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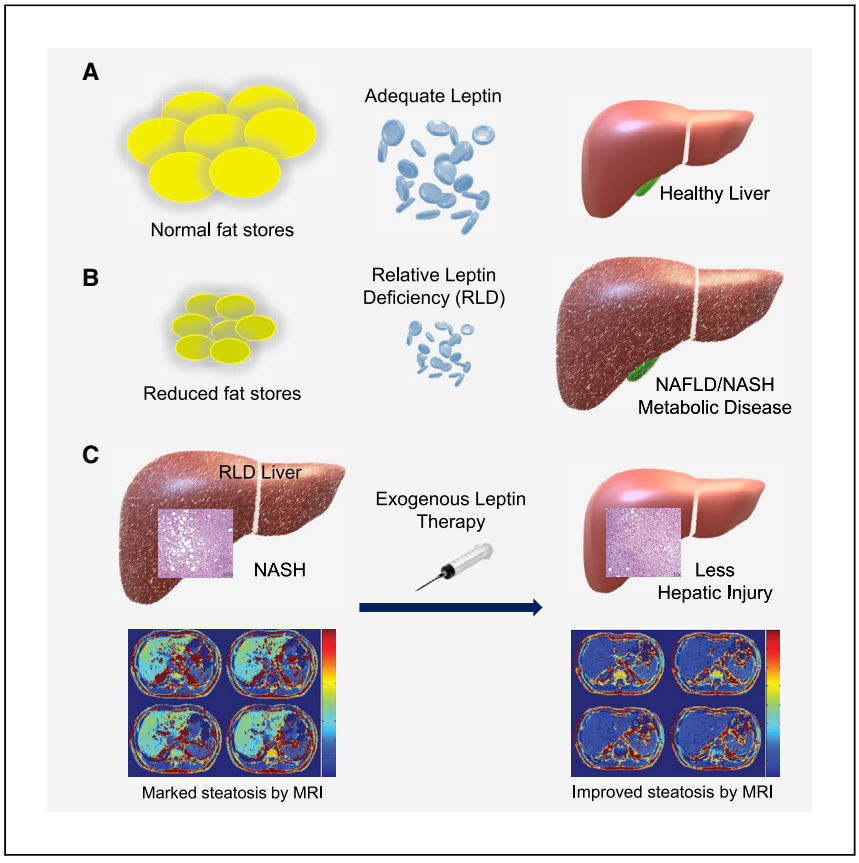
Metreleptin therapy for nonalcoholic steatohepatitis: Open-label therapy interventions in two different clinical settings



In two different clinical settings with relative leptin deficiency, Akinci et al. show that exogenous leptin therapy resulted in significant decreases in liver fat content on MRI and global nonalcoholic steatohepatitis scores on liver biopsies, suggesting the therapeutic window for leptin therapy may be wider than severe leptin deficiency.

Translation to Patients

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Highlights

Some patients with nonalcoholic steatohepatitis have relatively low leptin levels

In this subgroup, leptin therapy leads to reduced hepatic fat and lower NASH scores

Similar improvements are observed in a group of subjects with partial lipodystrophy

The therapeutic benefit of leptin may go beyond severe leptin deficiency

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Metreleptin therapy for nonalcoholic steatohepatitis: Open-label therapy interventions in two different clinical settings

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SUMMARY

Background: Recombinant leptin therapy reverses nonalcoholic steatohepatitis (NASH) in leptin-deficient lipodystrophy. We inquired if leptin therapy would improve NASH in more common forms of this heterogeneous condition.

Methods: Nine male patients with relative leptin deficiency (level, <25th percentile of body mass index- and sex-matched United States population) and biopsy-proven NASH and 23 patients with partial lipodystrophy and NASH were recruited for 2 distinctive open-label trials. Participants received leptin therapy in the form of metreleptin for 12 months. The primary endpoints were the global NASH scores from paired liver biopsies scored blindly.

Findings: Of 9 participants recruited in the relative leptin deficiency treatment study, 7 completed 12 months of therapy. Mean global NASH scores were reduced from 8 $\pm$ 3 to 5 $\pm$ 2 (range, from 1 to 6; $p = 0.004$). In the partial lipodystrophy study, 19 of 22 subjects completed 12 months of treatment and 18 completed a second liver biopsy. Global NASH scores also reduced significantly from 6 $\pm$ 2 to 5 $\pm$ 2 (range, from -2 to 4; $p = 0.008$). In both studies, the predominant changes were in steatosis and hepatic injury scores.

Conclusions: Our findings show that patients with NASH associated with both relative leptin deficiency and partial lipodystrophy have reductions in hepatic steatosis and injury in response to exogenous leptin therapy. Moreover, leptin deficiency may have regulatory effects in mediating fat deposition and ensuing injury in the liver.

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Context and significance

Fat goes to the liver when the body has limited fat storage capacity, damaging liver cells. Fat stores are communicated to the rest of the body through the fat cell hormone leptin. We have been interested in identifying groups of patients with fatty liver disease and relatively low leptin levels in order to test if exogenous administration of recombinant leptin would impact the amount of fat and the degree of cell damage and scarring in the liver. In this report, we describe the improvement observed in 2 of these subgroups with less fat and cellular injury after a year of therapy with leptin, suggesting that there may be a role for boosting leptin levels or leptin signal for fatty liver disease in select circumstances.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) covers a spectrum of diseases that range from simple hepatic steatosis to the more aggressive nonalcoholic steatohepatitis (NASH), which involves inflammation and/or fibrosis.[1] NAFLD is an emerging public health problem that is being recognized in up to one-third of the general population,[2–4] including teenagers and young adults. NAFLD is now considered the leading cause of abnormal liver function tests and chronic liver disease in adults from the United States.[1,3]

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An estimated 20% to 30% of patients with simple steatosis progress to NASH, which in turn can progress to cirrhosis, hepatocellular cancer, and liver failure.[1,5] Hepatic steatosis also appears to forecast future diabetes and cardiovascular disease.[6] Currently, there is no approved drug for the treatment of NAFLD, and lifestyle modifications remain the standard of care. Diet and exercise in association with weight loss appear to be effective in ameliorating steatosis in humans with or without diabetes, but long-term adherence to lifestyle interventions is difficult and costly even if successful.[7,8] Although vitamin E and thiazolidinediones have shown some encouraging preliminary results, there are potential drawbacks for their long-term use.[8,9] Obeticholic acid, a selective farnesoid X receptor (FXR) agonist, demonstrated improved histological features of NASH over 72 weeks in a phase 2 study of patients with noncirrhotic NASH;[10] however, obeticholic acid is not currently approved for this indication.[11] Given that there are no approved therapies to slow or halt the progression of NASH and that the extent of the affected portion of the population is quite large, new therapies are urgently needed to treat these conditions.

Leptin plays a central role in the regulation of body weight and adaptations to caloric restriction.[12] In addition to its crucial role in regulating energy expenditure, it also plays an important role in the regulation of insulin sensitivity.[13,14] We and others have previously shown that patients with non-HIV lipodystrophy, a rare heterogeneous cluster of syndromes characterized by a paucity of adipose tissue, insulin resistance, and hypertriglyceridemia due to either genetic or other acquired reasons, showed a marked amelioration in NASH scores,[15,16] in addition to the metabolic improvement when treated with recombinant leptin.[17]

In this study, we primarily sought to determine if leptin could play a role in the treatment of more commonly observed forms of NASH. Because the past therapeutic experience of leptin in obesity was disappointing due to postulated leptin resistance,[18] our approach was to identify NASH patients with relative leptin deficiency (RLD) among a cohort of biopsy-proven NASH patients followed at our institution who were nondiabetic and noncirrhotic. RLD was defined as a leptin level in the lower 25th percentile for BMI. We then initiated a 12-month, open-label, prospective pilot study of therapy with recombinant methionyl-human leptin (metreleptin [Myalept] in 9 male patients with NASH and RLD and assessed clinical and histological responses. We also provided data on potential molecular pathways in the liver following metreleptin treatment. Results from this small study may provide a rationale to investigate the therapeutic utility for this peptide or other analogs for the treatment of NASH on a larger scale.

In a separate cohort, we examined the efficacy of metreleptin in NASH associated with partial lipodystrophy (PL). Most prior studies evaluating the effects of metreleptin on metabolic and hepatic parameters included only those patients with baseline low leptin levels. We aimed to further characterize the effects of metreleptin therapy on the liver disease associated with PL in a more diverse group of patients than previously studied. The simultaneous presentation of both cohorts provides comprehensive documentation of systematic liver biopsy investigations in humans who have been treated with metreleptin for 12 months.

RESULTS

The RLD study

Demographics and disease characteristics of RLD

Fifty nondiabetic, noncirrhotic subjects with biopsy-proven NASH who were followed in our health care system (University of Michigan Medical School, Ann Arbor,

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Michigan) on a regular basis were identified from 2005–2009. Most of the cohort (47/50) were Caucasian. The mean age was 38 ± 9 (range, 29–63) years; 27 (54%) were male and 23 (46%) were female. The mean BMI was 31.6 ± 5.5 kg/m^2 (range, 24.5–42.0 kg/m^2), and the mean leptin level was 26.2 ± 14.0 ng/mL (range, 2.1–72.9 ng/mL).

Figure 1A demonstrates the relationship for the entire cohort between leptin levels and body adiposity as measured by a dual-energy X-ray absorptiometry (DEXA) scan. As in many other populations, leptin levels demonstrate a significant relationship with body adiposity in both men ($r = 0.430$, $p = 0.030$) and women ($r = 0.540$, $p = 0.010$) with NASH.

Subjects with RLD (n = 11 [22%]) were defined based on criteria in Table S1. This group classification was based on leptin levels measured by the historical Linco radioimmunoassay (RIA) (see STAR Methods). Subjects with RLD were mostly male (10/11) with lower subcutaneous fat volumes on a single-slice computed tomography (CT) study. There was a trend toward higher levels of circulating free fatty acid levels in the RLD group, but the differences were not statistically significant (data not shown).

These results established the feasibility of a pilot intervention study in the RLD group. It was interesting that when subjects with NASH were evaluated for leptinemia, almost 40% of our predominantly Caucasian male population met the definition of RLD, whereas there was only 1 female who met the definition. This finding of sexual dimorphism suggested that there was a difference in the threshold for hepatic fat deposition and subsequent NASH between the 2 sexes and confirmed to us that the 2 sexes should be studied separately. Given that it was feasible to treat a small cohort among the males, we decided to focus on males in the subsequent intervention study.

Study design, population baseline characteristics, and flow through the study

Nine male subjects from the RLD group with biopsy-proven NASH in the above cross-sectional study were included in the open-label pilot treatment study and were the only recruitment pool used for this study (Figure 1B). The definition of NASH grading is presented in Table S2. The hypothesis to be tested was that metreleptin would ameliorate the global NASH scores after 12 months of treatment in the RLD population with NASH. Eight participants would have given us 80% power with an alpha of 0.05 to detect the same degree of improvement noted in the NIH cohort,[17] with a mean effect size of 3-point reduction in global NASH score and an SD of 2. We aimed to recruit 10 of the 11 subjects (all males) identified to display RLD, but we could enroll only 9 from 2009 to 2011. One patient from the recruitment pool did not want to participate, as he wanted to pursue metabolic surgery for his high BMI. The study was completed in 2012. Treatment was delivered at a dose of 0.1 mg/kg/day in an open-label fashion by daily subcutaneous, patient self-administered injections. The maximum dose was 10 mg/day. The mean metreleptin dose was 9.02 ± 0.74 mg/day. Secondary outcomes were hepatic fat percent by proton density fat fraction as well as spectroscopy, body weight, and body fat. Other endpoints of interest were metabolic parameters of insulin sensitivity, fasting glucose, and resting energy expenditure (REE). Molecular changes in the liver, incretin hormone levels, and markers of inflammation, as well as circulating free fatty acid species, were studied as exploratory outcomes.

Pre-treatment characteristics of participants are presented in Table 1. The mean age of the subjects at enrollment was 43 ± 6, ranging from 32 to 53 years. The mean BMI

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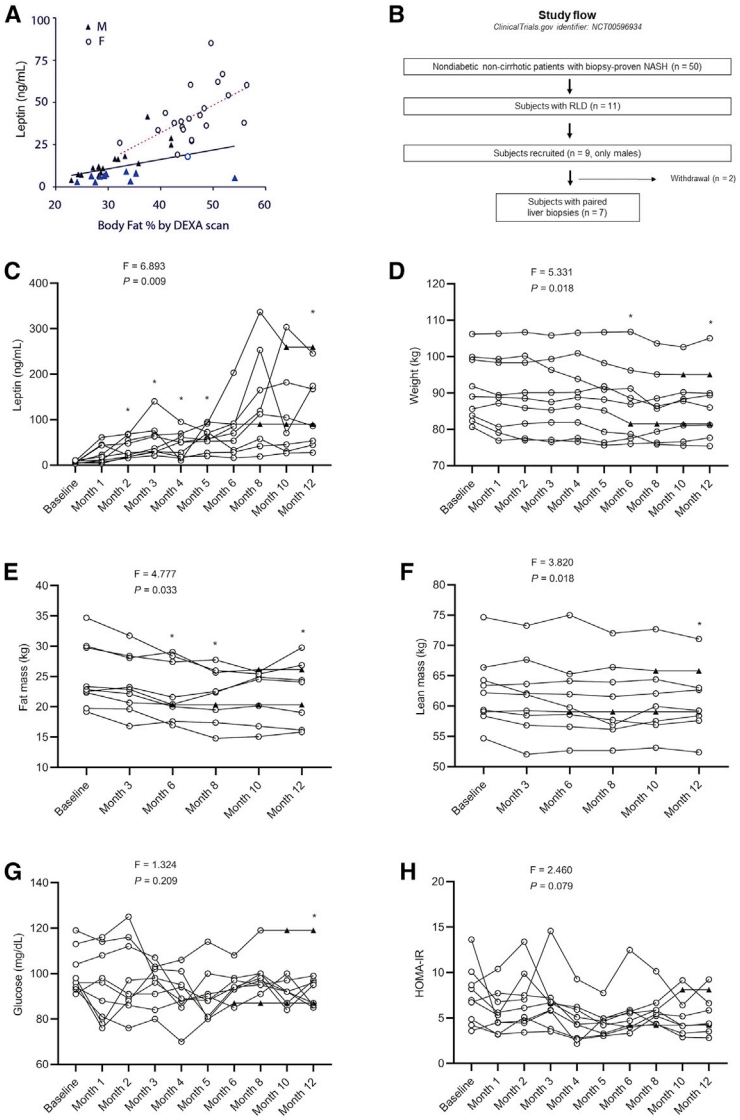

Figure 1. Correlation of circulating leptin levels with adiposity in patients with NASH, the RLD study design, and the effect of metreleptin therapy on metabolic parameters in the RLD study

(A) Serum leptin levels against total body fat percentage determined by DEXA scan in 50 NASH patients. F, females (r = 0.540, p = 0.010); M, males (r = 0.430, p = 0.030). Blue symbols show 11 patients with RLD.

(B) The RLD study design. For metabolic and body composition parameters, data were carried forward. For liver related parameters, a decision was made to conduct only completer analysis because liver biopsy was done only at baseline and at month 12, and withdrawal reasons may have confounded the observations.

(C) Effect of metreleptin therapy on leptin levels throughout the study period. Note that levels after month 3 may be confounded by circulating antidrug antibodies.

(D–H) Changes from baseline in weight (D), fat percentage (E), lean body mass percentage (F), glucose (G), and HOMA-IR (H) studied at the indicated times. We report the F-statistic and p value from a repeated-measures ANOVA. *, indicates p values that are significant versus baseline with post hoc paired sample t test after multiplicity correction. A paired t test was used to compare month 12 values to baseline (without multiplicity correction), as the change at 12 months versus baseline was a prespecified endpoint. ▲▲▲, shows specific data points where the last observation was carried forward.

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Table 1. Characteristics of subjects at baseline and 12 months after metreleptin treatment in the RLD study (n = 9)

Parameters	Baseline	Month 12	p value
Body weight (kg)	90.9 ± 8.7	86.8 ± 9.3	0.012
Total fat mass (kg)	24.9 ± 5.3	22.5 ± 4.9	0.048
Total lean mass (kg)	62.5 ± 5.8	61.0 ± 5.4	0.010
Leptin (ng/mL)	7.0 ± 2.4	127.5 ± 87	0.003
Glucose (mg/dL)	100 ± 10	95 ± 11	0.038
Triglycerides (mg/dL)	129 ± 66	148 ± 69	0.440
HOMA-IR	7.43 ± 3.16	5.45 ± 2.17	0.097
FFA (mEq/L)	0.44 ± 0.15	0.55 ± 0.31	0.414
REE (kcal)	1746 ± 307	1795 ± 247	0.475
RQ	0.80 ± 0.05	0.80 ± 0.05	0.710
Adiponectin (μg/mL)	6.44 ± 2.35	6.42 ± 2.32	0.962
GIP (pg/mL)	52.68 ± 37.29	27.77 ± 7.60	0.044
GLP-1 (pg/mL)	4.81 ± 2.16	4.62 ± 1.83	0.420
Ghrelin (pg/mL)	645.26 ± 497.24	786.20 ± 457.40	0.256
IL-6 (ng/mL)	1.71 ± 1.20	1.34 ± 0.99	0.373
sLEPR (ng/mL)	19.90 ± 6.1	19.83 ± 4.84	0.957

Mean ± SD values are shown (even for nonnormally distributed data). p values were calculated by using a paired-sample t test. Log transformation was applied if needed. FFA, free fatty acids; HOMA-IR, homeostatic model assessment of insulin resistance; GIP, glucose-dependent insulinotropic peptide; GLP-1, Glucagon-like peptide 1; REE, resting energy expenditure; RQ, Respiratory quotient; sLEPR, soluble leptin receptor.

was 28.2 ± 1.6 kg/m^2, ranging from 26.4 to 30.8 kg/m^2 (i.e., overweight or grade 1 obesity). Baseline circulating leptin levels as measured on an enzyme-linked immunosorbent assay (ELISA) (see STAR Methods) ranged from 3.6 to 10.9 ng/dL; values from the cross-sectional RLD study were slightly lower, as is typical for the change in methodology. Seven of the 9 subjects were on lipid-lowering agents (fibrates and/or statin or fish oil) for dyslipidemia. Three of the 9 participants had impaired fasting glucose levels, and 1 patient was treated with metformin for impaired glucose tolerance. Two of the 9 subjects were on antihypertensive treatment. Baseline medications were kept constant during the 1-year study period except for incidental use of analgesics and/or antibiotics for minor non-study-related intercurrent events. Seven of the nine participants completed one year of the study while two discontinued the treatment at 6 and 10 months of the protocol respectively (Figure 1B). One patient withdrew due to the inability to comply with daily injections and study visits, and the other due to the discovery of exclusion criterion (substantial alcohol intake) by the study team.

Effects of metreleptin on body composition and vital signs

Table 1 also compares the characteristics of subjects before and 12 months after metreleptin. Metreleptin therapy resulted in an increase in measured leptin levels when compared to baseline (Figure 1C), though these levels are confounded with the presence of antidrug antibodies after month 3 (Table S3). Participants lost an average of 4.4% ± 2.7% and 4.5% ± 3.9% of body weight at 6 and 12 months, respectively. Weight loss was statistically significant at months 6 ($p = 0.002$) and 12 ($p = 0.012$) compared to baseline (Figure 1D). The predominant weight loss was from the fat compartment ($p = 0.002$ at month 6, $p = 0.009$ at month 8, and $p = 0.048$ at month 12; Figure 1E), but there was loss of lean mass as well ($p = 0.010$ at month 12; Figure 1F). Blood pressure and heart rate were not impacted by metreleptin therapy, although some subjects were on blood pressure medications (data not shown).

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Effects of metreleptin on metabolic parameters, circulating hormones, and cytokines

Leptin slightly reduced fasting glucose levels ($p = 0.038$ at month 12; Figure 1G). A slight improvement was observed in insulin sensitivity, as evidenced by a reduction in homeostatic model assessment of insulin resistance (HOMA-IR) (Figure 1H); however, it was not statistically significant. Reported food intake on average was reduced by 19% from baseline at 3 and 6 months, but this did not reach statistical significance due to the large variation in the food intake (data not shown in figures, but available in Data S1). Despite the decrease in body weight and improvement in insulin sensitivity, REE and respiratory quotient (RQ) remained similar throughout the treatment period (Table 1).

The incretin glucose-dependent insulinotropic peptide (GIP) showed a trend toward time-dependent decline ($p = 0.044$ at 12 months; Figure S1A). Notably, another gut hormone, ghrelin, demonstrated an increase at 6 months ($p = 0.006$; Figure S1B). There was a trend toward an increase in adiponectin levels (Figure S1C). The change in interleukin-6 (IL-6) was not statistically significant (Figure S1D). No significant change was observed in soluble leptin receptor (sLEPR) levels (Table 1).

Effects of metreleptin on liver parameters and histologic features

As mentioned above, 7 participants underwent paired liver biopsies at baseline and month 12 (Figure 1B). Serum liver enzymes aspartate aminotransferase (AST) ($p = 0.002$ at month 6; Figure 2A) and alanine aminotransferase (ALT) ($p < 0.001$ at month 6; Figure 2B) were significantly lower at 6 months but not significantly different at the end of the study. The mean levels of AST and ALT were 36 ± 10 IU/L and 51 ± 21 IU/L at baseline and 35 ± 14 IU/L and 44 ± 29 IU/L at month 12, respectively. There were statistically significant decreases in hepatic fat content by magnetic resonance imaging (MRI) measurements at both 6 ($p = 0.009$) and 12 months ($p = 0.034$) (Figure 2C). The mean levels of hepatic fat (using MRI Dixon method) were $19\% \pm 8\%$ at baseline and $13\% \pm 9\%$ at month 12. The mean NASH scores in the 7 subjects at baseline and month 12 were 8 ± 3 and 5 ± 2, respectively, with a range of decrease by 1 to 6 points ($p = 0.004$; Figure 2D). All subjects demonstrated a decrease in NASH scores (Table S4). Five of the 7 subjects who completed 12 months of therapy had a minimum 3-point reduction in their global total NASH scores. An example of paired biopsies from a representative patient is shown along with matched MRI and Magnetic Resonance spectroscopy (MRS) examinations in Figure 2E. In addition to improvement in steatosis with weight loss, improvement of inflammation scores was observed even in participants without weight loss. One of the 7 subjects demonstrated an improvement of fibrosis and 1 experienced worsening of fibrosis (Figure 2F). The findings of key trial outcomes reported as mean percent changes from baseline are presented in Table S4. The full trial clinical database is available in the attached data file labeled Data S1.

Effects of metreleptin on hepatic gene expression and fatty acid analysis

To explore potential changes in liver gene expression following leptin treatment, we subjected messenger ribonucleic acid (mRNA) isolated from liver biopsies from 6 individuals (1 sample did not yield high-quality RNA) to quantitative profiling by using microarrays. Enriched pathways following leptin treatment were identified by LR path.[19] Pathways associated with protease activity and proteosome were found to be significantly (false discovery rate [FDR], <0.1) upregulated following leptin treatment, whereas G-protein-coupled olfactory receptor signaling pathways were downregulated (Figure S2A). A full dataset is also available as an additional file labeled Data S2. We also used quantitative PCR to directly assess the mRNA levels

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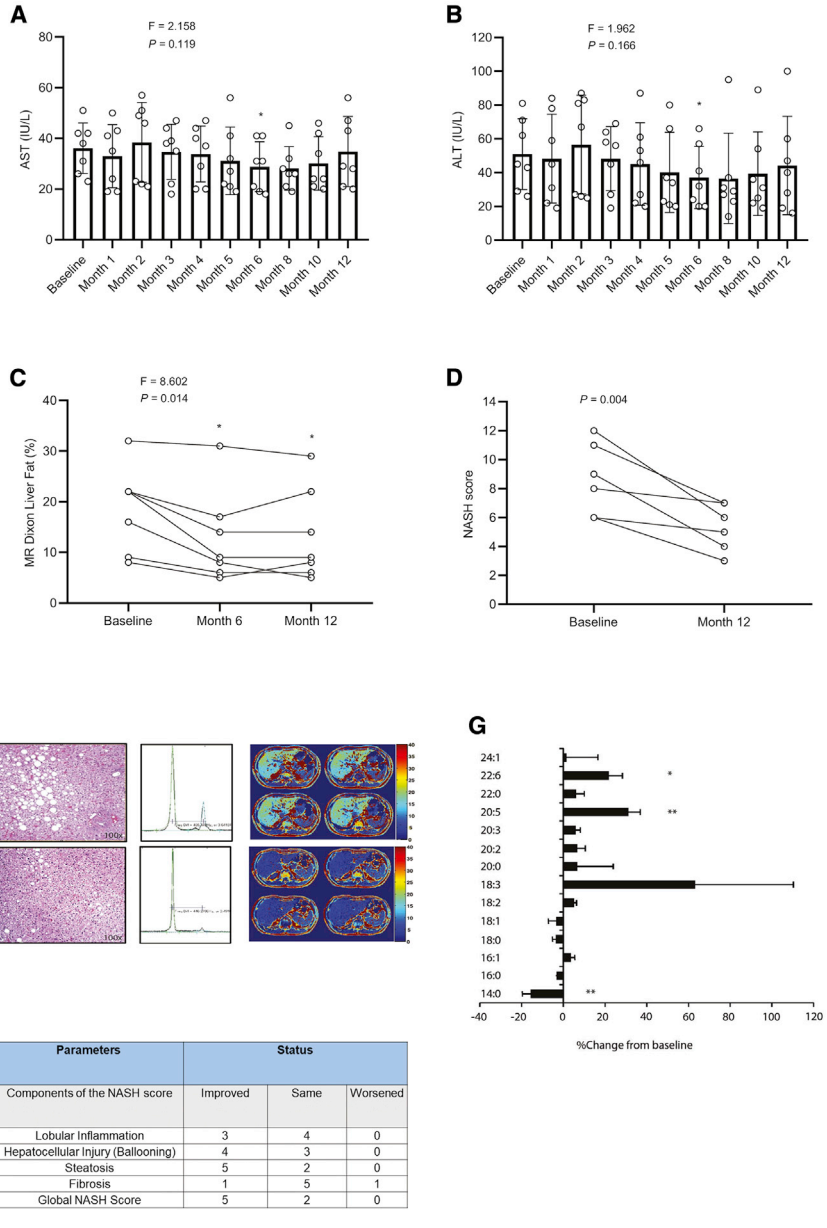

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Parameters	Status		
Components of the NASH score	Improved	Same	Worsened
Lobular Inflammation	3	4	0
Hepatocellular Injury (Ballooning)	4	3	0
Steatosis	5	2	0
Fibrosis	1	5	1
Global NASH Score	5	2	0

Figure 2. Effect of metreleptin therapy on liver parameters in the RLD study

(A–C) Effect of metreleptin on aspartate aminotransferase (AST) (A), alanine aminotransferase (ALT) (B), and liver fat percentage as determined by MRI over time (C).

(D) Individual NASH scores at baseline and 12 months. We report the F-statistic and p value from a repeated-measures ANOVA. *, indicates p values that are significant versus baseline with post hoc paired sample t test after multiplicity correction. Paired t test was used to compare month 12 values to baseline (without multiplicity correction), as the change at 12 months versus baseline was a prespecified endpoint.

(E) H&E stains of liver biopsies before and after 1 year of therapy with leptin. Note the marked improvement in steatosis on biopsy, magnetic resonance (MR) images, and spectroscopy (15% fat at baseline versus 5% fat after treatment with metreleptin).

(F) Components of NASH score at 12 months compared to baseline.

(G) Percent change in different free fatty acid species relative to total free fatty acid levels compared to baseline after 6 months of metreleptin therapy. *$p < 0.05$ and **$p < 0.01$ versus baseline.

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of several genes previously identified as being regulated by leptin in the mouse model.[20] Sterol regulatory element-binding protein 1 (SREBP1) is a transcription factor that regulates several genes associated with *de novo* lipogenesis, including stearoyl-coenzyme A (CoA) desaturase 1 (SCD-1) that causes the mono-desaturation of palmitic and stearic acid. There was a trend to decreased SREBP1 mRNA levels and SCD-1 levels (Figure S2B), but they did not reach significance ($p > 0.05$ for all). In addition, in keeping with reduced lipogenesis, there was a trend toward decreased ATP citrate lyase and fatty acid synthase expression. Furthermore, we interrogated the FXR/retinoid X receptor (RXR) pathway due to its importance in NAFLD pathophysiology, which showed a numerical decrease in the average expression of CYP27A1, but it did not reach statistical significance. We also assessed total fatty acids derived from total lipids in the peripheral plasma of subjects at baseline and at 1 and 6 months of metreleptin treatment. Data at 1 month did not reveal significant changes compared to baseline. We found a decrease in 14:0 fatty acids and increases in polyunsaturated fatty acids at 6 months (Figure 2G). There were minimal changes in the 16:1/16:0 and 18:1/18:0 ratios, suggesting that overall SCD-1 activity was not changed in response to metreleptin. These results suggest that metreleptin treatment has a minimal direct effect on these parameters in these subjects, likely due to dietary variability and treatment for hyperlipidemia.

Adverse events

Two of the 9 participants exposed to metreleptin treatment in this study experienced transient skin reactions at the injection sites. One patient had moderate and 1 patient had mild localized reactions that occurred within 2 to 4 weeks of treatment and abated spontaneously within 6 weeks of emergence. Leptin-binding antibodies and neutralizing activity were measured from samples collected at baseline and 3, 6, and 12 months by using an assay developed by Amylin Pharmaceuticals[21] after the trial was completed. All subjects had evidence for leptin-binding antibodies by 6 months, and they were sustained at 12 months (Table S3). None of the samples demonstrated *in vitro* neutralizing activity. One patient developed lymphadenopathy on the right side of the neck and axillae emerging at 11 months of treatment. Biopsy and clinical evaluation determined that the patient developed acute toxoplasmosis due to presumptive exposure to stray cats; the patient completed 12 months of the study treatment period.

The PL study

Study design, population baseline characteristics, and flow through the study

An NIDDK-funded open-label prospective 1-year intervention study was conducted to determine systematically whether leptin improves NASH histopathology associated with PL (University of Michigan Medical School, Ann Arbor, Michigan). For this study, we *a priori* defined at least 2 points of improvement in the total NASH score as a clinical response in 12 months. With a difference of 2, a baseline mean value of 8, and with a baseline SD of 2, a total of 17 subjects yielded 80% power with 5% of the significance level. Assuming a 15% possible dropout rate, we aimed to recruit a minimum of 20 and no more than 24 subjects.

Twenty-three participants with physician-diagnosed PL were enrolled and completed baseline study procedures. A detailed description of the baseline parameters of these subjects was previously published.[22] One patient was excluded, as her baseline liver biopsy did not meet the histopathological definition of NASH. The remaining 22 participants had biopsy-proven NASH and continued the protocol, receiving at least 1 dose of metreleptin (Supplemental information). Baseline characteristics of the participants receiving at least 1 dose of the drug in the study are presented in Data S3. The

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mean age of 22 subjects (17 females and 5 males) was 43 ± 16 years (range, 12–64). Nineteen subjects were Caucasian, 2 related subjects were Hispanic with mixed racial background, and 1 subject was African American. Twenty-one had familial PL (FPLD), and 1 subject had acquired PL (APL). Pathogenic variants in the lamin A/C (*LMNA*) gene were found in 7 of 22 participants receiving at least 1 dose of the drug. Two related subjects had the p.E1067K variant of the polymerase (DNA-directed), delta 1, catalytic subunit-1 (*POLD1*) gene as previously reported.[22] Additionally, several subjects had variants of unknown significance noted in several genes such as fibrillin-1 (*FBN1*), sterol regulatory element-binding transcription factor 1 (*SERBF1*), and dual-specificity tyrosine phosphorylation-regulated kinase 1b (*DYRK1B*) as shown in Data S3.

Median baseline leptin levels were 16.2 ng/mL (range, 4.8–67.1 ng/ml) with a mean ± SD of 22.3 ± 16.6 ng/mL. These levels were measured by an ELISA (see STAR Methods), and these subjects did not have severe leptin deficiency analogous to generalized lipodystrophy but instead had a broader range of leptinemia.

The mean baseline total NASH score was 6 ± 2 (details of the NASH scoring system are presented in Table S2). Individual-level data are further presented in the additional data file labeled as Data S3.

Nineteen subjects completed 12 months of metreleptin treatment (mean age, 43 ± 17 years; range, 12–64; 16 females and 3 males), and 18 subjects completed a second liver biopsy (mean age, 43 ± 17 years; range, 12–64; 15 females and 3 males). One participant did not complete the second biopsy due to initiation of anticoagulation during the study (Figure 3A).

The starting dose of metreleptin was 2.5 mg daily in the male subjects and 5 mg in the female subjects. Average metreleptin dose used per subject across the study was 7.04 ± 1.99 mg/day. The maximum dose was 10 mg/day. We made every attempt to keep the metabolic therapy stable with the exception of down-titration of metabolic treatments to avoid hypoglycemia. There were a few cases of subjects who had minor increases in their glucose-lowering treatments, as detailed in Data S3. Given the complicated multi-system disease, some patients did initiate additional therapies for intercurrent illness, such as the discovery of coronary artery disease in one subject (patient 22), necessitating the initiation of anticoagulant medication.

Effects of metreleptin on metabolic parameters

Nineteen participants were treated with metreleptin for 12 months (Figures 3A and 3B). Table 2 shows metabolic parameters before and 12 months after metreleptin treatment. Temporal changes in several metabolic parameters over 12 months in response to metreleptin are shown in Figures 3C–3F. We observed a significant decrease in fasting triglycerides, liver enzymes (ALT and AST), and REE 12 months after metreleptin. Although fluctuations were observed on an individual basis, the decrease in triglyceride levels was significant both during and at the end of the treatment period (Figure 3C). Fasting glucose and HbA1c levels also tended to be decreased after treatment, but the effect was not statistically significant (Table 2; Figure 3D). Liver enzymes showed mild reductions over time, and the differences were significant at month 12 (Figures 3E and 3F). REE decreased after metreleptin therapy ($p = 0.004$; Table 2).

Effects of metreleptin on liver parameters and histologic features

In the evaluable 19 patients with paired MRIs, liver fat (quantified by MRI Dixon method) decreased from baseline mean 13% ± 7% to 8% ± 5% after 12 months of

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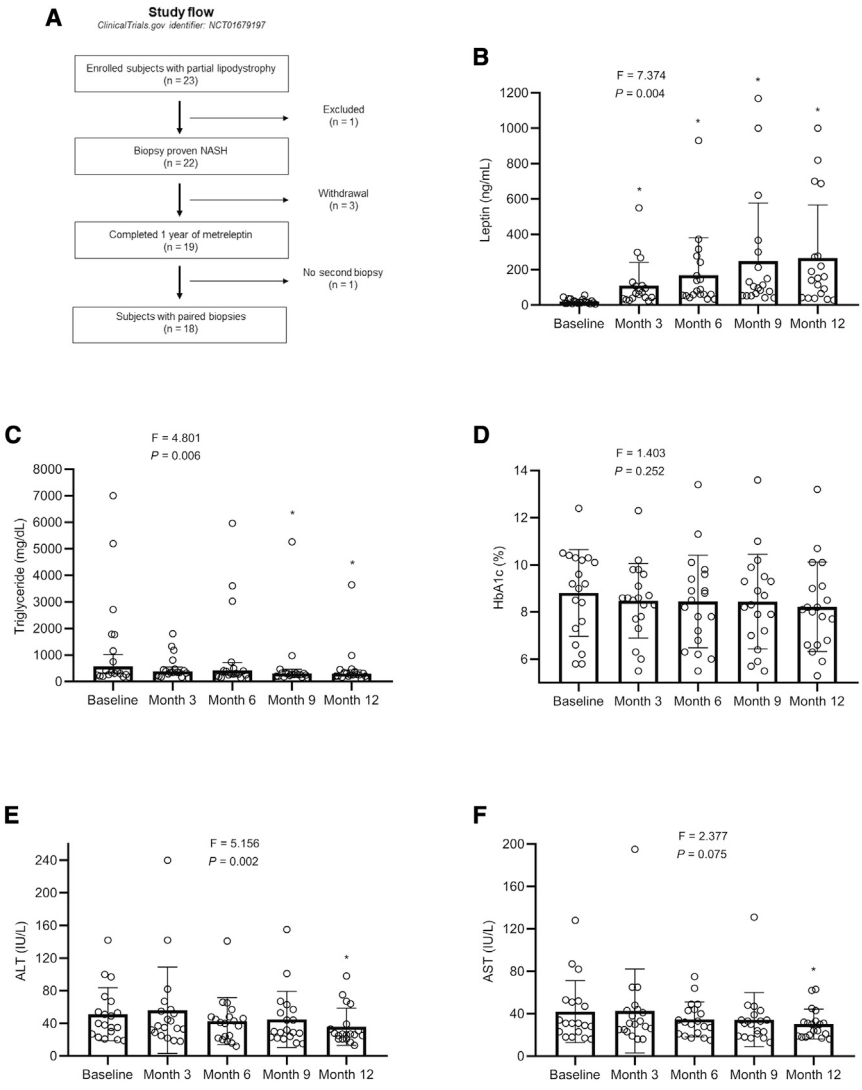

Figure 3. Leptin levels, study design, and the effect of metreleptin on metabolic parameters and liver enzymes over the 12-month treatment period in the PL study

(A) Leptin levels throughout the PL study period. Leptin levels were measured from 3 samples measured 30 min apart at baseline and 3, 6, 9, and 12 months after metreleptin treatment. The levels shown are average leptin levels. The F-statistic and p value are reported from a repeated-measures ANOVA. *, indicates p values that are significant versus baseline with post hoc paired sample t test after multiplicity correction. Paired t test was used to compare month 12 values to baseline (without multiplicity correction), as the change at 12 months versus baseline was a prespecified endpoint.

(B) Patient progression through the PL study. A total of 23 patients with partial lipodystrophy were enrolled and 22 had biopsy-proven NASH. Of the 22 patients, 3 withdrew from the study and 19 completed 1 year of metreleptin treatment. A total of 18 patients completed the 12-month post-treatment biopsy.

(C–F) Triglyceride (C), HbA1c (D), ALT (E), and AST (F) levels in subjects with partial lipodystrophy treated with metreleptin for 1 year. The F-statistic and p value are reported from a repeated-measures ANOVA. *, indicates p values that are significant versus baseline with post hoc paired sample t test after multiplicity correction. Paired t test was used to compare month 12 values to baseline (without multiplicity correction), as the change at 12 months versus baseline was a prespecified endpoint. Tests were run on log transformed data for triglycerides, ALT, and AST. Triglycerides are shown geometric mean with 95% confidence intervals (CIs); otherwise, the data are reported as mean ± standard deviation (SD). The last observed nonmissing values (month 6) were used to fill in missing values at the month 9 visit in 1 subject (subject ID: 21) who missed month 9 visit but completed the study protocol.

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Table 2. Comparison of study parameters at baseline and 12 months after metreleptin treatment in the PL study

Parameters	n[a]	Baseline	Month 12	p value
Body weight (kg)	19	75.6 ± 21.8	75.0 ± 23.1	0.494
Body mass index (kg/m^2)	19	26.8 ± 5.8	26.5 ± 6.3	0.270
Waist-to-hip ratio	19	1.00 ± 0.08	0.98 ± 0.08	0.079
FMR (%fat trunk/ %fat legs)	19	1.8 ± 0.5	1.7 ± 0.7	0.537
Systolic blood pressure (mmHg)	19	126 ± 16	128 ± 17	0.583
Diastolic blood pressure (mmHg)	19	74 ± 11	73 ± 12	0.653
Hemoglobin A1c (%)	19	8.9 ± 1.9	8.2 ± 1.9	0.077
Glucose (mg/dL)	19	193 ± 84	164 ± 67	0.055
Triglyceride (mg/dL)[b]	19	576 (327 - 1016)	301 (206 - 441)	0.014
AST (IU/L)[b]	19	42 ± 29	30 ± 14	0.043
ALT (IU/L)[b]	19	51 ± 33	36 ± 23	0.004
REE (kcal)	19	1851 ± 333	1719 ± 369	0.004
RQ	19	0.77 ± 0.07	0.75 ± 0.06	0.305
Liver fat (Dixon MR method) (%)	19	13 ± 7	8 ± 5	0.001
NAS	18	5 ± 1	4 ± 1	< 0.001
NASH score	18	6 ± 2	5 ± 2	0.008
Total intake (grams)[c]	18	2791 ± 1141	2668 ± 675	0.809
Total energy intake (kcal)	18	1710 ± 412	1540 ± 531	0.253
Total fat intake (grams)	18	73 ± 26	64 ± 24	0.175
Total carbohydrate intake (grams)	18	184 ± 49	166 ± 74	0.372
Total protein intake (grams)	18	88 ± 18	84 ± 28	0.802

Nineteen participants completed 1 year of metreleptin treatment and 18 completed a second liver biopsy. One subject did not complete the second biopsy due to use of anticoagulation. Energy intake was assessed in 18 subjects, as 1 subject (patient 1) did not return baseline food records. ALT, alanine aminotransferase; AST, aspartate aminotransferase; FMR, fat mass ratio; REE, resting energy expenditure; RQ, respiratory quotient; NAS, nonalcoholic fatty liver disease activity score, the sum of scores for steatosis, lobular inflammation, and ballooning; NASH score, nonalcoholic steatohepatitis score equals the sum of scores for steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis.

[a]Number of participants with data available at baseline and 12 months for each outcome.

[b]Tests were run on log-transformed data. Data are presented as mean ± standard deviation (SD). Triglycerides are shown as geometric mean with 95% confidence intervals.

[c]Total intake includes food, water, and other beverages. Levels are compared by using paired-sample t test.

metreleptin (n = 19; p = 0.001; Table 2; Figure 4A). NASH scores (the primary endpoint for this study) were compared in 18 subjects who completed the baseline and 1-year biopsies and showed a significant decrease from 6 ± 2 to 5 ± 2 (p = 0.008; Figure 4B). NAFLD activity scores (NASs) also decreased from 5 ± 2 to 4 ± 1 (p < 0.001; Figure 4C). Figure 4D shows the change in the components of the NASH score after metreleptin. Trends for reduction in steatosis (p = 0.072) and lobular inflammation (p = 0.063) as well as significant reduction in hepatocellular injury (p = 0.008) were noted, whereas fibrosis score did not change significantly (p = 0.727) after treatment with metreleptin. An illustrative case is presented in Figure 4E. Of 18 subjects who completed 12 months of therapy, 13 had improvements in NASs and NASH scores (Figure 4F). Steatosis and hepatocellular injury improved in 8 participants, and lobular inflammation improved in 5 subjects, although 2 subjects showed worsening of steatosis. Three of the 18 subjects demonstrated an improvement of fibrosis and 5 experienced worsening of fibrosis (none equaled to or more than 2 points of worsening). Individual liver biopsies are presented in Data S3.

In the 18 subjects with paired liver biopsies available, liver fat as measured by the MRI Dixon method decreased from baseline at 13% ± 7% to 8% ± 5% after 12 months of metreleptin (p = 0.001). The summary of key efficacy measurements

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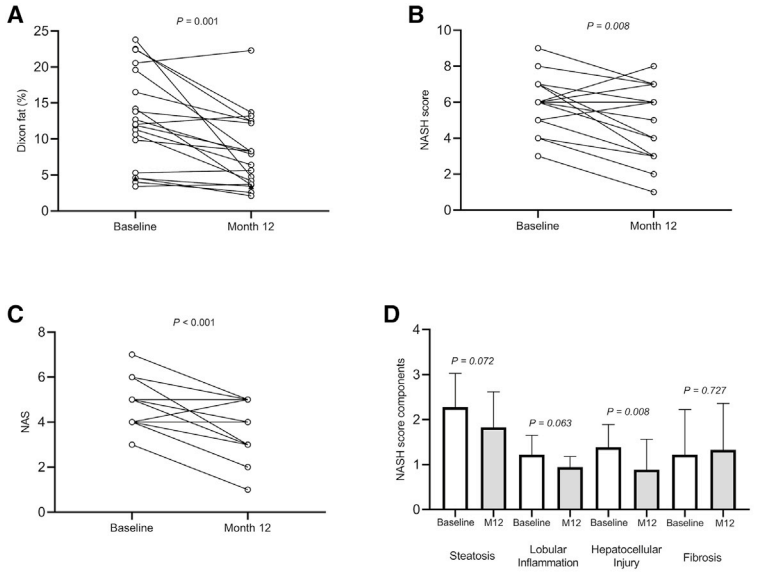

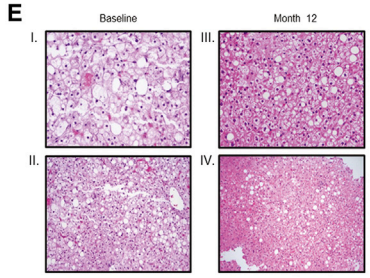

F

Parameters	Status		
Components of the NASH score	Improved	Same	Worsened
Lobular Inflammation	5	13	0
Hepatocellular Injury (Ballooning)	8	10	0
Steatosis	8	8	2
Fibrosis	3	10	5
NAS	13	4	1
Global NASH score	13	1	4

G

Parameters	Baseline (n = 18)	12 months (n = 18)	*P* value
Body weight (kg)	76.3 ± 22.3	75.7 ± 23.5	0.554
Body mass index (kg/m²)	26.8 ± 5.9	26.5 ± 6.5	0.322
Waist-to-hip ratio	1.00 ± 0.08	0.98 ± 0.08	0.049
Fat mass ratio (%fat trunk/ %fat legs)	1.65 ± 0.31	1.57 ± 0.28	0.049
Systolic blood pressure (mmHg)	125 ± 16	129 ± 17	0.412
Diastolic blood pressure (mmHg)	73 ± 11	73 ± 12	0.940
Hemoglobin A1c (%)	9.0 ± 1.8	8.3 ± 1.9	0.066
Glucose (mg/dL)	197 ± 84	166 ± 68	0.053
#Triglycerides (mg/dL)	602 (332 - 1091)	304 (204 - 455)	0.014
*Aspartate aminotransferase (IU/L)	43 ± 29	31 ± 14	0.046
*Alanine aminotransferase (IU/L)	53 ± 33	37 ± 23	0.006
Resting energy expenditure (kcal)	1855 ± 342	1731 ± 376	0.008
Respiratory quotient	0.77 ± 0.08	0.75 ± 0.06	0.407
*Total intake (grams)	2822 ± 1169	2725 ± 677	0.765
Total energy intake (kcal)	1711 ± 424	1607 ± 521	0.394
Total fat intake (grams)	73 ± 26	66 ± 23	0.270
Total carbohydrate intake (grams)	184 ± 50	173 ± 75	0.534
Total protein intake (grams)	88 ± 18	87 ± 28	0.919

Figure 4. Liver fat quantification and histopathological features of NASH after 1 year of metreleptin therapy in subjects with PL

(A) Liver fat quantification, using MR Dixon method, decreased from baseline at 13% ± 7% to 8% ± 5% after 12 months of metreleptin in subjects who completed 1 year of follow-up (n = 19; p = 0.001). Liver fat of 18 subjects with paired liver biopsies showed a reduction from baseline at 13% ± 7% to 8% ± 5% after 12 months of metreleptin (n = 18, p = 0.001).

(B) NASH score comparisons at baseline and after 12 months of metreleptin therapy of 18 subjects with 1-year biopsies show a decrease in global NASH scores from 6 ± 2 to 5 ± 2 (p = 0.008).

(C) During the 1-year follow-up, NASs also decreased from 5 ± 1 to 4 ± 1 (p < 0.001).

(D) Different components of the NASH score before and after metreleptin treatment. p values were obtained with Wilcoxon signed-rank test.

(E) H&E staining of the liver biopsy specimens from a 14-year-old female participant. I. Baseline biopsy at 200× magnification. II. 12-month biopsy at 200× magnification. III. Baseline biopsy at 100× magnification. IV.12-month biopsy at 100× magnification. Note the marked steatosis and hepatocyte ballooning at baseline state, which significantly improve by 12 months.

(F) Components of NASH score at 12 months compared to baseline in the PL study (n = 18).

(G) Metabolic parameters before and after metreleptin in PL patients with baseline and 12-month liver biopsies. Energy intake is reported in 17 patients. *Total intake includes food, water, and other beverages. Levels were compared by using paired sample t test. #Tests were run on log-transformed data. Data are presented as mean ± SD. Triglycerides are reported as geometric mean and 95% CIs.

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with percent change from baseline is presented in Table S5. Mean changes in specific metabolic characteristics of these 18 participants with paired liver biopsies are presented in Figure 4G.

Adverse events

All participants who received at least 1 dose of metreleptin (n = 22) reported some type of adverse event during the treatment period, although only 13 of the 22 subjects had at least 1 adverse event that was possibly or probably related to metreleptin treatment (Supplemental information). Eleven subjects experienced an upper respiratory tract infection, 6 had hypoglycemia, 5 had diarrhea, and 4 had reactions at the site of metreleptin injections. Three participants each also experienced urinary tract infections, dizziness, asthma exacerbation, abdominal pain, and nausea. Three subjects experienced skin rashes at other sites away from the immediate injection sites. One of these participants, who had remote history of skin granulomas prior to the start of the drug, developed recurrent granulomas starting at 6 weeks of exposure and withdrew from the study of her own volition. She was ultimately diagnosed with systemic sarcoidosis. This event was deemed unlikely related to metreleptin by subspecialty physicians who evaluated her (and not by primary investigator), owing in part to the presence of the history of dermal granulomatous disease prior to the initiation of the drug. Events that were deemed to be likely or definitely related to metreleptin treatment included hypoglycemia and injection site reactions. No serious hematologic events or development of neutralizing antibodies to leptin were observed during the reported 12-month treatment period even though 1 participant subsequently developed neutralizing antibody during the extension phase at 18 months of treatment, and her complicated clinical course is reported elsewhere.[23]

DISCUSSION

In this paper evaluating the effects of exogenous leptin therapy in the metabolic liver diseases of 2 distinctive groups of participants, we saw an amelioration in the histopathological features of NASH both in a group of males with common NASH and RLD without lipodystrophy, as well as in a group of participants with PL irrespective of their circulating leptin levels. These results document improvements, particularly in the degree of steatosis and hepatic injury, and provide further support for the hypothesis that the therapeutic utility of exogenous leptin therapy may extend beyond severe leptin deficiency. In addition, limited liver gene expression studies demonstrated a profile that was largely confirmatory of earlier observations in the rodent studies.

Do the subgroups we defined as RLD and PL lie across a continuous metabolic spectrum?

One noticeable characteristic of the RLD subgroup was relative reduction in the subcutaneous fat and preservation of the visceral fat. Although the RLD study historically strived to enroll subjects with NASH and RLD of both sexes, we were not able to identify a sufficient number of female subjects who met this definition among patients with biopsy-proven NASH without obvious clinical lipodystrophy.

This is in contrast to the overwhelming female preponderance among individuals who are correctly diagnosed with PL. PL refers to a heterogeneous cluster of diseases characterized by a relative paucity of peripheral fat depots coupled with insulin resistance and metabolic dyslipidemia. It is much easier clinically to appreciate the absence of peripheral (and especially lower extremity) fat in females. Our suspicion is that females who are more in the RLD category may have already received a diagnosis of PL by their metabolic specialists and making such a diagnosis is more difficult in males. Also, having no diabetes was a prerequisite to be included in the cross-sectional RLD study.

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Therefore, female patients with NASH may have already developed diabetes, as we know that fat loss from the peripheral compartments have far more severe metabolic consequences in females compared to males.[24] To that end, we have worked hard to come up with objective diagnostic criteria for PL. Currently having a mid-thigh skinfold thickness of less than 11 mm in males and 22 mm in females is accepted as sufficient evidence for the presence of PL[25,26]. However, a quick glance at the fat distribution patterns shown by the fat shadow images of our PL patients (presented in Data S3) highlights the heterogeneity of fat distribution patterns as well as the degree of residual fat in the PL population.

Although none of the cases included in our RLD cohort met the formal diagnostic criteria for PL, their body fat distribution characteristics suggested a more central deposition, and as such, they may represent a cluster of cases who resemble the PL cases in some metabolic features. If one can view common truncal obesity and metabolic syndrome/type 2 diabetes as a continuous spectrum, the PL cases with known single-gene causes likely represent the most extreme form of this phenotype.

In fact, females are reported to be protected from common NASH in younger ages, and there appears to be ethnic and racial factors that determine adipose tissue storage capacity and expandability that, in turn, may govern the threshold for NASH.[24,27] Further studies are needed to really underpin the mechanisms that determine how NASH develops in the two sexes across different racial and ethnic groups and how leptin availability may play a role in the different subgroups, as well as how genotypic factors can modify the clinical presentations and treatments.

Observations in the RLD cohort: Where do they fit?

It was of particular importance that in the RLD cohort, we observed a reduction in body weight and in both fat and lean mass. The earlier phase 2 studies with metreleptin in obesity demonstrated that there was heterogeneity in how individuals responded to exogenous leptin therapy,[18,28] with the challenge lying in predicting those who would demonstrate a biological response. Published phase 2 studies with monotherapy were not large enough to determine predictors of response.[18,29,30,31] However, a post hoc pooled subgroup analysis showed that metreleptin reduced weight in adults with low baseline leptin levels that was confirmed in a subsequent study in adults with low baseline leptin and BMI of 27.5–38.0 kg/m.[2,28] Other data from a trial using a combination of metreleptin with pramlintide suggested that individuals with lower grade obesity (specifically those with BMI of <35 kg/m^2) respond more robustly with weight loss.[32] This relatively lower BMI is concordant with the notion that the group that benefits the most from metreleptin therapy may have a limited capacity for adipose tissue expansion. However, other factors may influence leptin levels that include hormonal state,[33] iron stores,[34] other genetic influences of mitochondrial function,[35] and other factors that provide transcriptional control.[36] The decrease in body weight and adiposity was evident quite early on in the treatment in the 5 participants in our RLD cohort who demonstrated this response (as early as 3 months). Pursuant to weight loss, we also observed an increase in circulating ghrelin concentration at 6 months and a decrease in GIP levels by 12 months. These limited observations suggest that there may be specific regulators of the therapeutic response to metreleptin as well as adaptive changes. Although these points deserve further evaluation, we could not identify a baseline or early predictor of treatment response outside the clinical parameters tested, possibly due to our small sample size.

The hepatic gene expression analysis is also quite limited by our small sample size, although we did find a significant increase in the protease/proteasome pathway, but

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the mechanism for the change is unclear. In mouse models of fatty liver disease, proteasome function is decreased significantly in association with fat accumulation.[37] The increase we noted after metreleptin treatment may either be a primary effect of metreleptin therapy or a secondary adaptation to changes in metabolic improvement or weight reduction. Regulated cellular protein turnover by the ubiquitin-proteasome system can affect multiple homeostatic systems, including glucose and lipid metabolism.[38,39] The NAFLD risk variant of *PNPLA3* has been suggested to disrupt the degradation of the wild-type protein, resulting in impaired mobilization of triglycerides from lipid droplets.[40] We also observed a significant decrease in olfactory receptors and G-protein signaling. These receptors are found in multiple tissues outside of the olfactory system, are evolutionarily conserved, and may be related to the control of meta-inflammation.[41] The latter may involve adaptations to environmental changes, including energy control or conservation pathways.[42]

The trend toward a decrease in hepatic gene expression of SREBP-1c and SCD-1 in response to metreleptin therapy is consistent with previously published results.[20,43,44] SREBPs comprise a subclass of basic-helix–loop–helix–leucine zipper transcription factors that regulate expression of genes required to maintain cellular lipid homeostasis. SREBP-1c is the main isoform in the liver, and its upregulation has been implicated in the development of NAFLD.[45] SCD is a key enzyme involved in the biosynthesis of unsaturated fatty acids and is regulated by SREBP-1c. SCD-1 is the main isoform in the liver and has a key role in the promotion of hepatic *de novo* triglyceride synthesis.[46,47] Both SREBP-1c and SCD-1 are downregulated in rodent models by leptin administration therapy in concordance with the improvement in hepatic steatosis.[43,44] In addition, there is evidence that serum fatty acid composition is associated with insulin resistance and hepatic steatosis.[48–50] Thus, our suggested finding of a decrease in 14:0 fatty acids and slight increases in polyunsaturated fatty acids can be interpreted as a reflection of the overall metabolic improvement.

Previous *in vitro* studies have linked leptin to the development of hepatic fibrosis through the activation of hepatic stellate cells.[51,52] Some previous rodent studies have also suggested this idea.[53,54] In the RLD study, 5 out of 7 subjects had no change in fibrosis, except 1 showing worsening and another 1 demonstrating an improvement in fibrosis score. In addition, leptin has previously been associated with increased blood pressure.[55] It has been suggested that obesity-associated hypertension is mediated through leptin, as it may act centrally to impact sympathetic nervous system overactivity.[56] Similar to patients with lipodystrophy treated with metreleptin, the range of leptinemia achieved in this study is consistent with levels seen in obesity, although the reliability of leptin levels after metreleptin treatment is questionable due to frequent antibody development that would interfere with measured leptin levels. Nevertheless, metreleptin treatment was reported not to cause an increase in blood pressure in subjects with lipodystrophy.[57] Similarly, in this study, we observed no significant changes in blood pressure during metreleptin treatment. It is possible that the careful selection of subjects to those with RLD mitigated the potential of any side effects on doses aimed to reach physiological levels.

Because neutralizing antibodies were seen in patients who were treated during an extension open-label phase of obesity trial using metreleptin with pramlintide,[21] the development of metreleptin treatment programs for obesity and related disorders were halted while the development of the program for lipodystrophy continued. There are concerns that neutralizing the native leptin signaling could result in worsening metabolic parameters or immune status, although direct evidence for this is very limited. Therefore, studies in populations such as

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NASH/NAFLD should move forward only after careful risk-benefit analyses and preferably after characterization of the full *in vivo* effects of the neutralizing antibodies.[58] None of the subjects in this study were noted to develop neutralizing antibodies, although they all demonstrated some level of antidrug antibodies that affected the measurement of the leptin levels using the immunoassay.

Our observations in the PL cohort: Building upon what is known

In the PL study, we observed an overall favorable effect of metreleptin on PL-associated NASH. The primary study endpoint, global NASH scores, as well as NASs and levels of transaminases, significantly improved during the study. Global NASH scores improved, with reductions in steatosis, inflammation, and hepatic injury components. Overall, there were no significant changes in the fibrosis scores, although 5 individual subjects experienced 1 point worsening in the fibrosis scores. As discussed above with the RLD study, leptin had profibrotic effects in previous *in vitro* and rodent studies.[51,52,53,54] Whether this small degree of worsening in the liver fibrosis is of clinical importance is not certain. Natural disease progression despite metreleptin therapy and/or sampling-related factors may have played a role in the small changes in serial biopsies. Liver fat content decreased on MR assessment even in 4 of 5 individuals with a slight increase in fibrosis score. Despite the open-label design, our dataset is the largest dataset of systematic biopsies performed after 1 year of therapy in individuals with PL. Previous liver directed reports in lipodystrophy by Javor et al.,[15] Safar Zadeh et al.,[16] and Brown et al.[59] included fewer individuals with PL, and the timing of biopsies showed a wide range with not all first biopsies being performed at baseline and the second biopsies being performed anywhere between 3 months to 5 years after initiation of metreleptin. In our study, we obtained paired biopsies from 18 subjects with PL at baseline and 12 months of treatment.

As reported in previous studies, the metabolic abnormalities of leptin deficiency noted in severe generalized lipodystrophy can be reversed by exogenous leptin therapy.[17,60] As a result of these previous studies, metreleptin is now an FDA-approved treatment and indicated as an adjunct to diet to treat the metabolic complications in patients with congenital or acquired generalized lipodystrophy in the United States.[61] Metreleptin is also indicated for treatment of patients with PL who are older than age 12 in Europe when other metabolic therapies fail to achieve metabolic control.[62]

Our current PL study differs from previous studies in that we have presented findings on the treatment of participants with PL with quite variable leptin levels. Overall, the metabolic improvement seen in this study is in line with previous reports.[63] The metabolic effects reported here are not as robust and are more variable than seen in generalized lipodystrophy[17,60,64] but are consistent with other studies in PL.[60,63] Park et al.[65] reported that long-term leptin replacement was associated with metabolic benefits in subjects with PL, which was later supported by several other studies.[66,67] Diker-Cohen et al.[68] reported that metreleptin effectively reduced HbA1c and triglycerides in subjects with PL who had severe baseline metabolic abnormalities or low leptin.

Our data show that one does not have to observe an improvement in triglyceride or glucose control to see an improvement in liver-related parameters. Similar to our findings, Simha et al.[69] reported that both FPLD subjects (caused by pathogenic variants of the *LMNA* gene) with severe (mean leptin level, 1.9 ng/mL) and moderate (mean leptin level, 5.3 ng/mL) hypoleptinemia could benefit from metreleptin therapy in terms of decreasing triglyceride levels and hepatic steatosis but not glucose and HbA1c levels. In a recent study, Baykal et al.[70] showed that metreleptin reduced *de novo* lipogenesis in subjects with lipodystrophy across a broad range of leptinemia (endogenous leptin

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ranging from 0.5 to 35.7 ng/mL) despite a lack of significant improvement in triglycerides. Our previous work also suggested the best candidates for metreleptin treatment were PL subjects with an earlier onset of disease, subjects with *LMNA* pathogenic variants or other clear genetic abnormalities, and those with severe metabolic abnormalities.[71] The current dataset expands upon these observations and suggests that some of the PL patients may have important histopathological improvements in their liver-related parameters over a 12-month treatment period.

The neutralizing antibodies are noted in patients with lipodystrophy[21] and may attenuate the treatment response to metreleptin. In our PL cohort, none of the patients developed *in vitro* neutralizing antibodies during the 1-year treatment period, but 1 patient who continued on the treatment beyond 12 months for clinical benefit developed this at 18 months of therapy with loss of metabolic control.[23]

Limitations of study

The major limitations of these studies are the open-label designs, the small sample sizes, and the lack of placebo control. We also followed the designed protocols in the dosing regimen and used the maximum practicable dose as the ultimate ceiling in daily dose. It is possible that a higher level of efficacy could be noted if higher doses could be used in both cohorts.

The lack of a control group makes it difficult to interpret if the noted improvements were a direct effect of metreleptin or indirect effects due to reduction in food intake and/or weight loss, particularly in the RLD study, and it is impossible to eliminate the possible effect of other confounding factors. Background medications and recruitment related factors, among others, may have also contributed additional confounders. Although we endeavored not to increase or change background medications during the 12 months of the studies, this was not always achievable; in the PL study, we had to change some of the concomitant metabolic medications (even though the majority of the changes made were down-titrations), and this may have impacted some of our observations.

Keeping in mind that these types of studies are essentially uncontrolled and biased, our findings are still of importance because they lay the foundational groundwork for concepts of RLD and/or "preserved leptin responsiveness" in metabolic diseases. Both RLD and preserved leptin responsiveness can be analogous to how the concepts of "relative insulin deficiency" and "adequate therapeutic response to insulin therapy" are generally viewed in garden variety type 2 diabetes.

Overall conclusions

In conclusion, given the previously and currently noted response of patients with lipodystrophy to metreleptin and the encouraging results we have seen in the RLD study, we believe that it may be time to consider the therapeutic benefit of the leptin pathway more broadly, by carefully evaluating its efficacy in subpopulations with common metabolic diseases such as NASH who may be amenable to treatment.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability

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- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Description of overall study designs
  - The design of the treatment study in RLD (ClinicalTrials.gov identifier: NCT00596934)
- METHOD DETAILS
  - Study Assessment methods
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Analysis methods
  - Missing data
  - Data presentation
- ADDITIONAL RESOURCES

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.medj.2021.04.001.

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AUTHOR CONTRIBUTIONS

B.A. reanalyzed data from all three studies, created the figures, worked on the manuscript draft, revised further versions of the manuscript, and took the lead in the integration of two different manuscripts from the two cohorts. A.S. cleaned up the database, analyzed the data, and wrote the first draft of the RLD studies. N.A. followed recruited subjects in

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the PL study, collected data, performed first-pass data analyses, and drafted the first version of the PL manuscript. N.H.E., A.H.N., and A.E. conducted participant visits and provided critical feedback on manuscript versions and the conduct of the study. R.M. performed initial data analysis in the PL study. A.H.N. coordinated the visits and compiled the data. R.H. and D.R. followed subjects and performed data entry for further analyses. H.K.H., T.L.C., B.M.K., and C.S.M. provided key pieces of data, provided guidance during the conduct of the study, and approved the last version of the manuscript. A.R.R., M.K.T., and J.W.I. provided genetic sequencing data and critical analyses for genetic findings. H.S.C. followed the participants for liver disease and performed liver biopsies. C.L.B. also provided key pieces of data, provided guidance to the execution of the study, and contributed to the writing of the manuscript. E.A.O. designed the study, obtained funding, executed the study, provided clinical care to the participants, oversaw data analyses, and critically revised all versions of the manuscript. She also followed the participants clinically and performed all regulatory reporting and correspondence. E.A.O. is responsible for the integrity of the data and the conduct of the study. All authors read and provided critical input on different versions of the manuscript and approved the final version.

DECLARATION OF INTERESTS

Drug support was provided by mainly Amylin Pharmaceuticals Inc (later Ltd), the manufacturer of metreleptin at the time of the study, currently owned by Amryt Pharmaceuticals. The earlier writing support was provided by Aegerion Pharmaceuticals that owned metreleptin from 2015 to 2019. The following individual-level author conflicts are also reported. E.A.O. reports the conflicts grant support: Aegerion Pharmaceuticals (now Amryt Pharmaceuticals), Ionis Pharmaceuticals, Akcea Therapeutics, Gemphire Therapeutics, GI Dynamics (current), and AstraZeneca (2015–2017); consultant or advisor: AstraZeneca, Thera Therapeutics, and BMS (past); Aegerion Pharmaceuticals (now Amryt Pharmaceuticals), Akcea Therapeutics, Ionis Pharmaceuticals, and Regeneron Pharmaceuticals (current); drug support: Aegerion Pharmaceuticals (now Amryt Pharmaceuticals), Akcea Therapeutics, and Rhythm Pharmaceuticals (all current); other support: Aegerion Pharmaceuticals (now Amryt Pharmaceuticals) and Regeneron Pharmaceuticals (current). B.A. has attended Scientific Advisory Board Meetings organized by Aegerion Pharmaceuticals (now Amryt Pharmaceuticals) and Regeneron Pharmaceuticals and has received honoraria as a speaker from AstraZeneca, Lilly, MSD, Novartis, Novo Nordisk, Boehringer-Ingelheim, Servier, and Sanofi-Aventis. C.S.M. has served as a consultant for Coherus, Servier, Aegerion (now Amryt Pharmaceuticals), Regeneron, Genfit, and NovoNordisk; has received grants through his Institution by Coherus, Novo Nordisk, and Esai; and is shareholder of Coherus and Pangea, Inc.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of human subjects as much as scientifically applicable. The RLD study focused only on male subjects for scientific reasons mentioned in the paper. One or more authors of the paper self-identify as belonging to a minority group underrepresented in science. One or more of the authors of this paper received support from a program designed to increase minority representation in science.

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STAR★METHODS

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Elif A. Oral (eliforal@med.umich.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique code. The datasets obtained during the RLD study including the mRNA profiling data are provided in full. The lipidomics datasets can be provided upon request. We are currently conducting additional analyses from the PL study database but, we provided full data for results reported in the manuscript including liver biopsy images.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Description of overall study designs

Cross-sectional study in NASH. Fifty non-diabetic, non-cirrhotic subjects with biopsy-proven NASH were studied in the context of a cross-sectional study focusing on describing the various metabolic, histopathological, and body composition characteristics of subjects with biopsy-proven NASH. Patients were enrolled between 2005 and 2009. Subjects were excluded from the study for the following reasons: other causes of chronic liver diseases, alcohol use more than 40 g per week ($\geq$5 drinks per week) within the past 3 months or a history of long-term alcohol abuse or dependence in the past, use of medications reported to cause steatosis (including amiodarone, steroids, tamoxifen, valproic acid, methotrexate, and IV tetracycline) within the past 3 months or for more than 6 months in the past 2 years, weight reduction surgery within 1 year or jejunoileal bypass in the past, weight loss medication or participation in a weight loss program in the past 3 months, evidence of hepatic decompensation, HIV antibody positive, pregnancy, or breast-feeding. Adults with diabetes were not included in this study because possible changes in anti-diabetic therapy and glycemic control could confound the analysis.

All subjects underwent same-day anthropometric, laboratory, and radiological investigations. Measurements of the waist and hip circumference, weight, height, BMI, and blood pressure were performed. Laboratory testing for metabolic abnormalities including fasting triglyceride, glucose and insulin was obtained. The measurement of insulin resistance was determined by the HOMA index ([(fasting insulin*fasting glucose/18)/22.5]). Whole-body DEXA scanning using a dual X-ray absorptiometry (Lunar, General Electric, Madison, WI) was used for the estimation of fat and lean body mass. A non-contrast abdominal CT (single slice) scan at the level of L4 was performed for the measurement of visceral fat mass and abdominal non-visceral fat mass. Abdominal subcutaneous fat mass was estimated as the difference between abdominal and visceral fat.

Stored fasting sera were obtained on these subjects after a minimum of 8 hours of physical rest and analyzed for circulating leptin levels. These levels were then linked with the existing database on the individuals' various clinical, biochemical, radiological, and histological characteristics. We classified the subjects studied into three subgroups according to the following criteria: (1) RLD: subjects with level < 25th percentile of NHANES III population based on their sex and BMI; (2) normal leptin levels (NLL): subjects with levels

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falling between 25th and 75th percentile of NHANES III population based on sex and BMI and (3) relative leptin excess (RLE): subjects with levels > 75th percentile of NHANES III population based on sex and BMI. Table S1 summarizes the cut-offs for the 25th percentile for the National Health and Nutrition Examination Survey (NHANES) III population (complete data courteously provided by Dr. Ruhl).

The design of the treatment study in RLD (ClinicalTrials.gov identifier: NCT00596934)

Nine subjects from the RLD group agreed to participate in a prospective, open-label pilot study to test the efficacy of metreleptin. Patients were enrolled from 2009 to 2011. The primary outcome was determined as the global NASH score, obtained from histopathological assessments of the liver biopsy samples (Table S2). Changes in individual components of NASH and fibrosis scores were studied in a blinded fashion to assess the role of metreleptin on liver histopathology. Participants were admitted to the Michigan Clinical Research Unit for two days to undergo baseline tests for metabolic state and body composition and a liver biopsy. During this hospital stay, standardized weight maintenance diets of 50% carbohydrates, 20% protein, and 30% fat were served as soon as the tests of the day were completed. Following the completion of all baseline testing, subjects were started on metreleptin at a dose of 0.1 mg/kg/day given once a day subcutaneously. There was no dose adjustment throughout the 12-month period. The maximum dose that was administered was 10 mg/day due to practicability as one vial produced 10 mg to be administered. Metreleptin was provided primarily by Amylin Pharmaceuticals, San Diego, CA, and is currently owned by Amryt Pharmaceuticals, Dublin, Ireland. All medical therapies at baseline in these subjects were noted and remained unchanged for the 12 months of the study. Participants were seen monthly for the first 6 months and then every 2 months to assess the safety and tolerability of metreleptin. Blood was collected for metabolic parameters at each visit. Body composition was assessed using MRI and DEXA at baseline, 6 months, and 12 months. Liver fat using MRI was assessed at baseline, 6 months, and 12 months. A liver biopsy was repeated at 12 months. The 12-month studies were conducted as inpatients over two days using the aforementioned standardized study feeding routines.

The design of the treatment study in PL (ClinicalTrials.gov identifier: NCT01679197). This next study was designed as an open-label study performed at the University of Michigan, Ann Arbor, MI which was funded by the NIDDK (R01 DK088114) and approved by the University of Michigan IRBMED. Patients were enrolled between 2012 and 2015. Subjects enrolled in the study had the diagnosis of acquired or inherited PL made by physician assessment. The molecular basis of disease was determined using resources described in our previous work.[22] The presence of liver disease was assessed by ultrasound or prior liver biopsy demonstrating fatty liver disease. Exclusion criteria included presence of human immunodeficiency virus, advanced liver disease (labs demonstrating abnormal synthetic function), viral hepatitis, alcohol consumption greater than 40 g per week, end-stage renal disease, active cancer, greater than New York Heart Association class 2 congestive heart failure, known allergy to *Escherichia coli* derived proteins or hypersensitivity to any component of metreleptin treatment. Informed consent was obtained from all subjects or guardians. History and physical examinations were performed, and previous medical records were examined when possible. The first subject was enrolled on October 8, 2012, and data collection for this study was completed on April 14, 2016.

The primary outcome was determined as the global NASH score, obtained from histopathological assessments of the liver biopsy samples (Table S2). Changes in

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individual components of NASH and fibrosis scores were studied in a blinded fashion to assess the role of metreleptin on liver histopathology. Secondary outcomes were determined as the impact of metreleptin therapy on hepatic fat percent via proton density fat fraction obtained by magnetic resonance imaging, HbA1c and lipid levels as well as energy expenditure.

Following baseline study procedures, participants began metreleptin (recombinant human leptin) therapy. Metreleptin was provided by Amylin Pharmaceuticals (San Diego, CA), later manufactured by Bristol Myers Squibb and Aegerion Pharmaceuticals, and currently Amryt Pharmaceuticals. Participants self-administered subcutaneous injections after reconstitution once a day. Metreleptin doses were started at a dose of 2.5 mg per day in males and 5 mg per day in females. The dose was increased to a maximum of 10 mg daily (in single or divided doses based on individual's preference) after 2 weeks to 6 months at investigator discretion. We aimed for this higher dose as this was the dose used in the RLD pilot study evaluating the leptin effects in the liver. The dose increases were made at 1.25 to 2.5 mg increments depending on the principal investigator's opinion on the tolerability of such an increase.

Concomitant glucose-lowering treatments were reduced to avoid hypoglycemia. In some patients, minor changes in the glucose-lowering treatments were noted at their follow-up visits, initiated by their local physicians. We made every attempt to keep these treatments as stable as possible. Intercurrent medical events were treated as required. Participants were instructed to keep symptom sheets and safety visits were conducted by phone. Study visits were scheduled at 3 months, 6 months 9 months, and 12 months after the baseline visit and study drug initiation.

Study approval. All study protocols were approved by the University of Michigan Medical School Institutional Review Board and all participants gave informed consent.

METHOD DETAILS

Study Assessment methods

Energy intake and resting energy expenditure. Energy intake was assessed using 3-day food records in the PL study. Subjects were asked to record the type and amount of food and beverage consumed for two consecutive weekdays and one weekend day using standardized measures. Records were reviewed by study dieticians. Food intake data were analyzed, and energy and nutrient intake were calculated using the Nutritionist V Diet Analysis software (First DataBank Inc., San Bruno, CA). A diet aimed at weight maintenance was prescribed by a study dietician. This diet was well-balanced (50% carbohydrate, 20% protein and 30% fat) consisting of calculated weight maintenance calories per day. Alcohol intake was limited to 1 serving of alcoholic drink per week and participants were asked to avoid alcohol consumption 72 hours before scheduled study visits. They were instructed to consume a standardized dinner meal prior to the evening before study visits (750 calories, same composition as previously noted).

Resting energy expenditure (REE) was measured after 30 minutes of rest using a microprocessor-controlled indirect calorimetry device (Sensormedics, Yorba Linda, CA) that measures oxygen consumption and carbon dioxide production.[72] Respiratory quotient (RQ) was the ratio of oxygen consumed over carbon dioxide produced.

Leptin measurements. In the cross-sectional study, leptin levels were measured from a single fasting sample after 10 hours of fasting using radioimmunoassay

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(RIA), originally manufactured by Linco (St. Charles, MO) and then Millipore. Subjects with RLD were defined based on criteria in Table S1. The levels on the RLD trial were measured all together using the ELISA assay (EMDMilipore, Billerica, MA, USA). Leptin levels were measured by the latter ELISA kit in the PL study. In both of the treatment studies, three samples were drawn 30 minutes apart and averaged to compensate for the pulsatility seen in serum leptin levels across the intervention study periods.

Adipokine measurements. Adiponectin levels were measured as previously described using a commercially available kit by Linco Inc (St Charles, MO). Other cytokines and soluble receptors, as well as soluble leptin receptor levels, were measured with the Quantikine ELISA (R&D Systems, Minneapolis, MN).

Determination of other circulating hormone levels. In order to understand the pathways by which leptin effect can be mediated, the following circulating factors were measured using standardized commercially available kits (Millipore, Billerica, MA): ghrelin, glucagon-like peptide-1, and GIP using samples pretreated with protease and DPPIV inhibitor. The tubes for the incretin hormones contained Pefabloc SC (Sigma-Aldrich, St. Louis, MO) and DPP-IV inhibitor (EMD Millipore, Billerica, MA) to inhibit DPP-IV and other proteases.

Assessment of body composition. Body composition was evaluated using skin thickness measurements and waist and hip circumferences using standardized techniques. Dual X-ray absorptiometry (DEXA) (GE Lunar Prodigy, model PA +41744, Madison, WI) was used to estimate fat and lean body mass.[73] Data from DEXA evaluation was used to calculate FMR, a ratio of the percentage of the trunk fat mass to the percentage of the lower limb fat mass. Fat shadow images were generated by processing the DEXA scan files (.dfb) and analyzed by using enCore v14.10 as described previously.[74]

MRI for measurement of hepatic fat content. To evaluate hepatic fat content, MR imaging using quantitative multi-echo Dixon method and multi-echo MR-spectroscopy was utilized and this method has been described previously.[22,75] Briefly, three-dimensional volumes were acquired with geometry: nominal field-of-view (FOV) = 400mm x 350mm; in-plane acquired spatial resolution = 2.5mm reconstructed to 2mm resolution; and 56 axial 5mm thick slices centered mid-liver. Six gradient-recalled-echoes within echo times (TE) = 0.96ms – 4.46ms, short repetition time (TR) = 5.6ms, flip-angle = 3o and parallel imaging acceleration = 2 afforded single breath-hold acquisitions. Quantitative proton density fat-fraction (PDFF) maps were reconstructed on the MRI system using a 6-peak complex signal decay model.

Liver biopsies. Percutaneous liver biopsy specimens were obtained at baseline and 12 months of therapy. Histological features of NAFLD/NASH were studied using the validated NASH-clinical research network (CRN) scoring system in a blinded fashion. This scoring system comprises 14 histologic features, 4 of which (steatosis [0-3], lobular inflammation [0-2], hepatocellular ballooning [0-3], and fibrosis [0-4]) are evaluated semiquantitatively (Table S2). NAFLD activity score (NAS) is the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores, and this, together with the fibrosis score, constitutes the total NASH score.

Transcriptional profiling. RNA from 5-20 mg of flash-frozen liver biopsy samples were isolated using a QIAGEN RNeasy kit followed by treatment with RNase-free DNase (Ambion). The RNA concentration was assessed by Nanodrop (Thermo Fisher

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Scientific). RNA was then hybridized to the Human HT-12 BeadChip (Illumina), which interrogates 47 323 transcripts. The microarray hybridizations and normalizations were performed by the DNA sequencing Core at the University of Michigan according to the manufacturer's instructions. Raw data were background corrected using a normal-exponential convolution model based on negative control probes, quantile normalized, and log-2 transformed. Pathway enrichment between paired biopsies was performed using LRpath[19] genes, using p values and fold-change comparing baseline and post-treatment samples. We tested for enrichment of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for humans.

RT-PCR analyses. RNA was extracted as above. A 2-μg aliquot of total RNA was reverse-transcribed to generate single-strand complementary DNA (cDNA). Real-time PCR was carried out using an Opticon Cycler (MJ Research) using conditions specific for each primer set. Two microliters of cDNA were then amplified in a final volume of 25 μL using the QIAGEN CyberGreen Quantitec system. After an initial denaturation (95°C for 15 min), amplification was performed for 35 cycles. Amplification of 18S RNA was used to normalize each sample for reverse transcription efficiency. In general, each primer set was chosen to amplify a 3′ domain of the mRNA of interest and, where possible, the PCR product would cross a predicted intron to examine the genomic contamination of the PCR reaction. Duplicate samples of RNA from each biopsy were individually quantified.

Measurement of plasma fatty acids. Fatty acid analyses were done in the Metabolomics Core of the Michigan Metabolomics and Obesity Center. Plasma samples were collected in EDTA-containing tubes and immediately centrifuged at 3500 rpm for 5 minutes; plasma was subsequently stored at –20°C until analysis was performed. Total lipids were extracted from the plasma according to the method of Bligh and Dyer.[76] Heptadecanoic acid was added as an internal standard for the quantization of fatty acids. Subclasses of lipids were then isolated by thin layer chromatography and the fatty acid composition analyzed by gas chromatography on an Omega Wax 250 capillary column (Supelco) following methylation. Column temperature was increased from 60° to 240°C at a rate of 30° per minute. The column was kept at 240° for 8 minutes for a total run time of 14 minutes. Relative abundance of 18 different fatty acid species was done by comparison of retention times with known standards obtained from Nu-Chek Prep (Elysian, MN).

Lipidomic profiling of plasma. Liquid chromatography-mass spectrometry-based shotgun lipidomics using a TripleTOF 5600 was applied for lipid identification. Detailed methods can be found in Afshinia et al.[77] Missing values for lipids were imputed using the K nearest-neighbor method.[78] The data were log2 transformed and normalization a cross-contribution compensating multiple internal standard normalization method.[79] A compound-by-compound t test was applied to identify differentially regulated lipids that passed the nominal threshold *P value* of < 0.05, followed by the Benjamini-Hochberg correction for false discovery rate (FDR) to account for multiple comparisons.[79]

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis methods

Statistical analyses were performed using GraphPad Prism version 8 (La Jolla, CA), SAS version 9.2 (Cary, NC), and SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). The primary outcome variable of the open-label RLD clinical trial was the change in global NASH scores. Two-tailed *P value* of < 0.05 was considered significant as

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was decided a *priori*. A chi-square test was used to compare categorical variables. The independent samples t test was used to compare independent groups. The repeated-measures ANOVA was used to compare variables that were based on repeated observations. Differences in each collected parameter were evaluated using a paired test (compared to baseline). *P value*s are marked if they are significant after multiplicity correction. Paired t test was used to compare month-12 values to baseline (without multiplicity correction) as the change at 12 months versus baseline was a prespecified endpoint. Different components of the NASH score before and after metreleptin treatment were compared by Wilcoxon matched-pairs signed rank test. Log transformation was applied if needed. If data were skewed, nonparametric tests were used as needed.

Missing data

The last observation carried forward (LOCF) imputation method was used as repeated-measures were taken per subject by longitudinal time points. In the RLD study, the last observed non-missing values (month 6 and month 10, respectively) were used to fill in missing values in two subjects who dropped out of the study before the final visit was completed. For liver-biopsy-related parameters, the analyses were completed only in completers due to the potential confounding effects of the underlying reasons for drop-out. For the PL study, the last observed non-missing values (month 6) were used to fill in missing values at month 9 visit in one subject (subject ID: 21) who missed month 9 visit but completed the study protocol.

Data presentation

Data were presented as mean $\pm$ standard deviation (SD) or frequency unless otherwise stated. Triglycerides were reported as geometric mean with 95% confidence intervals for the PL study because the distribution was far more skewed than what was observed in the RLD study.

ADDITIONAL RESOURCES

The RLD study: ClinicalTrials.gov identifier: NCT00596934.

https://clinicaltrials.gov/ct2/show/NCT00596934

The PL study: ClinicalTrials.gov identifier: NCT01679197.

https://clinicaltrials.gov/ct2/show/NCT01679197