Downregulation of hepatic lipopolysaccharide binding protein improves lipogenesis-induced liver lipid accumulation

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Circulating lipopolysaccharide-binding protein (LBP) is increased in individuals with liver steatosis. We aimed to evaluate the possible impact of liver LBP downregulation using lipid nanoparticle-containing chemically modified LBP small interfering RNA (siRNA) (LNP-Lbp UNA-siRNA) on the development of fatty liver. Weekly LNP-Lbp UNA-siRNA was administered to mice fed a standard Chow diet, a high-fat and high-sucrose diet, and a methionine- and choline-deficient diet (MCD). In mice fed a high-fat and high-sucrose diet, which displayed induced liver lipogenesis, LBP downregulation led to reduced liver lipid accumulation, lipogenesis (mainly stearoyl-coenzyme A desaturase 1 [Scd1]) and lipid peroxidation-associated oxidative stress markers. LNP-Lbp UNA-siRNA also resulted in significantly decreased blood glucose levels during an insulin tolerance test. In mice fed a standard Chow diet or an MCD, in which liver lipogenesis was not induced or was inhibited (especially Scd1 mRNA), liver LBP downregulation did not impact on liver steatosis. The link between hepatocyte LBP and lipogenesis was further confirmed in palmitate-treated Hepa1-6 cells, in primary human hepatocytes, and in subjects with morbid obesity. Altogether, these data indicate that siRNA against liver Lbp mRNA constitutes a potential target therapy for obesity-associated fatty liver through the modulation of hepatic Scd1.

INTRODUCTION

Metabolic-associated fatty liver disease (MAFLD) is one of the main consequences of the overall burden of obesity worldwide1–3 and contributes to aggravate other obesity-associated metabolic disturbances, such as insulin resistance, dyslipidemia, and type 2 diabetes, a common pathophysiology.4 In the last decade, circulating lipopolysaccharide binding protein (LBP) has been identified as a relevant component of innate immunity linked to obesity, insulin resistance and metabolic syndrome.5–9 Circulating LBP has also been described to be associated with liver fat accumulation in children and adults.10–13 Raised LBP seems to be causal because LBP KO mice fed a western style diet showed decreased liver steatosis.14 The liver is the main source of circulating LBP,15,16 being also present in adipose tissue.17 To the best of our knowledge, the specific role of liver LBP in MAFLD has not been yet investigated.

Lipid-enabled and unlocked nucleic acid modified RNA (LUNAR®) is a safe, reproducible, and effective lipid nanoparticle (LNP) RNA delivery platform that specifically can be used to perform gene knock-down in liver.18 In the current study, we aimed to evaluate the specific impact of liver LBP knockdown in liver steatosis across the spectrum of fatness and explore the use of LBP as a therapeutic target using LUNAR technology. To that end, we studied the role of liver-specific LBP downregulation in vivo using different approaches to achieve liver steatosis, such as high-fat and high-sucrose diet and methionine- and choline-deficient diet (MCD). Given the scarcity of pharmacological therapies specific for MAFLD, the identification of new molecular targets is imperative.

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RESULTS

Lbp UNA-siRNA delivered through LNPs reduces liver Lbp gene expression and plasma LBP in vivo

To test Lbp siRNA efficacy in vitro, we screened three commercially available small interfering RNAs (siRNAs; si69107, si69108, si69109) at different doses in the Hepa1-6 cells. To increase siRNA stability and efficacy and to reduce the number of intravenous injections and potential immune response in vivo, we also tested the strategic introduction of chemical modifications in the three siRNAs. Both unmodified (si69108) and unlocked nucleomonomer agent (UNA)-containing chemically modified siRNA (UNA-si69108) sequences resulted in the most significant Lbp gene knockdown (Figure 1A) and were therefore selected for further in vivo studies, after being formulated in LUNAR LNPs (Arcturus Therapeutics), as detailed in the experimental procedures. This system has been shown to deliver payloads specifically to the mouse hepatocytes.18 Ten-week-old male mice were intravenously injected with vehicle (phosphate buffer saline), LUNAR with unmodified siRNA (LNPN- si69108, 1 and 3 mg/kg), or LUNAR with chemically modified siRNA (LNPN-UNA-si69108, 1 and 3 mg/kg). Three days after injection, plasma LBP levels were significantly decreased in the groups treated with LNPN-UNA-si69108 but not in those treated with LNPN-si69108 (Figure 1B), and this effect was maintained until day 6. At day 10, serum LBP levels were significantly lower only at the highest dose (3 mg/kg), returning to baseline levels 12 days after injection (Figure 1B). To confirm the dose-dependent inhibition of liver Lbp gene expression by LNPN-UNA-si69108, a second intravenous injection was performed a week later in the same group of mice, at 1 and 3 mg/kg, and the mice were sacrificed after 3 days. Liver Lbp gene-expression analysis confirmed the dose-dependent inhibition

Figure 1. In vitro validation of Lbp siRNAs and in vivo effect of Lbp UNA-siRNA delivered through lipid nanoparticles

(A) Effect of several doses of chemically unmodified and modified (UNA) siRNA (si69107, si69108, si69109) against Lbp mRNA sequence in Hepa1-6 cell line. (B) Effect of chemically unmodified (1 and 3 mg/kg) and chemically modified (UNA; 1 and 3 mg/kg) LNP-Lbp siRNA on plasma LBP at days 3, 6, 10, and 12 after injection. (C) Effect of chemically modified (UNA; 1 and 3 mg/kg) LNP-Lbp siRNA on liver Lbp gene expression 3 days after second injection. *p < 0.05, **p < 0.01, and ***p < 0.001 compared with 0 pmol/L or vehicle. (D) Effects of 6-month LNPN-Lbp UNA-siRNA administration in HFHS-fed mice on liver, inguinal WAT (iWAT), and perigonadal WAT (pgWAT) Lbp mRNA levels and plasma Lbp concentration in male and female mice. *p < 0.05, **p < 0.01, and ***p < 0.001 compared with CD, †p < 0.05, ‡p < 0.01, and §§p < 0.001 compared with HFHS.
of liver Lbp mRNA levels by LNP-UNA-si69108 (Figure 1C). Considering the data from this in vivo pilot experiment and to avoid side effects that may arise using higher doses, the dose of 3 mg/kg was selected for the following experiments.

**Delivery of LNP-Lbp UNA-siRNA prevents liver lipid accumulation and lipogenesis induced by high-fat and high-sucrose diet**

We first evaluated whether the treatment of mice with LNP-UNA-si69108 could prevent lipid accumulation in the liver under obesogenic conditions. To set up this mouse model, mice were fed a high-fat and high-sucrose diet (HFHS) to promote liver steatosis (Figures S1A–S1C).

**Diet effect**

In both males and females, HFHS-induced obesity did not significantly change liver Lbp mRNA levels (Figure 1D). In males, an HFHS resulted in a significant increased hepatic lipid accumulation (Figures 2 and S2), including lipid droplet count and area (Figure 2A) and triglycerides (Figure 2B) and signs of liver damage, such as increased hepatocyte vacuolation (with a histopathology score ranging between 4 and 5), serum alanine transaminase (ALT) levels, and expression of some inflammatory markers (Itgax and Ccl2 mRNA) (Figures S2A–S2C). Liver Lbp mRNA was increased by 2-fold in females compared with males fed a control diet or an HFHS (Figure 1D).

In line with this increased liver Lbp mRNA levels in females, liver lipid droplet counts and area, liver triglycerides, and hepatocyte vacuolation were all higher in female mice versus males fed a standard diet (Figures 2I, 2J, and S2F). In sharp contrast, the increase in lipid accumulation, ALT, and liver Itgax and Ccl2 mRNA levels in mice fed an HFHS diet was lower in females versus males (Figures 2I, 2J, and S2F–S2H). These data confirmed the resistance of females to the adverse effects of an HFHS diet. HFHS increased some markers of liver lipid accumulation and lipogenesis induced by an HFHS diet (A–P) Effects of 6-months LNP-Lbp UNA-siRNA administration on representative standard Masson’s trichrome staining histological liver slides (20×), lipid droplet (LD) count and area (A and L), liver triglycerides (B and J), malonyl-CoA levels (C and K), expression of lipogenic (D and L) genes, FAS and ACC protein (E, F, M, and N), ACC activity (G and O), and MDA levels (H and P) in HFHS-fed male (A–H) and female (I–P) mice. *p < 0.05, **p < 0.01, and ***p < 0.001 compared with CD; †p < 0.05, ††p < 0.01, and †††p < 0.001 compared with HFHS.
lipogenesis and oxidative stress-related genes (including Scd1, Gsta3, and Gpx4 mRNA, malonyl-coenzyme A [CoA], FAS, total ACC protein, and ACC activity) in females (Figures 2K–O and S2J).

Effects of LBP downregulation
LNP-UNA-si69108 significantly decreased liver Lbp mRNA levels in males fed an HFHS diet (84.6%, p < 0.0001) (Figure 1D), in parallel to decreased liver lipid droplet count and area, liver triglycerides, and a trend to decreased hepatocyte vacuolation (Figures 2A, 2B, and S2A) without significant effects on serum ALT levels or gene expression related to liver inflammation/liver damage (Figures S2B and S2C). Markers of liver lipogenesis (such as malonyl-CoA levels, Fasn, Acaca, Scd1, and Sreb1 gene expression, FAS protein, total ACC levels, and ACC activity [reduced phosphorylation of Ser79 in ACC]) were all significantly increased in HFHS-fed male mice and were significantly prevented by LNP-UNA-si69108 administration (Figures 2C–2G). This intervention also prevented an increased expression of genes related to NADPH production (isoctirate dehydrogenase 1 [Idh1] and cytosolic malic enzyme [Me1]) (Figure S2D), which also are important to sustain liver lipogenesis,21–23 and attenuated oxidative stress markers induced by lipid peroxidation (Gsta3, Gpx4, and Scd2 mRNA and malondialdehyde [MDA] levels) (Figures 2H and S2E). In females, LNP-UNA-si69108 administration also decreased liver Lbp mRNA levels (85.7%, p < 0.0001) (Figure 1D), liver triglycerides (Figure 2J), Scd1 mRNA, malonyl-CoA, and MDA levels and attenuated the increase in ACC protein and activity (increased phosphorylation of Ser79 ACC/total ACC ratio indicates reduced ACC activity) (Figures 2K, 2L, and 2N–2P).

These data indicate that weekly LNP-UNA-si69108 treatment for 6 months is efficacious in reducing lipid accumulation, without significant changes in liver damage (Figures S2A–S2C and S2F–S2H) or body weight (Figure S3A), compared with HFHS control mice.

LNP-Lbp UNA-siRNA treatment reduces liver lipid accumulation and lipogenesis in mice with established obesity
After observing that Lbp UNA-siRNA prevented lipid accumulation in mice fed an HFHS diet, we evaluated whether this therapy could reduce liver fat accumulation in mice with obesity induced by an HFHS diet, in which liver lipogenesis (Scd1 mRNA) was also induced. Once the mice reached a plateau of increased fat mass, weekly Lbp UNA-siRNA administration was initiated and maintained for 8 weeks (Figures S1D, S3B, and 3). This intervention in obese animals also led to reduced liver Lbp mRNA levels and parallel decreased liver lipid accumulation (lipid droplet count and area and triglycerides) both in males (Figures 3A–3C) and females (Figures 3I–3K). We also observed decreased hepatocyte vacuolation in females, with a non-significant decrease in fibrosis score and ALT levels both in males and females, without any effects on expression of liver inflammation/liver damage-related genes (except for a significant reduction in Il6 mRNA levels in males) (Figure S4). Again, Lbp UNA-siRNA also resulted in decreased lipogenic pathway, significantly reducing Fasn, Acaca, Scd1, Sreb1 mRNA, malonyl-CoA, and ACC protein in males (Figures 3D–3G) and Acaca, Scd1 mRNA, malonyl-CoA, and ACC activity in females (Figures 3L–3O). Liver MDA levels, Idh1 and Me1 (in both sexes), and Gpx4 and Sod2 mRNAs (in males) also decreased significantly (Figures 3H, 3P, S4D, S4E, S4I, and S4J).

These experiments confirmed that Lbp UNA-siRNA administration is effective in liver fat reduction in an obesity and insulin-resistance context, where the lipogenic pathway has been induced, through the specific inhibition of this pathway.

LNP-Lbp UNA-siRNA improves insulin action in HFHS-fed mice and in mice with established obesity
During development of HFHS-induced obesity, we observed increased insulin levels at weeks 4 and 8 in male compared with female mice (Figure 4A) and also in HFHS compared with control diet (CD) at week 8 but only in males (Figure 4A). Of note, LNP-UNA-si69108 treatment significantly reduced insulin increase from weeks 4 to 8 in male mice (Figure 4B). In addition, fasting glucose and homeostatic model assessment for insulin resistance (HOMAIR) were also significantly increased in male compared with female mice (Figures 4C and 4D). LNP-UNA-si69108 intervention decreased HOMAIG (Figure 4D) and improved insulin sensitivity during an insulin tolerance test (ITT) in males. This effect was not observed in females (Figures 4E and 4F).

In line with this, in mice with established obesity treated with LNP-UNA-si69108 for 8 weeks, glycemia was reduced at 30, 45, and 90 min in males and at 60 and 90 min in females, and glycemia area under the curve (AUC) was improved during ITT (Figures 4G and 4H). Similar to the previous experiment, when male and female mice were compared, impaired ITTs in male mice were found (Figures 4G and 4H).

Thus, females were more resistant to the metabolic derangement induced by HFHS, with LNP-Lbp UNA-siRNA able to improve insulin action in insulin-resistant male mice.

LNP-non-targeting UNA-siRNA did not impact on liver parameters
To discriminate off-target effects of LNP UNA-siRNA, the impact of 6-week LNP non-targeting UNA-siRNA administration on Lbp and liver parameters was assayed. No significant differences on liver Lbp gene expression and serum LBP levels, neither on liver triglycerides, lipogenesis, and markers of oxidative stress, inflammation, and liver damage, were found in mice treated with LNP non-targeting UNA-siRNA (3 mg/kg) compared with vehicle (Figure S5). These results indicated that changes on the different liver parameters were not related to the repeated dosing of the LUNAR LNPs but were specific to the Lbp knockdown.

LNP-Lbp UNA-siRNA requires the induction of hepatic Scd1 to impact on liver lipid accumulation
Current data point to the attenuation of HFHS-induced liver lipogenesis after LBP downregulation. We next studied whether the latter could also target non-alcoholic steatohepatitis (NASH) in another established mice model, the MCD24 (Figure 5). Under the MCD, weekly LNP-UNA-si69108 administration blunted liver Lbp mRNA
Scd1 mRNA was markedly suppressed (Figure 5B). Surprisingly, Lbp downregulation did not impact liver lipogenesis (Figures 5B–5G), MDA (Figure 5H), or liver triglycerides levels (Figure 5I). These data suggest that LBP knockdown prevents the worsening of liver steatosis only when liver lipogenesis/Scd1 has been previously triggered.

Lbp gene knockdown attenuated the detrimental effects of palmitate in murine hepatoma cell line

To gain insight in the potential mechanisms that underlie the positive effects of Lbp depletion on liver metabolism, in vitro experiments were performed in the murine Hepa1-6 cell line after palmitate versus vehicle (bovine serum albumin [BSA]). Lbp gene knockdown was performed using lentiviral particles with Lbp-specific short hairpin RNA (shRNA) (Figure 6A). In normal conditions (BSA), Lbp gene knockdown led to decreased Scd1, Me1, Gpx4, and Sod2, without significant effects on ACC and AMPK activity (Figures 6B–6H). Of note, palmitate (200 μM, 24 h) administration in these cells resulted in increased expression of Lbp in parallel to increased lipogenesis (including Srebf1, Scd1, Idh1, and Me1 mRNA and ACC protein and activity), oxidative stress (such as expression of Gpx4 and Sod2 mRNA), and ER stress (Synn1, Atf6, and Hspa5 mRNA) (Figures 6A–6I). Otherwise, palmitate attenuated AMPK activity (Figure 6F) and decreased metabolic activity as shown using an MTT assay (Figure 6J). Under these conditions of palmitate excess, Lbp gene knockdown prevented most of these negative effects, resulting in decreased lipogenesis (reducing Srebf1, Scd1, Idh1, and Me1 mRNA and ACC protein activity), oxidative stress (Gpx4 and Sod2 mRNA), and ER stress (Synn1, Atf6, and Hspa5 mRNA) (Figures 6A–6I).
gene knockdown reduced lipid accumulation in primary human hepatocytes

In primary human hepatocytes, LBP gene knockdown resulted in reduced intracellular lipid accumulation (Figures 7A and 7B) in parallel to decreased SCD1 mRNA levels (Figure 7C), and increased expression of β-oxidation (CPT1A and PPARA)-related genes and AMPK activity (Figures 7D–7F). LBP gene knockdown did not impact on expression of inflammation (IL6 and IL8)-related genes (Figures 7G and 7H).

Plasma LBP levels are associated with de novo lipogenic genes expression and hepatic steatosis in morbidly obese human subjects

Finally, we tested whether our observations could have translational implications in subjects with morbid obesity. Anthropometric and clinical parameters are shown in Table S1. Circulating LBP was increased in patients with non-alcoholic fatty liver disease (NAFLD) (Figure 8A). Similar to HFHS-fed mice (Figure 1D), liver LBP mRNA was not associated with NAFLD (Figure 8B). Increased plasma LBP levels were found in those participants with an increased ballooning degeneration score (Table S2). Circulating LBP concentration was positively correlated with liver LBP (r = 0.33, p = 0.01; Figure 8C). Most importantly, liver LBP mRNA and plasma LBP levels were positively correlated with expression of de novo lipogenesis-related genes (such as FASN, ACACA, and SREBF1) (Figures 8D–8G).

DISCUSSION

To the best of our knowledge, the possible impact of Lbp on diet-induced liver steatosis has only been explored in the LBP KO mouse model. The current study was designed to specifically inhibit Lbp in the liver of wild-type mice. Thus, this would be the first study, to our knowledge, testing the therapeutic potential of liver Lbp for fatty liver. The delivery of LNPs containing Lbp UNA-siRNA had a preventive impact on insulin resistance and glucose homeostasis in mice with established obesity.
effect on liver lipid accumulation after an HFHS diet in mice and reduced lipid accumulation in mice with established obesity. However, additional experiments in mice with established liver lipidosis should be required to further demonstrate this therapeutic effect. The consistency in the reduction of stearoyl-CoA desaturase (Scd1) mRNA levels in all the experimental models we used suggests that part of the beneficial effects of the treatment could be through Scd1 suppression. In fact, Scd1 is a key gene involved in triglyceride biosynthesis and upregulated in liver steatosis, and targeting of Scd1 has been shown to prevent and attenuate NAFLD progression through the inhibition of liver de novo lipogenesis and increased oxidative phosphorylation activity. In agreement with current findings, the ablation of the Scd1 gene is associated with increased phosphorylation and activity of AMPK in parallel to decreased ACC activity and malonyl-CoA levels.

Consistent with previous observations showing that disturbances in lipid metabolism and oxidative stress play an important role in MAFLD pathogenesis, we also found that markers of ROS production (MDA) associated with lipid peroxidation were significantly increased after HFHS and attenuated by LNP-Lbp UNA-siRNA administration. Peroxisomal oxidation of fatty acids is the most important contributor to ROS production in rat livers after HFHS and in patients with increased intrahepatic fat content.

A potential mechanism that would explain the beneficial effects of liver LBP depletion would be the prevention of HFHS-induced liver lipogenesis and, specially, the increased levels of Scd1. In fact, LBP depletion did not impact liver lipid accumulation in those experimental models in which liver lipogenesis/Scd1 was not triggered (standard diet) or was inhibited (MCD diet). Further confirming
these findings, the association between LBP and lipogenesis was observed in the livers from subjects with morbid obesity.

A major challenge for using LNPs as a therapeutic delivery system is the potential induction of liver damage and undesired stimulation of immune response. However, the approval of the first LNP-based siRNA therapeutic, patisiran, and more recently the LNP-mRNA based coronavirus 2019 (COVID-19) vaccine Comirnaty supports the potential of our approach as a treatment in humans. We have previously shown that the chemical properties of the LUNAR system make it a successful, safe, and potent siRNA delivery system. Here, we demonstrated that long-term treatment (6 months) with repeated LNP-Lbp UNA-siRNA weekly doses was well tolerated by the mouse liver and did not have any impact on serum ALT levels or liver fibrosis score, supporting the safety of this siRNA delivery system.

Since liver lipid accumulation is considered an important contributor to obesity-associated hepatic insulin resistance, the potential therapeutic effects of LNP-Lbp UNA-siRNA might be extended to improve insulin action. In fact, we found that LNP-Lbp UNA-siRNA also resulted in a slight, but significant, decrease of glycemia during ITT and HOMA IR, indicating positive effects improving insulin action, mainly in male mice, possibly as consequence of the reduction of liver lipid accumulation. In agreement with these findings, a recent study demonstrated that the inhibition of circulating and liver LBP in non-obese mice fed with chow standard diet resulted in improved glucose levels and glucose tolerance. The effects of a high-fat diet or in obese mice were not evaluated in this latter study. Male mice were more sensitive to HFHS-associated metabolic disturbances, showing insulin resistance as soon as week 8. LNP-Lbp UNA-siRNA administration resulted in significant metabolic benefits, reducing hyperinsulinemia, improving insulin action, and decreasing liver lipid accumulation.

**Figure 6. Impact of Lbp gene knockdown in Hepa1-6 cells**

(A–G) Effects of Lbp gene knockdown in vehicle (BSA5%, 24 h)- and palmitate-treated (200 μM, 24 h) Hepa1-6 cells on Lbp (A), Srebf1 (B), and Scd1 (C) gene expression, ACC protein (D), ACC and AMPK activity (E and F), NADPH production-, antioxidant response-, and ER stress (Syvn1, Atf6, and Hspa5)-related gene expression (G–I), and cellular viability and activity (MTT assay) (J). *p < 0.05, **p < 0.01, and ***p < 0.001 compared with shC; y p < 0.05, yy p < 0.01, and yyyy p < 0.001 compared with BSA.
accumulation in male mice but not in those with a metabolic healthy phenotype (females).

Taking into account other liver parameters, while LNP-Lbp UNA-siRNA administration for 8 weeks led to a non-significant decrease in fibrosis score, long-term (6 months) LNP-Lbp UNA-siRNA administration did not modify the fibrosis score compared with untreated mice fed with the same obesogenic (HFHS) diet. In addition, while LNP-Lbp UNA-siRNA administration for 8 weeks or 6 months in HFHS-fed mice decreased liver lipid accumulation, this intervention did not impact on the expression of liver damage and inflammation-related genes. These data suggest a disconnection between liver lipid accumulation and fibrosis/inflammation, which might be explained by the induction of lipophagy. Data from in vitro experiments in human hepatocytes suggest that findings from HFHS-fed mice might be translated to humans. Specifically, we found that LBP gene knockdown in primary human hepatocytes attenuated intracellular lipid accumulation in parallel to decreased SCD1 gene expression, and increased expression of β-oxidation-related genes and AMPK activity, without significant effects on proinflammatory cytokines.

It is important to note that the inhibition of liver LBP biosynthesis might increase the susceptibility to bacterial infection. For this reason, a precise control of LBP depletion is required. The current study demonstrates a reversible treatment, which allows a precise modulation with controlled amounts of dosing, to inhibit transient LBP production in those situations in which this protein could have a negative impact on health, such as in obesity.

To sum up, altogether current results substantiated the importance of liver Lbp gene knockdown through LNPs in the prevention and therapy of obesity-associated fatty liver, impacting on lipogenesis and constituting a potential target for MAFLD therapy.

MATERIALS AND METHODS

In vitro experiments for Lbp siRNA selection
Silencer Select Pre-designed siRNA assays si69107, si69108 and si69109 targeting mouse Lbp mRNA were purchased from Ambion (Life Technologies). Those same siRNA sequences were chemically modified by introducing 2′-O-methyl RNA bases, phosphorothioate linkages, and UNA to generate UNA siRNA (Integrated DNA Technologies, Coralville, IA, USA). siRNA sequences and chemical modifications are detailed in Table S3. Hepa1-6 cells were seeded on 96-well plates the day before transfection at a density of 10^4 cells per well. Following vendor recommended protocol, Lipofectamine RNAiMax was used to transfect siRNAs at different concentrations.
After 48 h, cells were washed with PBS, and EZCt Cells2Ct Direct Lysis Buffer (Lifeome, Oceanside, CA, USA) was added following the manufacturer’s suggested protocol.

**Preparation of LUNAR-Lbp UNA-siRNA**

Using LUNAR technology, a proprietary lipid enabled nucleic acid delivery platform, Arcturus Therapeutics (San Diego, CA, USA) produced LUNAR particles encapsulating Lbp-UNA siRNA as described previously. LUNAR is composed of four lipid components: Proprietary Arcturus Therapeutics’s lipid (ATX), cholesterol, a phospholipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and a pegylated lipid. The ATX lipid contains an ionizable amino head group and a biodegradable lipid backbone. The ionizable amino head group provides the lipid with a pKₐ of <7. At acidic pH, the amino group is protonated and interacts with the negatively charged RNA, thus forming nanoparticles and encapsulating the RNA. However, at physiological pH, which is above the pKₐ of the amino head group, LUNAR nanoparticles stand neutral charge, attenuating the toxicity commonly observed with positively charged cationic transfection vectors. The pH sensitivity of the amino head group also enables protonation of the lipid once inside the endosomes, thereby promoting their interaction with the oppositely charged anionic endosomal lipids, causing destabilization of the endosomal membrane and release of RNA payload into the cytosol. In addition, ester groups, incorporated into the lipidic backbone of ATX lipids, produce ester bonds that have good chemical stability at physiological pH but can be easily cleaved by esterases inside tissue and intracellular compartments once the load has been delivered. Finally, the remnant hydrophilic cleavage products can be quickly metabolized. 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 (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: 2,000) (NOF America, White Plains, NY, USA). Lipid solution was then combined with UNA siRNA solution using a Nanoassemblr microfluidic device (Precision NanoSystems, Vancouver, BC, Canada) at a flow rate ratio of 1:3 ethanol/aqueous phases. The mixed material was then diluted with 3 × volume of 10 mM Tris 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 (mPES Kros membranes, 100 Kd MWCO, Spectrum Laboratories, Rancho Dominguez, CA, USA), followed by diafiltration against 10 volumes of 10 mM Tris buffer (pH 7.4) containing 9% sucrose. Post diafiltration, formulations were then concentrated to

Figure 8. Association of liver LBP with expression of de novo lipogenesis genes in morbidly obese human subjects

(A and B) Plasma LBP concentration (A) and liver, SAT, and VAT LBP mRNA levels (B) according to NAFLD. (C) Bivariate correlations between liver LBP mRNA levels and plasma LBP concentration in morbidly obese participants. (D–F) Bivariate correlations between liver LBP and SREBF1 (D), FASN (E), and ACACA (F) mRNA levels. (G) Bivariate correlations between plasma LBP concentration and liver FASN mRNA levels.
desired UNA-siRNA concentration, followed by filling into vials and freezing. Formulations were characterized for particle size, UNA-siRNA content, and encapsulation efficiency, as detailed in Table S4. 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 with the total RNA content that is obtained upon lysis of the particles by 1% Triton X-100 (Ft), where the percentage of encapsulation = (Ft – Fi)/Ft × 100.

Mice experiments

Pilot experiment

Eight-week-old male C57/BL6J mice (n = 25) were housed for 2 weeks under standard conditions of light (12-h light/12-h dark cycle) and temperature (22°C ± 1°C). Next, one intravenous injection into the tail of the following treatments was performed: (1) vehicle (PBS), (2–3) chemically unmodified (1 and 3 mg/kg), and (4–5) UNA-containing chemically modified (1 and 3 mg/kg) Lbp siRNA complexed by LUNAR delivery platform. The effect of this injection was evaluated at day 3, 6, 10, and 12, and blood was collected in citrate tubes for preparation of plasma each day. The weight of mice was measured at 8 (day – 14 of injection) and 10 (day 0 of injections) weeks and 12 days after injections. Finally, at week 12, in the same mice, a second injection was performed, and 3 days later, mice were killed by suffocation under sedation, and liver was removed, immediately frozen in liquid nitrogen, and stored at –80°C until processing for RNA extraction.

Metabolic experiment

Eight-week-old male and female C57/BL6J mice (n = 46) were housed for 25 weeks under standard conditions of light (12-h light/12-h dark cycle) and temperature (22°C ± 1°C) in the following experimental conditions: (1) non-treated, CD-fed mice (TD.120455, 3.3 Kcal/g, ENVIGO) for 25 weeks; (2) non-treated, HFHS diet-fed mice (HFHS/obese vehicle; TD.08811, 4.7 Kcal/g, ENVIGO) for 25 weeks; (3) weekly LUNAR-Lbp UNA-siRNA-treated (3 mg/kg), HFHS-fed mice for 25 weeks (HFHS + siLbp); and (4) weekly LUNAR-Lbp UNA-siRNA-treated (3 mg/kg), HFHS-fed mice only in the last 8 weeks (obese + siLbp) (Figures S1A–S1D). To discriminate off-target effects from the carrier (LNPs), mice from the non-treated experimental conditions (1–2 or 4 in the first 16 weeks) were injected weekly with the corresponding buffer as the vehicle. Body weight was recorded weekly. Serum and plasma were collected at 4 and 8 weeks. ITT was performed at week 20. Briefly, insulin (Actrapid; Novo Nordisk Pharma A/S, Bagsvaerd, Denmark) in saline solution was administered intraperitoneally (0.75 UI/kg) to mice, and glycemia in blood obtained from the tail was measured 15, 30, 45, 60, and 90 min after glucose injection. At week 25, after overnight fasting, mice were sacrificed by suffocation under sedation. Then, blood serum and plasma, liver, inguinal WAT (iWAT), and perigonadal WAT (pgWAT) were collected, immediately frozen in liquid nitrogen, and stored at –80°C until processing for RNA or protein analysis. A piece of liver was also fixed with 4% formalin for 24 h and then stored in 70% ethanol at 4°C for histological analysis.

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). In pilot and metabolic experiments, animal protocols were approved by the Ethical Committee for Animal Experimentation of Barcelona Science Park (PCB) and the University of Barcelona (experimental project approval number: 9955).

Standard control and MCD experiment

Eight-week-old male C57BL/6J mice (n = 28) were kept under 12-h light/12-h dark cycle and had ad libitum access to standard diet or MCD diet (A02082002BR, Research Diets) for 6 weeks. In the study, mice received weekly intravenous injections of non-targeting control siRNA (siRNA control) or siRNA-Lbp (3 mg/kg) for 6 weeks, and we also used the corresponding buffer as the vehicle to discriminate off-target effects from the non-targeting control siRNA (Figure S5). Sequence and validation of this non-silencing control siRNA with the same LUNAR formulation was previously reported. Food intake and body weight were monitored weekly. In these experiments, animal protocols were approved by the Ethical Committee for Animal Experimentation of the University of Santiago de Compostela.

Lentiviral shRNA-Lbp particles production

Four different shLbp (clone set against mouse Lbp, NM_008489.2) primer sequences and random negative control (NC) sequences that did not have targets for any gene were synthesized by Tebu-bio (Tebu-bio, Spain, SL). Lentivirus-targeted Lbp was obtained by co-transfection 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. shRNA sequence for knockdown Lbp gene is detailed in Table S3. Obtained lentiviruses were used to treat Hepa1-6 cell line. Lentivirus effectiveness was confirmed in Hepa1-6 cells.

In vitro experiments with lentiviral shRNA-Lbp particles

The mouse hepatoma Hepa1-6 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4,500 mg/L glucose, 10% fetal bovine serum (Gibco), 100 units/mL penicillin and streptomycin, 1% glutamine, and 1% sodium pyruvate, at 37°C and 5% CO2 atmosphere. Gene silencing was achieved using Lbp-targeted and control shRNA lentiviral particles. Stable clones expressing the shRNA were selected by puromycin dihydrochloride. Treatments were performed in Hepa1-6 for 24 h after seeding. Fatty acid accumulation was induced by palmitate exposure as follows: 27.84 mg of palmitate (Sigma, St. Louis, MO, USA) were dissolved in 1 mL sterile water to make a 100 mM stock solution. An aliquot of 5% BSA was prepared in serum-free DMEM. One-hundred mM palmitate stock solution and 5% BSA were mixed for at least 1 h at 40°C to obtain a 5 mM solution. Hepa1-6 cells were treated with 0.2 mM palmitate for 24 h. BSA-supplemented medium was used as vehicle. Four biological replicates were collected from 2 independent experiments.
In vitro experiments in primary human hepatocytes

Commercially available (Innoprot, Bizkaia, Spain) primary human hepatocytes (HHs) were cultured with hepatocyte medium (Innoprot) supplemented with 5% fetal bovine serum (FBS), 1% hepatocyte growth supplement (i.e., an optimized combination of growth factors, hormones, and proteins required for primary hepatocyte cultures), and 100 units/mL penicillin and streptomycin (P/S). HHs were grown on fibronectin pre-coated dishes at 37°C and 5% CO2 atmosphere. Twenty-four h after seeding, HHs were forward transected with siRNAs for 72 h. Briefly, the siRNA (Sigma-Aldrich, St. Louis, MO, USA) against LBP and Lipofectamine RNAiMAX (Life Technologies, Darmstadt, Germany) were diluted separately with Opti-MEM I Reduced Serum Medium (Life Technologies, Darmstadt, Germany) and mixed by pipetting afterward. The siRNA-RNAiMAX complexes were left to incubate for 20 min at room temperature and subsequently added on the top of the adherent cells drop-wise. The final concentrations of Lipofectamine RNAiMAX and esiRNAs were 1.6 μl/cm2 and 75 nM, respectively, in 24-well cell culture plates, and the final amount of medium per well was 1 mL. Hepatocytes were harvested 72 h after transfection without changing cell culture medium. Transfection efficiency was assessed by real-time PCR. The siRNAs (Sigma-Aldrich) used were human LBP (SA-SI_Hs02_0033699) and the MISSION siRNA Universal Negative Control #1 (Sigma-Aldrich, SIC001) as non-targeting siRNA. For quantification of lipid droplets, at the end of the experiment, HHs were fixed with paraformaldehyde 4% for 1 h. Cells were dipped in 60% isopropanol before they were dried and stained with oil red O (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature. Pictures were taken using Nikon Eclipse Ts2 inverted microscope (100× total magnification). Absorbance was measured at 500 nm. All experimental conditions were tested in 6 biological replicates.

Gene-expression analysis

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, USA). Gene expression was assessed by real-time PCR using a LightCycler 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, Waltham, MA, USA) used were as follows: endogenous control 18S and target gene mouse LBP (Lbp; Mm00493139_m1); fatty acid synthase (Fasn; Mm00662319_m1); stearoyl-CoA desaturase 1 (Scd1; Mm00772290_m1); acetyl-CoA carboxylase alpha (Acaca; Mm01304257_m1); sterol regulatory element binding transcription factor 1 (Srebf1; Mm00550338_m1); isocitrate dehydrogenase 1 (NADP(+)-dependent, cytosolic (Me1; Mm00782380_s1); glutathione S-transferase, alpha 3 (Gsta3; Mm00497498_m1); glutathione peroxidase 4 (Gpx4; Mm00515041_m1); superoxide dismutase 2, mitochondrial (Sod2; Mm01313000_m1); synovial apoptosis inhibitor 1, synoviolin (Ssyn1; Mm00511995_m1); activating transcription factor 6 (Atf6; Mm01295316_m1); and heat shock protein 5 (Hspa5; Mm00517691_m1). In humans, peptidylprolyl isomerase A (cyclophilin A) (4333763, PPIA as endogenous control); LBP (Lbp; Hs01084621_m1); fatty acid synthase (FASN; Hs00188012_m1); acetyl-CoA carboxylase alpha (ACACA; Hs01046047_m1); sterol regulatory element binding transcription factor 1 (SREBF1; Hs02561944_s1); stearoyl-CoA desaturase (SCD1; Hs01682761_m1); peroxisome proliferator activated receptor alpha (PPARA; Hs00947536_m1); carnitine palmitoyltransferase 1A (CPT1A; Hs00912671_m1); interleukin-6 (IL-6; Hs00985639_m1); and C-X-C motif chemokine ligand 8 (CXCL8 or IL-8; Hs00174103_m1) were used.

The information of all primer/probe sets used is available on the Thermo Fisher Scientific website and can be obtained using the assay reference for each gene.

Histological procedures

Masson's trichrome staining

Tissue processing and a standard Masson's trichrome staining was performed at Allele Biotechnologies (San Diego, CA, USA). After stain, slides were examined by a certified pathologist at HistoTox Labs (Boulder, CO, USA), who evaluated and quantified the degree of fibrosis. LD area and number were assessed using Fiji (NIH). A minimum of four random regions of interest (ROIs) of 151.719 μm² were created from each image, and the area and number of the LDs contained in the ROI was retrieved and analyzed.

Quantification of triglycerides in liver

Liver (−20 mg) samples 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 × g for 5 min. Triglycerides were measured in the supernatant, which was diluted 10-fold with water before assay, using the Triglyceride Quanti kit (Biovision, Milpitas, CA, USA) following the manufacturer’s protocol.

Malonyl-CoA measurement

Liver (−20 mg) was homogenized in PBS and centrifuged for 20 min at 2,000–3,000 RPM, and supernatant was used to measure malonyl-CoA levels using Mouse Malonyl-CoA ELISA KIT (cat. no. EK18900, Signalway Antibody, Greenbelt, MD, USA) and strictly following the manufacturer’s protocol.

ACC and AMPK activity

ACC and AMPK activities were inferred through the ratio of phosphorylation in serine 79 for ACC (pSer79 ACC) and in threonine 172 for AMPK (pThr172 AMPK) versus total ACC and AMPK protein amount, respectively. Increased pSer79 ACC /total ACC indicates decreased ACC activity, whereas increased pThr172 AMPK /AMPK is proportional to AMPK activity. pSer79 ACC, total ACC, pThr172 AMPK, and AMPK were measured by PathScan Phospho-Acetyl-CoA Carboxylase (Ser79) Sandwich ELISA Kit (#9868C); PathScan Total Acetyl-CoA Carboxylase Sandwich ELISA Kit (#9766C); PathScan Phospho-AMPK (Thr172) Sandwich ELISA Kit (#9795C); and PathScan total...
AMPKz Sandwich ELISA Kit (#7961C, Cell Signaling, Danvers, MA, USA). Liver (~30 mg) and Hepa1-6 cells (~3.5 × 10⁵ cells) were homogenized in PathScan Sandwich ELISA Lysis Buffer (1 ×) containing phenylmethylsulfonyl fluoride (0.2 mM, P7626, Merck Life Science, Madrid, Spain). Tissue lysates were centrifuged at 1,500 × g for 5 min at 4°C to remove tissue and cell debris, protein content was quantified using the Lowry method, and tissue and cell lysates were diluted at the same recommended protein concentration (0.3 mg/mL for total ACC and AMPKz and 1 mg/mL for phosphorylated ACC and AMPKz). Test procedure performed following the manufacturer’s protocol.

**Western blot analysis**
Liver (30 mg) was homogenized in lysis cell 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). Tissue lysates were centrifuged at 1,500 × g for 5 min at 4°C to remove cell debris, and protein content was quantified using the Lowry 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 4°C to primary antibodies anti-fatty acid synthase (FAS [C20G5], rabbit monoclonal antibody [mAb] #3180) at 1/1,000 dilution (Cell Signaling, Hilden, the Netherlands) with intra- and interassay coefficients of variation <8%. Serum glucose levels were measured in duplicate by the glucose oxidase method with a Beckman Glucose Analyzer 2 (Beckman Instruments, Brea, CA, USA). 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). Glycosylated hemoglobin (HbA₁c) was measured by the high-performance liquid chromatography method (Bio-Rad, Muenchen, Germany), and autoanalyzer Jokoh HS-10, respectively. Intra- and inter assay CVs were <4% for all these tests. C-reactive protein (ultrasensitive assay; 110 Beckman, Fullerton, CA, USA) was determined by a routine laboratory test.

**Human study**
Fifty-eight morbidly obese subjects were recruited from the ongoing multicenter FLORINASH Project. Participants were recruited at the Endocrinology Service of the Hospital Universitari Dr. Josep Trueta (Girona, Spain). Anthropometric and biological parameters are described in Table S1. Inclusion criteria were age 30 to 65 years and ability to understand study procedures. Exclusion criteria were systemic diseases, infection in the previous month, serious chronic illness, >20 g ethanol intake/day, or use of medications that might interfere with insulin action. Liver and subcutaneous and adipose tissue (SAT and VAT, respectively) tissue samples were collected and snap frozen in liquid nitrogen for gene-expression analysis. Liver samples were also fixed in formalin for the histological assessment. Fixed samples were stained with hematoxylin and eosin and Masson’s trichrome stain. All samples were evaluated by the same pathologist according to the degree of steatosis. Then, participants were stratified as subjects without significant steatosis (<5%), “borderline” (5%–33%), and subjects with significant steatosis (>33% of fat). Additional exclusion criteria in this subgroup of participants included cirrhosis or bridging fibrosis, a liver biopsy less than 2-cm long, and the use of statins. This study was carried out in accordance with the recommendations of the ethical committee of the Hospital of Girona Dr. Josep Trueta. The protocol was approved by the ethical committee of the Hospital of Girona Dr. Josep Trueta (approval number: 27052013). All subjects gave written informed consent in accordance with the Declaration of Helsinki after the purpose of the study was explained to them.

**Serum/plasma measurements**
Plasma LBP (HK205-02, LBP mouse ELISA kit, Hycult Biotech, Plymouth Meeting, PA, USA), insulin (90080, Crystal Chem, Zaandam, the Netherlands), glucose (Accutrend; Roche Diagnostics, Mannheim, Germany), and ALT activity (Alfa Wasserman/Vet Axxel Reagent SA1046) were measured using commercial kits according to manufacturer’s instructions.

In 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%. Serum glucose levels were measured in duplicate by the glucose oxidase method with a Beckman glucose analyzer 2 (Beckman Instruments, Brea, CA, USA). 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). Glycosylated hemoglobin (HbA₁c) was measured by the high-performance liquid chromatography method (Bio-Rad, Muenchen, Germany), and autoanalyzer Jokoh HS-10, respectively. Intra- and inter assay CVs were <4% for all these tests. C-reactive protein (ultrasensitive assay; 110 Beckman, Fullerton, CA, USA) was determined by a routine laboratory test.

**Statistical analysis**
Statistical analyses were performed using the SPSS 12.0 software. 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 analyzed by simple correlation (using Spearman’s and Pearson’s tests). One-factor ANOVA was used to compare clinical variables and liver LBP gene expression relative to parameters related to liver pathology. In mice experiments, 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.
Data availability statement
All data generated or analyzed during this study are included in this published article (and its supplemental information).

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.08.003.

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AUTHOR CONTRIBUTIONS
R.D.-T., M.G., J.M.F.-R., and J.M.M.-N. participated in study design and analysis of data. J.L., R.D.-T., F.C., A.G.-N., E.M., N.D., S.M.-R., R.M., F.O., A.C.-N., N.O.-C., P.P.K., and J.M.M.-N. participated in acquisition of data. J.L., R.D.-T., A.G.-N., E.M., N.D., W.R., K.T., P.C., F.V., M.L., M.G., J.M.F.-R., and J.M.M.-N. participated in interpretation of data. R.D.-T. and J.M.M.-N. wrote and edited the manuscript. M.L., K.T., F.V., M.G., and 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
R.D.-T., R.M., P.P.K., K.T., and P.C. are employees of Arcturus Therapeutics. The authors declared no additional conflict of interest.

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