IF mice compared with the *ad libitum*-fed mice. Accordingly, plasma levels of leptin were 50% higher in the IF mice than in the *ad libitum*-fed mice. In addition, the IF mice showed increased plasma levels of total cholesterol (37%), VLDL-cholesterol (195%) and LDL-cholesterol (50%). As expected, in wild-type mice, the IF regimen decreased plasma cholesterol levels and epididymal fat mass. Glucose homeostasis was also disturbed by the IF regimen in LDL-receptor knockout mice. Elevated levels of glycaemia (40%), insulinaemia (50%), glucose intolerance and insulin resistance were observed in the IF mice. Systemic inflammatory markers, TNF- $\alpha$  and C-reactive protein, were significantly increased and spontaneous atherosclerosis development were markedly increased (3-fold) in the IF mice. In conclusion, the IF regimen induced obesity and diabetes and worsened the development of spontaneous atherosclerosis in LDL-receptor knockout mice. Although being efficient in a wild-type background, this type of food restriction is not beneficial in the context of genetic hypercholesterolaemia.

**Key words:** LDL receptor: Energy restriction: Adiposity: Body composition: Insulin resistance: Glucose tolerance

Moderate reductions in food/energy ingestion (20–40%) have been widely associated with increases in lifespan and reductions in the risks of ageing-related chronic disease in animal models<sup>(1)</sup>. A reduction in energy intake promotes several metabolic responses related to substrate mobilisation mediated by hormonal alterations that are well characterised in energy restriction (ER), such as decreased body fat, inflammation and oxidative stress, and increased insulin sensitivity<sup>(1)</sup>. In human subjects, studies of ER have indicated improvements in surrogate markers of ageing, such as glycaemia, blood pressure and cholesterol concentrations<sup>(2,3)</sup>. It has been postulated that physiological changes elicited by ER contribute towards a condition of robust health, and that these same changes may trigger greater longevity in certain species<sup>(1)</sup>.

ER also modulates the cellular redox balance. Rats under ER have been reported to show diminished production of

mitochondrial reactive oxygen species (ROS) in several tissues<sup>(4–6)</sup>. The reduction in mitochondrial electrochemical membrane potential may be one mechanism responsible for the decreased production of ROS in rats under ER<sup>(7)</sup>, as this would lead to an acceleration of respiration rates. Another antioxidant mechanism of ER is the increase in mitochondrial biogenesis and consequent higher electron transport efficiency<sup>(8)</sup>.

A widely used ER protocol is obtained by fasting animals every other day, which is also called intermittent fasting (IF). Several studies have demonstrated that IF decreases the risk factors of CVD and diabetes in human subjects and rodents<sup>(9)</sup>, reduces the production of liver mitochondrial reactive oxygen in mice<sup>(10)</sup> and increases the lifespan of rodents<sup>(11)</sup>.

It has previously been demonstrated by our group that LDL-receptor knockout mice, a model of familial

**Abbreviations:** ER, energy restriction; IF, intermittent fasting; KO-AL, knockout mice fed *ad libitum*; KO-IF, knockout mice under intermittent fasting; ROS, reactive oxygen species; SREBP, sterol response element-binding proteins.

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hypercholesterolaemia, show tissue mitochondrial oxidative stress<sup>(12)</sup> and glucose intolerance<sup>(13)</sup>. Furthermore, these mice show hypertension<sup>(14)</sup>. Therefore, in the present study, we hypothesised that IF-induced ER would ameliorate metabolic disturbances in LDL-receptor knockout mice, and reduce susceptibility to atherosclerosis in this context.

## Experimental methods

### Animals

LDL-receptor knockout mice and control wild-type mice (C57BL6/J) founders were purchased from Jackson Laboratory. The animal experiments were approved by the University's Committee for Ethics in Animal Experimentation (protocol no. 1969-1; CEUA/UNICAMP). The animals had free access to a standard laboratory rodent chow diet (Nuvital CR1; Quintia, S.A.), and were housed at  $22 \pm 1^\circ\text{C}$  on a 12 h light–12 h dark cycle. The IF protocol used was an every-other-day fasting regimen in which male mice had free access to food only on alternate days over 12 weeks from 8 weeks of age.

### Plasma biochemical analysis

Blood samples were collected from either the retro-orbital plexus or the tail tip of anaesthetised mice. Unless specified, blood samples were obtained after 12 h of fasting. Total cholesterol, TAG and NEFA concentrations were measured in fresh plasma samples using standard commercial kits (Roche-Hitachi<sup>®</sup> and Wako<sup>®</sup>). Glucose levels were measured using a hand-held glucometer (Accu-Chek Advantage; Roche Diagnostic). Plasma insulin, leptin and adiponectin (EMD Millipore Corporation), TNF- $\alpha$  (eBioscience, Inc.) and C-reactive protein (Immuno-Biological Laboratories, Inc.) levels were measured by ELISA. Plasma total antioxidant capacity was determined by an enzymatic assay (Cayman Chemical Company). Plasma lipoprotein levels were determined by fast protein liquid chromatography (Amersham-Pharmacia Biotech)<sup>(15)</sup>.

### Body composition analysis

Mice as well as their food intake were weighed once per week. The epididymal adipose tissue and liver mass of mice were determined gravimetrically. Carcass composition of mice was determined as previously described in detail by Salerno *et al.*<sup>(16)</sup>. Liver lipids were extracted using the Folch method<sup>(17)</sup>. Liver cholesterol and TAG contents were determined using colorimetric enzymatic assays (Roche-Hitachi<sup>®</sup>) after dissolving the lipid extracts in a triton-containing buffer.

### Liver VLDL-TAG secretion

After 12 h of fasting, a base line blood sample was harvested from the tail tip of mice. Then, mice received an intraperitoneal injection of Triton WR 1339 (Sigma), 500 mg/kg in saline solution, to inhibit lipoprotein lipase activity and TAG hydrolysis and clearance. At 2 h after the injection of Triton, another blood sample was obtained to determine TAG

levels. VLDL-TAG secretion was calculated by the difference in TAG concentrations between values at 2 h and baseline. In order to measure the rate of VLDL-TAG secretion during the fed state, an oral glucose load (1.5 g/kg) was given to 12 h fasted mice 30 min before the administration of Triton.

### Adipocyte isolation

Adipocytes of mice were isolated using modifications of the established protocol for rat adipocytes<sup>(18)</sup>. Briefly, subcutaneous fat samples were cut into small pieces, and the fragments were digested at  $37^\circ\text{C}$  with collagenase II (1 mg/ml; Sigma-Aldrich) in Krebs–Ringer bicarbonate buffer containing fatty acid-free albumin (KRBA, 3%) and glucose (6 mM) at pH 7.4. After 45 min of incubation under continuous vigorous shaking, the fat cells were filtered through a nylon mesh and washed three times with KRBA to eliminate the stroma-vascular fraction and collagenase. The cells were counted in a Neubauer chamber. Adipocyte area ( $\mu\text{m}^2$ ) was analysed (at least 100 adipocytes per mice) with software ImageJ (1.45h; National Institutes of Health).

### Oral glucose tolerance and insulin tolerance tests

For the oral glucose tolerance test, mice were fasted for 12 h, and blood samples were harvested before and after an oral glucose load of 1.5 g/kg body weight. For the insulin tolerance test, blood samples of the fed mice harvested before and after an insulin injection (intraperitoneal) of 3.5 pmol/kg body weight (Biohulin<sup>®</sup>R; Biobrás)<sup>(13)</sup>.

### In vivo CO<sub>2</sub> production rates

Mice were adapted to the respirometer chamber for 5 d. After the adaptation period, CO<sub>2</sub> production of each mouse was measured in a temperature-monitored respirometer<sup>(19)</sup>. CO<sub>2</sub> expiration was quantified during a period of 5 min once per d, between 09.00 and 11.00 hours, for 3 d. The production rate of CO<sub>2</sub> of each mouse was calculated as the average of the 3 d measurements.

### VLDL oxidative susceptibility

Plasma VLDL fractions were obtained from 12 h fasting mice by ultracentrifugation ( $d < 1.006$ , 50 min, 140 000 rpm,  $16^\circ\text{C}$ , micro-ultracentrifuge Hitachi model CS150GXL; Hitachi Koki Co., Ltd), and used in the assay on the basis of TAG concentrations (100  $\mu\text{g}/\text{ml}$ ). CuSO<sub>4</sub>-induced oxidation (40  $\mu\text{M}$ ,  $37^\circ\text{C}$ ) was measured by detecting the formation of conjugated dienes at 234 nm over time (Spectrophotometer Fusion<sup>™</sup>; Packard BioScience Co.)<sup>(20)</sup>.

### Liver protein carbonyl content

Liver protein carbonyl content was estimated according to Reznick & Packer<sup>(21)</sup>, as modified by Schild *et al.*<sup>(22)</sup>. Liver homogenate samples were treated with 10 mM-dinitrophenylhydrazine in 2.5 M-HCl for 1 h at room temperature. The reaction

was stopped by the addition of 20% TCA. The pellets were washed twice with absolute ethanol–ethyl acetate (1:1) and once with 10% TCA. The protein pellets were dissolved in 6M-guanidine hydrochloride, and absorption at 370 nm was determined. Carbonyl content was calculated using the molar absorption coefficient of aliphatic hydrazones of 0.022/μm per cm.

*Isolation of mouse liver mitochondria*

Mitochondria were isolated by conventional differential centrifugation at 4°C<sup>(23)</sup>. Isolated mitochondria were kept over ice and used within 90 min of preparation.

*Reactive oxygen species production*

ROS production by mitochondria was monitored using the membrane-permeable fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate according to Garcia-Ruiz *et al.*<sup>(24)</sup>. The rate of H<sub>2</sub>O<sub>2</sub> produced by isolated mitochondria was determined by measuring the conversion of Amplex red (Molecular Probes, Invitrogen), in the presence of horseradish peroxidase, to highly fluorescent resorufin<sup>(25)</sup>.

*Histological analysis of atherosclerosis*

The heart of anaesthetised mice was perfused *in situ* with PBS followed by 10% PBS-buffered formaldehyde. The heart samples were then embedded in the Tissue-Tek<sup>®</sup> OCT compound (Sakura) and frozen at -50°C. Oil red O stainings were performed according to Paigen *et al.*<sup>(26)</sup>. The lipid-stained lesions were quantified, as described by Rubin *et al.*<sup>(27)</sup>, using software ImageJ (1.45h; National Institutes of Health) for image analysis. The slides were read without group identification (blind). The areas of lesions were expressed as the sum of lesions in six 10 μm sections, 80 μm apart, along a total aorta length of 480 μm. The segment chosen for analysis started at the aortic sinus.

*Statistical analysis*

Results are presented as means with their standard errors. Comparisons between the groups were analysed by unpaired Student's *t* test and correlation analyses by Spearman's *r* test. The level of significance was set at *P* ≤ 0.025.

**Results**

Body weight and food intake were measured weekly during a period of 3 months in hypercholesterolaemic LDL-receptor knockout mice under IF (KO-IF) or fed *ad libitum* (KO-AL). As expected, the KO-IF mice showed a reduction of 20% in cumulative food intake (263 (SEM 2.3) *v.* 326 (SEM 3.3)g, *P* < 0.0001). Weight gain was reduced by 20% (3.6 (SEM 0.4) *v.* 4.5 (SEM 0.3)g, *P* = 0.043), and food efficiency (weight gain/food intake × 100) (1.39 (SEM 0.15) *v.* 1.42 (SEM 0.08)) was similar between the groups.

Possible parallel changes in body metabolism were evaluated indirectly by measuring CO<sub>2</sub> production *in vivo*, since the IF regimen changes the feeding behaviour. The KO-IF mice showed marked variations in CO<sub>2</sub> production rates (g CO<sub>2</sub>/kg per h) during the fasting and fed days. Compared with the KO-AL mice (12.4 (SEM 0.23)), the KO-IF mice showed a 22% decrease (9.7 (SEM 0.25), *P* < 0.0001) and 10% increase (13.7 (SEM 0.45), *P* = 0.02) in CO<sub>2</sub> production rates during the fasting and fed days, respectively. The KO-IF mice exhibited changes in their body metabolic rates by 40% between the fasting- and fed-day measurements.

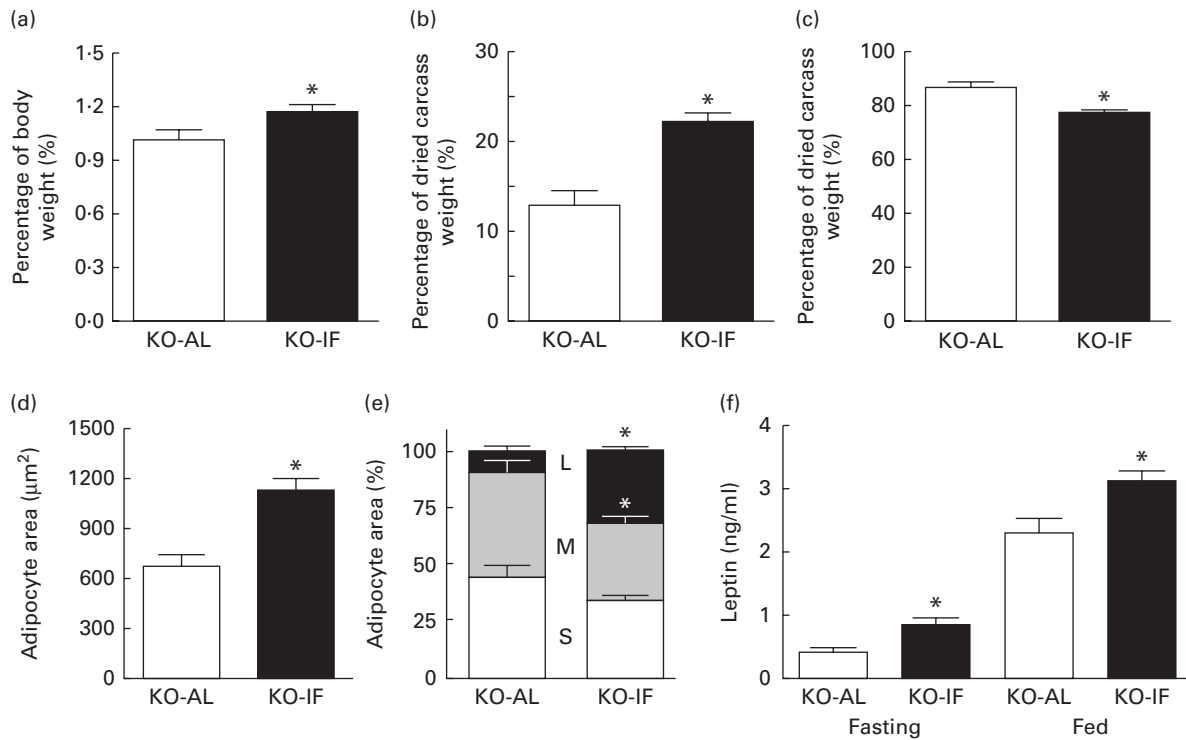
The IF regimen induced changes in plasma lipids and cholesterol distribution in plasma lipoproteins, which are shown in Table 1. The KO-IF mice showed a marked elevation of 37% in total plasma cholesterol, corresponding to increases of 195% in VLDL-cholesterol and 50% in LDL-cholesterol. The plasma levels of HDL-cholesterol, TAG and NEFA were similar between the KO-IF and KO-AL mice (Table 1). The rates of VLDL-TAG secretion in the fasted state were similar between the KO-AL and KO-IF groups (8.2 (SEM 1.5) *v.* 7.6 (SEM 0.6) mg/l per min, respectively). However, in the fed state (after an oral glucose dose), the rate of VLDL-TAG secretion was significantly increased by 32% (*P* = 0.04) in KO-IF mice compared with KO-AL mice (24.6 (SEM 2.4) *v.* 18.6 (SEM 1.6) mg/l per min, respectively). In agreement with this, the hepatic TAG content in KO-IF mice was 35% lower than that in KO-AL mice (15.8 (SEM 1.7) *v.* 21.3 (SEM 2.3) mg/g). The IF regimen did not change the liver mass (4.28 (SEM 0.19) *v.* 4.29 (SEM 0.13)% body weight) and the hepatic cholesterol content (2.07 (SEM 0.13) *v.* 2.18 (SEM 0.16) mg/g).

The IF effects on body composition and adiposity are shown in Fig. 1. Based on the body composition, the KO-IF mice showed an obesity phenotype with significant increases of 15% in the epididymal adipose tissue (Fig. 1(a)) and 72% in the carcass fat content (Fig. 1(b)), as well as a decrease of 11% in the body lean mass relative to the KO-AL mice (Fig. 1(c)). In addition, the mean size of subcutaneous adipocyte was

**Table 1.** Plasma lipids, glucose and insulin in LDL-receptor knockout mice under an intermittent fasting regimen (KO-IF) and fed *ad libitum* (KO-AL) over 3 months (Mean values with their standard errors)

	KO-AL			KO-IF		
	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>
Cholesterol (mmol/l)	5.66	0.20	15	7.76*	0.27	15
VLDL-cholesterol (mmol/l)	0.34	0.04	8	1.00*	0.13	10
LDL-cholesterol (mmol/l)	2.69	0.08	8	4.03*	0.09	10
HDL-cholesterol (mmol/l)	2.62	0.05	8	2.73	0.13	10
TAG (mmol/l)	1.35	0.10	16	1.47	0.09	15
NEFA (mmol/l)	0.62	0.05	17	0.62	0.05	14
Glucose (mmol/l)						
Fed	8.00	0.22	27	7.89	0.28	25
Fasting	4.78	0.15	16	6.78*	0.34	15
Insulin (ng/ml)						
Fed	1.12	0.14	7	1.78*	0.22	8
Fasting	0.36	0.01	7	0.54*	0.06	8
HOMA-IR index	14.2	1.7	7	27.1*	3.5	8

HOMA-IR, homeostatic model assessment for insulin resistance. \*Mean values were significantly different from those of the KO-AL group (*P* < 0.025; Student's *t*test).



**Fig. 1.** (a) Epididymal adipose tissue, (b) carcass fat, (c) lean mass, (d) subcutaneous adipocyte area and (e) adipocyte size distribution and (f) plasma leptin in LDL-receptor knockout mice under an intermittent fasting regimen (KO-IF) and fed *ad libitum* (KO-AL) over 3 months. L, large adipocyte (> 1400 µm<sup>2</sup>); M, medium adipocyte (465–1400 µm<sup>2</sup>); S, small adipocyte (< 465 µm<sup>2</sup>). Values are means of eleven to twenty-two mice per group, with their standard errors represented by vertical bars. \*Mean values were significantly different from those of the KO-AL group ( $P < 0.025$ ; Student's *t* test).

68% greater in KO-IF mice than in KO-AL mice (Fig. 1(d)). The adipocyte size distribution shows that IF caused a reduction of 25% in the number of medium-sized adipocytes and an increase of 260% in large-sized adipocytes (Fig. 1(e)). Furthermore, the fasting and fed plasma levels of leptin were, respectively, 50 and 35% higher in KO-IF mice than in KO-AL mice (Fig. 1(f)). The plasma levels of adiponectin were similar in KO-AL and KO-IF mice (7.68 (SEM 0.33) *v.* 8.45 (SEM 0.28) µg/ml, respectively).

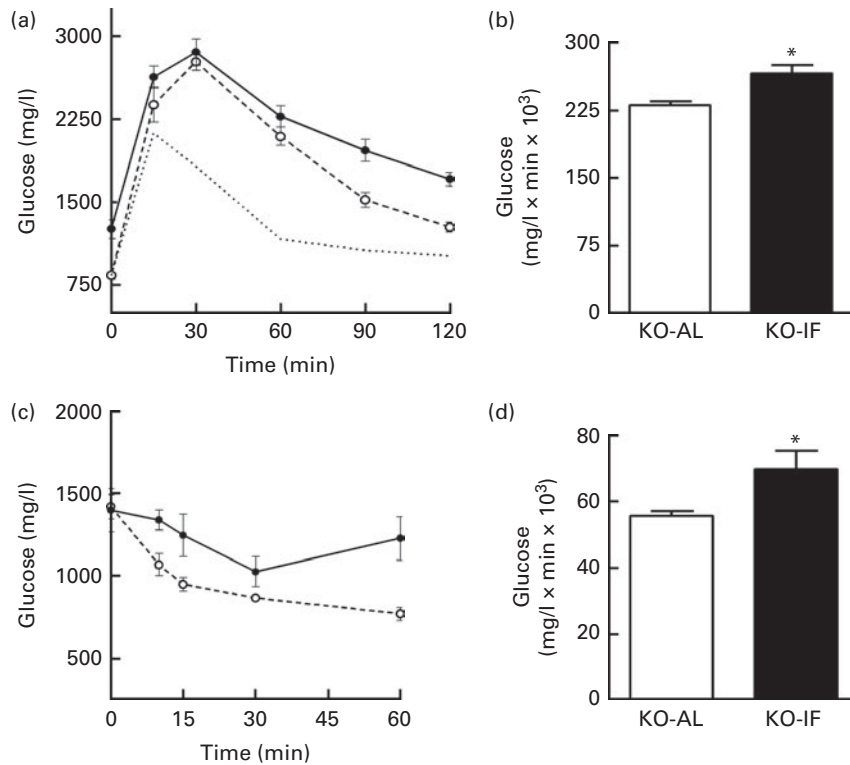
The increases in cholesterolaemia and adipose mass in response to IF were quite unexpected. Thus, the control wild-type mice (C57BL6/J) under the same feeding protocol were also studied. As expected, cholesterolaemia was reduced by 28% ( $n$  9,  $P < 0.0001$ ) and the epididymal fat pad was decreased by 24% ( $n$  9,  $P < 0.01$ ). Therefore, we attribute the IF responses of LDL-receptor knockout mice to their specific genetic defect.

The effects of IF on glucose homeostasis in LDL-receptor knockout mice are shown in Table 1. The baseline fasting glycaemia levels were 42% higher in KO-IF mice than in KO-AL mice, while in the fed state, the levels of glycaemia were similar in both groups. In addition, there were increases of 50 and 60% in the fasting and fed plasma levels of insulin, respectively, in KO-IF mice relative to the KO-AL mice. The homeostatic model assessment for insulin resistance index was approximately 2-fold higher in KO-IF mice than in KO-AL mice, indicating that the IF regimen induced insulin resistance (Table 1). Accordingly, after an oral glucose tolerance test, the KO-IF mice showed increased levels of glycaemia at 90 and

120 min post-glucose loading, and a significant increase of 15% in the area under the glycaemia curve (Fig. 2(a) and (b)). In addition, the KO-IF mice clearly showed an insulin resistance response after the insulin tolerance test, exhibiting an increase of 25% in the area under the glycaemia curve after the insulin injection (Fig. 2(c) and (d)). Therefore, the IF regimen worsened glucose homeostasis in these hypercholesterolaemic mice, causing peripheral insulin resistance.

Correlation analyses showed that the carcass fat content was positively correlated with fasting glycaemia ( $R$  0.65,  $P = 0.01$ ) and with the degree of glucose intolerance ( $R$  0.88,  $P < 0.0001$ ). In addition, the lean body mass was inversely correlated with fasting glucose ( $R$  -0.61,  $P = 0.02$ ) and with the degree of glucose intolerance ( $R$  -0.86,  $P = 0.0001$ ).

The effects of IF on the markers of oxidative stress are shown in Table 2. The plasma total antioxidant capacity of KO-IF mice was similar to that of KO-AL mice. However, the plasma VLDL of KO-IF mice took a longer time to be oxidised by CuSO<sub>4</sub> than that of KO-AL mice, indicating protection against oxidative insult. Compared with KO-AL mice, KO-IF mice showed the same amounts of liver protein oxidation, as indicated by the unaffected protein carbonyl content. However, the overall liver mitochondrial ROS production, indicated by the oxidation of the probe 2',7'-dichlorodihydrofluorescein, was reduced by 43% in KO-IF mice relative to KO-AL mice (Table 2). Thus, the IF regimen exerted beneficial effects on the systemic and tissue redox state in hypercholesterolaemic mice.



**Fig. 2.** (a, b) Glucose tolerance test (GTT) and (c, d) insulin tolerance test (ITT) in LDL-receptor knockout mice under an intermittent fasting regimen (KO-IF, ●) and fed *ad libitum* (KO-AL, ○) over 3 months. (a) 12 h fasted mice received an oral glucose load of 1.5 g/kg. The GTT was also performed in wild-type C57BL6/J mice (WT, .....), as a reference. (b) Fed mice received an intraperitoneal injection of regular insulin (3.5 pmol/kg). (b) AUC GTT curves and (d) AUC ITT curves. Values are means of nine to ten mice per group, with their standard errors represented by vertical bars. \* Mean values were significantly different from those of the KO-AL group ( $P < 0.025$ ; Student's *t* test).

The effects of the IF regimen on systemic inflammation markers and on the development of spontaneous atherosclerosis were evaluated. The IF regimen promoted a pro-inflammatory profile in hypercholesterolaemic mice, as shown by a 28% increase in plasma TNF- $\alpha$  levels (Fig. 3(a)), and a 23% increase in plasma C-reactive protein levels (Fig. 3(b)) in KO-IF mice compared with the KO-AL mice. Spontaneous atherosclerosis in the aortic root was markedly aggravated in KO-IF mice, which showed a 3-fold increase in the average size of the lesions (Fig. 3(c) and (d)). The atherosclerosis lesion size was positively correlated with plasma cholesterol

levels ( $R$  0.60,  $P=0.0009$ ), carcass fat content ( $R$  0.60,  $P=0.008$ ), plasma leptin levels ( $R$  0.56,  $P=0.01$ ) and plasma TNF- $\alpha$  levels ( $R$  0.85,  $P=0.004$ ). All of these correlations remained statistically significant after adjusting the analyses for plasma cholesterol concentrations.

## Discussion

In the present study, we hypothesised that LDL-receptor knockout mice could benefit from IF-induced ER. However, IF actually worsened the metabolic syndrome features of

**Table 2.** Oxidative stress markers in LDL-receptor knockout mice under an intermittent fasting regimen (KO-IF) and fed *ad libitum* (KO-AL) over 3 months  
(Mean values with their standard errors)

	KO-AL			KO-IF		
	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>
Plasma total antioxidant capacity (mmol/l)	1.02	0.04	8	1.08	0.06	8
Susceptibility of VLDL to oxidation† (lag time in min)	75	4.7	9	94*	6.5	11
Liver protein carbonyl content (nmol/mg)	8.0	1.0	8	7.4	1.7	7
Liver mitochondrial ROS production						
H <sub>2</sub> DCF oxidation‡	2.13	1.0	6	1.22*	1.0	6
H <sub>2</sub> O <sub>2</sub> production§	228	3.1	5	207	16.1	5

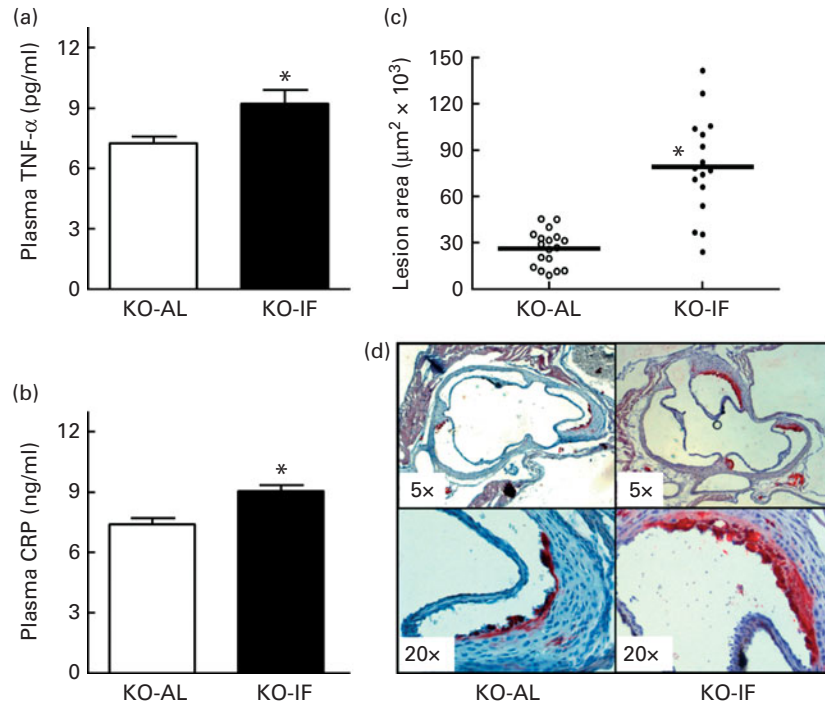
ROS, reactive oxygen species; H<sub>2</sub>DCF, 2',7'-dichlorodihydrofluorescein.

\* Mean values were significantly different from those of the KO-AL group ( $P < 0.025$ ; Student's *t* test).

† VLDL was oxidised at 37°C using 40  $\mu$ M-copper sulphate.

‡ nmol H<sub>2</sub>DCF/mg protein per min.

§ nmol H<sub>2</sub>O<sub>2</sub>/mg protein per min.



**Fig. 3.** (a) Plasma TNF- $\alpha$ , (b) C-reactive protein (CRP), (c) areas of aortic atherosclerotic lesions and (d) representative histological sections in LDL-receptor knockout mice under an intermittent fasting regimen (KO-IF) and fed *ad libitum* (KO-AL) over 3 months. Images with 5 $\times$  and 20 $\times$  magnification. Values are means of seven to eighteen mice per group, with their standard errors represented by vertical bars. \* Mean values were significantly different from those of the KO-AL group ( $P < 0.025$ ; Student's *t* test).

these models, precipitating the appearance of obesity and diabetes and markedly aggravating atherosclerosis. These results are genotype dependent since wild-type mice responded to IF with significant decreases in plasma cholesterol levels and body fat mass.

Varady *et al.*<sup>(28)</sup> showed cardioprotective effects on C57BL6 mice under alternate-day fasting, such as reductions in cholesterol and TAG plasma concentrations and reductions in aortic smooth muscle cell proliferation. In the present study, despite the reduction in cumulative food intake, the KO-IF mice showed a marked elevation in the plasma levels of total cholesterol and VLDL- and LDL-cholesterol, an increased rate of liver VLDL-TAG secretion and a concomitant reduction in the liver content of TAG. These data are consistent with the stimulated rates of liver TAG synthesis and secretion during the fed day. In accordance with the present results, Bruss *et al.*<sup>(29)</sup> demonstrated that 30% ER in C57BL6 mice stimulates the rates of VLDL secretion for the first 6 h after the commencement of feeding<sup>(29)</sup>. Increased VLDL-TAG secretion shown in KO-IF mice resulted in further elevation of its product LDL that accumulated in the vascular compartment due to the lack of LDL receptors. Besides increasing LDL (and hence hypercholesterolaemia), the increase in VLDL secretion has another consequence, that is, providing more substrates (NEFA) that accumulate and expand the adipose tissues. Thus, in addition to worsening hypercholesterolaemia, the IF regimen generated an obesity profile showing augmented visceral adipose mass, fat carcass content, adipocyte size and plasma leptin levels.

In addition to the increased availability of plasma TAG-derived NEFA to the adipose tissue, lipid synthesis rates in the adipose tissue may also be enhanced. Bruss *et al.*<sup>(29)</sup> showed that energy-restricted mice showed an increase in newly synthesised fatty acids stored in the epididymal and subcutaneous adipose tissues in the first hours after feeding<sup>(29)</sup>. Increased lipogenesis occurs in the tissues of LDL-receptor knockout mice<sup>(20)</sup> because this animal model has an already activated state of sterol response element-binding proteins (SREBP), transcription factors that up-regulate the expression of genes for both cholesterol and fatty acid synthesis<sup>(12,30,31)</sup>. Therefore, cycles of energy depletion during the fasting day and energy refuelling after the feeding day may be fully compensated in a healthy context; however, cholesterol-deprived tissues found in this LDL-receptor knockout model plus the alternate energy intake may overactivate SREBP, exacerbating lipogenesis in liver and adipose tissues. Lean and obese C57BL6 control mice submitted to 30% of ER per d respond with significant reductions in both fat mass (4%) and lean mass (8–10%)<sup>(32)</sup>. In another study using intermittent feeding, C57BL6 mice showed a reduction in visceral adipose tissue, but no overall change in body fat mass<sup>(28)</sup>. In the present study, control C57BL6 mice submitted to the IF regimen also lost fat mass. Conversely, LDL-receptor knockout mice under IF exhibited a differential substrate partitioning, leading to a reduction in lean mass but the preservation of adipose tissue mass. These findings may be explained by the genotype-dependent increased liver lipid synthesis and secretion that

are exacerbated during the fed state and stored in the adipose tissue.

It has previously been demonstrated that ER decreases plasma glucose and insulin concentrations<sup>(33,34)</sup> and improves glucose tolerance and insulin sensitivity<sup>(35)</sup>. Conversely, LDL-receptor knockout mice under IF became more intolerant to glucose and acquired insulin resistance, exhibiting a typical diabetic phenotype. It has consistently been shown that obesity and the insulin resistant state are deeply linked<sup>(36)</sup>. In the present study, the carcass fat content positively correlated with fasting glycaemia and with the degree of glucose intolerance, demonstrating the link between the disturbed glucose homeostasis and obesity in IF LDL-receptor knockout mice. In addition, it is possible that the reduced body lean mass contributes to lower glucose clearance, causing glucose intolerance and muscle insulin resistance. The higher plasma levels of insulin (approximately 50%) would contribute to the preservation of adipose tissue mass. In fact, lean body mass was negatively correlated with fasting glucose and with the degree of glucose intolerance.

Previous studies have demonstrated that ER in rodents minimises oxidative stress by reducing mitochondrial ROS production<sup>(4–6,10)</sup>. The present study shows that IF ameliorates at least two oxidative stress markers, the susceptibility of VLDL to oxidation and the rate of mitochondrial ROS generation. The higher resistance of VLDL to oxidation may indicate that this lipoprotein is secreted with fewer oxidised components, or that it may have acquired more antioxidants during the hepatic assembly process.

The levels of systemic markers of inflammation, TNF- $\alpha$  and PCR, were determined since inflammation is always present in obesity and atherosclerosis<sup>(37–39)</sup>, and ER has been shown to be anti-inflammatory<sup>(40,41)</sup>. However, these markers were instead increased in the plasma of KO-IF mice. The presence of hypercholesterolaemia, obesity, insulin resistance and systemic inflammation led to severe advancement of atherosclerosis in KO-IF mice. All of these disturbances have been shown to be directly involved in all phases of disease development<sup>(37,42,43)</sup>. Importantly, the atherosclerosis lesion size in KO-IF mice is positively correlated with carcass fat content, plasma leptin and TNF- $\alpha$  level, even after adjusting for plasma cholesterol levels, which reinforces the close association between inflammation, obesity and atherosclerosis.

The IF regimen in rodents typically leads to increased food consumption above the energy needs on the days when food is provided, in contrast to humans who only slightly consume in excess of energy needs on the 'feed' days of IF<sup>(44)</sup>. It is unclear from the present study as to what the respective contributions of fasting and hyperphagia were to the cardiometabolic derangements observed in the LDL-receptor knockout mice. If hyperphagia is important, then, in humans who do not exhibit it<sup>(44)</sup>, these disturbances may not occur. We believe that hyperphagia is particularly noxious in the case of LDL-receptor deficiency.

In conclusion, ER promoted by the IF regimen induced obesity and diabetes and worsened the development of spontaneous atherosclerosis in LDL-receptor knockout mice. Therefore, the reported benefits of food restriction are not

applicable in the context of genetic hypercholesterolaemia due to the defective LDL receptor.

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The authors' contributions are as follows: G. G. D. designed and conducted the research, analysed the data, wrote the paper and had primary responsibility for the final content; J. C. R., C. J. F. L., B. A. P. and H. F. R. conducted the research and analysed the data; A. E. V. analysed the data and reviewed the paper; H. C. F. O. was involved in the project conception, designed the research, analysed the data, wrote the paper and had primary responsibility for the final content. All authors read and approved the final manuscript.

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