Downregulation of peripheral lipopolysaccharide binding protein impacts on perigonadal adipose tissue only in female mice

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ABSTRACT

Background and aims: The sexual dimorphism in fat-mass distribution and circulating leptin and insulin levels is well known, influencing the progression of obesity-associated metabolic disease. Here, we aimed to investigate the possible role of lipopolysaccharide-binding protein (LBP) in this sexual dimorphism.
Methods: The relationship between plasma LBP and fat mass was evaluated in 145 subjects. The effects of Lbp downregulation, using lipid encapsulated unlocked nucleomonomer agent containing chemically modified-siRNA delivery system, were evaluated in mice.
Results: Plasma LBP levels were associated with fat mass and leptin levels in women with obesity, but not in men with obesity. In mice, plasma LBP downregulation led to reduced weight, fat mass and leptin gain after a high-fat and high-sucrose diet (HFHS) in females, in parallel to increased expression of adipogenic and thermogenic genes in visceral adipose tissue. This was not observed in males. Plasma LBP downregulation avoided the increase in serum LPS levels in HFHS-fed male and female mice. Serum LPS levels were positively correlated with body weight and fat mass gain, and negatively with markers of adipose tissue function only in female mice. The sexually dimorphic effects were replicated in mice with established obesity. Of note, LBP downregulation led to recovery of estrogen receptor alpha (*Esr1*) mRNA levels in females but not in males.
Conclusion: LBP seems to exert a negative feedback on ERα-mediated estrogen action, impacting on genes involved in thermogenesis. The known decreased estrogen action and negative effects of metabolic endotoxemia may be targeted through LBP downregulation.

1. Introduction

Obesity is the most important global factor in the progression of metabolic diseases, contributing to the overall burden of disease worldwide [1]. The regulation of body adiposity occurs through coordinated actions of peripheral and central mechanisms. Leptin, secreted

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from white adipocytes in direct proportion to fat content, decreases food intake and increases energy expenditure [2]. Insulin is also secreted in direct proportion to white fat [3].

Chronic inflammation has been recognized in the past decades as an important pathophysiological factor in the development of obesity-associated metabolic complications in general, and insulin resistance in particular. Cross talk between microbes or their components and the host innate immune system is critical to regulate host metabolism. LPS-binding protein (LBP) is a relevant component of innate immunity synthesized in response to lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria and important trigger of obesity-driven low-grade inflammation [4–10].

There is evidence in animal models and humans that while the male brain is relatively more sensitive to the metabolic action of insulin, the female brain is relatively more sensitive to the metabolic action of leptin [11–13]. It has been shown that leptin is a better correlate of body fat in females and insulin is a better correlate of body fat in males [11]. As leptin and insulin, plasma LBP circulates in proportion to fat mass [6,7].

Women are known to mount stronger pro-inflammatory responses during infections [14]. A recent research disclosed that the two sexes present differential sensitivity to LPS administration as an immune stressor, being female rats more robust against LPS-induced anorexia [15,16]. Thus, it is expected that females and males will respond differently to down-regulation-induced effects of LPS action.

These intriguing studies led us to hypothesize unprecedented interactions among plasma LBP, fat mass and insulin resistance according to sex. To investigate this hypothesis, in the current study, we aimed to examine the relationship between plasma LBP, fat mass and systemic insulin sensitivity in non-obese and obese men and women, and to test the impact of plasma Lbp depletion using lipid encapsulated unlocked nucleomonomer agent (UNA)-containing chemically modified-siRNA delivery system [17] on fat mass and adipose tissue adipogenesis in male and female mice.

2. Materials and methods

2.1. Human study

From January 2016 to October 2017, a cross-sectional case-control study was undertaken in the Endocrinology Department of Josep Trueta University Hospital. We included 64 non obese (BMI 18.5-<30 kg/m^2) and 81 obese (BMI≥30 kg/m^2) participants, with an age range of 27.2–66.6 years. Exclusion criteria were: previous type 2 diabetes mellitus, chronic inflammatory systemic diseases, acute or chronic infections in the previous month; severe disorders of eating behavior or major psychiatric antecedents; neurological diseases, history of trauma or injured brain, language disorders; and excessive alcohol intake (≥ 40 g OH/day in women or 80 g OH/day in men). Body fat composition was estimated using Bio-electrical impedance analysis (BC-418, Tanita Corporation of America, Illinois, USA).

Insulin action was determined by hyperinsulinemic-euglycemic clamp. After an overnight fast, two catheters were inserted into an antecubital vein, one for each arm, used to administer constant infusions of glucose and insulin and to obtain arterialized venous blood samples. A 2-h hyperinsulinemic-euglycemic clamp was initiated by a two-step primed infusion of insulin (80 mU/m^2/min for 5 min, 60 mU/m^2/min for 5 min) immediately followed by a continuous infusion of insulin at a rate of 40 mU/m^2/min (regular insulin [Actrapid; Novo Nordisk, Plainsboro, NJ]). Glucose infusion began at minute 4 at an initial perfusion rate of 2 mg/kg/min being then adjusted to maintain plasma glucose concentration at 88.3–99.1 mg/dL. Blood samples were collected every 5 min for determination of plasma glucose and insulin. Insulin sensitivity was assessed as the mean glucose infusion rate during the last 40 min. In the stationary equilibrium, the amount of glucose administered (M) equals the glucose taken by the body tissues and is a measure of overall insulin sensitivity.

All subjects gave written informed consent, validated and approved by the ethical committee of the Hospital of Girona "Dr Josep Trueta", after they were explained the purpose of the study.

2.2. Lipid encapsulated Lbp UNA-siRNA nanoparticles biosynthesis

Using LUNAR® technology, a proprietary lipid enabled nucleic acid delivery platform, Arcturus Therapeutics (San Diego, CA) produced LUNAR® particles encapsulating *Lbp*-UNA siRNA as described previously [17]. Briefly, UNA siRNA was dissolved in 2 mM citrate buffer, pH 3.5. Lipids at the desired molar ratio were dissolved in ethanol. The molar ratio of the constituent lipids is 58% ATX (proprietary ionizable amino lipid), 7% DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) (Avanti Polar Lipids, Alabaster, AL, USA), 33.5% cholesterol (Avanti Polar Lipids, Alabaster, AL, USA), and 1.5% DMG-PEG (1,2-Dimyristoyl-sn-glycerol, methoxypolyethylene glycol, PEG chain Molecular weight: 2000) (NOF America Corporation, White Plains, NY). Lipid solution was then combined with UNA siRNA solution using a Nano-assemblr® microfluidic device (Precision NanoSystems Inc., Vancouver, Canada) at a flow rate ratio of 1:3 ethanol:aqueous phases. The mixed material was then diluted with 3X volume of 10 mMTris buffer, pH 7.4 containing 9% sucrose, reducing the ethanol content to 6.25%. The diluted formulation was then concentrated by tangential flow filtration using hollow fiber membranes (mPESKros membranes, 100 Kd MWCO, Spectrum Laboratories, Inc., Rancho Dominguez, California), followed by diafiltration against 10 volumes of 10 mM Tris buffer, pH 7.4 containing 9% sucrose. Post diafiltration, formulations were then concentrated to desired UNA-siRNA concentration followed by filling into vials and freezing. Formulations were characterized for particle size, UNA-siRNA content and encapsulation efficiency. Particle size was determined by dynamic light scattering (ZEN3600, Malvern Instruments). Encapsulation efficiency was calculated by determining unencapsulated UNA-siRNA content by measuring the fluorescence upon the addition of RiboGreen (Molecular Probes) to the particles (Fi) and comparing this value to the total RNA content that is obtained upon lysis of the particles by 1% Triton X-100 (Ft), where % encapsulation = (Ft − Fi)/Ft × 100.

2.3. Mice experiments

Standard control diet experiment. Eight-week-old male (N = 14) and female C57BL/6 J mice (N = 16) were kept under 12 h light/dark cycle and had ad libitum access to standard laboratory diet (SD, SAFE A04: 3.1% fat, 59,9% carbohydrates, 16,1% proteins, 2.791 kcal/g; Scientific Animal Food & Engineering; Nantes, France) for 6 weeks. In the study, mice received weekly intravenous injections of siRNA-control or siRNA-Lbp (3 mg/kg) for 6 weeks, using the corresponding buffer as the vehicle to discriminate effects from the carrier. Food intake and body weight were weekly monitored. At the end of experiment, after overnight fasting, mice were sacrificed by suffocation under sedation. Then, blood serum, and inguinal (i) and perigonadal (pg) white adipose tissue (WAT) were collected, immediately frozen in liquid-nitrogen, and stored at −80 °C until processing for RNA or protein analysis. Animal protocols were approved by the Committee at the University of Santiago de Compostela.

High-fat and high-sucrose diet experiment. Eight-week-old male (N = 18) and female (N = 18) C57/BL6Jmice were housed during 25 weeks under standard conditions of light (12-hour light/12-hour dark cycle) and temperature (22 ± 1 °C) in the following experimental conditions: (i) Non-treated control diet (CD)-fed mice (TD.120455, 3.3 Kcal/g, ENVIGO) during 25 weeks (n = 6 in males and n = 6 in females); (ii) Non-treated high-fat and high-sucrose (HFHS, TD.08811, 4.7 Kcal/g, ENVIGO)-fed mice during 25 weeks (HFHS) (n = 6 in males and n = 6 in females); (iii) Weekly LUNAR®-*Lbp* UNA-siRNA (3 mg/kg) treated HFHS-fed mice during 25 weeks (HFHS+siLbp) (n = 6 in males and n = 6 in females). The number of mice per cage was the same in male and female groups and in each experimental condition.

Effect of siRNA-Lbp in diet-induced obese mice. Eight-week-old male (N = 12) and female (N = 9) C57/BL6J mice were housed under standard conditions of light (12-hour light/12-hour dark cycle) and temperature (22 ± 1 °C) and fed with HFHS during 25 weeks. At week 17 of HFHS diet, when female mice exceeded 30 g and males 45 g and the body weight stabilized, weekly LUNAR®-*Lbp* UNA-siRNA (3 mg/kg) or vehicle administration during 8 weeks was performed in 25-week-old HFHS-fed mice.

Body weight and food consumption were weekly reported. Body composition (fat and lean mass) was measured by EchoMRI at week 5, 14 and 24. Blood (~180 µl), which was handled to obtain plasma, was collected by facial puncture at week 4 and 8 to monitor circulating Lbp levels. At week 25, after overnight fasting, mice were sacrificed by suffocation under sedation. The blood was drawn by arterial puncture once the animal was anaesthetized and before euthanasia. Then, blood was handled to obtain serum and plasma, and inguinal (i) and perigonadal (pg) white adipose tissue (WAT) were collected, immediately frozen in liquid-nitrogen, and stored at −80 ºC until processing for RNA or protein analysis. Animal protocols were approved by the Ethical Committee for Animal Experimentation of Barcelona Science Park (PCB).

In all mice experiments, the research was conducted in accordance with the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU).

2.4. Gene expression

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 Light Cycler® 480 Real-Time PCR System (Roche Diagnostics SL, Barcelona, Spain), using TaqMan®technology suitable for relative genetic expression quantification. The commercially available and pre-validated TaqMan® primer/probe sets (Thermo Fisher Scientific Inc, MA, USA) used were as follows: Endogenous control *18 S*, and target gene mouse lipopolysaccharide binding protein (*Lbp,* Mm00493139_m1); fatty acid synthase (*Fasn,* Mm00662319_m1); stearoyl-Coenzyme A desaturase 1 (*Scd1,* Mm00772290_m1); perilipin 1 (*Plin1,* Mm00558672_m1); leptin (*Lep,* Mm00434759_m1); peroxisome proliferator activated receptor gamma (*PPARγ,* Mm00440940_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 syntethase long-chain family member 1 (*Acsl1,* Mm00484217_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* or *Mcp1,* Mm00441242_m1), estrogen receptor 1 (alpha) (*Esr1,* Mm_00433149_m1); and estrogen receptor 2 (beta) (*Esr2,* Mm00599821_m1).

2.5. Western blot analysis

Dissected perigonadal WAT was homogenized using an IKA T25 digital ULTRA-TURRAX homogenizer in lysis buffer (50 mmol/l Tris-HCl pH 7.4, 150 mmol/l NaCl, 1.5 mmol/l MgCl2, 1 mmol/l EDTA, 1 mmol/l EGTA, 40 mmol/l β-glycerophosphate, 2 mmol/l Na3VO4, 1 mmol/l PMSF, 1 mmol/l DTT) containing complete protease inhibitor cocktail (Roche Applied Science). Lysates were centrifuged at 1500 x g for 5 min at 4 °C to remove intact cells, and protein content was quantified using the Bradford method. For Western blotting, proteins (40 µg) were resolved by SDS-PAGE and transferred to a PVDF membrane

(Immobilon; Millipore). Membranes were exposed overnight at 4ºC to primary antibodies anti-UCP1 at 1/1000 dilution (ab10983, Abcam Plc, Cambridge, UK), anti-ERα at 1/1000 dilution (MA5-13191, Thermo Fisher Scientific Inc, MA, USA) and anti-β-actin at 1/5000 (A5441, Sigma-Aldrich) 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 HRP substrate (Millipore) and analyzed with a Luminescent Image Analyzer LAS-3000 (Fujifilm Life Science, Tokyo, Japan).

2.6. Serum/plasma measurements

Human study. Plasma LBP was measured by human LBP enzyme-linked immunosorbent assay (ELISA) kit (HK315-02, HyCult Biotechnology, Huden, the Netherlands) with intra- and interassay coefficients of variation < 8%. Plasma leptin levels were measured by Human Leptin ELISA kit (RAB0333-1KT, Sigma-Aldrich). Serum glucose levels were measured in duplicate by the glucose oxidase method with a Beckman Glucose Analyzer 2 (Beckman Instruments, Brea, CA). The coefficient of variation (CV) was 1.9%. Total serum cholesterol was measured through the reaction of cholesterol esterase/ oxidase/ peroxidase, using a BM/ Hitachi 747. HDL cholesterol was quantified after precipitation with polyethylene glycol at room temperature. Total serum triglycerides were measured through the reaction of glycerol-phosphate-oxidase and peroxidase by routine laboratory tests on a Hitachi 917 instrument (Roche, Mannheim, Germany).

Mice experiments. Plasma or serum LBP (HK205-02, LBP mouse ELISA kit, HyCult Biotech *Inc.,* PA, USA), leptin (90030) and adiponectin (80569, Crystal Chem, Zaandam, Netherlands) were measured using commercial kits according to manufacturer's instructions. Serum LPS levels were analyzed by LAL chromogenic Endpoint Assay (HIT302, HyCult Biotech Inc*., PA, USA*) following manufacturer's instructions. Prior to serum LPS analysis, serum endotoxin inhibiting compounds were neutralized by heating the sample at 75 °C for 5 min.

2.7. Statistical analysis

Statistical analyses were performed using the SPSS 12.0 software. In mice experiments, all results are expressed as means ± SEM. In mice experiments, differences were tested for statistical significance using one-factor ANOVA, student's unpaired and paired *t*-tests, and non-parametric tests (Mann–Whitney U test) were used in those groups with higher data variability. In human study, unless otherwise stated, descriptive results of continuous variables are expressed as mean and SD for Gaussian variables or median and interquartile range for non-Gaussian variables. The relation between variables was analysed by simple correlation (using Spearman's and Pearson's tests). One-factor ANOVA and Student's unpaired *t*-tests were used to compare clinical variables and plasma LBP concentration according to obesity. Levels of statistical significance were set at p < 0.05.

3. Results

3.1. Plasma LBP concentration is associated with adiposity differently in men and women

We studied a cohort of 145 subjects (44 men and 101 women) (Supplementary Table 1). Plasma LBP levels were significantly increased in obese men and women (Fig. 1A). Of note, in subjects with obesity, plasma LBP levels were significantly increased in pre-menopausal women compared to post-menopausal women or men in parallel to estradiol levels (Fig. 1B).

We observed that in women, whether with or without obesity, plasma LBP concentration was linearly associated with fat mass and leptin in a continuous manner (Fig. 1C). In men, however, there was a
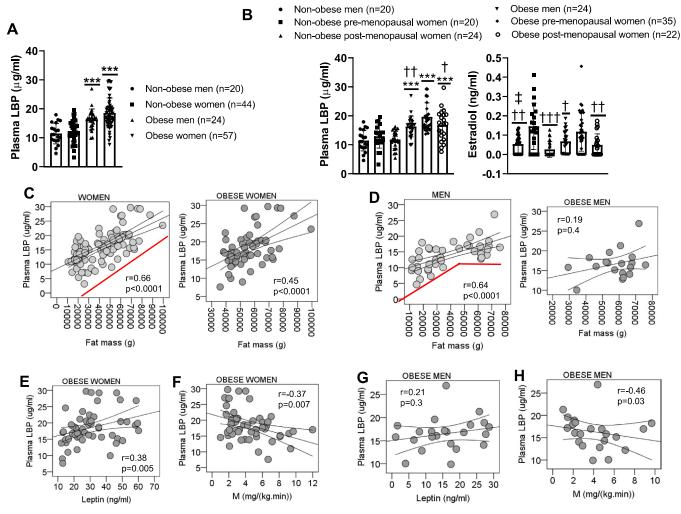
Fig. 1. A) Plasma LBP according to obesity in men and women. B) Plasma LBP and estradiol levels according to obesity and menopause. ***p < 0.001 compared to non-obese subjects. †p < 0.05, ††p < 0.01 and †††p < 0.001 compared to pre-menopausal women. ‡p < 0.05 compared to post-menopausal women. C–H) Bivariate correlations between plasma LBP and fat mass, leptin and insulin sensitivity in obese women (C,E,F) and men (D,G,H). The discontinuous red line indicates the trend of the association between plasma LBP and fat mass in all participants. M: Insulin sensitivity measured by hyperinsulinemic-euglycemic clamp.

plateau of fat mass and leptin above which LBP did no longer increase proportionally (Fig. 1D). In obese participants, a negative correlation between plasma LBP and insulin sensitivity was observed in both men and women, whereas only in women, plasma LBP was positively correlated with adiposity measures (BMI, fat mass, percent total fat and waist circumference) and leptin levels (Table 1, Fig. 1C–H). In multiple linear regression analyses, fat mass (β = 0.37, t = 2.46, p = 0.01) was the unique factor significantly associated with plasma LBP levels in women after controlling for age (β = −0.09, t = −0.62, p = 0.5) and insulin action during the clamp (β = −0.25, t = −1.94, p = 0.06). These findings suggest gender-related differences in the associations among LBP and fat mass in obese subjects. In addition, a sexual dimorphism in the correlation between plasma LBP and fat mass according to fat distribution were found (Table 1). While in both non-obese and obese men, plasma LBP concentration were correlated to android, but not gynoid, fat mass, in obese women (but not in non-obese), plasma LBP concentration were correlated with gynoid and android fat mass (Table 1). We thus decided to study whether LBP depletion could impact fat mass and adipose tissue differently in male and female mice in obesity.

3.2. Plasma LBP depletion attenuates body weight and fat mass gain in high-fat and high-sucrose diet–fed female mice

A pilot experiment, in which several doses of LNP-Lbp UNA-siRNA (1

and 3 mg/kg) were tested, indicated that the optimal dose to achieve a significant depletion in plasma LBP levels was 3 mg/kg. In mice fed a high-fat and high-sucrose diet (HFHS) during 6 months, lipid encapsulated Lbp UNA-siRNA administration resulted in a significant decrease of plasma LBP levels (Fig. 2A).

Despite similar weight gain (Suppl. Fig. 1A), important differences between females and males were found regarding food consumption (Fig. 2B). Females treated with LNP-Lbp UNA-siRNA had increased food consumption, remaining unchanged in males (Fig. 2B). The maximum increase in food consumption in females was found in weeks 5–15 (2.3 fold-increase, p < 0.0001) although it remained significantly higher until week 25 (Fig. 2B).

The significant increase in food intake between weeks 5–14 did not reflect an increase in body weight and fat mass gain in the same period in treated females (Fig. 2C–D and Suppl. Fig. 1B–C). On the contrary, at the end of the experiment, female treated with LNP-Lbp UNA-siRNA exhibited less body weight gain and fat accumulation (Fig. 2C–D and Suppl. Fig. 1B–C), without significant changes on lean mass (Fig. 2E and Suppl. Fig. 1D). In frank contrast, food intake, body weight gain, fat and lean mass gain under HFHS was not significantly affected by LNP- LBP UNA-siRNA in males (Fig. 2B–E and Suppl. Fig. 1).

Table 1

Bivariate correlations between plasma LBP (µg/ml) and anthropometric and clinical parameters in non-obese and obese participants according to gender.

Men	All		Non-obese		Obese	
N = 44	r	p	r	p	r	p
Age (years)	0.11	0.4	0.38	0.09	-0.04	0.9
BMI (kg/m2)	0.54	<0.0001	-0.01	0.9	0.15	0.5
Fat mass (kg)	0.64	<0.0001	0.37	0.1	0.19	0.4
Lean mass (kg)	0.37	**0.01**	-0.65	**0.002**	0.42	0.05
Percent total fat (%)	0.62	<0.0001	0.53	**0.01**	0.01	0.9
Gynoid fat mass (g)	0.58	<0.0001	0.17	0.4	0.07	0.8
Android fat mass (g)	0.71	<0.0001	0.48	**0.03**	0.47	**0.03**
Waist circumference (cm)	0.66	<0.0001	0.35	0.1	0.38	0.06
Total cholesterol (mg/dl)	-0.09	0.6	0.55	**0.01**	-0.36	0.08
HDL cholesterol (mg/dl)	-0.19	0.2	0.18	0.4	-0.14	0.5
LDL cholesterol (mg/dl)	-0.01	0.9	0.37	0.1	-0.17	0.4
Fasting triglycerides (mg/dl)	0.08	0.6	0.42	0.06	-0.22	0.3
Fasting glucose (mg/dl)	0.20	0.2	0.41	0.07	0.17	0.4
M (mg/(kg·min))	-0.53	**0.001**	-0.31	0.2	-0.46	**0.03**
Leptin (ng/ml)	0.61	<0.0001	0.34	0.1	0.22	0.3
Women	**All**		**Non-obese**		**Obese**	
N = 101	r	p	r	p	r	p
Age (years)	-0.29	**0.003**	-0.08	0.6	-0.17	0.2
BMI (kg/m2)	0.64	<0.0001	-0.04	0.8	0.48	**<0.0001**
Fat mass (kg)	0.66	<0.0001	0.11	0.5	0.45	**<0.0001**
Lean mass (kg)	0.49	<0.0001	-0.09	0.6	0.17	0.2
Percent total fat (%)	0.66	<0.0001	0.14	0.4	0.46	**<0.0001**
Gynoid fat mass (g)	0.66	<0.0001	0.09	0.5	0.46	**<0.0001**
Android fat mass (g)	0.65	<0.0001	0.12	0.4	0.47	**<0.0001**
Waist circumference (cm)	0.61	<0.0001	-0.08	0.6	0.35	**0.009**
Total cholesterol (mg/dl)	-0.19	0.06	-0.12	0.4	-0.09	0.5
HDL cholesterol (mg/dl)	-0.38	<0.0001	0.01	0.9	-0.16	0.2
LDL cholesterol (mg/dl)	-0.04	0.7	-0.01	0.9	-0.03	0.8
Fasting triglycerides (mg/dl)	0.22	**0.02**	0.03	0.8	-0.03	0.8
Fasting glucose (mg/dl)	-0.06	0.6	-0.05	0.7	-0.23	0.09
M (mg/(kg·min))	-0.59	<0.0001	-0.03	0.8	-0.37	**0.007**
Leptin (ng/ml)	0.67	<0.0001	0.37	**0.02**	0.38	**0.005**

M: Insulin sensitivity measured by hyperinsulinemic-euglycemic clamp.

3.3. Plasma LBP depletion attenuates the increase in circulating leptin levels in high-fat and high-sucrose diet–fed female mice

Similar to body weight or fat mass, no significant differences on circulating leptin levels were found in treated and untreated animals (with siRNA LBP, Fig. 3A). Even though, the increase in circulating leptin levels between week 4 and 25 observed in mice fed with HFHS was attenuated in females, but not males, treated with LNP-Lbp UNA-siRNA (Fig. 3B).

3.4. Plasma LBP depletion prevents the increase in serum LPS levels in high-fat and high-sucrose diet–fed female mice

Since increased intestinal LPS translocation in mice fed with high-fat diet is associated to body weight and fat mass gain [18,19], the impact of plasma LBP depletion on serum LPS levels were also evaluated. In both males and females, LNP-Lbp UNA-siRNA administration avoided the increase in serum LPS levels in HFHS-fed mice (Fig. 3 C). Interestingly, serum LPS levels were positively correlated with body weight and fat mass gain during the last 10 weeks in females, and with the percent change in leptin in both male and female mice (Table 2).

3.5. Plasma LBP depletion enhances circulating adiponectin levels in high-fat and high-sucrose diet–fed female mice

Interestingly, LNP-Lbp UNA-siRNA administration prevented the obesity-associated reduction in circulating adiponectin, being this adipokine significantly increased compared to HFHS controls in females at week 25 (Fig. 3D), preventing the decrease in circulating adiponectin levels between week 4 and 25 observed in mice fed with HFHS in both female and male mice (Fig. 3E). Serum LPS levels were negatively correlated with the percent change in circulating adiponectin levels in both male and female mice (Table 2). Since adiponectin is a specific marker of adipose tissue physiology [20], the impact of plasma LBP depletion on markers of adipose tissue function was then investigated.

3.6. Plasma LBP depletion improves expression of adipogenic and thermogenic genes in visceral adipose tissue from HFHS–fed female mice

Consistent with circulating levels of adiponectin (Fig. 3D–E), LNP-Lbp UNA-siRNA administration led to a significantly increased adipogenic (Adipoq, Slc2a4, Fabp4, Scd1, Plin1, Pparg) and lipolytic (Lipe, Mgll, Acsl1) gene expression in perigonadal (pg) WAT from HFHS-fed female mice, without any effects on obesity (Lbp, Lep)- and inflammation (Il6, Tnf, Ccl2)-related genes (Fig. 4A–D). In addition, the lack of major changes in body weight after LBP depletion, despite significant hyperphagia observed in females prompted us to investigate possible thermogenic effect. Our data showed that plasma LBP depletion increased the expression of key browning markers in the pgWAT of female mice, such as Ucp1, Nrg4 and Adbr3, as well as UCP1 protein levels, when compared to HFHS control mice (Fig. 4E–F).

In male mice, similar to body weight or fat mass gain, adipose tissue-related gene expression profile did not change significantly after LNP-Lbp UNA-siRNA administration (Fig. 4).

Serum LPS levels also were negatively correlated with pgWAT Adipoq, Slc2a4, Lipe, Ucp1 and Nrg4 gene expression in female, but not in male mice (Table 2).

To gain insight in this sexual dimorphism, gene expression of estrogen receptors as mediators of estrogen action were analysed. The most abundant estrogen receptor in adipose tissue was estrogen receptor alpha (Esr1) (Suppl. Fig. 2A), through which estrogen is known to impact on adipose tissue [21,22]. Of note, Lbp depletion led to increased pgWAT Esr1 mRNA in female mice in negative correlation with serum LPS levels (Table 2), putatively restoring the estrogen action in perigonadal adipose tissue under HFHS (Fig. 4G), and no significant differences in pgWAT Esr2 mRNA were observed (Suppl. Fig. 2B).

Otherwise, no significant effects of LNP-Lbp UNA-siRNA treatment were observed in inguinal (i) WAT (Suppl. Fig. 2B and Suppl. Fig. 3).

3.7. Short-term (8 weeks) effect of plasma LBP depletion on adiposity and adipose tissue in obese mice

To strengthen these findings, the effect of LNP-Lbp UNA-siRNA treatment during 8 weeks in 25-week-old diet-induced obese mice was also investigated. Similar to long-term (25 weeks) treatment, in male obese mice, after 8 weeks of treatment of LNP-Lbp UNA-siRNA no significant differences on food intake, body weight and fat and lean mass were observed, whereas female obese mice only displayed a non-significant reduction in fat mass (p = 0.07) (Fig. 5A–E). In both male and female obese mice, plasma LBP depletion resulted in decreased serum LPS levels (Fig. 5F). In relation to adipose tissue, similar findings were observed after 8 weeks of LNP-Lbp UNA-siRNA administration in obese mice, resulting in increased Ucp1, Nrg4, Adipoq, Slc2a4, Fabp4, Fasn, Lipe and Esr1 gene expression and UCP1 and ERα protein levels, but decreased Lbp and Ccl2 gene expression in female pgWAT (Fig. 5G–N). Similar to previous experiment, no significant effects of
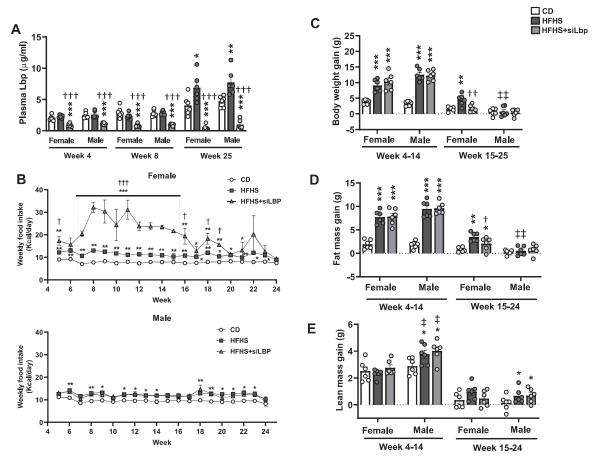
Fig. 2. A–D) Effects of 6 months-LNP-Lbp UNA-siRNA administration on plasma LBP levels at 4, 8 and 25 weeks (A), food intake (B), body weight gain (C), fat mass gain (D), and lean mass gain (E). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to CD; †$p < 0.05$, ††$p < 0.01$ and †††$p < 0.001$ compared to HFHS; ‡$p < 0.05$, ‡‡$p < 0.01$ and ‡‡‡$p < 0.001$ compared to female mice.

LNP-Lbp UNA-siRNA treatment on iWAT were observed (Suppl. Fig. 2C and Suppl. Fig. 4).

Taken together, these experiments showed that circulating LBP depletion led to reduced fat mass during obesity progression and improved gene expression markers of visceral adipose tissue function in females (adipogenic, catabolic and thermogenic-related genes), possibly in part through reversal of HFHS-induced reduction in estrogen action in this fat depot. Then, to elucidate which of these processes could depend on the restoration of estrogen action in adipose tissue, we evaluated the impact of circulating LBP depletion in mice fed a standard diet, in which the action of estrogen on adipose tissue was preserved.

3.8. Circulating LBP depletion increased expression of adipogenic genes in visceral adipose tissue from standard diet-fed female mice

In both male and female mice fed a standard diet (STD) during 6 weeks, LNP-Lbp UNA-siRNA administration resulted in a significant decrease of serum LBP levels (Fig. 6A), without any effects on food intake or body weight (Fig. 6B–C). However, in females, but not in males, this intervention resulted in increased expression of some adipogenic genes (*Slc2a4, Fasn, Plin1* and *Pparg*), *Mgll* and Nrg4 in pgWAT, without any effects on *Ucp1, Adrb3, Esr1* and *Esr2* mRNA levels (Fig. 6D–I and Suppl. Fig. 2D).

Otherwise, LNP-Lbp UNA-siRNA administration decreased pgWAT *Adipoq, Slc2a4* and *Il6* gene expression in males (Fig. 6D–E).

Under a standard diet, no significant effects of LNP-Lbp UNA-siRNA treatment on iWAT were observed (Suppl. Fig. 2D and Suppl. Fig. 5).

4. Discussion

To the best of our knowledge this is the first study showing sexual dimorphism in plasma LBP metabolic actions on fat accretion and obesity-associated adipose tissue dysfunction.

A negative correlation between plasma LBP and insulin sensitivity without associations with adiposity was observed in men with obesity, whereas circulating LBP was associated with fat mass after controlling for age and insulin action in women with obesity. Among subjects without obesity, plasma LBP positively correlated with leptin in women, but not in men. These findings suggest gender-related differences in the possible metabolic actions of LBP. Supporting these observations, experiments in mice demonstrated that LNP-Lbp UNA-siRNA administration resulted in raised food consumption, decreased fat mass and plasma leptin gain and increased markers of adipose tissue function only in females. These data confirm an important impact of LBP on fat mass and adipose tissue physiology in females.

It is important to note that the effects of LBP depletion on physiological parameters started to be observed from week 6 of LNP-Lbp UNA-siRNA administration, suggesting these effects would become evident when the negative impact of the HFHS diet would begin to manifest. The induction of intestinal LPS translocation in mice fed with high-fat diet has been demonstrated in previous studies [18,19]. Of note, in the present study we found that plasma Lbp depletion avoided the increase of serum LPS levels in HFHS-induced obesity in both male and female mice. Serum LPS levels were positively correlated with the increase in adiposity parameters, such as body weight, fat mass and circulating leptin, but negatively with circulating adiponectin and gene expression markers of adipose tissue function in females, and only associated with
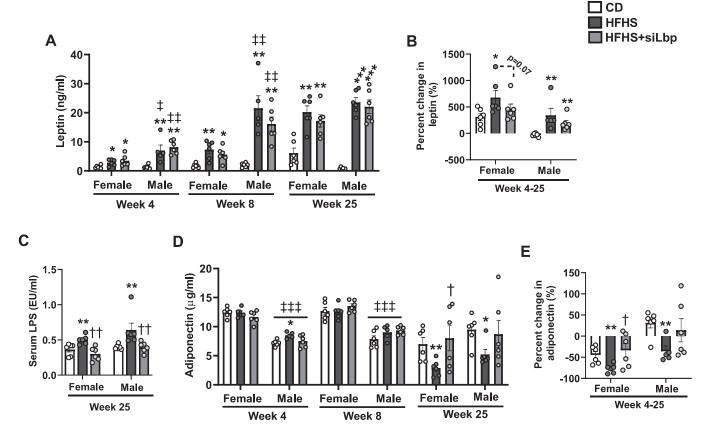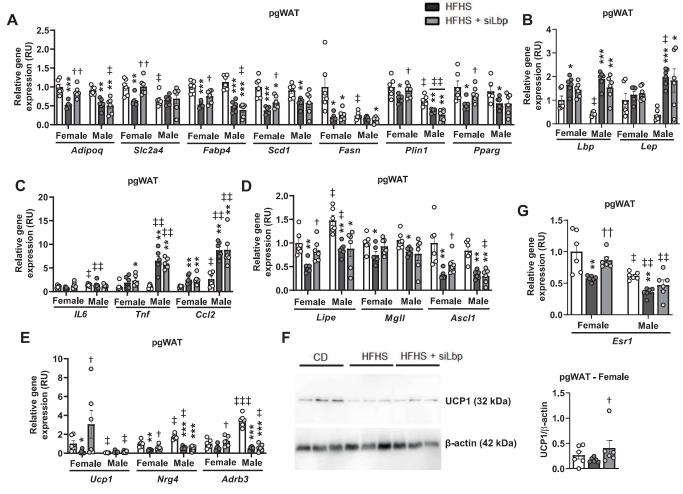
Fig. 3. A–B) Effects of 6 months-LNP-Lbp UNA-siRNA administration on circulating leptin at 4, 8 and 25 weeks (A) and the percent change in plasma leptin between week 4 and 25 (B). C) Effects of 6 months-LNP-Lbp UNA-siRNA administration on serum LPS at 25 weeks. D–E) Effects of 6 months-LNP-Lbp UNA-siRNA administration on circulating adiponectin at 4, 8 and 25 weeks (D) and the percent change in plasma adiponectin between week 4 and 25 (E). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to CD; †$p < 0.05$ compared to HFHS; ‡$p < 0.05$, ‡‡$p < 0.01$ and ‡‡‡$p < 0.001$ compared to female mice.

Table 2

Bivariate correlations between plasma LPS (EU/ml) and metabolic parameters and pgWAT gene expression in male and female mice in high-fat and high-sucrose diet experiment.

	Male mice (N = 18)		Female mice (N = 18)	
	r	p	r	p
Body weight gain (g) - week 4–14	0.12	0.6	0.08	0.7
Body weight gain (g) - week 15–25	0.09	0.7	0.63	**0.006**
Fat mass gain (g) - week 4–14	0.27	0.3	0.14	0.5
Fat mass gain (g) - week 15–24	-0.06	0.8	0.57	**0.01**
Percent change in leptin (%)	0.55	**0.02**	0.61	**0.007**
Percent change in adiponectin (%)	-0.54	**0.02**	-0.51	**0.03**
pgWAT *Adipoq* (RU)	-0.26	0.3	-0.58	**0.01**
pgWAT *Slc2a4* (RU)	-0.04	0.8	-0.48	**0.04**
pgWAT *Fabp4* (RU)	-0.24	0.3	-0.47	0.05
pgWAT *Scd1* (RU)	-0.34	0.2	-0.42	0.08
pgWAT *Fasn* (RU)	0.15	0.6	-0.26	0.3
pgWAT *Plin1* (RU)	-0.13	0.6	-0.34	0.1
pgWAT *Pparg* (RU)	-0.22	0.4	-0.27	0.2
pgWAT *Lbp* (RU)	0.51	**0.03**	0.32	0.2
pgWAT *Lep* (RU)	0.37	0.1	-0.08	0.7
pgWAT *Il6* (RU)	-0.1	0.7	-0.41	0.09
pgWAT *Tnf* (RU)	-0.06	0.8	-0.18	0.4
pgWAT *Ccl2* (RU)	0.05	0.8	0.03	0.9
pgWAT *Lipe* (RU)	-0.43	0.07	-0.51	**0.03**
pgWAT *Mgll* (RU)	-0.41	0.09	-0.31	0.2
pgWAT *Ascl1* (RU)	-0.37	0.1	-0.41	0.09
pgWAT *Esr1* (RU)	0.27	0.3	-0.65	**0.004**
pgWAT *Ucp1* (RU)	0.26	0.3	-0.51	**0.03**
pgWAT *Nrg4* (RU)	-0.29	0.2	-0.54	**0.02**
pgWAT *Adrb3* (RU)	-0.21	0.4	-0.31	0.2

the percent change in circulating leptin and adiponectin in males. These findings led to suggest that the beneficial effects of plasma Lbp depletion on body weight and fat mass in obese female mice could be explained in part by the attenuation of serum LPS levels. Supporting this suggestion, other interventions that improve obesity-associated metabolic disturbances and reduced plasma LBP levels, such as caloric restriction, also resulted in decreased circulating LPS levels [23]. Interestingly, the beneficial effects of caloric restriction were reverted after LPS reconstitution, for instance avoiding the caloric restriction-induced fat browning [23], and confirming the previously reported negative effects of metabolic endotoxemia on body weight, fat mass, glucose tolerance and adipose tissue physiology [19,24,25]. Strikingly, the protective effect of the attenuation of serum LPS levels on body weight and fat mass were not observed in obese male mice. This discrepancy may be due to the fact that the greater obesogenic impact of HFHS in male mice may dilute the obesogenic effect of LPS. Supporting this idea, the obesogenic effects of LPS infusion were reported in normal diet-, but not in high-fat diet-fed male mice [19].

Plasma LBP depletion led to increased expression of the browning marker *Ucp1*, factors that promote browning (*Adbr3, Nrg4*) and transporters that facilitate the capture of energy substrates lipid (*Lipe* and *Slc2a4*) in female perigonadal fat. In line with this, previous studies in KO mice or in adipose *Lbp* gene knockdown demonstrated enhanced markers of WAT browning in absence of adipose Lbp [26,27], and *Lbp* gene knockdown in perigonadal WAT increased browning-related gene expression in female, but not in male mice [27]. In all the experiments, increased pgWAT *Ucp1* mRNA levels were found in female compared to male mice. In agreement with current findings, female sex hormones induced sympathetic signaling sensitivity in female perigonadal WAT [28,29]. Kim et al. demonstrated that female sex hormones can

Fig. 4. A–F) Effects of 6 months-LNP-Lbp UNA-siRNA administration on pgWAT expression of adipogenic- (*Adipoq, Slc2a4, Fabp4, Scd1, Fasn, Plin1, Pparg*) (A), obesity- (*Lbp, Lep*) (B), inflammation- (*Il6, Tnf, Ccl2*) (C), lipolysis- (*Lipe, Mgll, Ascl1*) (D) and browning- (*Ucp1, Nrg4, Adrb3*) (E) related genes, on UCP1 protein levels (F) and on *Esr1* mRNA levels (G) in HFHS-fed mice. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to CD; †$p < 0.05$ and ††$p < 0.01$ compared to HFHS; ‡$p < 0.05$, ‡‡$p < 0.01$ and ‡‡‡$p < 0.001$ compared to female mice.

specifically enhance thermogenic response in perigonadal, but not inguinal, WAT in response to browning stimuli [28]. Wang et al. also observed increased browning capacity in female peri-ovarian white adipose tissue compared to male epididymal WAT [30]. Oestrogens also suppressed alpha 2 adrenergic receptors in parallel to increased beta 3 adrenergic receptor availability in isolated adipocytes, sensitizing them to sympathetic signaling and promoting browning [29].

Although UCP1 expression was affected by LBP deletion in the females, more evidence is needed to relate an effect on thermogenesis, since adipose tissue thermogenesis does not depend exclusively of UCP1 [31,32]. Furthermore, two recent studies point to that WAT thermogenesis might have a minor impact on HFD diet-induced weight gain prevention [33,34]. Therefore, an association between WAT browning activity and enhanced food consumption in 6-months LNP-Lbp UNA-siRNA intervention, and the resistance to fat accretion and body weight gain in female mice in early life months cannot be made. The absence of measurements of energy balance by indirect calorimetry and energy loss in the feces are a limitation of the current study. These should be considered in further experiments to understand the disconnection between food intake and changes in fat mass and body weight in LNP-Lbp UNA-siRNA–treated female mice in obesogenic conditions.

Mechanistically, the current results suggest that the sexual dimorphism might be explained by the effects of plasma LBP downregulation on the expression of Esr1 in pgWAT. Estrogen receptors are known to

impact on adipose tissue function [21] and adipose tissue browning [35]. Selective-activation of ERα results in browning of adipose tissue while mice lacking ERα are cold intolerant [35]. Specific white and brown adipocyte Esr1 gene deletion resulted in impaired mitochondrial function and biogenesis [22]. In fact, the expression of *ESR1/Esr1* in adipose tissue from mice and humans positively associated with genes involved in mitochondrial function and with markers of metabolic health (insulin sensitivity, glucose uptake) [22]. It is well known that an optimal mitochondrial function and biogenesis is crucial to maintain adipose tissue physiology [36].

Similar to HFHS diet (used in the current study), other experimental models that represses ESR1, such as mitochondrial carrier homolog 2 overexpression, led to increased fat accumulation in *Caenorhabditis elegans*, cultured adipocytes, and mice [37]. Estradiol promotes adipogenesis and glucose uptake through enhancing *Slc2a4* expression and GLUT4 translocation to the cell membrane by ESR1-dependent mechanisms [38–40]. ERα KO mice displayed increased leptin, but decreased adiponectin levels [41], indicating adipose tissue dysfunction [20] and supporting the relevance of ERα in adipose tissue physiology. In humans, increased proportion in ERα vs. ERβ protein in the nuclear fraction of subcutaneous adipose tissue was associated with greater reduction of adipose tissue insulin resistance in response to estradiol [42]. Importantly, the positive effects of estradiol through ERα on adipocytes were attenuated when these cells were exposed to LPS [43].
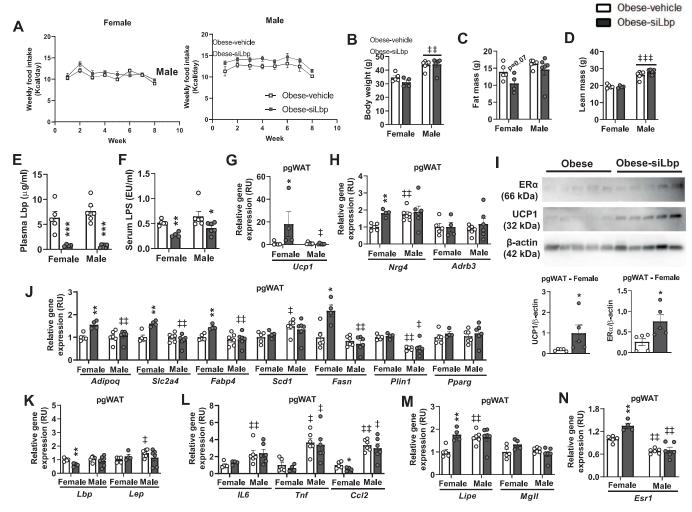
Fig. 5. A–L) Effects of 8 weeks-LNP-Lbp UNA-siRNA administration on food intake (A), body weight (B), fat (C) and lean (D) mass, plasma Lbp (E), serum LPS (F) and on pgWAT *Ucp1, Nrg4* and *Adbr3* gene expression (G-H) and on UCP1 and ERα protein levels (I), and on expression of adipogenic- (*Adipoq, Slc2a4, Fabp4, Scd1, Fasn, Plin1, Pparg*) (J), obesity- (*Lbp, Lep*) (K), inflammation- (*Il6, Tnf, Ccl2*) (L), lipolysis- (*Lipe, Mgll*) (M) related genes and *Esr1* mRNA (N) in diet-induced obese mice. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to obese-vehicle; ‡p < 0.05, ‡‡p < 0.01 and ‡‡‡p < 0.001 compared to female mice.

Taking into account the increase in LPS levels (metabolic endotoxemia) after HFHS diet [44–46], the relevance of LBP on LPS action in adipocytes [47], and the inhibitory effect of LPS on ERα in these cells [43], a scenario in which HFHS-promoted obesogenic conditions led to decreased ERα in inguinal and perigonadal WAT can be envisioned. LBP downregulation prevents this decrease in perigonadal WAT, putatively preserving the estrogen action only in this fat depot. Of note, estrogen is known to increase liver LBP production via ERα [48,49], suggesting an estrogen to LBP axis in which LBP exerts a negative feedback on ERα-mediated estrogen action. In line with this, plasma LBP levels were significantly increased in pre-menopausal women compared to post-menopausal women or men in parallel to estradiol levels (current study). However, to confirm this hypothesis additional experiments with ERα KO or ovariectomized mice and controlling the stage of female cycle and hormone levels should be performed.

The increased food intake observed in female mice after LBP depletion could be explained by alternative mechanisms. A recent study demonstrated that the combination of low-dose LPS administration and western-style diet (similar to HFHS diet used in current study) resulted in increased food intake in pregnant mice [50]. Since increased plasma LBP levels attenuated LPS response [51,52], we hypothesize that plasma LBP depletion might increase LPS exposure and enhanced LPS-induced hyperphagia in female mice fed a high fat diet. Even though, to study the impact of LBP depletion on food intake in a more accurate way, a pair-fed group should be included in further studies.

Another important finding, also associated to sexual dimorphism, was the impact of circulating LBP depletion on expression of adipogenic genes in visceral adipose tissue. Specifically, we found that LNP-Lbp UNA-siRNA administration in females fed with HFHS diet resulted in a significant improvement on gene expression markers of adipocyte function and adipogenesis in perigonadal WAT. Even though, early obesity-associated fat mass expansion is associated to increased adipogenesis, long-term diet-induced obesity results in adipose tissue dysfunction, which is characterized by decreased expression markers of adipogenesis-related genes [53–55], as it was shown in Fig. 4A. Reinforcing these findings, LNP-Lbp UNA-siRNA administration prevented the reduction of adiponectin (the most important circulating marker of adipogenesis) in obesity [20], and attenuated the increase of plasma leptin levels, a relevant circulating marker of obesity and fat mass [56]. Again, these effects were more pronounced in females.

In addition, circulating LBP depletion also slightly increased some gene expression markers of adipocyte function in pgWAT (including *Slc2a4, Fasn, Plin1* and *Pparg*) in female mice fed with standard control diet (in non-obesogenic conditions). The negative impact of Lbp on adipogenesis and markers of adipocyte function has been previously observed in several in vitro experimental models, including bone marrow mesenchymal stem cells [57], 3T3-L1 and 3T3-F442A cell lines [47] and human preadipocytes [58].
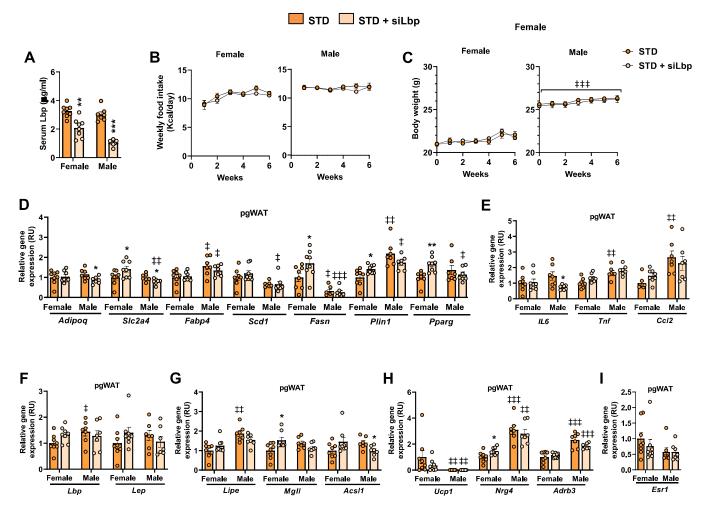
Fig. 6. A–H) Effects of 6 weeks-LNP-Lbp UNA-siRNA administration on serum Lbp (A), food intake (B), body weight (C), and on pgWAT expression of adipogenic- (*Adipoq, Slc2a4, Fabp4, Scd1, Fasn, Plin1, Pparg*) (D), inflammation- (*Il6, Tnf, Ccl2*) (E), obesity- (*Lbp, Lep*) (F), lipolysis- (*Lipe, Mgll, Acsl1*) (G) and browning- (*Ucp1, Nrg4, Adrb3*) (H) related genes and *Esr1* mRNA (I) in mice fed with standard diet. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to STD-vehicle; ‡p < 0.05, ‡‡p < 0.01 and ‡‡‡p < 0.001 compared to female mice.

Therapeutically, this study demonstrated that LNP-Lbp UNA-siRNA delivery system is an accurate and effective way to deplete plasma LBP concentration.

In conclusion, the observations in men and women and the pre-clinical study provide evidence about the potential utility of plasma LBP depletion (using a lipid encapsulated UNA-siRNA delivery system) as a new therapeutic approach in the prevention of obesity-associated adiposity in females. The known decreased estrogen action [59] and the negative effects of metabolic endotoxemia (LPS) [23,24] may be targeted through LBP downregulation. Further studies to understand the sex-dependent effects of plasma LBP depletion are required.

CRediT authorship contribution statement

MG, JMF-R and **JMM-N** participated in study design and analysis of data. **FC, RD-T, AG-N, EM, ND, SM-R, RM, JL, FO, MA-R, NO-C, PPK** and **JMM-N** participated in acquisition of data. **RD-T, FC, JL, WR, KT, PC, FV, ML, JMF-R, MG** and **JMM-N** participated in interpretation of data. **JMF-R, RD-T** and **JMM-N** wrote and edited the manuscript. **AG-N, FV, ML** and **MG** revised the manuscript critically for important intellectual content. All authors participated in final approval of the version to be published.

Conflict of interest statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ramon Díaz-Trelles, Rajesh Mukthavaram, Priya P. Karmali, Kiyoshi Tachikawa and Pad Chivukula are employees of Arcturus Therapeutics. The authors declared no additional conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113156.

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