No effect of administration of unacylated ghrelin on subcutaneous PC3 xenograft growth in a *Rag1*$^{-/-}$ mouse model of metabolic dysfunction

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Conflict of interest

The authors declare no conflict of interest.

Abstract

Ghrelin is a peptide hormone which, when acylated, regulates appetite, energy balance and a range of other biological processes. Ghrelin predominately circulates in its unacylated form (unacylated ghrelin; UAG). UAG has a number of functions independent of acylated ghrelin, including modulation of metabolic parameters and cancer progression. UAG has also been postulated to antagonise some of the metabolic effects of acyl-ghrelin, including its effects on glucose and insulin regulation. In this study, *Rag1*$^{-/-}$ mice with high-fat diet-induced obesity and hyperinsulinaemia were subcutaneously implanted with PC3 prostate cancer xenografts to investigate the effect of UAG treatment on metabolic parameters and xenograft growth. Daily intraperitoneal injection of 100 μg/kg UAG had no effect on xenograft tumour growth in mice fed normal rodent chow or 23% high-fat diet. UAG significantly improved glucose tolerance in host *Rag1*$^{-/-}$ mice on a high-fat diet, but did not significantly improve other metabolic parameters. We hypothesise that UAG is not likely to be an effective treatment for prostate cancer, with or without associated metabolic syndrome.

Introduction

The peptide hormone ghrelin is a circulating appetite-stimulating hormone which regulates a number of other biological processes [1-3], including metabolism and energy balance [1-4], and diseases such as cancer [5]. Ghrelin acts via its cognate receptor, the growth hormone secretagogue receptor 1a (GHSR1a), a G protein-coupled receptor [6], and one or more unknown alternative receptors [7-10]. In order to activate GHSR1a at physiological concentrations, ghrelin must be acylated at its third residue, a serine [11, 12], by the enzyme ghrelin *O*-acyl transferase (GOAT) [11, 12].

The major circulating form of ghrelin is its unmodified form, unacylated ghrelin (UAG). UAG, which does not directly stimulate feeding [5], was initially considered to be functionally inactive, but is now appreciated to bind to and activate a distinct, unknown receptor [4, 13-18] and have a number of functions [19-22]. UAG plays roles in the regulation of glucose and energy balance and has effects on cell proliferation [19-23]. Importantly, it may oppose some of the effects of acyl-ghrelin [16, 24-26] preventing the rise in circulating glucose and insulin associated with acyl-ghrelin administration in rodents [22, 26, 27]. From these studies, it is apparent that UAG is an endocrine hormone in its own right [20]. UAG and the truncated, cyclised UAG analogue AZP-531 prevented the development of pre-diabetes in C57BL/6 mice fed a high-fat diet for two weeks, highlighting a potential of unacylated forms of ghrelin as treatments for metabolic syndrome [27]. In human trials, UAG had similar effects, improving glycaemic control and insulin sensitivity in patients with type 2 diabetes mellitus [28] and improving glucose handling and reducing free fatty acids in healthy subjects when administered overnight as a continuous infusion [29]. AZP-531 also had beneficial effects on glucose balance and led to weight loss in patients with type 2 diabetes mellitus in a phase I clinical trial [30]. Similar benefits have been observed in

patients with Prader-Willi syndrome, a genetic disorder associated with hyperghrelinaemia and obesity [31].

Close to two decades of work has firmly established a role for the ghrelin axis in cancer [5, 32-35]. This includes prostate cancer, a classical endocrine-related cancer and the most commonly diagnosed cancer in American men after skin cancer [36], where acyl-ghrelin increases cell proliferation and migration [5, 14, 37-46]. UAG also has functional effects in several cancers, including prostate cancer [5, 32-34, 43]. In the PC3 prostate cancer cell line, UAG has a biphasic effect, reducing cell proliferation at supraphysiological levels (10nM-1μM) [14].

Studies investigating the role of UAG in prostate cancer have been limited to *in vitro* experiments. *In vivo* studies are required, however, as obesity and overweight and co-morbidities, including hyperinsulinaemia, are now recognised as critical risk factors for numerous cancers [47-49]. These include cancer types with high-prevalence and mortality, such as tumours of the prostate, endometrium, breast, and gastrointestinal system [47-55]. Obesity and increased body mass have been associated with increased risk of advanced prostate cancer, more aggressive and high-grade disease and increased risk of death from prostate cancer [56-59]. Castration-resistant prostate cancer (CRPC) occurs when prostate cancer recurs after remission from androgen-targeted therapies (ATT) [60]. Treatments for CRPC are limited and this stage of the disease often results in the formation of painful, metastatic bone lesions and associated morbidity and mortality [61-63]. Metabolic syndrome and hyperinsulinaemia are common side effects of ATT [64, 65] and may also further accelerate the progression to CRPC [48, 58, 66-68]. As UAG reduces prostate cancer proliferation *in vitro* [14] and has potential beneficial metabolic effects *in vivo*, we examined the effect of UAG in our model of metabolic dysfunction: *Rag1*$^{-/-}$ mice fed a high-fat diet, with subcutaneous prostate cancer cell line xenografts [69].

Materials and Methods

Cell Culture

Human prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The PC3 prostate cancer cell line was cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) and supplemented with 10% (v/v) Fetal Calf serum (FCS) (Thermo Fisher Scientific, Waltham, MA, USA), 50 units/ml penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific). Cells were tested negative for *Mycoplasma*.

Hyperinsulinaemic *Rag1*$^{-/-}$ mouse model treated with unacylated ghrelin (UAG)

To determine the metabolic effect of UAG in an engraftable mouse model of hyperinsulinaemia [69], male recombination-activation gene deficient mice (B6.SVJ129-*Rag1*^{tm1Bal}/Arc; *Rag1*$^{-/-}$) (Jackson Laboratories, supplied by Animal Resource Centre, Murdoch, WA, Australia) were weaned onto a diet of low-fat normal chow (LFD) or a Western-style, high-fat diet (HFD; 23% fat, SF04-027, Specialty Feeds, WA) [69]. After two weeks on the diet, mice were subcutaneously injected into the left flank with 1×10^6 PC3 cells diluted 1:1 in growth factor reduced, phenol red-free Matrigel (Corning, NY, USA). Tumours were allowed to grow until a volume of approximately 50-100 mm^3 was reached, when mice were randomly divided into two experimental groups. Mice then received daily intraperitoneal injections of 100 µg/kg UAG (Mimotopes, Mulgrave, Vic, Australia) (n=6 HFD, n=10 LFD) (a dose previously determined to inhibit breast cancer growth *in vivo* [8]) or phosphate buffered saline (PBS) control (n=8 HFD, n=10 LFD) for 16 days. Tumour

124 volume was calculated by measuring subcutaneous tumour length and width twice weekly
125 using digital calipers (ProSciTech, Kirwan, QLD, Australia). Tumour volume was calculated
126 using the equation 'tumour volume = (width × length^2)/2' [70]. Bodyweight was measured
127 twice weekly. At endpoint, tumours and adipose tissue (epididymal fat pad and interscapular
128 brown adipose tissue) were excised and weighed. Fasting blood glucose was measured at
129 endpoint and blood was collected by cardiac puncture for serum biochemical measurements.
130 Surrogate indices of insulin resistance, insulin sensitivity, and steady state β-cell function
131 were determined using the homeostatic model for assessment calculator (HOMA2), available
132 from the Oxford Centre for Diabetes, Endocrinology and Metabolism [71], using measured
133 fasting glucose and insulin levels.
134

135 ## Statistics

136 Statistical analyses were performed using GraphPad Prism v.6.01 software (GraphPad
137 Software, Inc., San Diego, CA). Kruskal-Wallis (three or more groups) and Mann-Whitney
138 *U*-test (two groups) tests used for non-normally distributed data, while a two-way ANOVA
139 with Tukey's post-hoc test used for normally distributed data. $P \leq 0.05$ was considered to be
140 statistically significant.

141 # Results

142 ## No effect of intraperitoneal administration of UAG on PC3
143 ## xenograft growth in obese, hyperinsulinaemic *Rag1*$^{-/-}$ mice

144 No significant differences in tumour volume over the treatment period or tumour
145 weight (P=0.57) and volume at endpoint (P=0.55) were observed between UAG-treated and
146 untreated obese mice (14 days of treatment) (Mann-Whitney test, Fig. 1A-C). An increase in
147 insulin sensitivity (P=0.70) and reduction in body weight (P=0.08), epididymal fat pad

148 weight (*P*=0.26), interscapular brown adipose tissue weight (*P*=0.12), fasting blood glucose
149 (*P*=0.50), fasting blood insulin (*P*=0.90), insulin resistance (*P*=0.70), and steady-state β-cell
150 function (*P*=0.22) was observed in the UAG treatment group in HFD-fed *Rag1*$^{-/-}$ mice –
151 however, these changes were not statistically significant (Fig. 1D-L). There was a significant
152 difference in blood glucose at 30 minutes following glucose challenge in HFD-fed UAG-
153 treated mice compared to PBS controls at endpoint (after 16 days of treatment) (21.6 ±
154 1.2mM, *n*=6 vs 25.4 ± 1.9mM, *n=5*, *P*=0.02, two-way ANOVA with post-hoc test, Fig. 1G),
155 however, this was not observed at other time points, suggesting no major change in glucose
156 tolerance. There was no significant difference in fasting blood glucose (*P*=0.50), blood
157 insulin concentration (*P* = 0.90, Mann-Whitney test, Fig. 1I), insulin resistance (*P* = 0.70,
158 Mann-Whitney test, Fig. 1J), or insulin sensitivity (*P=0.70*, Mann-Whitney test, Fig. 1L).
159
160 **Fig. 1. Unacylated ghrelin (UAG) affects glucose tolerance but has no effect on tumour**
161 **volume or on other metabolic parameters.** *Rag1*$^{-/-}$ mice fed a 23% high-fat diet (HFD) or
162 low-fat diet (LFD) were injected with subcutaneous PC3 xenografts and administered UAG
163 (100μg/kg/day, i.p.) (*n*=6 HFD, *n*=10 LFD) or PBS control (*n*=8 HFD, *n*=10 LFD) once
164 tumours were palpable. Mean ± s.e.m. * *P*≤0.05. (A) Tumour volume (mm^3) measured over
165 time (*P*=0.57), (B) tumour volume (mm^3) (*P*=0.55) and (C) tumour weight (g) measured at
166 experimental endpoint were not significantly different between UAG- and PBS-treated mice
167 fed HFD or LFD. Mean ± s.e.m. Mann-Whitney test. (D) Body weight (g) of mice at
168 endpoint (*P*=0.08), (E) epididymal fat pad weight (g) (*P*=0.26) and (F) interscapular brown
169 adipose tissue weight (g) (*P*=0.12) were not significantly different in UAG-treated mice
170 compared to PBS-treated mice. Mean ± s.e.m. Mann-Whitney test. (G) HFD-fed UAG-
171 treated mice (*n*=6) had significantly lower blood glucose 30 min post-glucose challenge
172 compared to HFD-fed PBS treated mice (*n*=8), determined by intraperitoneal glucose

tolerance test (IPGTT). Mean ± s.e.m. Two-way ANOVA. * P=0.025. (H) Fasting blood glucose (mM) (P=0.50) and (I) fasting blood insulin (ng/ml) were not altered in UAG-treated compared to PBS-treated mice on either diet. Mean ± s.e.m. P=0.90. Mann-Whitney test. (J) Insulin resistance (HOMA-IR) (P=0.70), (K) steady state β-cell function (HOMA%B) (P=0.22) and (L) insulin sensitivity (HOMA%S) (P=0.70) were not altered in UAG-treated compared to PBS-treated mice on either diet. Mean ± s.e.m. Mann-Whitney test.

Discussion

It has recently been recognised that UAG can under some conditions act as a functional ghrelin inhibitor, reducing ghrelin-mediated increases in plasma glucose [22, 26, 28, 72] and lipid [27, 29]. As the ghrelin axis also plays a role in the progression of a number of endocrine-related cancers [5, 32-34], including prostate cancer [5, 43], we hypothesised that UAG may have beneficial effects in advanced prostate cancer associated with metabolic syndrome. To evaluate this hypothesis, we examined the effect of UAG on a prostate cancer cell line *in vivo*.

In our diet-induced hyperinsulinaemic *Rag1*$^{-/-}$ mouse model [69], we investigated the effect of supraphysiological systemic UAG treatment (100μg/kg/day) on metabolic parameters and PC3 prostate cancer xenograft growth. No differences in metabolic parameters (fasting blood glucose, fasting blood insulin, insulin resistance, steady-state β-cell function, and insulin sensitivity) were observed with UAG treatment in HFD-fed mice. Other studies have found that UAG prevents insulin resistance and hyperglycaemia in short-term HFD-fed mice [73], observations which may stem from the ability of UAG to cross the blood-brain barrier and oppose the central actions of ghrelin on energy homeostasis [74]. Furthermore, in human clinical trials UAG improved glucose and lipid metabolism in healthy [29] and diabetic patients [28]. In our study, a decrease in bodyweight, epididymal fat pad

198 weight, and interscapular brown adipose tissue was observed in HFD-fed UAG treated mice
199 but this difference was not statistically significant. UAG did significantly reduce blood
200 glucose levels at 30 minutes post-glucose challenge in HFD, but not LFD-fed mice, however.
201 This is similar to other studies, which have only found positive effects of UAG on glucose
202 tolerance in obese patients [72]. Similarly, in clinical trials, AZP-531 (a cyclised, truncated
203 analogue of UAG) improved food-related behaviour, waist circumference, and glucose
204 tolerance in Prader-Willi syndrome patients, but had no effect on body weight [31]. AZP-531
205 also prevents HFD-induced weight gain, insulin resistance, and impairment of glucose
206 tolerance in mice [27].

207 To the best of our knowledge, this is the first report on the effects of UAG on cancer
208 cell line xenograft growth *in vivo*. While our study and others show somewhat promising
209 effects of UAG treatment on metabolic parameters, systemic UAG administration had no
210 effect on prostate tumour xenograft size in mice fed a low-fat or high-fat diet. While
211 preliminary, our study suggests that UAG administration, or targeting of endocrine UAG,
212 may have limited therapeutic potential for prostate cancer, in patients with and without
213 symptoms of metabolic syndrome.

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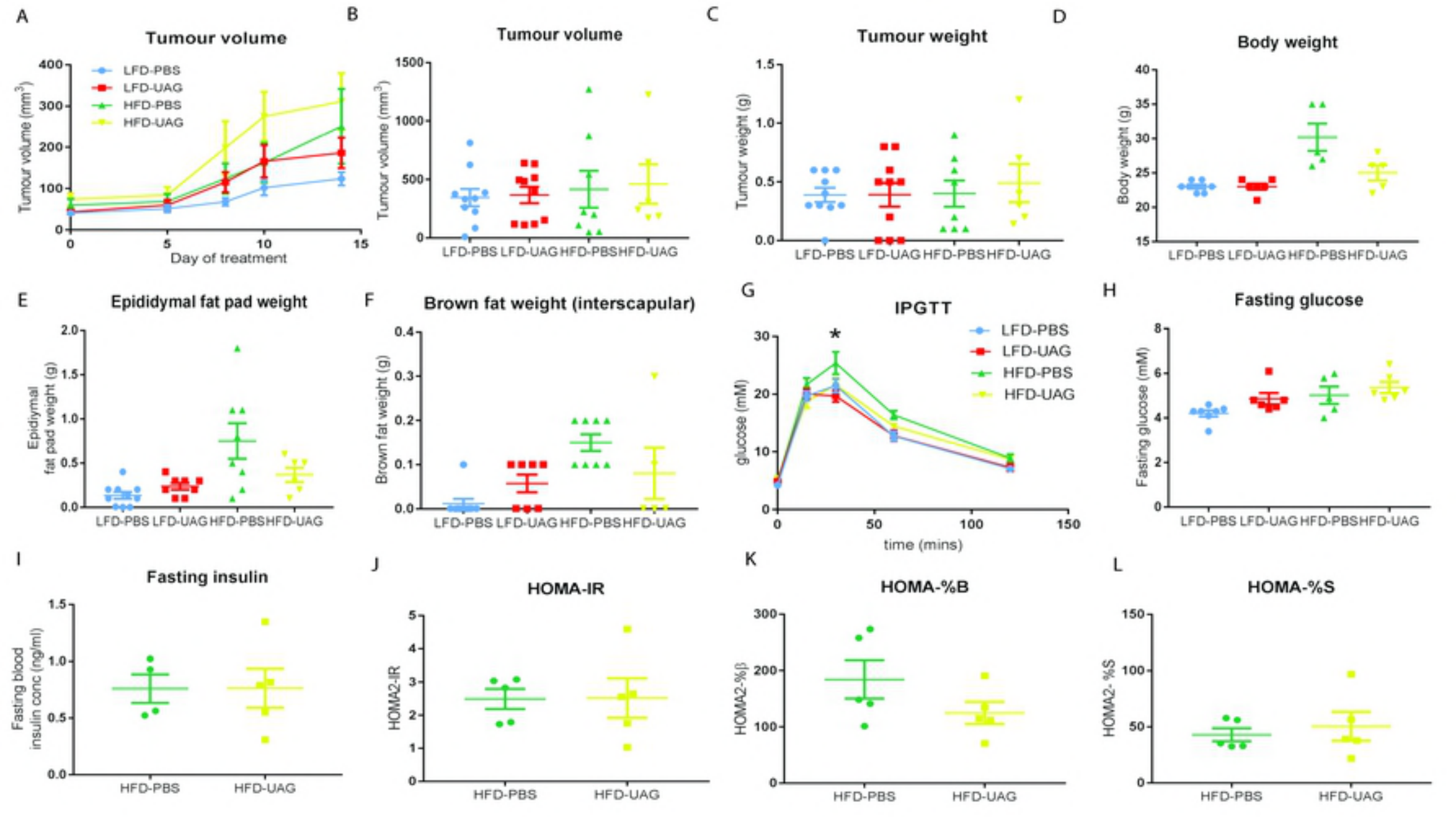
A
Tumour volume
LFD-PBS
LFD-UAG
HFD-PBS
HFD-UAG
Tumour volume (mm3)
Day of treatment
B
Tumour volume
Tumour volume (mm3)
LFD-PBS LFD-UAG HFD-PBS HFD-UAG
C
Tumour weight
Tumour weight (g)
LFD-PBS LFD-UAG HFD-PBS HFD-UAG
D
Body weight
Body weight (g)
LFD-PBS LFD-UAG HFD-PBS HFD-UAG
E
Epididymal fat pad weight
Epididymal fat pad weight (g)
LFD-PBS LFD-UAG HFD-PBS HFD-UAG
F
Brown fat weight (interscapular)
Brown fat weight (g)
LFD-PBS LFD-UAG HFD-PBS HFD-UAG
G
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LFD-PBS
LFD-UAG
HFD-PBS
HFD-UAG
glucose (mM)
time (mins)
H
Fasting glucose
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LFD-PBS LFD-UAG HFD-PBS HFD-UAG
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Fasting insulin
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