

Metreleptin improves insulin sensitivity independent of food intake in humans with lipodystrophy

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Endocrinology

Metabolism

BACKGROUND. Recombinant leptin (metreleptin) ameliorates hyperphagia and metabolic abnormalities in leptin-deficient humans with lipodystrophy. We aimed to determine whether metreleptin improves glucose and lipid metabolism in humans when food intake is held constant.

METHODS. Patients with lipodystrophy were hospitalized for 19 days with food intake held constant by controlled diet in an inpatient metabolic ward. In a non-randomized cross-over design, previously metreleptin-treated patients ($n = 8$) were continued on-metreleptin for five days, and off-metreleptin for the next 14 days (withdrawal cohort). This order was reversed in metreleptin-naïve patients ($n = 14$), who were restudied after six months of metreleptin treatment on an ad libitum diet (initiation cohort). Outcomes included insulin sensitivity by hyperinsulinemic-euglycemic clamp, fasting glucose and triglycerides, lipolysis measured using isotopic tracers, and liver fat by magnetic resonance spectroscopy.

RESULTS. With food intake constant, peripheral insulin sensitivity decreased by 41% after stopping metreleptin for 14 days (withdrawal cohort) and increased by 32% after starting metreleptin for 14 days (initiation cohort). In the initiation cohort only, metreleptin decreased fasting glucose by 11%, triglycerides by 41%, and increased hepatic insulin sensitivity. Liver fat decreased from 21.8% to 18.7%. In the initiation cohort, lipolysis did not change independent of food intake, but decreased after six months on metreleptin on an ad libitum diet by 30% (palmitate turnover) to 35% (glycerol turnover).

CONCLUSION. Using [...]

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1 **Metreleptin improves insulin sensitivity independent of food intake in humans**
2 **with lipodystrophy**

3

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29 in the design, conduct, analysis, interpretation, or decision to publish the study.

30 **Abstract**

31

32 **Background.** Recombinant leptin (metreleptin) ameliorates hyperphagia and metabolic
33 abnormalities in leptin-deficient humans with lipodystrophy. We aimed to determine whether
34 metreleptin improves glucose and lipid metabolism in humans when food intake is held constant.

35

36 **Methods.** Patients with lipodystrophy were hospitalized for 19 days with food intake held constant
37 by controlled diet in an inpatient metabolic ward. In a non-randomized cross-over design,
38 previously metreleptin-treated patients (N=8) were continued on-metreleptin for five days, and
39 off-metreleptin for the next 14 days (withdrawal cohort). This order was reversed in metreleptin-
40 naïve patients (N=14), who were restudied after six months of metreleptin treatment on an *ad*
41 *libitum* diet (initiation cohort). Outcomes included insulin sensitivity by hyperinsulinemic-
42 euglycemic clamp, fasting glucose and triglycerides, lipolysis measured using isotopic tracers, and
43 liver fat by magnetic resonance spectroscopy.

44

45 **Results.** With food intake constant, peripheral insulin sensitivity decreased by 41% after stopping
46 metreleptin for 14 days (withdrawal cohort) and increased by 32% after starting metreleptin for 14
47 days (initiation cohort). In the initiation cohort only, metreleptin decreased fasting glucose by 11%,
48 triglycerides by 41%, and increased hepatic insulin sensitivity. Liver fat decreased from 21.8% to
49 18.7%. In the initiation cohort, lipolysis did not change independent of food intake, but decreased
50 after six months on metreleptin on an *ad libitum* diet by 30% (palmitate turnover) to 35% (glycerol
51 turnover).

52

53 **Conclusion.** Using lipodystrophy as a human model of leptin deficiency and replacement, we
54 showed that metreleptin improves insulin sensitivity, and decreases hepatic and circulating
55 triglycerides, independent of its effects on food intake.

56

57 **Trial registration.** [ClinicalTrials.gov, NCT01778556](https://clinicaltrials.gov/ct2/show/study/NCT01778556)

58

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60 of Diabetes and Digestive and Kidney Diseases.

61 **Introduction**

62

63 Leptin is an adipocyte-derived hormone that signals overall body energy sufficiency (1) and acute
64 energy balance (2). Leptin deficient states, such as starvation or mutations in the leptin gene, lead
65 to hyperphagia. An additional leptin deficient state is lipodystrophy, in which a deficiency of
66 adipose tissue results in hypoleptinemia, which induces hyperphagia, with energy intake ~40%
67 higher than predicted (3). The excess caloric intake is stored as ectopic fat in liver and muscle,
68 causing severe insulin resistance and diabetes, along with hypertriglyceridemia, low levels of high-
69 density lipoprotein cholesterol (HDL-C), and non-alcoholic fatty liver disease (NAFLD) (4, 5).
70 Therefore, patients with lipodystrophy can serve both as a model of leptin-deficiency and
71 replacement, as well as metabolic disease that is analogous to, albeit more severe than, that seen
72 in patients with obesity-associated metabolic syndrome.

73

74 Treatment with metreleptin, a recombinant analog of human leptin, in humans with lipodystrophy
75 ameliorates hyperphagia, ectopic lipid storage, hypertriglyceridemia, insulin resistance, and
76 reproductive dysfunction (4, 6-9). The reduction in food intake is likely responsible for part of the
77 observed improvements in glucose and lipid metabolism. Rodent studies in leptin-treated ob/ob
78 mice and n-SREBP-1c lipodystrophic mice showed an additional reduction in glucose and insulin
79 levels compared to pair-fed controls, suggesting that leptin has a hypoglycemic effect independent
80 of its effects on food intake (10, 11). Whether leptin has these energy intake independent effects
81 in humans has not previously been determined.

82

83 Using lipodystrophy as a human model of leptin deficiency and replacement, we conducted a non-
84 randomized crossover study to determine the energy intake independent effects of leptin on
85 glucose and lipid metabolism. We hypothesized that, during constant food intake, patients with
86 lipodystrophy would have greater insulin sensitivity and reduced lipolysis during the period of
87 leptin replacement versus the leptin-deficient state. Patients with no prior exposure to metreleptin
88 constituted the initiation cohort, and patients already undergoing metreleptin treatment prior to our
89 study constituted the withdrawal cohort. All patients were hospitalized for 19 days with energy
90 and macronutrient intake held constant by controlled diet in an inpatient metabolic ward during
91 metreleptin treated and untreated periods. The withdrawal cohort was on-metreleptin for five days,
92 and off-metreleptin for the next 14 days. This order was reversed in the initiation cohort, who were
93 restudied after six months of metreleptin on an *ad libitum* diet (Figure 1).

94 **Results**

95 *Study participants.* Twenty-five patients with lipodystrophy were enrolled. The flow of
96 participants in this non-randomized crossover study is shown in Figure 2. Of the fifteen initiation
97 subjects, one did not complete study procedures for the short-term, controlled food-intake portion
98 of the study but completed six-month follow-up, and one completed the short-term study but was
99 excluded from analysis of the six-month data due to non-compliance with the study drug. In the
100 withdrawal cohort, eight subjects completed the study and were included in the analysis.

101

102 Baseline characteristics of subjects are shown in Table 1. Of the 15 subjects in the initiation group,
103 three had generalized lipodystrophy and 12 had partial lipodystrophy. Nine were non-Hispanic
104 White, four were Hispanic White, one was Asian, and one was Other race. In the withdrawal
105 cohort, all eight patients had generalized lipodystrophy. Four were non-Hispanic White, two were
106 African-American, and two were Hispanic White. The majority were female (~70%) in both
107 groups. At baseline, the initiation cohort had an endogenous leptin level of 9.5 ± 10.2 ng/dL.
108 Seventy-one percent were taking insulin with a mean insulin dose among insulin users of $225 \pm$
109 136 units per day. In contrast, the withdrawal cohort had a lower endogenous leptin level prior to
110 metreleptin therapy of 1.2 ± 0.5 ng/dL, reflecting greater leptin insufficiency in patients with
111 generalized lipodystrophy, and had an average of 7.7 ± 4.7 (range 0.9-14.5) years of prior
112 metreleptin treatment. None were taking insulin. The expected relationship between fat mass and
113 the log of endogenous leptin was observed in the combined cohorts ($R^2 = 0.69$, $p < 0.0001$), with
114 no difference by sex, cohort (initiation versus withdrawal), or lipodystrophy type (generalized
115 versus partial).

116

117 *Short-term effects of metreleptin independent of food intake*

118 *Food intake, diet, and body composition.* During the 19-day inpatient stay, patients were required
119 to consume all study-provided food and forbidden to consume any outside food. Any uneaten
120 portions of the study diet were weighed, and uneaten nutrients were replaced at the next meal when
121 possible. Energy intake and macronutrient content were successfully held constant in the off-
122 versus on-metreleptin periods in both groups (Table 2). Furthermore, multivariate analyses showed
123 that the effects of metreleptin on outcome measures of interest were not significantly influenced
124 by actual caloric intake during the off and on metreleptin periods in either cohort (Supplemental
125 Table 1).

126
127 In the initiation cohort, body weight and fat mass significantly decreased by 0.7 kg and 0.3 kg,
128 respectively, after two weeks on metreleptin. There was no change in body weight or fat mass in
129 the withdrawal cohort, and no change in lean mass or percent body fat in either group.

130
131 *Peripheral insulin sensitivity was greater on metreleptin therapy independent of food intake in*
132 *both initiation and withdrawal cohorts. Short-term metreleptin therapy increased hepatic insulin*
133 *sensitivity independent of food intake in the initiation cohort only.* In the initiation cohort,
134 peripheral insulin sensitivity measured by hyperinsulinemic-euglycemic clamp increased from 4.4
135 ± 2.3 mg/kg_{FFM}/min at the end of Period 1 pre-metreleptin, to 5.8 ± 2.2 mg/kg_{FFM}/min at the end
136 of Period 2 on-metreleptin ($p=0.001$) (Figure 3). Similarly, in the withdrawal cohort, peripheral
137 insulin sensitivity decreased from 10.9 ± 4.1 mg/kg_{FFM}/min at the end of Period 1 on-metreleptin
138 to 6.4 ± 1.8 mg/kg_{FFM}/min ($p=0.01$) at the end of Period 2 after metreleptin withdrawal (Figure 3).
139 The magnitude of the increase in insulin sensitivity in the on- versus off-metreleptin condition was

140 greater in the leptin withdrawal cohort. In the withdrawal cohort, there was a correlation between
141 the reduction in peripheral insulin sensitivity after metreleptin withdrawal, and increases in fasting
142 glucose (p=0.014) and c-peptide (p=0.006).

143
144 In the initiation cohort, hepatic insulin sensitivity measured as insulin-mediated suppression of
145 hepatic glucose production (HGP) increased from $61 \pm 23\%$ at the end of Period 1 to $75 \pm 33\%$
146 (p=0.008) at the end of Period 2 (Figure 3). Suppression of HGP did not change in the withdrawal
147 cohort (Figure 3).

148
149 Changes in hepatic triglyceride content significantly predicted changes in both peripheral and
150 hepatic insulin sensitivity with metreleptin in the initiation cohort, only (Supplemental Tables 2
151 and 3). Moreover, changes in peripheral and hepatic insulin sensitivity with metreleptin in the
152 initiation cohort were no longer statistically significant after adjustment for changes in hepatic
153 triglyceride content. By contrast, intramyocellular triglyceride content was not a significant
154 predictor of either peripheral or hepatic insulin sensitivity in most models, and improvement in
155 peripheral and hepatic insulin sensitivity with metreleptin remained statistically significant after
156 adjustment for intramyocellular triglyceride content (Supplemental Tables 2 and 4). Changes in
157 body composition did not predict changes in insulin sensitivity with metreleptin, and
158 improvements in insulin sensitivity remained statistically significant after adjustment for body
159 composition.

160
161 *Short-term metreleptin therapy decreased fasting glucose and glucosuria independent of food*
162 *intake in the initiation cohort.* In the initiation cohort, fasting glucose decreased from 152 ± 42

163 mg/dL at the end of Period 1 pre-metreleptin to 136 ± 34 mg/dL ($p=0.003$) at the end of Period 2
164 on-metreleptin (Figure 3). In addition, 24-hour urine glucose excretion decreased from 2.0
165 [0.2,10.3] g/24h at the end of Period 1 pre-metreleptin to 1.2 [0.2,7.2] g/24h ($p=0.049$) at the end
166 of Period 2 on-metreleptin (Table 3). HbA1c decreased from $8.7 \pm 2.0\%$ at the end of Period 1 pre-
167 metreleptin to $8.0 \pm 1.3\%$ ($p=0.002$) at the end of Period 2 on-metreleptin. However, because the
168 initial HbA1c reflected glycemic control for the three months prior to the study, this change cannot
169 be considered as independent of food intake. Relative to hospital admission when patients were on
170 an *ad libitum* diet, mean insulin dose in these patients decreased by 95 ± 126 units per day at the
171 end of Period 2 on metreleptin ($p=0.04$); however, there was no significant change in insulin dose
172 or insulin secretion (measured as fasting C-peptide) independent of food intake. Fasting glucose,
173 HbA1c, C-peptide, and urine glucose excretion did not change in the withdrawal cohort.

174

175 *Short-term metreleptin therapy decreased triglycerides and total cholesterol independent of food*
176 *intake in the initiation cohort, but did not change HDL-C, free fatty acids, or LDL-C.* In the
177 initiation cohort, triglycerides decreased from 556 [224,1144] (geometric mean [25th,75th
178 percentile]) mg/dL at the end of Period 1 pre-metreleptin to 335 [162,611] mg/dL at the end of
179 Period 2 on-metreleptin ($p=0.01$) (Figure 4). Total cholesterol also decreased from 241 ± 116
180 mg/dL at the end of Period 1 to 171 ± 48 mg/dL at the end of Period 2 ($p=0.002$) (Table 3). In the
181 withdrawal cohort, triglycerides and total cholesterol did not change. The magnitude of the
182 decrease in total cholesterol in the on- versus off-metreleptin condition was greater in the leptin
183 initiation cohort. Free fatty acids, HDL-C, and LDL-C did not change in either the initiation or
184 withdrawal cohort (Table 3).

185

186 *Short-term metreleptin therapy did not change lipolysis independent of food intake.* Lipolysis was
187 quantified by infusing D₅-glycerol and ¹³C₁₆-palmitate to measure turnover through isotope
188 dilution studies. In the initiation and withdrawal cohorts, short-term metreleptin with food intake
189 held constant did not change the endogenous rate of appearance (Ra) of glycerol or palmitate
190 (Figure 4).

191
192 *Short-term metreleptin therapy decreased hepatic triglyceride content independent of food intake*
193 *in the initiation cohort.* In the initiation cohort, there was a reduction in liver fat from 21.8 ± 10.9%
194 at the end of Period 1 pre-metreleptin to 18.7 ± 12.5% (p=0.03) at the end of Period 2 on-
195 metreleptin (Figure 4). Liver fat did not change in the withdrawal cohort independent of food
196 intake. Short-term metreleptin did not change either extramyocellular (EMCL) or intramyocellular
197 lipid (IMCL) content independent of food intake in either the initiation or withdrawal cohorts
198 (Table 3).

199
200 *Short-term metreleptin therapy decreased total and resting energy expenditure independent of*
201 *food intake in the initiation cohort.* In the initiation cohort, total energy expenditure decreased
202 from 2463 ± 362 kcal/day at the end of Period 1 pre-metreleptin to 2319 ± 400 kcal/day at the end
203 of Period 2 on-metreleptin (p=0.001). Resting energy expenditure also decreased in this cohort
204 from 1855 ± 289 kcal/day to 1736 ± 308 kcal/day (p=0.01); this change was no longer statistically
205 significant after adjusting for changes in lean and fat mass. Non-resting energy expenditure (total
206 minus resting) did not change in the initiation cohort. Total, resting, and non-resting energy
207 expenditure did not change in the withdrawal cohort independent of food intake.

208

209 *Long-term effects of metreleptin while on an ad libitum diet*

210 To study the long-term effects of metreleptin, the initiation cohort returned for a follow-up visit
211 after 6.8 ± 1.0 months of metreleptin therapy. At this visit and during the six months prior, patients
212 were on an *ad libitum* diet, thus any observed effects of metreleptin were not independent of food
213 intake.

214
215 *Long-term metreleptin therapy decreased body weight, fat mass, lean mass, and percent body fat*
216 *in the initiation cohort.* At the six-month follow-up visit for the initiation cohort while on an *ad*
217 *libitum* diet, body weight decreased from 73.8 ± 16.0 kg pre-metreleptin to 70.8 ± 16.8 kg
218 ($p=0.005$), fat mass decreased from 18.3 ± 10.6 kg to 15.5 ± 10.0 kg ($p=0.028$), lean mass
219 decreased from 53.1 ± 9.2 kg to 51.5 ± 9.4 kg ($p=0.002$), and percent body fat decreased from 24.3
220 $\pm 10.8\%$ to $21.3 \pm 10.6\%$ ($p=0.02$).

221
222 *Long-term metreleptin therapy maintained improvements in peripheral and hepatic insulin*
223 *sensitivity.* At the six-month follow-up visit for the initiation cohort while on an *ad libitum* diet,
224 peripheral insulin sensitivity improvement was maintained at 8.0 ± 4.0 mg/kg_{FFM}/min ($p=0.01$ vs
225 Period 1). There was no further increase in peripheral insulin sensitivity at six-month follow-up
226 relative to Period 2 in the unadjusted analysis ($p=0.09$, Figure 3), although this difference was
227 significant after adjustment for covariates ($p=0.048$, Supplemental Table 1). Similarly, hepatic
228 insulin sensitivity improvement was maintained at the six-month follow up visit at $86 \pm 18\%$
229 suppression of HGP ($p=0.02$ vs Period 1), but there was no further increase in hepatic insulin
230 sensitivity relative to Period 2 (Figure 3).

231

232 Changes in hepatic triglyceride content significantly predicted long-term changes in peripheral
233 insulin sensitivity with metreleptin (Period 1 versus six-month follow-up, and Period 2 versus 6-
234 month follow-up, Supplemental Tables 2 and 3). Hepatic triglyceride content also significantly
235 predicted change in hepatic insulin sensitivity from Period 2 to six-month follow-up
236 (Supplemental Tables 2 and 3). Changes in both peripheral and hepatic insulin sensitivity
237 (Period 1 versus 6-month follow-up) were no longer statistically significant after adjustment for
238 changes hepatic triglyceride content (Supplemental Table 3). Intramyocellular triglyceride
239 content was not a significant predictor of long-term change in peripheral or hepatic insulin
240 sensitivity in most models (Supplemental Tables 2 and 4).

241

242 *Long-term metreleptin therapy maintained improvements in fasting glucose and HbA1c.* At the
243 six-month follow-up for the initiation cohort while on an *ad libitum* diet, the reduction in fasting
244 glucose was maintained at 126 ± 26 mg/dL ($p=0.02$ vs Period 1), and HbA1c reduction was also
245 maintained at $6.9 \pm 1.4\%$ ($p=0.01$ vs Period 1), but there were no further decreases relative to
246 Period 2 (Figure 3). Reductions in glycemia in the initiation cohort were observed despite
247 decreases in insulin doses in nine of 10 insulin-treated subjects. Relative to hospital admission,
248 mean insulin dose in these patients decreased by 112 ± 109 units per day (a 50% reduction) at six-
249 month follow-up ($p=0.01$). Two subjects discontinued insulin use by their six-month follow-up.
250 The mean number of diabetes medications (insulin + other agents) did not change after six months.
251 There were no significant changes in C-peptide during the study.

252

253 *Long-term metreleptin therapy maintained improvements in triglycerides and total cholesterol.* At
254 the six-month follow-up for the initiation cohort while on an *ad libitum* diet, the reduction in

255 triglycerides was maintained at 304 [122,547] (p=0.24 vs Period 1, Figure 4), and reduction in
256 total cholesterol was maintained at 129 ± 32 (p=0.02 vs Period 1) but there were no further
257 decreases relative to Period 2. Free fatty acids, HDL-C, and LDL-C did not change in either the
258 initiation or withdrawal cohort (Table 3). The mean number of lipid-lowering medications did not
259 change during the study.

260
261 *Long-term metreleptin therapy decreased glycerol and palmitate turnover in the initiation cohort.*

262 At six-month follow-up for the initiation cohort while on an *ad libitum* diet, palmitate turnover
263 decreased by 30% from 3.2 ± 1.3 $\mu\text{mol/kgLBM}/\text{min}$ prior to metreleptin in Period 1 to 2.2 ± 0.7
264 $\mu\text{mol/kgLBM}/\text{min}$ (p=0.02), and glycerol turnover decreased by 35% from 4.5 ± 2.3
265 $\mu\text{mol/kgLBM}/\text{min}$ prior to metreleptin in Period 1 to 2.9 ± 0.7 $\mu\text{mol/kgLBM}/\text{min}$ (p=0.02), indicating
266 a decrease in lipolysis (Figure 4).

267

268 *Long-term metreleptin therapy maintained reduction in liver fat, and reduced ALT and AST. At*

269 six-month follow-up for the initiation cohort while on an *ad libitum* diet, reduction in liver fat was
270 maintained at $13.6 \pm 9.7\%$ (p=0.006 vs Period 1), but there was no further improvement relative
271 to Period 2 (Figure 4). ALT and AST were measured on study entry (prior to the controlled diet)
272 but not at the end of Period 1, thus any changes observed were not independent of food intake.
273 Mean ALT was elevated at study entry (pre-metreleptin) at 64 ± 54 U/L (normal ≤ 41 in males
274 over 18 years, ≤ 33 in females over 18 years, ≤ 30 in children), decreased non-significantly to 43
275 ± 23 after 2 weeks, and decreased significantly to 26 ± 13 after six months of metreleptin relative
276 to study entry (p=0.004). Mean AST was borderline elevated at study entry at 39 ± 25 U/L (normal
277 ≤ 40 in males over 18 years, ≤ 32 in females over 18 years, ≤ 40 in children), decreased

278 significantly to 30 ± 19 after 2 weeks, and further decreased to 22 ± 7 after six months of
279 metreleptin ($p=0.03$ relative to study entry; $p=0.04$ relative to 2 weeks).

280
281 *Long-term metreleptin therapy did not change IMCL, and had variable effects on EMCL.* IMCL
282 did not change in any muscle group after six months of metreleptin, but EMCL decreased in the
283 lateral vastus and tibialis anterior muscles and increased in the soleus muscle (Table 4).

284
285 *Long-term metreleptin therapy maintained reduction in total and resting energy expenditure.* At
286 six-month follow-up for the initiation cohort while on an *ad libitum* diet, reductions in total and
287 resting energy expenditure were maintained at 2296 ± 372 kcal/day and 1731 ± 236 kcal/day,
288 respectively ($p=0.02$ vs Period 1 for both), but there was no further change relative to Period 2.

289
290 *Adverse Events.* The following not-serious adverse events occurred in one subject each in the
291 initiation cohort during long-term metreleptin treatment, and were considered at least possibly
292 related to research: decreased appetite, weight loss, hair loss, hypoglycemia (in a subject treated
293 with insulin), injection site reaction, and menorrhagia. Serious adverse events considered not
294 related to research were: abdominal pain of unknown etiology ($n=1$) and angioedema secondary
295 to angiotensin converting enzyme inhibitor use ($n=1$). Serious adverse events considered at least
296 possibly related to research were: anemia secondary to menorrhagia (two events in one subject).

297 **Discussion**

298

299 In patients with lipodystrophy, metreleptin therapy ameliorates metabolic abnormalities by
300 reducing food intake (3, 12, 13), improving insulin resistance and diabetes (4, 13-15), and reducing
301 ectopic lipid (7). These improvements in glucose and lipid metabolism are likely due in part to the
302 reduction in food intake, but the clinical effects of metreleptin that are independent of changes in
303 food intake have been poorly explored in humans. A single patient with acquired, generalized
304 lipodystrophy was studied while taking metreleptin, and during metreleptin withdrawal, with
305 constant energy intake (13). Upon metreleptin withdrawal, this patient experienced no changes in
306 blood glucose, but a rise in serum insulin and triglycerides within one week (13). Although based
307 on a single subject, these data suggested that leptin affects both insulin resistance and lipid
308 metabolism independent of energy intake in humans. Our study demonstrates that metreleptin has
309 food-intake independent effects in humans with lipodystrophy to increase peripheral and hepatic
310 insulin sensitivity, and decrease fasting glucose, triglycerides, total cholesterol, and percent liver
311 fat. As expected, the magnitude of metreleptin's effects independent of food intake over 2 weeks
312 was smaller than the maximal effects of long-term metreleptin treatment during *ad libitum* food
313 intake shown in prior studies (Figure 5) (4, 7, 15).

314

315 Because leptin reduces appetite (13), its effects independent of food intake cannot be studied in a
316 free-living environment with *ad libitum* access to food. In this study, the tightly controlled nature
317 of a metabolic ward permitted meticulous control of dietary intake, and our data confirmed that
318 we successfully held food-intake constant for three weeks. It is likely that many of leptin's
319 biological effects require more than two weeks of treatment initiation or withdrawal to show

320 maximal changes and thus, our study may underestimate the biological effects of leptin that are
321 independent of food intake. Although it would have been informative to continue the study for a
322 longer duration, three weeks was the practical limit during which we could keep patients
323 hospitalized on a controlled diet.

324

325 The most consistent effect of metreleptin independent of food-intake was improvement in
326 peripheral insulin sensitivity, which was 32% greater in the initiation cohort and 41% greater in
327 the withdrawal cohort during the metreleptin treated periods. Hepatic insulin sensitivity was
328 higher during metreleptin treatment in the initiation cohort, only. Our human data are consistent
329 with prior findings in rodents, which showed that leptin improved peripheral and hepatic insulin
330 sensitivity by 12-33% and 32-41%, respectively, independent of food intake (16, 17). Consistent
331 with the improvements in insulin sensitivity, two weeks of metreleptin improved fasting glucose
332 by 11%. Similar ~42-53% reduction in fasting glucose has been observed in pair-fed, leptin-
333 deficient rodent studies (10, 11).

334

335 Based on prior data in humans (7, 18), we hypothesized that improved insulin sensitivity with
336 metreleptin would be due to reductions in ectopic triglyceride in liver and myocytes. However,
337 only reductions in hepatic triglyceride were observed. Numerous studies have demonstrated an
338 association between hepatic triglyceride content and peripheral insulin resistance, although the
339 direction of causality in this relationship is unclear (19-22). Although it is possible that lipid
340 laden hepatocytes secrete cytokines or other substances that increase muscle insulin resistance, it
341 is also possible that skeletal muscle or adipose tissue insulin resistance lead to hepatic
342 triglyceride accumulation through mechanisms such as increased free fatty acid delivery from

343 adipose tissue to liver, or increased de novo lipogenesis stimulated by hyperinsulinemia. In
344 rodents, a liver-targeted mitochondrial uncoupling agent led to decreases in both hepatic and
345 peripheral insulin resistance, supporting the notion of a causal relationship between hepatic
346 triglyceride and peripheral insulin resistance (23). Multivariate analyses in the current study also
347 support a stronger role for intrahepatic triglyceride (versus intramyocellular triglyceride) in
348 mediating both hepatic and peripheral insulin resistance, although we cannot prove a causal
349 relationship. Changes in hepatic triglyceride content, but not intramyocellular triglyceride
350 content, significantly predicted changes in both peripheral and hepatic insulin sensitivity, and
351 changes in insulin sensitivity were no longer statistically significant after adjustment for changes
352 in hepatic triglyceride. This suggests that reductions in hepatic triglyceride content with
353 metreleptin may have mediated the improvements in insulin sensitivity.

354
355 Although patients who initiated metreleptin lost a small amount of weight and fat mass during
356 metreleptin treatment with constant food intake, these changes in body composition did not
357 predict changes in insulin sensitivity, and thus the small reductions in weight and body fat
358 observed in the metreleptin-initiation cohort were not likely to have contributed to improved
359 insulin sensitivity. An unexpected finding was the decrease in resting and total energy
360 expenditure in the metreleptin-initiation cohort during constant food intake. Limited data in
361 patients with congenital leptin deficiency or weight loss have suggested that metreleptin either
362 does not change energy expenditure (24), or increases non-resting energy expenditure (25, 26).
363 The biology underlying the reduction in resting energy expenditure in this study remains to be
364 determined, but might include decreased urinary glucose loss, decreased energetic cost of hepatic
365 glucose production (27, 28), decreased patient movement during measurement of energy

366 expenditure after repeated testing (29), and that subjects were in slightly negative energy
367 balance. Regardless of the reason for decreased energy expenditure, it is clear that there was no
368 increase in energy expenditure with metreleptin that contributed to weight loss or improved
369 insulin sensitivity.

370

371 Our study shows that there are food-intake independent effects of metreleptin on lipid metabolism,
372 with a reduction in circulating and hepatic triglycerides and total cholesterol in humans with
373 lipodystrophy. Although rodent studies have not demonstrated clinically relevant changes in lipids
374 independent of food intake, mechanistic studies in rodents have suggested that these effects may
375 be mediated by increased expression of enzymes and transcription factors involved in fatty acid
376 oxidation (e.g. mitochondrial and peroxisomal acyl-coenzyme A oxidase, peroxisomal
377 proliferator-activated receptor-alpha) and decreased expression of those regulating fatty acid
378 synthesis (e.g. stearoyl-CoA desaturase-1) (30-32).

379

380 We found that metreleptin treatment for six-months while on an *ad libitum* diet decreased both
381 glycerol and palmitate turnover in subjects with lipodystrophy, indicating a reduction in lipolysis.
382 This reinforces data from a prior study in three subjects with lipodystrophy, in whom three to five
383 months of metreleptin non-significantly decreased glycerol turnover (7). In contrast, *in vitro* and
384 *in vivo* rodent studies have shown that leptin treatment reduces muscle, liver, and adipose
385 triglyceride content by increasing lipolysis and fatty acid oxidation (33-39). These lipolytic effects
386 of leptin have been shown in obese rodents with mutations in the leptin gene or leptin-receptor,
387 but not in rodents with lipodystrophy, suggesting that the observed lipolytic effects of leptin
388 require normal adipose depots. Contrary to the findings in obese rodent models, long-term

389 metreleptin had anti-lipolytic effects in subjects with lipodystrophy. Although humans with
390 lipodystrophy have a paucity of adipose tissue, these subjects are known to have elevated rates of
391 lipolysis compared to gender-, age-, and BMI-matched controls prior to metreleptin therapy,
392 presumably reflecting greater lipolysis in their residual fat mass (7). The effects of long-term
393 metreleptin to suppress lipolysis are presumably secondary to improved insulin sensitivity, and
394 hence increased insulin-mediated suppression of lipolysis. Given the hierarchy of physiologic
395 responses to insulin, with suppression of lipolysis being the most sensitive, followed by
396 suppression of hepatic glucose production, followed by glucose uptake in muscle, it is somewhat
397 surprising that short-term metreleptin treatment did suppress hepatic glucose production and
398 increase muscle glucose uptake, but did not decrease lipolysis. We speculate that the null effects
399 of short-term metreleptin on lipolysis may be due to opposing direct lipolytic effects of leptin,
400 versus indirect suppression of lipolysis mediated by improved insulin sensitivity.

401

402 A limitation of our study was the small number of participants, but lipodystrophy is a rare disorder.
403 We had limited success in demonstrating biological effects of metreleptin withdrawal independent
404 of food intake. Other than effects on peripheral insulin sensitivity, the withdrawal cohort did not
405 experience the food-intake independent effects of metreleptin therapy that were observed in the
406 initiation cohort. This may have been due to small sample size, as there were few statistical
407 differences for metabolic changes in the on- versus off-metreleptin periods between the withdrawal
408 and initiation cohorts. The lack of changes in the withdrawal cohort may also be due to two
409 biological factors. First, the withdrawal cohort had an average of 7.7 ± 4.7 (range 0.9-14.5) years
410 of prior metreleptin treatment, resulting in euglycemia and normal triglycerides despite their
411 lipodystrophy diagnosis. Second, two weeks of metreleptin withdrawal may have been insufficient

412 to detect metabolic changes in the withdrawal cohort. By contrast, the initiation cohort had no
413 exposure to metreleptin and worse metabolic profiles at baseline, allowing for metabolic changes
414 that were of greater magnitude. The two groups also differed in types of lipodystrophy. In the
415 withdrawal cohort, all subjects had generalized lipodystrophy, and in the initiation cohort, most
416 subjects had partial lipodystrophy. This difference is not a likely explanation for the lack of effects
417 in the withdrawal cohort because we would have expected greater effects in subjects with
418 generalized lipodystrophy who have lower endogenous leptin levels, but this was not observed.

419

420 By using lipodystrophy as a model for leptin-deficiency and replacement, we have successfully
421 demonstrated that metreleptin therapy has food-intake independent effects on glucose and lipid
422 metabolism in humans. In addition to serving as a model for leptin-deficiency, lipodystrophy is
423 also a more severe form of the obesity-associated metabolic syndrome. Although metreleptin
424 treatment has biological effects in states of chronic hypoleptinemia, it has little effect on appetite,
425 body weight, or hormonal axes in leptin replete subjects undergoing either mild, ongoing caloric
426 restriction, or acute, severe energy restriction (72 hour fast), despite the fact that caloric restriction
427 can acutely decrease leptin levels (40-43). This study provides evidence for food-intake
428 independent effects of metreleptin in leptin-deficient humans, but effects of leptin independent of
429 food intake have yet to be explored in leptin-sufficient human models such as the obesity-
430 associated metabolic syndrome.

431 **Methods**

432 *Study subjects.* This was a non-randomized, crossover group study. Two groups of patients aged
433 14 to 70 years with lipodystrophy were studied: leptin initiation and leptin withdrawal. Participants
434 were recruited by referral from November 2012 to January 2017. Leptin initiation subjects had no
435 prior exposure to exogenous metreleptin and leptin withdrawal subjects had taken a stable dose of
436 exogenous metreleptin for a minimum of four months prior to study participation. The flow chart
437 of study participants in each cohort is shown in Figure 1. Of the 25 patients enrolled, 15 were in
438 the leptin-initiation cohort and 10 were in the leptin-withdrawal cohort. In the leptin-initiation
439 cohort, one subject did not complete data collection for the short-term study, but continued study
440 drug and completed the long-term study, and another subject completed the short-term study but
441 was excluded from analysis of the long-term study because of non-compliance with metreleptin
442 therapy. In the leptin-withdrawal cohort, one subject withdrew consent and another subject was
443 excluded from analysis due to recurrent hypoglycemia during the short-term study. Therefore, 14
444 subjects in the leptin-initiation cohort and eight subjects in the leptin-withdrawal cohort were
445 included in final analysis.

446

447 *Inclusion/Exclusion criteria.* Eligibility was based on a clinical diagnosis of lipodystrophy, age \geq
448 14 years, and one or more metabolic abnormalities including diabetes mellitus defined by the 2007
449 American Diabetes Association criteria, insulin resistance (fasting insulin $\geq 30 \mu\text{IU/mL}$), or
450 hypertriglyceridemia (fasting triglyceride $> 200 \text{ mg/dL}$). Patients were also required to have low
451 endogenous serum leptin measured either at NIH or at an outside laboratory prior to metreleptin
452 treatment ($< 8 \text{ ng/mL}$ in males, $< 12 \text{ ng/mL}$ in females). Exclusion criteria included HIV-
453 associated lipodystrophy, active inflammatory disease or glucocorticoid use, and changes in

454 diabetes or lipid-lowering medications within the past six weeks. Because of the risk of worsening
455 metabolic status with metreleptin withdrawal, additional exclusion criteria applied to the leptin-
456 withdrawal cohort, including: age < 18 years, HbA1c \geq 9%, serum triglycerides > 800 mg/dL, > 1
457 lifetime episode of acute pancreatitis, or \geq 1 episode of pancreatitis while on metreleptin, lipase
458 greater than upper limit of normal at study entry, or known presence of neutralizing antibodies to
459 leptin.

460

461 *Study design.* The study design is shown in Figure 2. Initiation subjects were studied for the first
462 five days without metreleptin (Period 1), then treated with metreleptin (5 mg subcutaneously q12
463 hours) for the next 14 days (Period 2). Withdrawal subjects were studied for the first five days on
464 their home dose of metreleptin (Period 1), then withdrawn from metreleptin for the next 14 days
465 (Period 2). Metreleptin was donated by Aegerion Pharmaceuticals (Cambridge, MA). Subjects and
466 investigators were not blinded to the intervention. All subjects were hospitalized on the metabolic
467 unit of the NIH Clinical Center, and consumed a controlled diet provided by the metabolic kitchen.
468 The study diet was controlled for macronutrient content (20 \pm 5% protein, 25 \pm 5% fat, 55 \pm 5%
469 carbohydrate). Research dietitians used the Mifflin St. Jeor equations for males with an activity
470 factor of 1.5 to estimate total caloric requirements (for both male and female participants). Food
471 intake (total kilocalories and macronutrient content) was adjusted for body weight fluctuations to
472 ensure eucaloric feeding during Period 1 and then the energy was clamped for Period 2, in order
473 to assess leptin's effects independent of energy intake. Subjects were instructed in the importance
474 of eating 100% of food given, and not consuming any additional food. However, to determine
475 possible deviations from the study diet, any uneaten food was weighed and the uneaten kilocalories
476 were recorded. At the end of Period 2, metreleptin was restarted in subjects in the withdrawal

477 cohort at their previous doses. Patients in the initiation cohort continued self-administered
478 metreleptin treatment after discharge, and underwent follow-up evaluation after six months of
479 treatment on an *ad libitum* diet. For patients in the initiation cohort with partial lipodystrophy,
480 metreleptin was continued at a dose of 5 mg q12 hours. For patients in the initiation cohort with
481 generalized lipodystrophy, the metreleptin dose was lowered at the end of Period 2 to prevent
482 excessive weight loss during the six-month follow-up period.

483
484 Apart from insulin and sulfonylureas, subjects continued their pre-admission medications
485 throughout the study, including oral hypoglycemic agents, lipid-lowering medications, and other
486 medications either related or unrelated to lipodystrophy and its complications. Initiation subjects
487 taking insulin or sulfonylureas were at risk of hypoglycemia due to improved insulin sensitivity
488 after metreleptin. None of the withdrawal subjects were taking insulin. Glucose monitoring was
489 performed in subjects with diabetes prior to meals and at bedtime. Due to hypoglycemia risk,
490 insulin and sulfonylurea doses were reduced as needed to minimize hypoglycemia.

491
492 *Primary outcomes.* The aim of this study was to determine the energy intake-independent effects
493 of leptin on glucose and lipid metabolism in lipodystrophic subjects. The pre-specified primary
494 outcome for glucose metabolism was total body insulin sensitivity (measured as glucose disposal
495 rate during a hyperinsulinemic-euglycemic clamp), and for lipid metabolism was the rate of
496 lipolysis (measured using glycerol stable isotope tracers). For leptin initiation and withdrawal
497 cohorts, clinical values were collected at study entry, end of Period 1, and end of Period 2 (Figure
498 2). Additional clinical values were obtained from the leptin initiation cohort at the six-month
499 follow-up visit while on an *ad libitum* diet.

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Additional outcomes included serum leptin levels, anthropometric parameters (body mass index [BMI] and body fat percent), glycemic and lipid variables (fasting glucose, fasting insulin, fasting c-peptide, HbA1c, lipids, urinary glucose excretion, number of anti-diabetic and lipid-lowering medications, insulin use and average daily insulin dose among insulin users), hepatic insulin sensitivity (measured as suppression of endogenous glucose production during a hyperinsulinemic-euglycemic clamp), rates of lipolysis and fatty acid turnover (measured using glycerol and palmitate stable isotope tracers), and lipid content in liver and skeletal muscles (measured using magnetic resonance spectroscopy [MRS]).

Metabolites and hormones. Blood samples were obtained following an 8-12 hour fast. Urine was collected over 24-hour periods. Glucose, insulin, C-peptide, HbA1c, total cholesterol, HDL-C, LDL-C, triglycerides, and urinary glucose excretion were analyzed using standard techniques of the NIH Clinical Center laboratory. In the withdrawal cohort, endogenous leptin in fasting serum samples was measured prior to metreleptin initiation by radioimmunoassay (EMD-Millipore, Billerica MA). The intra and inter assay coefficient of variation were 9.3% and 9.6% respectively. Of note, these samples for measurement of endogenous leptin were collected immediately prior to metreleptin initiation, 0.9 to 14.5 years prior to participation in the current study, under other IRB-approved protocols. In both cohorts, leptin was also measured in fasting EDTA-plasma samples at the end of Periods 1 and 2, and again after six months of metreleptin in the initiation cohort, by ELISA (EMD-Millipore, Billerica MA). The intra and inter assay coefficient of variation were 3.9% and 4.8% respectively.

523 *Body composition.* A DXA scan was obtained to measure fat and lean body mass at the end of
524 Period 1 and Period 2 for both cohorts, and during the six-month follow-up for the initiation cohort
525 only (iDXA, GE Healthcare, Madison, WI).

526

527 *Energy expenditure.* Energy expenditure was measured at the end of Period 1 and Period 2 for both
528 cohorts, and during the six-month follow-up for the initiation cohort only. Resting energy
529 expenditure (REE) was measured using indirect calorimetry with a hood calorimeter (ParvoMedics
530 TrueOne2400, Sandy UT) upon awakening after a minimum 8-hour fast, in a resting supine
531 position. Twenty-four-hour total energy expenditure (TEE) was using a whole-room indirect
532 calorimeter (metabolic chamber) (44). Periods of exercise during the 24-hour metabolic chamber
533 stay were assessed using a microwave detection system; these periods were excluded from the
534 analysis of TEE, with data renormalized to a 24-hour period. Non-resting energy expenditure was
535 calculated as the difference between TEE and REE.

536

537 *MRI/MRS.* Hepatic triglyceride content was measured using MRS as previously described (45, 46).
538 Intramyocellular and extramyocellular triglyceride content in the vastus lateralis, anterior tibialis,
539 and soleus muscles were measured using MRS as previously described (46).

540

541 *Tracer dilution and clamp studies.* Following an overnight fast, stable isotope tracers were used to
542 measure glucose, glycerol, and palmitate turnover using the tracer dilution method. At 0500 hours,
543 one catheter was inserted into the forearm vein to infuse stable isotopically labeled tracers. A
544 second catheter was inserted into a vein in the contralateral hand or arm to obtain blood samples.
545 A primed, continuous infusion of [6,6-²H₂]glucose (priming dose 28 μmol/kg of body weight;

546 infusion rate 0.4 $\mu\text{mol/kg}$ of body weight/min for 180 min) was used to measure basal endogenous
547 production (Cambridge Isotope Laboratories). At 0700 hours, a primed, continuous infusion of
548 [$^2\text{H}_5$]glycerol (priming dose 0.045 $\mu\text{mol/kg}_{\text{BW}}$; infusion rate: 0.18 $\mu\text{mol/kg}_{\text{BW}}/\text{min}$) and an
549 unprimed infusion of [U- $^{13}\text{C}_{16}$]palmitate (infusion rate: 0.006 $\mu\text{mol/kg}_{\text{BW}}/\text{min}$) were administered
550 for 60 minutes to measure rate of lipolysis (Cambridge Isotope Laboratories).

551
552 At 0830 hours, a hyperinsulinemic-euglycemic clamp study began. Regular human insulin was
553 infused at a priming rate of 240 mU/m 2 /min for eight minutes, followed by a continuous insulin
554 infusion at 120 mU/m 2 /min for approximately 3 hours. [6,6- $^2\text{H}_2$]glucose was infused at 25% of the
555 baseline rate (0.1 $\mu\text{mol/kg}_{\text{BW}}/\text{min}$). Dextrose solution (20%) enriched with 2.5% [6,6- $^2\text{H}_2$]glucose
556 tracer was infused at a variable rate to maintain blood glucose at 100 ± 5 mg/dL. Due to severe
557 insulin resistance and hyperglycemia, two subjects maintained a steady state glucose of 132 ± 1.3
558 mg/dL for all visits. Blood samples (0.5 mL) were obtained every five to 10 min for analysis of
559 whole-blood glucose concentration, measured by an automated glucose analyzer (Yellow Springs
560 Instruments Co.). Blood samples for analysis of glucose, insulin, C-peptide, and [6,6- $^2\text{H}_2$]glucose
561 were collected every 10 minutes during steady-state (the final 30 minutes of the study).

562
563 *Liquid chromatography-mass spectrometry.* Isotope enrichment was measured using a Waters
564 Acquity UPLC and a Thermo Scientific Q-Exactive (high resolution – accurate mass). The
565 separation was on a Waters BEH Amide column (1.7 μm 2.1 x 100 mm) using solvent A (30%
566 ACN, 70% H $_2$ O, 0.1% NH $_3$) and solvent B (80% ACN, 20% H $_2$ O, 0.1% NH $_3$). The Q-Exactive
567 with HESI-II electrospray source negative ion used targeted-SIM mode at 70K resolution for
568 palmitate, 70K full scan for glycerol and targeted-SIM mode at 140K for glucose. Each targeted-

569 SIM was triggered by an inclusion list of the natural occurring molecule. Glucose was measured
570 at m/z 179.0556, [6,6-²H₂]glucose at 181.0684, glycerol at 91.0388, [²H₅]glycerol at 96.0700,
571 palmitate at 255.2336 and [U-¹³C]palmitate at 271.2874. Standards of 0 – 16.7 molar percent
572 enrichment (MPE) of [6,6-²H₂]glucose, 0 – 13.1 MPE [²H₅]glycerol, and 0 – 0.9 MPE [U-
573 ¹³C]palmitate were calibrated with R² > 0.99 (47).

574

575 *Calculations.* The rate of appearance of glucose, glycerol, and palmitate per kg of lean body mass
576 was calculated by measuring isotope enrichment using the single pool model (48). Peripheral
577 insulin sensitivity (M value) was calculated as the average glucose infusion rate during 30-minute
578 steady-state of the hyperinsulinemic-euglycemic clamp and corrected for fat-free mass (49).
579 Hepatic glucose production was calculated as the difference between basal glucose rate of
580 appearance and glucose infusion rate during clamp steady-state.

581

582 *Power and sample size calculations.* Power analyses were conducted a priori based on data from
583 previous human studies using leptin-deficient and replacement models and indicated that a sample
584 size of 10 subjects in each group (leptin-initiation and leptin-withdrawal) would provide 80%
585 power to detect significant differences between the off versus on metreleptin condition during
586 constant food intake for the following primary and secondary outcomes: peripheral insulin
587 sensitivity, hepatic insulin sensitivity, fasting plasma glucose, rate of lipolysis, and fasting
588 triglycerides. Given the limited pool of subjects with lipodystrophy already taking metreleptin
589 (leptin-withdrawal cohort) who met inclusion/exclusion criteria, we were unable to accrue the
590 target sample size of 10 for this group.

591

592 *Statistics.* For all outcomes, normally distributed data were reported as mean \pm SD. Non-normally
593 distributed data were reported as geometric mean [25th,75th percentiles]. Measurements in each of
594 the primary and secondary outcomes were analyzed to detect differences between Period 1 and
595 Period 2 for each cohort (leptin-initiation and leptin-withdrawal). For the leptin-initiation cohort,
596 secondary analyses were conducted to detect differences between Period 1 and six-month follow-
597 up, and between Period 2 and six-month follow-up using both multiple paired comparisons, as
598 well as linear mixed models with Bonferroni correction for multiple comparisons for pairs of
599 timepoints.

600
601 Data analysis for primary and secondary outcomes was done in two ways: without covariate
602 adjustment and with covariate adjustment. Potential covariates included in each model were:
603 baseline (pre-diet) value for the outcome, age, sex, race, type of lipodystrophy (partial versus
604 generalized, initiation cohort only), endogenous leptin level prior to metreleptin treatment, and
605 measured mean caloric intake during Period 1 and Period 2. For total body insulin sensitivity,
606 additional models were conducted including the above covariates plus body weight, fat mass, and
607 lean mass during the metreleptin treated and untreated conditions. For both hepatic and total body
608 insulin sensitivity, additional models were conducted including covariates age, sex, and hepatic
609 and intramyocellular triglyceride content (together and in separate models) during the metreleptin
610 treated and untreated conditions. For total, resting, and non-resting energy expenditure, models
611 included fat mass and lean body mass as covariates.

612
613 Unadjusted comparisons for each outcome were conducted using the paired t-test (for normally
614 distributed variables) or Wilcoxon paired test (for skewed variables). For adjusted comparisons

615 for each outcome, a variable selection for linear mixed model was conducted and then a final linear
616 mixed model with the selected covariates was performed to compare timepoints. With a single
617 exception, noted in the Results, adjustment for covariates did not alter the statistical significance
618 of any primary or secondary outcome. Therefore, only the unadjusted analyses are presented in the
619 Results and Figures. Linear mixed model analyses for covariate-adjusted analysis are presented in
620 Supplemental Tables 1-4. If significant differences were present in Period 1 versus Period 2 for an
621 outcome in either the initiation or withdrawal cohort, we compared the delta between Periods 1
622 and 2 for the two cohorts using 2-sample t-tests (for normally distributed variables) or Mann-
623 Whitney tests (for skewed variables). Only differences that were statistically significant are
624 mentioned in the Results.

625
626 For the two prespecified co-primary outcomes of total body insulin sensitivity and lipolysis
627 (glycerol Ra), a p-value <0.025 was considered statistically significant to account for multiple
628 comparisons. No multiplicity corrections were used for secondary outcomes, and a p-value <0.05
629 was considered statistically significant. All reported p-values are two-sided. Data analysis was
630 conducted by using SAS software (version 9.4, Cary, NC) and GraphPad Prism (version 7.00,
631 GraphPad Software, La Jolla California USA, www.graphpad.com).

632
633 *Study Approval.* The institutional review board of the National Institute of Diabetes and Digestive
634 and Kidney Diseases approved this study. All patients or legal guardians for those under 18 years
635 of age provided written informed consent before participation, and minor subjects provided written
636 assent. This study was registered at www.clinicaltrials.gov (trial ID NCT01778556).

637 **Author Contributions**

638 RJB initiated the investigation, led the clinical experiments and wrote, reviewed, and edited the
639 manuscript. AV obtained and analyzed the data, and wrote, edited, and reviewed the manuscript.
640 MS obtained data, and wrote, edited, and reviewed the manuscript. EC obtained data, and edited
641 and reviewed the manuscript. AG obtained and interpreted MRS data, and reviewed and edited the
642 manuscript. RJB and KYC obtained and interpreted energy expenditure data, and reviewed and
643 edited the manuscript. PW, HMG, HC, and MW obtained data, and edited and reviewed the
644 manuscript. AC and SB designed and implemented the controlled study diet and reviewed and
645 edited manuscript. AS provided statistical guidance prior to study implementation, conducted
646 statistical analyses, and edited and reviewed the manuscript. PG contributed to the design of the
647 study, and reviewed and edited the manuscript. All authors gave final approval of the version to
648 be published.

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655

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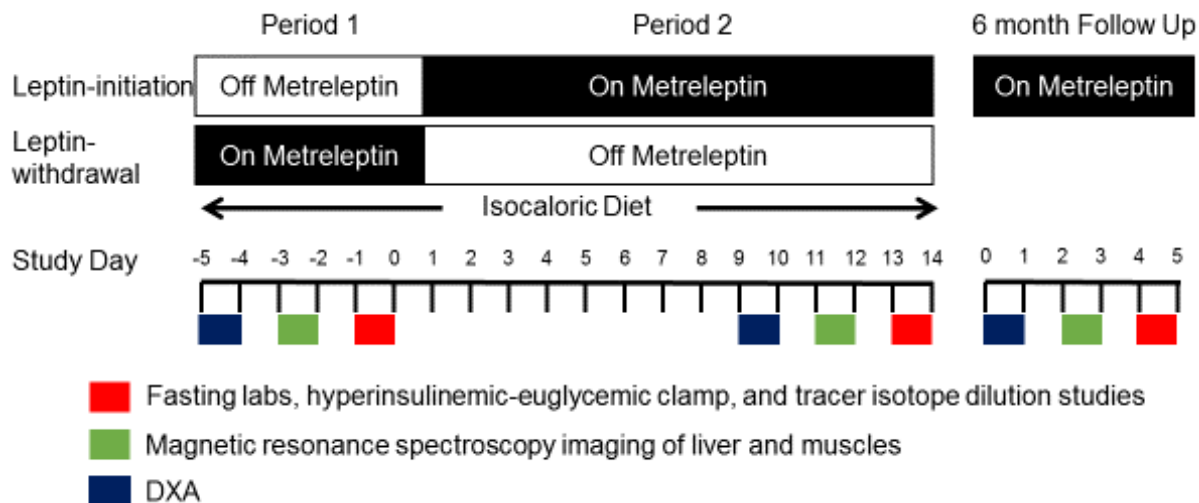
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794 **Figures and Figure Legends**

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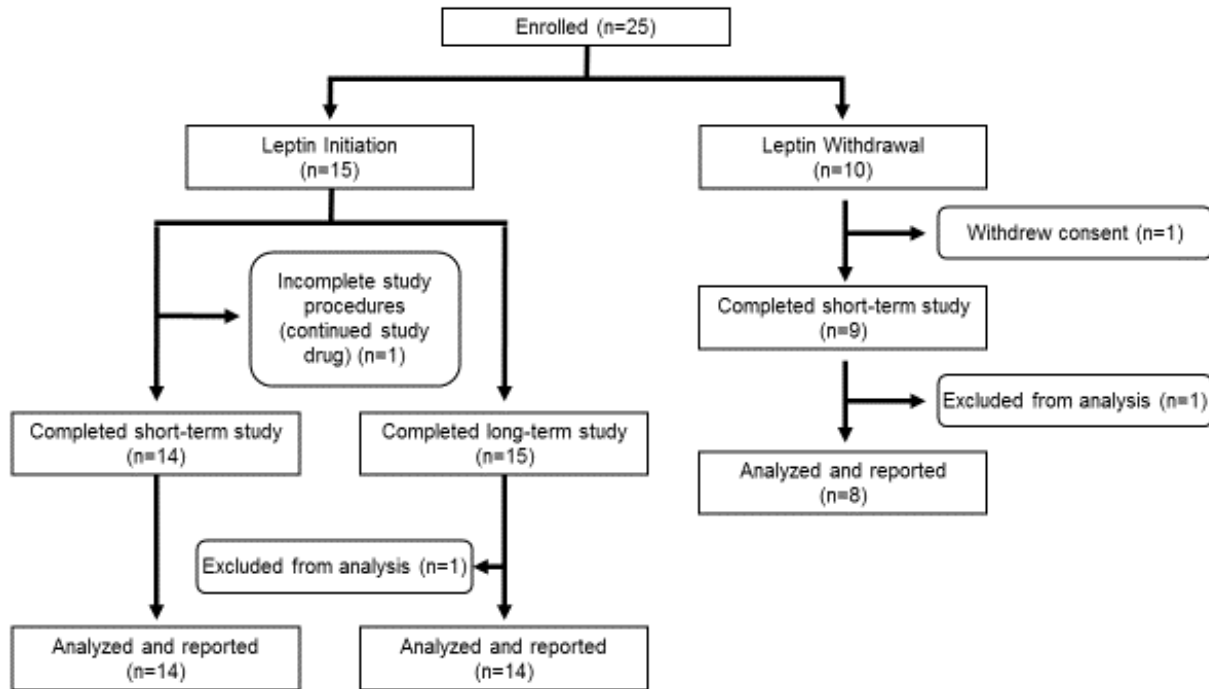
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798 **Figure 1. Study design.** The leptin-initiation cohort was untreated for first five days (Period 1),
799 then metreleptin was given for the following 14 days (Period 2). This order was reversed for leptin-
800 withdrawal cohort. During the short-term study, an isocaloric diet was maintained for both cohorts
801 to permit study of metreleptin’s effects during constant energy and macronutrient intake. During
802 both Periods 1 and 2, patients in both cohorts underwent a DXA scan, hyperinsulinemic-
803 euglycemic clamp, and MRS/MRI scan. This was repeated at a six-month follow-up visit in the
804 initiation cohort only during *ad libitum* diet.

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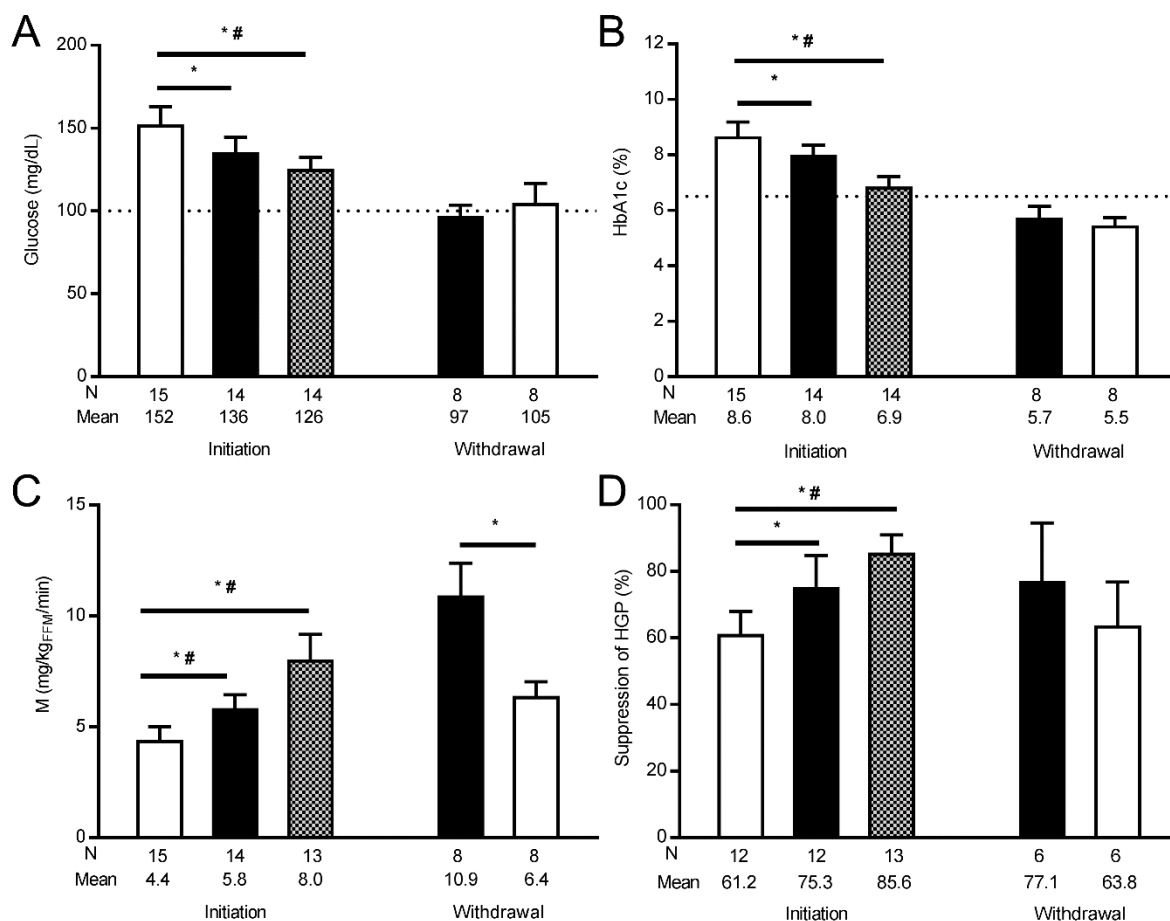
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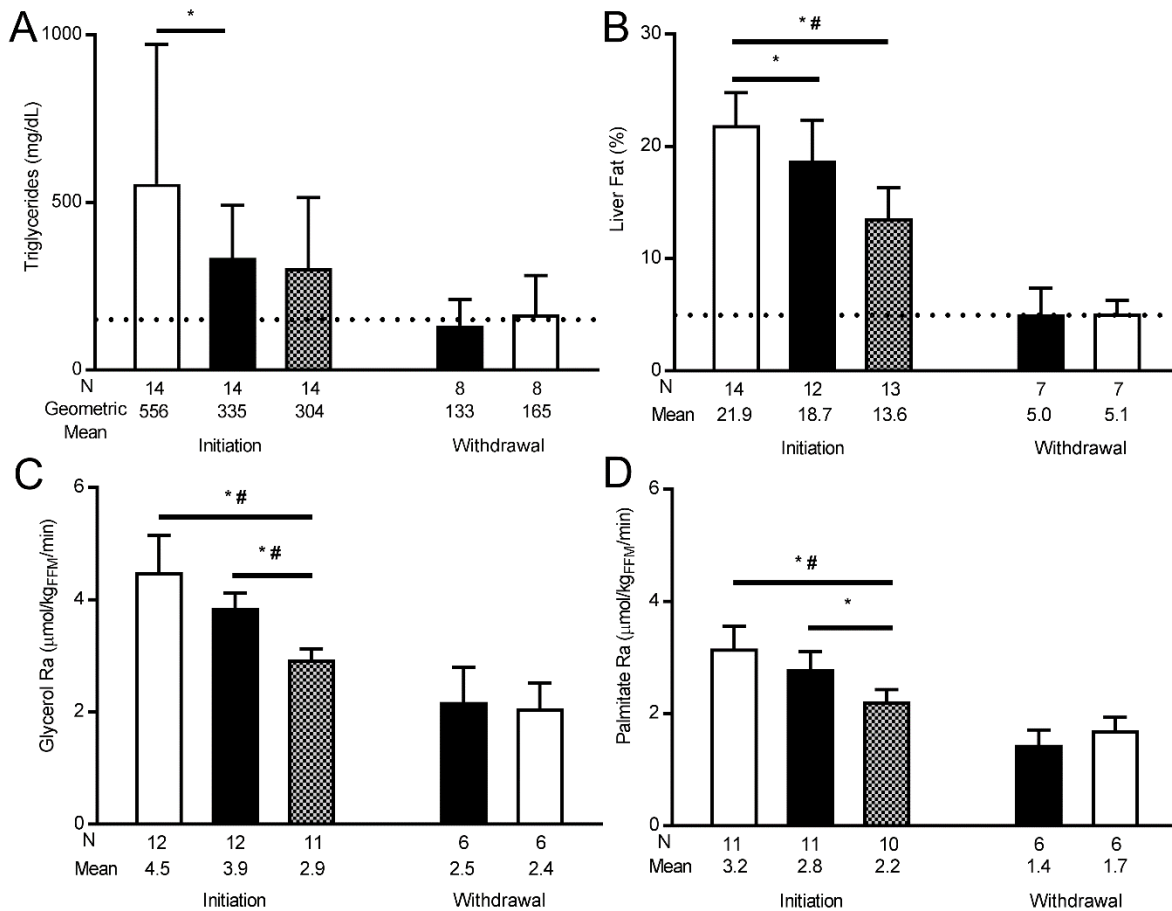
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809 **Figure 2. Study flow chart.** A total of 25 subjects were enrolled in the study, 15 in the initiation
 810 and 10 in the withdrawal cohort. In the initiation cohort, one subject did not have complete data
 811 collection for the short-term study, but completed the long-term study, and one subject was
 812 excluded from final analysis of the long-term study because of non-compliance with metreleptin.
 813 In the withdrawal cohort, one subject withdrew and another subject with type 1 diabetes was
 814 excluded from the analysis due to recurrent hypoglycemia during the short-term study.



815
 816 **Figure 3. Glucose control and insulin sensitivity improved in humans with lipodystrophy**
 817 **while on metformin independent of food intake.** (A) Fasting glucose levels in leptin-initiation
 818 and leptin-withdrawal subjects while off (white bars), on (black bars), and after six months on
 819 (gray bars) metformin. The dotted gray line indicates of the upper limit of normal (100 mg/dL).
 820 (B) Hemoglobin A1c. The dotted gray line indicates the threshold for diagnosis of diabetes (6.5%).
 821 (C) Whole-body insulin sensitivity reflected by the M value (hyperinsulinemic-euglycemic
 822 clamp). (D) Insulin-mediated suppression of hepatic glucose production (HGP) as an indicator of
 823 hepatic insulin sensitivity. Data shown represent the mean \pm SEM. The study was powered to
 824 detect differences between the off versus on leptin state (black versus white bars) during constant

825 food intake. * indicates $P < 0.05$ determined by 2-tailed t test or Wilcoxon matched-pairs signed
826 rank test between each pair of time points based on data distribution. # indicates $P < 0.05$ by linear
827 mixed model for all three timepoints with post-hoc pairwise Bonferroni correction in the leptin-
828 initiation cohort.



829
 830 **Figure 4. Triglycerides and liver fat decreased in humans with lipodystrophy while on**
 831 **metreleptin independent of food intake.** (A) Triglycerides of leptin-initiation subjects and leptin-
 832 withdrawal subjects while off (white bars), on (black bars), or after six months on (gray bars)
 833 metreleptin. The dotted line indicates the upper limit of normal (150 mg/dL). (B) Percent liver fat
 834 measured by magnetic resonance spectroscopy. The dotted line indicates upper limit of normal
 835 (5%). (C) Glycerol rate of appearance (Ra) in plasma. (D) Palmitate Ra in plasma. Data shown
 836 represent the mean \pm SEM or geometric mean \pm 95% CI (triglycerides). The study was powered
 837 to detect differences between the off versus on leptin state (black versus white bars) during
 838 constant food intake. * indicates $P < 0.05$ determined by 2-tailed t test or Wilcoxon matched-pairs

839 signed rank test between each pair of time points based on data distribution. # indicates $P < 0.05$
840 by linear mixed model for all three timepoints with post-hoc pairwise Bonferroni correction in the
841 leptin-initiation cohort.
842

	Effects of leptin independent of food intake	Maximal effects of leptin
Peripheral Insulin Sensitivity	↑	↑
Hepatic Insulin Sensitivity	↑	↑
Blood glucose	↓	↓
Lipolysis	↔	↓
Plasma triglycerides	↓	↓
Hepatic triglyceride	↓	↓
Intramyocellular lipid	↔	↓

843

844 **Figure 5. Effects of leptin in patients with lipodystrophy independent of food intake versus**

845 **maximal effects of leptin during *ad libitum* food intake.** The current study demonstrated

846 effects of leptin replacement with metreleptin with food intake held constant over 2 weeks.

847 These effects were smaller in magnitude than the maximal effects of metreleptin demonstrated in

848 long-term studies with ad libitum food intake.

849 **Table 1. Baseline characteristics in initiation and withdrawal cohorts**

Clinical Values	Initiation (n=15)	Withdrawal (n=8)
Type of lipodystrophy (Generalized/Partial)	(3/12)	(8/0)
Sub-type of lipodystrophy	3 CGL 12 FPL	7 CGL 1 AGL
Sex (Male/female)	(3/12)	(3/5)
Age (years)	32 ± 17	25 ± 6
Race/ethnicity	9 Caucasian 4 Hispanic 1 Asian 1 Other	4 Caucasian 2 African-American 2 Hispanic
Endogenous leptin level (ng/dL)	9.5 ± 10.2 ^A	1.2 ± 0.5 ^A
Duration of metreleptin treatment prior to study (years)	0	7.7 ± 4.7
Subjects on insulin (%)	71	0
Insulin dose (units per day, insulin users only)	225 ± 136	0
Number of diabetes medications	1.6 ± 1.2	0.4 ± 0.5
Number of lipid medications	1.8 ± 0.9	0.4 ± 0.7

850 Data represent mean ± SD except as noted. CGL: Congenital generalized lipodystrophy; FPL:
 851 Familial partial lipodystrophy; AGL: Acquired generalized lipodystrophy. ^AEndogenous leptin
 852 levels were measured by ELISA in the initiation cohort, and by RIA in the withdrawal cohort prior
 853 to metreleptin initiation.

854 **Table 2. Diet and body composition off- and on-metreleptin treatment.**

	Initiation (n=14)			Withdrawal (n=8)		
	OFF (Period 1)	ON (Period 2)	P	ON (Period 1)	OFF (Period 2)	P
Diet Composition						
Energy Intake (kcal)	2416 ± 312	2422 ± 370	0.85	2350 ± 501	2425 ± 525	0.38
Protein intake (%)	17.3 ± 1.1	17.4 ± 1.8	0.61	17.6 ± 2.1	17.6 ± 2.2	0.74
Carbohydrate intake (%)	52.2 ± 1.8	52.2 ± 1.8	0.44	53.2 ± 1.1	52.9 ± 1.3	0.26
Fat intake (%)	30.4 ± 1.0	30.0 ± 0.5	0.22	29.2 ± 1.9	29.5 ± 2.1	0.13
Body Composition						
Body Weight (kg)	73.8 ± 16.0	73.1 ± 15.8	0.04	59.3 ± 17.2	59.0 ± 16.7	0.53
BMI (kg/m ²)	25.5 ± 4.5	25.0 ± 4.7	0.01	19.8 ± 4.2	20.0 ± 4.1	0.41
Lean mass (kg)	53.1 ± 9.2	52.7 ± 9.3	0.32	54.3 ± 13.8	54.8 ± 13.1	0.81
Fat mass (kg)	18.3 ± 10.6	18.1 ± 10.6	0.02	4.1 ± 1.2	4.2 ± 0.9	0.76
Percent Fat Mass (%)	24.3 ± 10.8	24.1 ± 10.9	0.16	7.4 ± 1.6	7.6 ± 1.2	0.81
Plasma leptin (ng/dL)^A	9.5 ± 10.2	71.0 ± 25.3	0.0001	62.0 ± 79.4	3.7 ± 8.6	0.008

855 Data represent mean ± SD. ^AThe plasma leptin assay measures both endogenous leptin and

856 exogenous metreleptin.

857 **Table 3. Metabolic characteristics off- and on-metreleptin treatment.**

	Initiation (n=14)			Withdrawal (n=8)	
	OFF (Period 1)	ON (Period 2)	ON (6-month)	ON (Period 1)	OFF (Period 2)
Glycemic Parameters					
Fasting glucose (mg/dL)	152 ± 42	136 ± 34 ^A	126 ± 26 ^B	97 ± 18	105 ± 33
Fasting insulin (µU/mL)	40 [23,57]	33 [18,63]	25 [12,66]	20 [13,28]	31 [15,51]
Fasting c-peptide (ng/mL)	4.0 ± 1.6	4.2 ± 1.9	3.4 ± 1.9	3.5 ± 1.4	5.2 ± 2.2
Urinary glucose excretion (g/24h)	2.0 [0.2,10.3]	1.2 [0.2,7.2] ^A	0.4 [0.1,0.6]	0.2 [0.1,0.7]	0.3 [0.1,2.4]
Lipid Parameters					
Triglycerides (mg/dL)	556 [224,1144]	326 [162,660] ^A	304 [122,547]	133 [78,215]	165 [99,361]
Total cholesterol (mg/dL)	241 ± 116	171 ± 48 ^A	171 ± 58 ^B	129 ± 32	123 ± 29
LDL-C (mg/dL)	87 ± 34	78 ± 33	73 ± 32	68 ± 27	54 ± 24
HDL-C (mg/dL)	27 ± 5	25 ± 5	28 ± 5	32 ± 7	29 ± 8
FFA (mEq/L)	0.43 ± 0.17	0.42 ± 0.18	0.41 ± 0.10	0.20 ± 0.09	0.23 ± 0.06

858 FFA, free fatty acids. Data represent mean ± SD or geometric mean [25th,75th centile] based on
859 distribution of data. ^ASignificant difference between Period 1 vs Period 2, ^BSignificant difference
860 between Period 1 vs six-month visit. There were no significant differences between Period 2 vs
861 six-month visit in the initiation cohort.

862 **Table 4. Intramyocellular (IMCL) and extramyocellular (EMCL) lipid content in muscles**
 863 **during off- and on-metreleptin treatment.**

	Initiation (n=12)			Withdrawal (n=6)	
	OFF (Period 1)	ON (Period 2)	ON (6-month)	ON (Period 1)	OFF (Period 2)
IMCL (%)					
Lateral Vastus	7.7 ± 4.1	7.7 ± 3.5	7.2 ± 4.9	3.9 ± 3.4	3.7 ± 3.3
Tibialis Anterior	8.0 ± 4.7	7.9 ± 3.9	6.6 ± 3.4	4.9 ± 1.8	6.3 ± 3.7
Soleus	11.8 ± 6.3	18.0 ± 10.1	13.3 ± 7.6	7.9 ± 6.0	10.0 ± 7.8
EMCL (%)					
Lateral Vastus	19.4 ± 10.5	16.5 ± 11.8	13.4 ± 9.2 ^A	2.5 ± 2.2	4.4 ± 2.9
Tibialis Anterior	27.5 ± 16.4	28.6 ± 22.4	18.0 ± 12.8 ^A	5.0 ± 4.2	6.2 ± 3.9
Soleus	50.8 ± 25.7	41.5 ± 23.0	54.6 ± 39.1 ^B	5.4 ± 3.6	6.3 ± 2.8

864 Data show mean ± SD of all subjects. There were no significant differences between Period 1 vs
 865 Period 2, ^ASignificant decrease from Period 1 to six-month visit, and ^BSignificant increase from
 866 Period 2 to six-month visit.