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Original Article

Specific adipose tissue *Lbp* gene knockdown prevents diet-induced body weight gain, impacting fat accretion-related gene and protein expression

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Lipopolysaccharide binding protein (Lbp) has been recently identified as a relevant component of innate immunity response associated to adiposity. Here, we aimed to investigate the impact of adipose tissue Lbp on weight gain and white adipose tissue (WAT) in male and female mice fed an obesogenic diet. Specific adipose tissue *Lbp* gene knockdown was achieved through lentiviral particles containing shRNA-Lbp injected through surgery intervention. In males, WAT *Lbp* mRNA levels increased in parallel to fat accretion, and specific WAT *Lbp* gene knockdown led to reduced body weight gain, decreased fat accretion-related gene and protein expression, and increased inguinal WAT basal lipase activity, in parallel to lowered plasma free fatty acids, leptin, triglycerides but higher glycerol levels, resulting in slightly improved insulin action in the insulin tolerance test. In both males and females, inguinal WAT *Lbp* gene knockdown resulted in increased *Ucp1* and *Ppargc1a* mRNA and Ucp1 protein levels, confirming adipose Lbp as a WAT browning repressor. In perigonadal WAT, *Lbp* gene knockdown also resulted in increased *Ucp1* mRNA levels, but only in female mice, in which it was 500-fold increased. These data suggest specific adipose tissue *Lbp* gene knockdown as a possible therapeutic approach in the prevention of obesity-associated fat accretion.

INTRODUCTION

Obesity, a worldwide epidemic caused by disturbed energy balance (increased food energy intake and/or decreased energy expenditure) and characterized by adipose tissue enlargement and increased body fat accretion, is the most important factor in the progression of metabolic diseases, including type 2 diabetes, dyslipidemia, arterial hypertension, ischemic heart disease, non-alcoholic fatty liver disease, and some types of cancer, also contributing to the overall burden of disease worldwide.[1,2] Research in new therapeutic targets preventing weight and fat mass gain and attenuating obesity-associated fat accretion is mandatory to treat these metabolic diseases.

Lipopolysaccharide binding protein (LBP) has been recently identified as a relevant component of innate immunity response associated to obesity and insulin resistance.[3–9] Of note, previous observations in humans[10], and experiments in human and 3T3-L1 adipocytes[11,12] and in Lbp knockout (KO) mice[13] pointed to a possible role of Lbp in fat accretion, and suggested that this protein impacts negatively on adipose tissue physiology, attenuating browning/beiging but enhancing proinflammatory pathways. After liver, adipose tissue is the second source of plasma LBP levels.[10,11] Recently, we found that long-term high-fat diet feeding resulted in increased adipose tissue, but not liver, *Lbp* gene expression and protein levels in parallel to plasma LBP levels (J.M., unpublished data), reinforcing adipose tissue as an important source of increased plasma LBP concentration observed in obesity.[3–9] In fact, specific liver Lbp gene knockdown (KD) did not exert significant effects on body weight and fat mass accumulation.[14]

To gain insight in the possible role of adipose tissue in the relationship between LBP and obesity, here, we aimed to investigate the impact of specific adipose tissue *Lbp* gene KD using lentiviral particles on weight gain and white adipose tissue (WAT).

RESULTS

Short-term specific inguinal and perigonadal WAT *Lbp* gene knockdown impacts weight gain in male mice

Specific WAT *Lbp* gene KD was performed injecting lentiviral particles with shRNA scramble and shRNA against *Lbp* mRNA directly in inguinal (iWAT) and perigonadal (pgWAT) white adipose tissue through a surgical intervention as detailed in methods. The specificity

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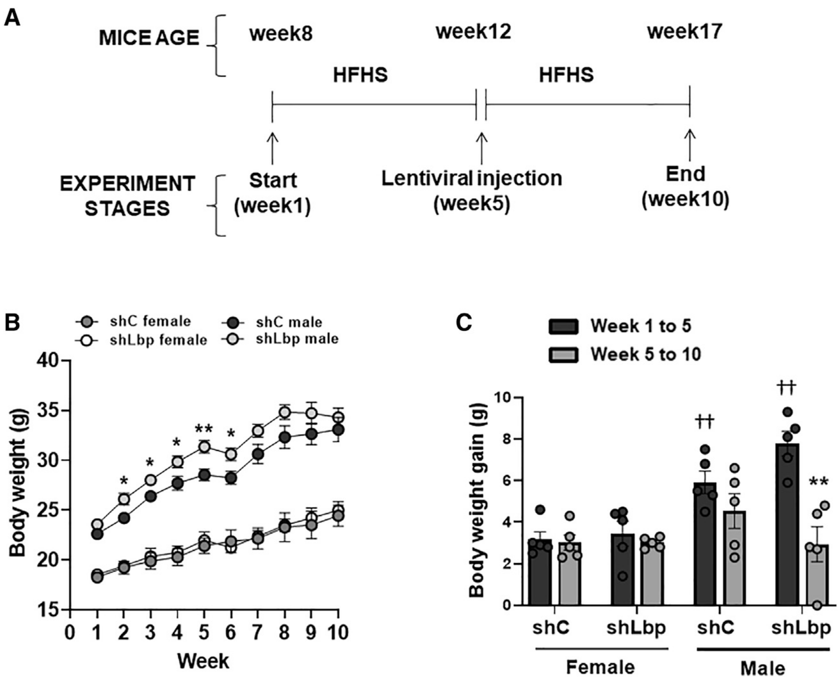

Figure 1. The impact of iWAT and pgWAT *Lbp* gene knockdown on body weight gain

(A) Representative diagram of specific iWAT and pgWAT *Lbp* gene knockdown experimental design. (B and C) Effects of specific iWAT and pgWAT *Lbp* gene knockdown on weekly body weight (B) and body weight gain (C). *$p < 0.05$, **$p < 0.01$ compared to shC (B) or week 1 to 5 (C); $^{††}p < 0.01$ compared to female mice; n = 5/group.

of shRNA against *Lbp* mRNA was confirmed *in vitro* in the murine Hepa1-6 cell line, demonstrating a significant decreased in *Lbp* mRNA levels (∼80%, $p < 0.0001$, data not shown).

In a pilot study, we found that with only one surgical intervention, an effective gene KD was observed until 5 weeks after surgical intervention, but weeks later the effect was lost (J.M., unpublished data). Thus, in this study this intervention was used to evaluate the short-term effects of WAT *Lbp* gene KD in adipose tissue physiology in 12-week-aged mice fed with a high-fat and high-sucrose (HFHS) diet (Figure 1A). Sexual dimorphism was observed in this experiment. Increased body weight gain was observed in male compared with female mice (Figures 1B and 1C), indicating increased fat accretion in males in this period. Similar to body weight and fat accretion (*Fabp4, Plin1, Slc2a4, Lipe, Mgll*)-related gene expression, *Lbp* gene expression was increased in male compared with female mice in both iWAT (Figure 2) and pgWAT (Figure 3). Specific iWAT *Lbp* gene KD (65% in males and 37% in females, $p < 0.0001$; Figure 2A) and pgWAT *Lbp* gene KD (70% in males and 55% in females, $p < 0.0001$; Figure 3A) resulted in a significant decreased body weight gain in male, but not in female mice (Figure 1C).

Short-term specific iWAT and pgWAT *Lbp* gene knockdown attenuates expression of fat accretion-related genes

Specific iWAT *Lbp* gene KD also resulted in decreased fat accretion-related gene expression (including *Fasn, Fabp4, Slc2a4*, and *Mgll*) in male mice, whereas no significant changes in the expression of these genes were observed in female mice (Figures 2A and 2B). Similar to iWAT, specific pgWAT *Lbp* gene KD (Figure 3A) resulted in decreased *Fasn, Acsl1, Plin1, Slc2a4, Lipe*, and *Mgll* gene expression in male mice (Figure 3B). Otherwise, this intervention increased pgWAT *Acsl1, Fabp4*, and *Slc2a4* in females (Figure 3B).

Short-term specific iWAT and pgWAT *Lbp* gene knockdown enhances thermogenic-related gene expression

Of interest, iWAT *Lbp* gene KD increased *Ucp1* and *Ppargc1a* mRNA in this fat depot in both male and female mice (Figure 2C). In pgWAT, *Lbp* gene KD also resulted in increased *Ucp1* and *Ppargc1a* mRNA levels, but only in female mice (Figure 3C). Interestingly, *Ucp1* mRNA levels were 500-fold increased in female compared with male pgWAT ($p < 0.0001$; Figure 3C), showing a sexual dimorphism in pgWAT *Ucp1* gene expression in young mice, which could explain the resistance to fat accretion and body weight gain in female mice (Figure 1C).

To strengthen these findings, we investigated whether gene expression changes observed in males after WAT *Lbp* gene KD were replicated at the protein level. Of note, WAT *Lbp* gene KD resulted in decreased WAT LBP protein (38% in iWAT and 51% in pgWAT, $p < 0.0001$) in parallel to decreased FAS and GLUT4 in both iWAT and pgWAT, and increased UCP1 protein levels only in iWAT (Figure 4).

Short-term specific iWAT and pgWAT *Lbp* gene knockdown did not impact inflammatory markers

This intervention did not have any effects on adipose tissue inflammatory markers, except for a significant reduction in pgWAT *Itgax* mRNA in females (Figures 2D and 3D).

Short-term specific WAT *Lbp* gene knockdown impacts iWAT basal lipase activity in male mice

In males, similar to expression of thermogenic genes, iWAT *Lbp* gene KD led to increased basal lipase activity in this fat depot (Figure 5A), without any effects in pgWAT (Figure 5B). Interestingly, basal lipase activity in both fat depots was significantly increased in females compared with males, and no significant differences in WAT basal lipase activity between control (shC) and WAT *Lbp* gene KD (shLbp) groups were found in females (Figures 5A and 5B).

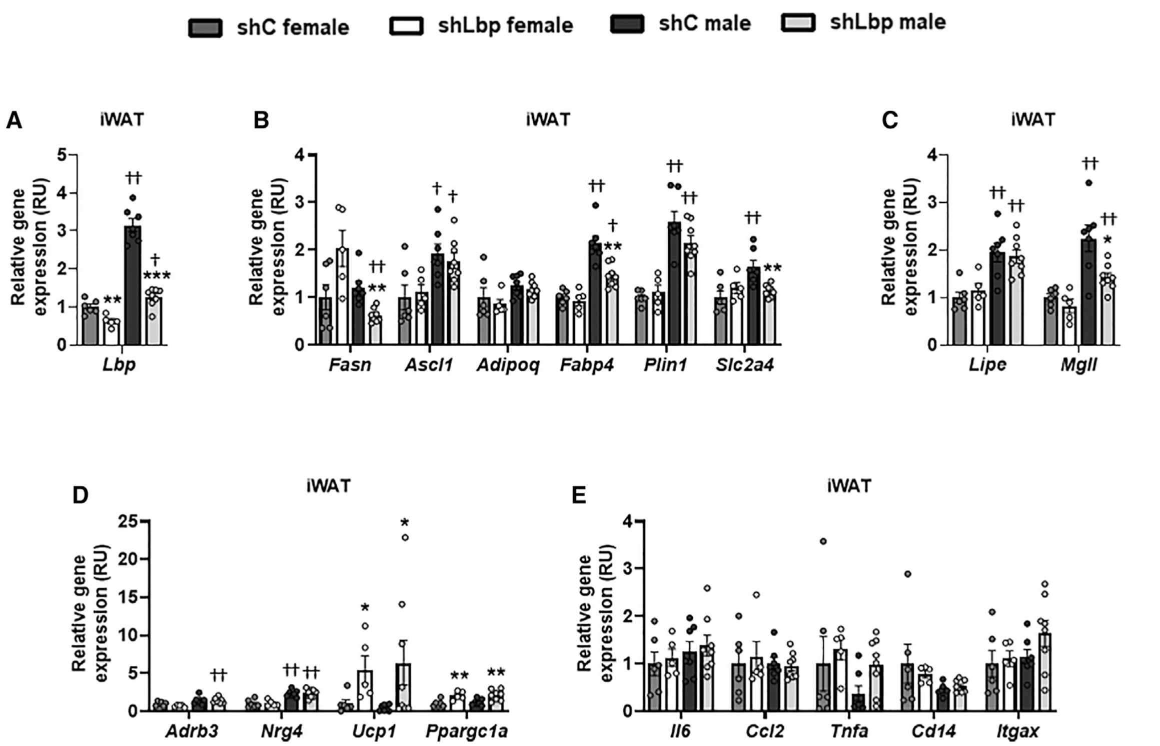

Figure 2. The impact of iWAT *Lbp* gene knockdown on expression of metabolic-related genes in this fat depot

(A) Effects of specific iWAT *Lbp* gene knockdown (A) on iWAT adipogenic (*Fasn, Ascl1, Adipoq, Fabp4, Plin1*, and *Slc2a4*) (B), lipolytic (*Lipe, Mgll*) (C), thermogenic (*Adrb3, Nrg4, Ucp1, Ppargc1a*) (D), and inflammatory (*Il6, Ccl2, Tnf, Cd14, Itgax*) (E) gene expression in HFHS-fed female and male mice. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to shC; †p < 0.05 and ††p < 0.01 compared to female mice; n = 5/group, five to eight tissue pieces were analyzed per experimental group.

Short-term specific WAT *Lbp* gene knockdown impacts plasma free fatty acids, glycerol, leptin, triglycerides, and insulin tolerance in male mice

After assessing the impact of short-term specific WAT *Lbp* gene KD on adipose tissue, the impact of this intervention on circulating parameters (including plasma LBP) and glucose and insulin tolerance was examined (Figures 5C–5E and 6). In males, short-term specific WAT *Lbp* gene KD resulted in decreased plasma free fatty acids (FFA), leptin, and triglycerides and increased glycerol levels, without significant differences on plasma adiponectin, LBP, insulin, 12-h and 4-h fasting glucose, or blood glucose during glucose tolerance test (GTT) (Figures 5C–5E and 6A–6H). In addition, this intervention slightly decreased blood glucose at 30 min during insulin tolerance test (ITT) and tended to attenuate glycemia (area under the curve, AUC) during ITT in male mice (Figure 6I). Otherwise, no significant impact of short-term specific WAT *Lbp* gene KD in these metabolic parameters was found in females (Figures 5C–5E and 6). Similar to body weight gain (Figure 1C), the increase in plasma FFA, leptin, triglycerides, 12-h fasting glucose, AUC glycemia during GTT and ITT and the decrease in glycerol observed in males compared with females was blunted in mice from specific WAT *Lbp* gene KD group (Figures 5C–5E and 6).

Next, to evaluate if the decrease in WAT *Lbp* mRNA levels was in proportion to body weight gain and metabolic parameters, non-parametric bivariate correlations (Spearman coefficient) were analyzed. Of note, both iWAT and pgWAT *Lbp* mRNA were positively correlated to body weight gain, FFA/glycerol ratio, plasma leptin, 4-h fasting glucose and AUC glycemia during ITT and negatively with plasma glycerol (only with iWAT *Lbp* mRNA) in male mice (Table 1). In females, pgWAT, but not iWAT, *Lbp* mRNA was positively correlated with FFA/glycerol ratio, plasma leptin, and AUC glycemia during ITT and negatively with plasma glycerol (Table 1). No significant correlations were identified between WAT *Lbp* mRNA and the other circulating metabolic parameters (FFA, triglycerides, adiponectin, insulin, or blood glucose during GTT) (Table 1).

Short-term specific iWAT and pgWAT *Lbp* gene knockdown did not impact liver metabolism-related gene expression and liver triglycerides

Since plasma LBP concentration was not affected by WAT *Lbp* gene KD (Figure 5D) and the liver is the largest source of circulating LBP,[11] the off-target effects of current intervention in liver and the association between liver *Lbp* mRNA and plasma LBP were then examined. Specific iWAT and pgWAT *Lbp* gene KD slightly decreased liver *Lbp* mRNA in females, without significant effects in males (Figure 7A). For both males and females, no significant effects of this intervention

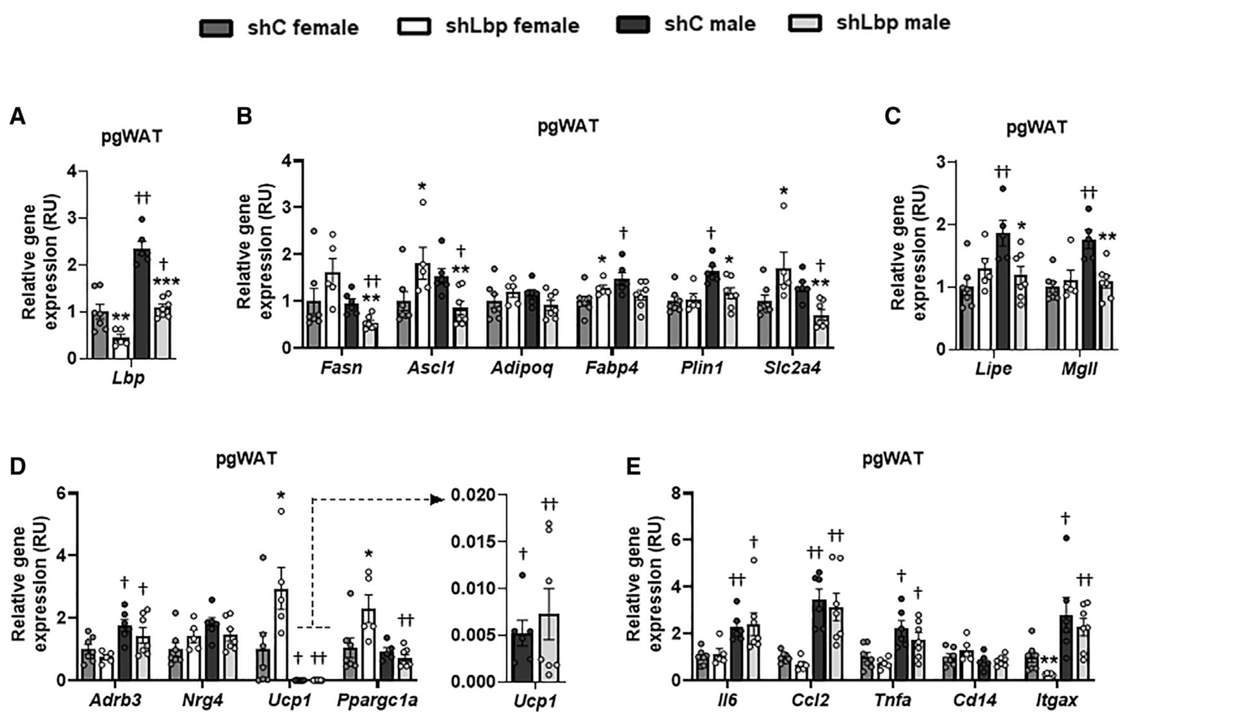

Figure 3. The impact of pgWAT *Lbp* gene knockdown on expression of metabolic-related genes in this fat depot

(A) Effects of specific pgWAT *Lbp* gene knockdown (A) on pgWAT adipogenic (*Fasn, Ascl1, Adipoq, Fabp4, Plin1*, and *Slc2a4*) (B), lipolytic (*Lipe, Mgll*) (C), thermogenic (*Adrb3, Nrg4, Ucp1, Ppargc1a*) (D), and inflammatory (*Il6, Ccl2, Tnf, Cd14, Itgax*) (E) gene expression in HFHS-fed female and male mice. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to shC; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ compared to female mice; n = 5/group, five to seven tissue pieces were analyzed per experimental group.

on expression of genes related to lipid metabolism (*Fasn, Acaca, Srebf1,* and *Scd1*), mitochondrial (*Ppargc1a*), fibrosis (*Tgfb1, Col4a1*), inflammation (*Itgax, Ccl2, Tnf,* and *Il6*) or oxidative stress (*Gpx4, Sod2*, except for *Gsta3* in females) (Figures 7B–7F) were found. However, the slightly decreased liver *Lbp* mRNA observed in females after lentiviral intervention in WAT was associated (Figure 7G) and significantly correlated with liver triglyceride accumulation (Figure 7H).

When the relationship between tissue *Lbp* mRNA and plasma LBP was further explored, we found that liver *Lbp* mRNA was positively correlated with plasma LBP in both males and females (Table 2), whereas iWAT *Lbp* mRNA was only positively correlated with circulating LBP levels in females, and pgWAT *Lbp* mRNA was not correlated (Table 2).

DISCUSSION

To the best of our knowledge this is the first study investigating the specific impact of WAT *Lbp* gene KD on high-fat diet-induced weight gain. Increased iWAT and pgWAT *Lbp* gene expression was observed in male young mice in association with increased body weight and expression of fat accretion- and lipolysis (*Lipe, Mgll*)-related genes, but decreased basal lipase activity in adipose tissue. Importantly, in these mice, specific iWAT and pgWAT *Lbp* gene KD resulted in decreased fat accretion- and lipolysis-related gene expression, but increased iWAT lipase activity in parallel to decreased body weight gain, plasma leptin, FFA and triglycerides, increased glycerol levels, and slightly improved blood glucose levels during ITT. As reported in previous studies,[15–17] basal lipolysis (inferred from WAT lipase activity) was inversely associated with expression of lipolysis-related genes, supporting that the interaction among lipases and other co-factors in the lipid droplet membrane is more important than gene expression in the regulation of lipolysis.[15,17] In addition, the association between fat accretion- and lipolysis-related gene expression was also previously observed.[15]

Since plasma leptin levels are proportional to fat mass,[18] these findings indirectly supported the impact of WAT *Lbp* gene KD on fat mass, serum lipid parameters, and insulin action, indicating that adipose tissue Lbp might exert a possible role in obesity-associated fat accretion and metabolic disturbances, as suggested in previous observational studies in humans and mice.[6,10,13] Even though current findings on body weight gain and circulating leptin levels suggest that adipose tissue *Lbp* gene KD might prevent diet-induced obesity, further studies are warranted.

Current data suggest that anti-obesity effects observed in LBP KO mice[13] might be caused by specific WAT Lbp gene depletion-induced browning. Confirming WAT LBP as a WAT browning repressor,[13] specific iWAT and pgWAT *Lbp* gene KD resulted in

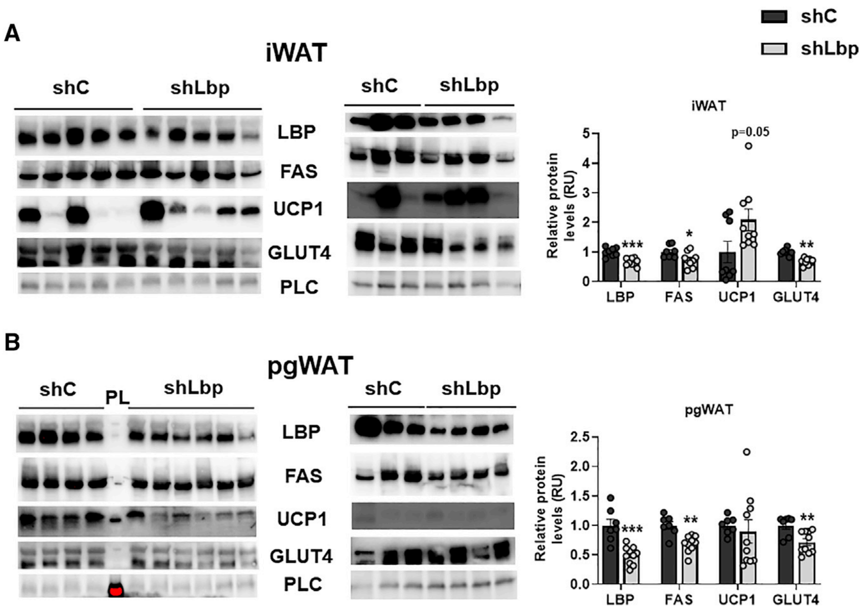

Figure 4. The impact of WAT *Lbp* gene knockdown on adipose LBP, FAS, UCP1, and GLUT4 protein levels

(A and B) Effects of specific iWAT (A) and pgWAT (B) *Lbp* gene knockdown on adipose tissue LBP, FAS, UCP1, and GLUT4 protein levels in male mice. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to shC. PLC: protein loading control. PL: protein ladder lane. The panels to the left and right of the protein annotations represents the two blots used for protein analysis; n = 5/group, seven to ten tissue pieces were analyzed per experimental group.

enhanced iWAT thermogenic (*Ucp1* and *Ppargc1a*) gene expression in male and female, and pgWAT *Ucp1* and *Ppargc1a* mRNA only in female mice. In males, this intervention also increased UCP1 protein levels in iWAT, but not in pgWAT. Interestingly, increased *Ucp1* mRNA levels in pgWAT were found in female compared with male mice. This sexual dimorphism could explain the resistance to fat accretion and body weight gain in female mice. In agreement with these findings, female sex hormones can specifically enhance thermogenic response in gonadal, but not inguinal, WAT in response to browning stimuli.[19] In fact, experiments in adipocytes have demonstrated that estrogen suppressed alpha 2 adrenergic receptor in parallel to increased beta 3 adrenergic receptor availability, sensitizing adipocytes to sympathetic signaling and promoting browning in these cells.[20] Supporting the impact of *Lbp* gene KD on pgWAT thermogenesis in females, increased gene expression markers of mitochondrial biogenesis (*Ppargc1a*), fatty acid degradation (*Ascl1*), and fatty acid (*Fabp4*) and glucose (*Slc2a4*) uptake were found after this intervention only in this group.

The high degree of inter-individual variation observed in *Ucp1* gene expression and UCP1 protein levels might reflect the heterogeneous dispersion of beige adipocytes within WAT.[21,22]

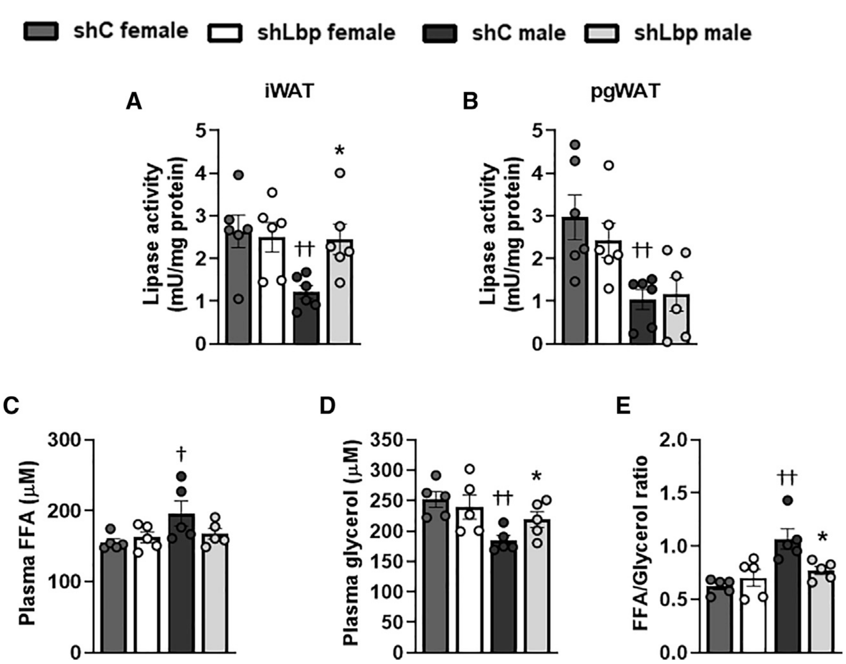

Figure 5. The impact of WAT *Lbp* gene knockdown on markers of WAT lipolysis

(A–E) Effects of specific iWAT and pgWAT *Lbp* gene knockdown on iWAT lipase activity (A), pgWAT lipase activity (B), plasma FFA (C), glycerol (D), and FFA/glycerol ratio (E). *p < 0.05 compared to shC; †p < 0.05 and ††p < 0.01 compared to female mice; n = 5/group, six tissue pieces were analyzed per experimental group.

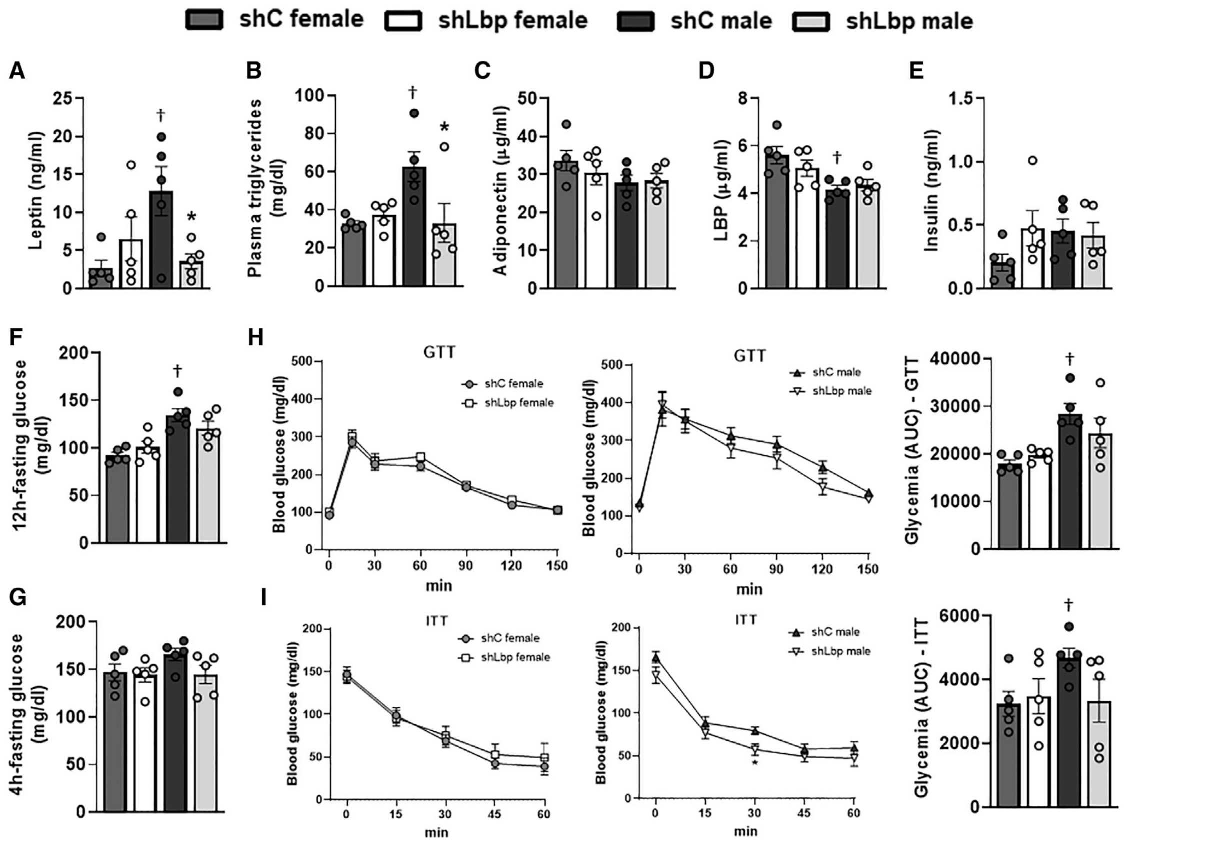

Figure 6. The impact of WAT *Lbp* gene knockdown on circulating metabolic parameters

(A–I) Effects of specific iWAT and pgWAT *Lbp* gene knockdown on plasma leptin (A), triglycerides (B), adiponectin (C), LBP (D), insulin (E), 12-h fasting glucose (F), 4-h fasting glucose (G), and blood glucose and glycemia (AUC) during GTT (H) and ITT (I). *p < 0.05 compared to shC; †p < 0.05 compared to female mice; n = 5/group.

It is important to note that thermogenic gene expression (*Ucp1* and *Pgc1a*) and UCP1 protein levels run in parallel to lipase activity in males. In fact, females had increased lipase activity in both iWAT and pgWAT compared with males, and *Lbp* gene KD in iWAT, but not in pgWAT, led to increased lipase activity in males. Interestingly, circulating plasma glycerol was also increased in males after downregulation of Lbp in WAT compared to control males, whereas plasma FFAs and FFA/glycerol ratio were reduced. This supports that lipid catabolism and beta oxidation were increased in iWAT from shLbp male mice.

Compared with males, females displayed increased plasma glycerol but reduced FFAs, also indicating increased lipid catabolism and beta oxidation in WAT. Similar to body weight, other metabolic parameters (such as plasma triglycerides and glycemia), or fat accretion-related gene expression, no significant differences on plasma glycerol and FFAs were found in females when comparing shC and shLbp. These data also suggest that, even though expression of thermogenic (*Ucp1* and *Ppargco1a*) genes were increased after WAT *Lbp* gene KD, the high basal lipid catabolism and the decreased body weight gain and leptin levels observed in control females compared with control males might mask the impact of WAT *Lbp* gene KD on weight gain and adipose tissue in females. However, this suggestion should be confirmed by the measurement of energy expenditure in further experiments. The absence of energy expenditure data is a limitation of the current study. Otherwise, WAT thermogenesis alone might not impact on high-fat diet-induced weight gain and fat accretion.[23]

Off-target effect of current intervention on liver *Lbp* mRNA levels was also examined. In males, WAT *Lbp* gene KD using lentiviral injections did not impact liver *Lbp* mRNA levels, whereas in females, this intervention resulted in a small but significant reduction in liver *Lbp* mRNA. The reduced fat depot content in female compared with male mice might explain this small off-target effect observed in females. Comparing WAT *Lbp* gene KD versus control group, no significant differences in plasma LBP were found. The strong correlation between plasma LBP and liver *Lbp* mRNA observed in the current study, which confirmed liver as the largest source of circulating LBP,[11] suggests that the off-target effect observed in females was minor and without any impact on plasma LBP levels.

For both male and female mice, specific iWAT and pgWAT *Lbp* gene KD did not impact liver lipid metabolism-, mitochondrial-, fibrosis-, inflammatory- nor oxidative stress-related gene expression. However,

Table 1. Bivariate correlations between iWAT and pgWAT *Lbp* mRNA levels and metabolic parameters in male and female mice

	iWAT *Lbp* mRNA		pgWAT *Lbp* mRNA	
Males	r	p	r	p
Body weight gain (g)[a]	0.84	**0.004**	0.62	0.06
Plasma FFA (μM)	0.31	0.4	0.61	0.06
Plasma glycerol (μM)	−0.82	**0.005**	−0.41	0.2
FFA/glycerol ratio	0.83	**0.004**	0.72	**0.02**
Plasma triglycerides (mg/dL)	0.57	0.08	0.16	0.6
Leptin (ng/mL)	0.67	**0.04**	0.85	**0.002**
Adiponectin (μg/mL)	0.23	0.5	0.32	0.3
Insulin (ng/mL)	0.05	0.8	−0.22	0.5
12-h fasting glucose (mg/dL)	0.39	0.2	0.2	0.6
4-h fasting glucose (mg/dL)	0.73	**0.02**	0.71	**0.02**
Glycemia (AUC), ITT	0.76	**0.01**	0.69	**0.03**
Glycemia (AUC), GTT	0.41	0.2	0.13	0.7
	iWAT *Lbp* mRNA		**pgWAT *Lbp* mRNA**	
Females	**r**	**p**	**r**	**p**
Body weight gain (g)[a]	0.27	0.4	0.28	0.4
Plasma FFA (μM)	0.32	0.4	0.31	0.4
Plasma glycerol (μM)	−0.61	0.06	−0.66	**0.04**
FFA/glycerol ratio	0.51	0.1	0.64	**0.04**
Plasma triglycerides (mg/dL)	−0.53	0.1	−0.13	0.7
Leptin (ng/mL)	0.57	0.08	0.82	**0.005**
Adiponectin (μg/mL)	−0.04	0.9	−0.23	0.5
Insulin (ng/mL)	−0.04	0.9	0.28	0.4
12-h fasting glucose (mg/dL)	0.28	0.4	0.58	0.07
4-h fasting glucose (mg/dL)	0.31	0.4	0.21	0.5
Glycemia (AUC), ITT	0.06	0.8	0.73	**0.02**
Glycemia (AUC), GTT	−0.46	0.2	0.27	0.4

[a]After lentiviral injection, from weeks 5 to 10.

the reduction in liver *Lbp* mRNA observed in females was correlated with liver triglyceride content. Even though specific liver *Lbp* gene KD did not exert significant effects on liver steatosis in mice fed with a chow diet,[14] current findings suggest a relationship between liver Lbp and triglyceride content in mice fed with a HFHS diet. Further studies focused on liver Lbp are required to investigate this relationship in depth.

While the current experimental approach allows for post-developmental short-term specific adipose tissue gene KD interventions, the lack of longer effects is a limitation of this study. For post-developmental long-term studies, the generation of a floxed-Lbp mouse model recombined with Adipoq-Cre needs to be further explored.

In conclusion, this study provides more evidence about the relevance of adipose tissue LBP in obesity, suggesting specific adipose tissue *Lbp* gene KD as a possible therapeutic approach in the prevention of obesity-associated fat accretion.

MATERIALS AND METHODS

Lentiviral shRNA-Lbp particles production

Four different short-hairpin-Lbp (clone set against mouse Lbp, NM_008,489.2) primer sequences and random negative control sequence that did not have targets for any gene were synthesized by Tebu-bio (Tebu-bio, Spain, SL). Lentivirus-targeted Lbp were obtained by cotransfection of shRNA plasmids against Lbp and a combination of packaging and envelope plasmid from Addgene (pCMV-VSV-G and pCMV-dR8.2 dvpr) into HEK293T using LipoD293 transfection reagent following manufacturers' instructions. Obtained lentiviruses were used to perform *in vivo* experiments, which effectiveness was previously confirmed in Hepa1-6 cells.

Mice experiments

Eight-week-old male and female C57BL/6J mice (n = 20) were fed a HFHS diet (TD.08811, 4.7 Kcal/g, ENVIGO) with water *ad libitum* for 4 weeks. Then, lentiviral injection in iWAT and gWAT was performed at week 12. To inject lentiviral particles in both iWAT and gWAT, mice were anesthetized by isoflurane before dissection of the skin and body wall. The lentiviral preparation ($1 \times 10^{7-8}$ plaque-forming units in a volume of 100 μL) was injected into the right and left gWAT and iWAT depot, which was distributed first in six injections of 10 μL for each gWAT, and then four injections of 8 μL for each iWAT. Each mouse was injected with ~185 μL of lentiviral preparation. Mice were randomly allocated to the treatment groups (shC group versus shLbp group, n = 10 mice/group) and fed with a HFHS diet. Insulin (ITT) and glucose tolerance tests (GTT) were performed at week 16. For GTT, glucose in aqueous solution was administered intraperitoneally (2.5 g glucose/kg) to overnight (12 h)-starved mice, and glycemia in blood obtained from the tail was measured 15, 30, 60, 90, 120, and 150 min after glucose injection. For ITT, insulin (Actrapid; Novo Nordisk Pharma A/S, Bagsvaerd, Denmark) in saline solution was administered intraperitoneally (0.75 UI/kg) to 4-h-starved mice, and glycemia in blood obtained from the tail was measured 15, 30, 45, 60, and 90 min after glucose injection. At week 17, mice were sacrificed by CO_2 inhalation. Then, blood serum and plasma were collected, and gWAT, iWAT and liver were rapidly dissected out, frozen in liquid nitrogen, and stored at −80°C until RNA or protein extraction and biochemical analysis.

In mice experiments, the research was conducted in accordance with the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU) and was approved by the Ethical Committee for Animal Experimentation of Barcelona Science Park (PCB).

Gene expression

WAT and liver RNA purification (isolation) was performed using the RNeasy Lipid Tissue Mini Kit (QIAgen, Izasa SA, Barcelona, Spain), and the integrity was checked by the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Gene expression was assessed by real-time PCR using a LightCycler 480 Real-Time PCR System (Roche

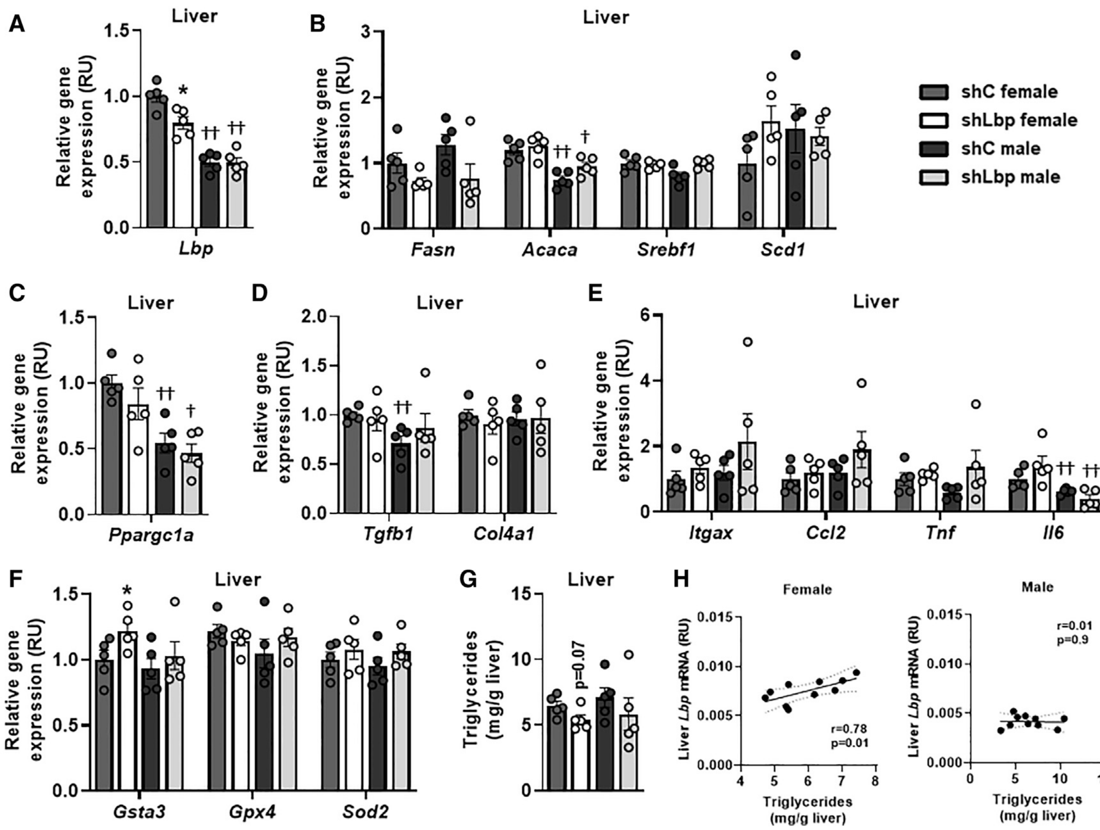

Figure 7. The impact of WAT *Lbp* gene knockdown on liver

(A–G) Effects of specific iWAT and pgWAT *Lbp* gene knockdown on liver *Lbp* (A), lipogenic (*Fasn, Acaca, Srebf1, Scd1*)-related (B), *Ppargc1a* (C), fibrosis (*Tgfb1, Col4a1*)-related (D), inflammatory (*Itgax, Ccl2, Tnf, Il6*)-related (E), and oxidative stress (*Gsta3, Gpx4, Sod2*)-related (F) gene expression and liver triglycerides (G). (H) Bivariate correlations between liver *Lbp* mRNA and liver triglycerides in female and male mice. *p < 0.05 compared to shC; †p < 0.05 and ††p < 0.01 compared to female mice; n = 5/group.

Diagnostics SL, Barcelona, Spain), using TaqMan technology suitable for relative genetic expression quantification. The commercially available and pre-validated TaqMan primer/probe sets used were as follows: Endogenous control *18S*, and target gene mouse LBP (*Lbp,* Mm00493139_m1); fatty acid synthase (*Fasn*, Mm00662319_m1); stearoyl-Coenzyme A desaturase 1 (*Scd1*, Mm00772290_m1); acetyl-Coenzyme A carboxylase alpha (*Acaca*, Mm01304257_m1); sterol regulatory element binding transcription factor 1 (*Srebf1*, Mm00550338_m1); perilipin 1 (*Plin1*, Mm00558672_m1); adiponectin (*Adipoq,* Mm00456425_m1); fatty acid binding protein 4, adipocyte, (*Fabp4,* Mm00445880_m1); solute carrier family 2 (facilitated glucose transporter), member 4 (*Slc2a4* or *Glut4*, Mm01245502_m1); lipase, hormone sensitive (*Lipe*, Mm00495359_m1); monoglyceride lipase (*Mgll*, Mm00449274_m1); acyl-CoA synthetase long-chain family member 1 (*Acsl1*, Mm00484217_m1); peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (*Ppargc1a*, Mm01208835_m1); uncoupling protein 1 (mitochondrial, proton carrier) (*Ucp1*, Mm01244861_m1); neuregulin 4 (*Nrg4*, Mm00446254_m1); adrenergic receptor, beta 3 (*Adrb3*, Mm02601819_g1); interleukin 6, (*Il6*, Mm00446190_m1); tumor necrosis factor (*Tnf*, Mm00443258_m1); chemokine (C-C motif) ligand 2 (*Ccl2*, Mm00441242_m1); CD14 antigen (*Cd14*, Mm01158466_g1); integrin alpha X (*Itgax*, Mm00498701_m1); collagen, type IV, alpha 1 (*Col4a1*, Mm01210125_m1); transforming growth factor, beta 1 (*Tgfb1*, Mm01178820_m1); glutathione S-transferase, alpha 3 (*Gsta3*, Mm00494798_m1); glutathione peroxidase 4 (*Gpx4*, Mm00515041_m1); and superoxide dismutase 2, mitochondrial (*Sod2*, Mm01313000_m1).

Protein analysis

Adipose tissue proteins were extracted directly in radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mmol/L NaCl, and 50 mmol/L Tris-HCl, pH 8.0), supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride). Cellular debris and lipids were eliminated by centrifugation of the solubilized samples at 12,000 *g* for 10 min at 4°C, recovering the soluble fraction. Protein concentration was determined using the RC/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). RIPA protein extracts (15 μg) were run in 12.5% SDS-PAGE and transferred to nitrocellulose

Table 2. Bivariate correlations between plasma LBP levels and liver, iWAT or pgWAT *Lbp* mRNA levels

	Plasma LBP (μg/mL)					
	All mice		Male		Female	
	r	p	r	P	r	p
Liver *Lbp* mRNA (RU)	0.82	**<0.0001**	0.73	**0.02**	0.65	**0.04**
iWAT *Lbp* mRNA (RU)	−0.34	0.1	0.02	0.9	0.83	**0.005**
pgWAT *Lbp* mRNA (RU)	−0.33	0.1	0.38	0.3	0.12	0.7

membrane by conventional procedures. After blocking with 5% BSA in TBS-Tween, membranes were exposed overnight at 4°C to primary antibodies anti-GLUT4 (Glut4 (1F8) Mouse mAb # 2213) and anti-FAS (Fatty Acid Synthase [C20G5] Rabbit mAb #3180) at 1/1000 dilution (Cell Signaling, Massachusetts, USA), anti-UCP1 at 1/1000 (Rabbit polyclonal to UCP1, ab10983, ABCAM netherlands) and anti-LBP at 1/1000 (Anti-mouse-LBP antibody [Ab] biG 33, Abnova, Taipei, Taiwan), both diluted in 1x PBS containing 0.1% Tween 20, following the recommendations of the manufacturer. After secondary antibody incubation (Anti mouse/Rabbit HRP), signals were detected using enhanced chemiluminescence horseradish peroxidase substrate (Millipore) and analyzed with a luminescent image analyzer ChemiDoc MP Imaging System (BIO-RAD Laboratories, California, USA). TGX Stain-Free gels (#4568096, BIO-RAD Laboratories, California, USA) were used as protein loading control.

Quantification of triglycerides in liver

30–40 mg of liver were homogenized using a TissueLyser LT in 400 μL of distilled water containing 5% Igepal CA-630, boiled for 5 min twice, and centrifuged at 13,000 xg for 5 min. Triglycerides were measured in the supernatant using Triglyceride Quantification Colorimetric/Fluorometric Kit (MAK266, MERCK LIFE SCIENCE, Madrid, Spain) according to manufacturer's instructions. Values were normalized by tissue weights used for the homogenizations.

Circulating parameters

Plasma LBP (HK205, LBP mouse ELISA kit, Hycult Biotech, PA, USA), insulin (0030-40-1, Mouse Insulin ELISA Kit, High Sensitivity, Quantitative, Life Technologies, Delhi, India), adiponectin (RD293023100R, Adiponectin Mouse ELISA, BioVendor - Laboratorni medicina, Brno, Czech Republic), leptin (RD291001200R, Leptin Mouse/Rat ELISA, BioVendor - Laboratorni medicina, Brno, Czech Republic), triglycerides (MAK266, MERCK LIFE SCIENCE, Madrid, Spain), WAT lipase activity (K723-100, Lipase Activity Colorimetric Assay kit II, Biovision, Inc., CA, USA), plasma free fatty acids (700310, Free Fatty Acid Fluorometric Assay Kit, Cayman chemical, MI, USA), plasma glycerol (10010755, Glycerol Colorimetric Assay Kit, Cayman chemical, MI, USA), and blood glucose (Accutrend; Roche Diagnostics, Mannheim, Germany) were measured using commercial kits according to manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the SPSS 12.0 software. All results are expressed as means ± SEM, and differences were tested for statistical significance using Student's unpaired and paired *t* tests, and non-parametric tests (Mann–Whitney U test). Levels of statistical significance were set at $p < 0.05$.

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AUTHOR CONTRIBUTIONS

J.M.F-R. and J.M.M-N. participated in study design and analysis of data. J.L., F.O., A.L., N.O-C., F.C., A.G-N., S.M-R., and J.M.M-N. participated in acquisition of data. J.L., W.R., F.V., M.G., J.M.F-R., and J.M.M-N. participated in interpretation of data. J.M.M-N. wrote and edited the manuscript. J.M.F-R. revised the manuscript critically for important intellectual content. All authors participated in final approval of the version to be published.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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