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Journal article

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1 **Effect of acute and short-term dietary fat ingestion on postprandial skeletal muscle protein**
2 **synthesis rates in middle-aged, overweight and obese men**

3

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14

15 **Running title:** Muscle protein synthesis and dietary fat in obesity

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21

22 **Abbreviations list:** HOMA-IR = Homeostatic Model Assessment of Insulin Resistance; FSR =
23 Fractional protein Synthetic Rate; RER = Respiratory Exchange Ratio; CHO = Carbohydrate; BMI =
24 Body Mass Index.

25

26

27 **Abstract**

28 Muscle anabolic resistance to dietary protein is associated with obesity and insulin resistance.
29 However, the contribution of excess consumption of fat to anabolic resistance is not well studied. The
30 aim of these studies was to test the hypothesis that acute and short-term dietary fat overload will
31 impair the skeletal muscle protein synthetic response to dietary protein ingestion. Eight
32 overweight/obese males [46.4±1.4 years, BMI 32.3±5.4 kg/m²] participated in the acute feeding study,
33 which consisted of 2 randomised crossover trials. On each occasion, subjects ingested an oral meal
34 (with and without fat emulsion) 4h before the coingestion of milk protein, intrinsically labelled with
35 [1-¹³C]phenylalanine, and dextrose. Nine overweight/obese males [44.0±1.7 years, BMI 30.1±1.1
36 kg/m²] participated in the chronic study, which consisted of a baseline 1-week isocaloric diet followed
37 by a 2-week high fat diet (+25% energy excess). Acutely, incorporation of dietary amino acids into the
38 skeletal muscle was 2-fold higher (P<0.05) in the lipid trial compared to control. There was no effect
39 of prior lipid ingestion on indices of insulin sensitivity (muscle glucose uptake, PDC activity and Akt
40 phosphorylation) in response to the protein/dextrose drink. Fat overfeeding had no effect on muscle
41 protein synthesis or glucose disposal in response to whey protein ingestion, despite increased muscle
42 DAG C16:0 (P=0.06) and ceramide C16:0 (P<0.01) levels. Neither acute nor short-term dietary fat
43 overload has a detrimental effect on skeletal muscle protein synthetic response to dietary protein
44 ingestion in overweight/obese men, suggesting dietary-induced accumulation of intramuscular lipids
45 *per se* is not associated with anabolic resistance.

46

47 **Keywords:** dietary fat; obesity; postprandial period; skeletal muscle protein synthesis; intramuscular
48 lipids.

49 **Introduction**

50 The inability of skeletal muscle to adequately synthesise new protein in response to anabolic stimuli
51 such as amino acids (termed 'anabolic resistance') is a key contributory factor to the muscle mass loss
52 observed in a variety of conditions such as ageing, type 2 diabetes (T2D), disuse, and critical illness
53 (36, 47). Skeletal muscle protein synthesis in response to amino acids appears to be negatively related
54 to whole body fat mass in obese insulin resistant humans (24). In agreement, overweight and obese
55 young men exhibit lower postprandial anabolic response to dietary protein ingestion when compared
56 with healthy lean men (4). It has also been suggested that a sedentary lifestyle and lack of physical
57 activity may be key parameters in the development of anabolic resistance in obese individuals (29).
58 Furthermore, skeletal muscles from individuals with higher leg fat mass are more resistant to the
59 anabolic response of amino acid ingestion in the presence of physiological hyperinsulinaemia when
60 compared with individuals with lower leg fat mass (33). In support of this, animals studies have shown
61 the time course of chronic high-fat overfeeding induced obesity and anabolic resistance in rats to
62 coincide with muscle lipid accumulation (32). Similarly, diet-induced obesity in mice was shown to
63 impair the activation of skeletal muscle protein synthesis in response to feeding of a mixed meal,
64 although basal (postabsorptive) rates of skeletal muscle protein synthesis were not affected (2).

65 Intramuscular accumulation of lipid species has been associated with the inability of skeletal muscle
66 glucose metabolism to respond adequately to insulin signalling (insulin resistance) (42) (37) (9).
67 Insulin signalling is also integral to skeletal muscle amino acid delivery, transport and metabolism, in
68 particular playing a permissive role in the regulation of muscle protein synthesis via activation of the
69 mammalian (mechanistic) target of rapamycin complex 1 (mTOR) pathway (24, 31). We have
70 previously shown in healthy young humans that elevating fatty acid availability through an infusion of
71 heparin plus Intralipid (triglyceride) emulsion in the presence of physiological hyperinsulinaemia
72 induces insulin resistance and impairs the skeletal muscle fractional protein synthetic rate (FSR) in
73 response to ingestion of 20 g amino acids (39). This anabolic resistance was mediated in part via the
74 repression of translation initiation at the level of the eukaryotic initiation factor (eIF) 4E binding
75 protein-1 (4E-BP1), a target for mTOR, but was independent of the phosphorylation of mTOR itself.

76 Lipid-induced insulin resistance was observed both at the level of insulin-stimulated glucose disposal
77 into peripheral tissues and its oxidation at the level of skeletal muscle pyruvate dehydrogenase
78 complex (PDC) activation (39).

79 We have recently demonstrated that obese individuals with moderate whole-body glucose intolerance,
80 insulin resistance and fat distribution in the lower legs do not exhibit a reduced skeletal muscle protein
81 synthetic response to dietary protein ingestion when compared to lean individuals (29). Thus, it is not
82 clear whether insulin resistance *per se* and/or the fat overload and accumulation of intramuscular lipids
83 are the driving forces for attenuated FSR. Furthermore, there is a paucity of studies investigating the
84 effects of oral fat overloads rich in saturated fatty acids (SFA) on anabolic resistance, particularly in
85 overweight/obese individuals. The fatty acid composition of such meals is an important factor, as high
86 dietary SFA is associated with insulin resistance in humans whereas meals and diets rich in mono- and
87 poly-unsaturated fatty acids content protect against the development of insulin resistance (27, 44). It
88 should also be noted that previous studies are either cross-sectional or acute (hours) investigations
89 performed under insulin clamp conditions, whereas chronic feeding studies are lacking.

90 The **aim** of the present studies was to test the hypothesis that both acute and short-term dietary fat
91 overload (defined as a state of physiologically relevant increase in dietary fat) with primarily saturated
92 fatty acids under real-life conditions in middle-aged overweight and obese individuals will impair the
93 skeletal muscle protein synthetic response to dietary protein ingestion.

94

95 **Subjects and Methods**

96 **Acute Feeding Study Subjects.** Eight, middle-aged, overweight/obese (n=4 and n=4, respectively) and
97 physically inactive males [46.4 ± 1.4 years, body mass 96.7 ± 5.3 kg, BMI 32.3 ± 5.4 kg/m², waist
98 circumference 107 ± 5 cm, and hip circumference 106 ± 5 cm] were recruited to participate in this
99 study. The HOMA-IR (fasting glucose x fasting insulin / 22.5), a surrogate index of insulin resistance,
100 at baseline was 4.7 ± 1.4 .

101 **Short-term (overfeeding) Study Subjects.** A different group of nine, middle-aged, overweight/obese
102 (n=3 and n=6, respectively) and physically inactive males [44.0 ± 1.7 years, body mass 97.0 ± 3.3 kg,
103 BMI 30.1 ± 1.1 kg/m², waist circumference 106 ± 3 cm, and hip circumference 105 ± 2 cm] were
104 recruited to participate in this study. The HOMA-IR at baseline was 2.2 ± 0.4 .

105 In both studies, subjects were informed about the nature and risks of the experimental procedures
106 before their written consent was obtained. The studies were approved by the University of Nottingham
107 Medical School Research Ethics Committee in observance of the present regulations imposed by the
108 Code of Ethics of World Medical Association (Declaration of Helsinki). All participants underwent an
109 individual medical screening, which involved completing health and physical activity questionnaires,
110 having a 12 lead ECG and blood pressure measured, and a blood sample taken for routine screening.
111 Individuals with history of cardiovascular disease, diabetes, musculoskeletal disorders, smoking and
112 excessive alcohol consumption (>28 units per week) were excluded from participation.

113 **Acute Feeding Study - Experimental Protocol.** This study was part of a bigger investigation
114 (ClinicalTrials.gov Identifier NCT03146286). All subjects underwent 2 experimental trials (lasting 7h
115 each), at least 2 weeks apart, after an overnight fast in a randomised crossover study. On each
116 occasion, subjects ingested an oral test meal 4 hours before (0h time point) the ingestion of a bolus of
117 milk protein (0.35 g/kg body mass), which was intrinsically labelled with [1-¹³C]phenylalanine, along
118 with 0.8 g/kg body mass of dextrose and 2 g of chocolate powder (containing 0.46 g protein, 0.2 g
119 CHO, 0.4 g fat) dissolved in 6 ml/kg body mass of water (4h time point). The oral test meal consisted
120 of a hot, chocolate-flavoured drink consisting of either a fat emulsion (lipid trial) or water (control

121 trial). The oral lipid load consisted of 0.7 g/kg body mass of palm stearin (containing ~65% of
122 saturated fat), 1g of monoglyceride (emulsifier), 5g of chocolate powder (containing 1.15 g protein,
123 0.5 g CHO, 1.0 g fat) and 1g of aspartame-based sweetener dissolved in 6 ml/kg body mass of water.
124 The palm stearin and monoglyceride were excluded from the control drink. The energy content and
125 macronutrient composition of the two test meals were: Control 0.16 kcal per kg body weight (57.7%
126 Fat, 29.5% Protein and 12.8% CHO); Lipid 6.22 kcal per kg body weight (98.9% Fat, 0.8% Protein and
127 0.3% CHO).

128

129 On each occasion, the subjects reported to the laboratory at 08.00 after an overnight fast (10-12h),
130 having abstained from heavy exercise and alcohol for the previous 48h. Subjects were then asked to
131 rest on a bed for 7h in a semi-supine position and an intravenous cannula was inserted retrograde into
132 the superficial hand vein of one arm for arterialised-venous blood sampling. The hand remained in a
133 hot air warming unit (50-55°C) throughout the trial, with hand and air temperature continuously
134 monitored (21). The cannula was kept patent via a saline drip. Blood samples were obtained at
135 baseline (0h) and 1h, 2h, 3h, 3.5h and 4h after ingestion of the test meal for the determination of blood
136 glucose, insulin, amino acids, free fatty acids (FFA), triglyceride (TAG) and urea concentrations.
137 Blood samples were also obtained every 15 min after ingestion of the milk protein and dextrose drink
138 for the determination of plasma phenylalanine, tyrosine and leucine concentrations, and [1-
139 ¹³C]phenylalanine enrichment in addition to the metabolites listed above. Resting energy expenditure,
140 RER and whole-body substrate oxidation rates were measured using indirect calorimetry for 20 min
141 before and 2.5h after the ingestion of the milk protein and dextrose drink. Urine samples were
142 obtained at 0h, 4h and 7h for the determination of nitrogen urea excretion rates, which were used to
143 correct indirect calorimetry data for protein oxidation rates to allow calculation of non-protein RER
144 and whole-body substrate oxidation rates using equations from (20). Muscle biopsy samples were
145 taken before (4h time point) and 3 hours after (7h time point) the ingestion of the milk protein and
146 dextrose drink in order to measure [1-¹³C]phenylalanine incorporation into the muscle myofibrillar

147 protein pool to provide an index of fractional protein synthetic rate (FSR) of dietary protein derived
148 amino acids (35).

149

150 In order to assess the effect of treatment on local muscle insulin sensitivity, on 2 separate subsequent
151 occasions, 5 of the 8 subjects [46.4 ± 1.4 years, body mass 96.7 ± 5.3 kg, BMI 32.3 ± 5.4 kg/m², waist
152 circumference 107 ± 5 cm, and hip circumference 106 ± 5 cm] repeated the 2 acute trials (without
153 undergoing skeletal muscle biopsies) and had 2 intravenous cannulae inserted retrograde into a
154 superficial hand vein of one arm for arterialized-venous blood sampling and into a deep antecubital
155 vein of the other arm for deep venous blood sampling using ultrasound guidance. As above, the hand
156 remained in a hot air warming unit (50 - 55°C) throughout the trial. Blood samples from each sampling
157 line were obtained at the same intervals described above for the determination of blood glucose
158 concentrations. Differences between arterialised venous (from the hand) and deep venous (from the
159 antecubital vein) glucose concentrations, along with measurements of blood flow of the brachial artery
160 (BF_{BA} ; expressed in ml·min⁻¹) using Doppler ultrasound, were used to determine rates of glucose (G)
161 uptake (expressed in $\mu\text{mol}\cdot\text{l}^{-1}$) across the forearm using the following equation: $G_{\text{uptake}} = ([G]_{\text{arterialised}} -$
162 $[G]_{\text{venous}}) \times BF_{BA}$. This provided an index of local muscle insulin sensitivity.

163 **Short-term (overfeeding) Study – Experimental Protocol.** All subjects consumed an isoenergetic diet
164 that matched their habitual total daily energy expenditure for 1 week (baseline isocaloric phase;
165 approx. 35%E fat, 47.5%E CHO and 17.5%E protein) before attending their first main experimental
166 visit. All food was provided for the participants via a delivery to their home or collection from the
167 laboratory. The energy intake for each subject was based on their habitual food intake as determined
168 using 3-day food diaries and matched to the individual's predicted total energy expenditure using the
169 Henry Equation (25) and a self-administered IPAQ questionnaire (15). Following the first
170 experimental visit, each subject continued to receive the same diet but with +25% energy excess
171 energy from fat (48-50%E fat, 37%E CHO and 13%-15%E protein) for 2 weeks (fat overfeeding
172 phase) before returning for the second experimental visit. Subjects received the 25% excess calories
173 during the overfeeding phase in the form of double cream (Sainsbury's, UK: 100ml: 47.5g fat, 2.6g
174 CHO, and 1.7g protein) that was ingested with their evening meal.

175

176 On each experimental visit, the subjects reported to the laboratory at 08.00 after an overnight fast (10-
177 12h), having abstained from heavy exercise and alcohol for the previous 48h, and rested in a semi-
178 supine position while cannulae were inserted into a superficial dorsal hand vein for arterialized-venous
179 blood sampling and in both forearm veins (antecubital fossa) for insulin, glucose and stable isotope
180 infusions. Baseline blood samples were obtained for the determination of blood glucose, insulin and
181 FFA concentrations. A 360-min primed (4mg/kg), continuous infusion of [6,6²H₂] glucose
182 (40µg/kg/min) was then initiated for the determination of glucose appearance (R_a) and glucose
183 disappearance (R_d) rates. Phenylalanine (L-[ring-²H₅]-phenylalanine) was also infused at 0.5/kg/hr for
184 360 min for the determination of skeletal muscle FSR. After the first 120 min of tracer infusion, a
185 hyperinsulinaemic (30mU/m²/min) euglycaemic clamp was commenced for the next 150 min, at which
186 point (270 min from the start of the tracer infusion) a 25 g whey protein isolate drink spiked with 6%
187 [²H₅]phenylalanine was ingested (MyProtein, UK). The insulin clamp was continued for a further 90
188 min until the end of the 360 min infusion period. The period between 120-270 min was used to assess
189 the effect of overfeeding on skeletal muscle insulin sensitivity, whereas the period between 270-360
190 min was used to assess the effect of treatment on muscle FSR. Muscle biopsy samples were taken
191 before (270 min time point) and after (360 min time point) the ingestion of the whey protein drink in
192 order to assess changes in the activation (phosphorylation) of key signaling proteins and measure
193 [²H₅]phenylalanine incorporation into the muscle protein pool.

194

195 Arterialised blood samples were obtained from the heated hand vein every 60 min for the first 120 min
196 and every 30 min during the insulin clamp to determine plasma [6,6²H₂] glucose enrichment. Further
197 blood samples were taken every 5 min during the insulin clamp for the determination of glucose
198 concentrations, and every 30 min over the entire period of infusion for the determination of serum
199 insulin and plasma FFA concentrations. Resting energy expenditure and whole-body substrate
200 oxidation rates were measured using indirect calorimetry for 15 min before the infusion of the tracer
201 (baseline), before the insulin clamp and during the last 15 min of the clamp.

202

203 **Blood and Urine Analysis.** In both studies, whole blood glucose concentrations were determined
204 using a Yellow Springs Instrument Analyzer (YSI, 2300 STAT PLUS). Serum was separated from one
205 aliquot of blood by centrifugation (15 min at 3,000 g) after being allowed to clot and analyzed for
206 insulin concentrations by radioimmunoassay (HI014K, Merck Millipore, MA, US) and TAG
207 concentrations by coupled enzymatic colorimetry using a clinical chemistry analyser (ABX Pentra
208 400, Horiba Ltd., Kyoto, JP). Another aliquot of blood was collected into a tube containing 30µl
209 EGTA glutathione and centrifuged immediately at 3,000g for 15 min at 4°C to obtain plasma that was
210 aliquoted into a tube containing tetrahydrolipostatin (30 µg/ml plasma) for the determination of FFA
211 using a commercially available kit (NEFA HR-2, Wako, Osaka, JPN). In the acute feeding study,
212 urinary and plasma urea concentrations were determined using a commercially available enzymatic
213 kinetic assay (Randox Cat# UR220). In both studies, after deproteinisation on ice with dry 5-
214 sulfosalicylic acid, another aliquot of plasma separated from EGTA treated blood was also analysed
215 for phenylalanine, tyrosine and leucine concentrations, and [1-¹³C]phenylalanine enrichment (acute
216 feeding study) and L-[ring-²H₅]-phenylalanine enrichment (short-term overfeeding study) by GC-MS
217 (Agilent 7890A GC/5975C; MSD, Little Falls, DE) after derivitisation with tert-butyl dimethylsilyl
218 (TBDMS) as previously described (11, 22).

219

220 **Skeletal muscle biopsy and analysis.** Muscle biopsies were obtained from the middle region of the
221 *vastus lateralis* muscle using the percutaneous needle biopsy technique as described previously (5). In
222 a given trial, muscle biopsies were taken from the same leg spaced by at least 3 cm. Muscle biopsies
223 were rapidly frozen in liquid nitrogen cooled isopentane and stored in liquid nitrogen for subsequent
224 analysis.

225

226 One portion of the frozen muscle sample (~50 mg) was freeze-dried, separated free of visible blood
227 and fat, and following ice-cold 2% perchloric acid extraction, analysed for intracellular tissue [1-
228 ¹³C]phenylalanine (acute feeding study) and L-[ring-²H₅]-phenylalanine (short-term overfeeding
229 study) incorporation into the myofibrillar protein pool (acute study) and muscle mixed protein pool
230 (short-term overfeeding study) in the same manner as the plasma samples. Amino acids were purified

231 from the remaining protein pellet as described previously (22) and used to determine the [1-
232 ¹³C]phenylalanine or L-[ring-²H₅]-phenylalanine enrichment using GC-MS.

233

234 In the acute study, muscle BCAA and their keto acids and short-chain acylcarnitines were analysed
235 using both hydrophilic interaction liquid chromatography (leucine, isoleucine and valine) and reversed
236 phase chromatography (KIC, KMV and KV) coupled to high resolution mass spectrometry. A single
237 extraction procedure using a mixture of isopropanol and acetonitrile containing isotopically labelled
238 internal standard for each metabolite of interest was used. Method validation in skeletal muscle and a
239 proxy matrix (7.5% BSA) showed excellent linearity ($R^2 > 0.99$), accuracy and precision, and
240 consistent levels of recovery across all metabolites.

241

242 **Western blotting.** In both studies, total muscle protein homogenates were extracted from another
243 portion (~30 mg) of the frozen muscle tissue by homogenisation in a HEPES phosphatase buffer in the
244 presence of protease and phosphatase inhibitors (P-8340, Sigma, UK) as described previously (42).
245 Protein was quantified using the bicinchonic acid (BCA) protein (Pierce, Perbio, Aalst, Belgium). The
246 muscle protein content of phosphorylated Akt (serine⁴⁷³, 1:500, Cat. No #9271), mTOR (serine²⁴⁴⁸,
247 1:1000, Cat. No #2971), 4E-BP1 (threonine^{37/46}, 1:500, Cat. No #9459) (all by Cell Signalling,
248 Beverly, MA USA) and Actin (1:5000, Cat. No #A2066, Sigma, Dorset, UK) were determined by
249 western blot analysis using an anti-rabbit horseradish peroxidase (1:2000, Cat. No #P0217, Dako,
250 Denmark) as a secondary antibody. All immunoreactive proteins were visualized using Amersham™
251 ECL™ Prime Western blotting detection reagent (GE Healthcare Life Sciences, Buckinghamshire,
252 UK), quantified by densitometry using the Quantity One 1-D Analysis Software version 4.5 (Bio-Rad
253 Laboratories, Inc., USA), and normalized to α -actin (Sigma-Aldrich Company Ltd., Dorset, UK).

254

255 **Muscle lipid content.** In both studies, intramyocellular lipid (IMCL) content, lipid droplet size and
256 number analysis was performed as previously described (12, 43). In the chronic (overfeeding) study,
257 quantification of the most abundant intramuscular DAG (diC16:0, C16:0/C18:1, diC18:1) and

258 ceramide (C16:0, C18:0, C24:0) species was performed in 5 mg freeze-dried muscle powder using
259 high-performance liquid chromatography tandem mass spectroscopy as previously described (7, 8,
260 12).

261

262 **Calculations.** In the short-term overfeeding study, calculations of glucose disposal were made at
263 steady state during the insulin clamp (210-270 min period). The modified Steele equations (19) were
264 used to calculate glucose appearance R_a during basal (0-120 min) and insulin stimulated (120-270
265 min) states. Hepatic glucose output (HGO) was calculated as the difference between R_a and glucose
266 infusion rate (GIR) during the clamp and, therefore, total rate of glucose disappearance (Rd), the true
267 measure of glucose disposal, was calculated as the sum of HGO and GIR.

268

269 Skeletal muscle FSR was calculated by dividing the increment in enrichment in the product (i.e.,
270 protein-bound [ring-²H₅]phenylalanine) by the enrichment of the precursor. Plasma and muscle free
271 [ring-²H₅]phenylalanine enrichments were used to provide an estimate of the lower and higher
272 boundaries of true FSR, respectively. The formula used was $FSR = [\Delta Ep / (E_{precursor} \times t)] \times 100$,
273 where ΔEp is the delta increment of protein-bound [ring-²H₅]phenylalanine during incorporation
274 periods, $E_{precursor}$ is the enrichment of the precursor used during the time period for amino acid
275 incorporation determination, and t denotes the time duration (h) between biopsies. Data were then
276 multiplied by 100 to express FSR as percentage per hour.

277

278 **Statistical analysis.** Data analysis was carried out using GraphPad Prism 7.0 Software (GraphPad
279 Software Inc., San Diego, CA). All blood and muscle data were analysed using a two-way (treatment x
280 sampling time) analysis of variance (ANOVA). When a significant difference was obtained with the
281 two-way ANOVA, data were further analysed with Student's paired t-tests using the Bonferroni
282 correction. [¹³C]phenylalanine incorporation into the myofibrillar protein pool was assessed using a
283 Student's paired t-test. Data are reported as means \pm SEM, and statistical significance was set at
284 $P < 0.05$.

285

286 **Results**

287 *Acute Feeding Study.*

288 **Blood glucose, serum insulin, serum TAG and plasma FFA.** Blood glucose and serum insulin
289 concentrations were similar between trials at baseline and during the first 4h following the ingestion of
290 the test meal (**Figure 1A and 1B**). Although circulating glucose and insulin increased (time effect
291 $P<0.0001$) in response to milk protein and dextrose ingestion at 4h, there were no differences between
292 trials at any time point during the last 3h.

293 Two-way ANOVA revealed significant interaction effects ($P<0.0001$) between trials (control vs. lipid)
294 and time (baseline vs. postprandial sampling times) for serum TAG and plasma FFA levels (**Figure**
295 **1C and 1D**). Specifically, serum TAG increased in response to the ingestion of the fat test meal and
296 decreased after the ingestion of the milk protein and dextrose solution, whereas values remained
297 unchanged throughout the control trial. As a result, serum TAG concentrations were higher at 2h, 3h,
298 3.5h and 4h following ingestion of the fat test meal and for the first 105 min after ingestion of the milk
299 protein and dextrose solution when compared to the control meal (**Figure 1C**). Plasma FFA
300 concentrations increased ($P<0.001$) between 3h and 4h after ingestion of the fat test meal and were
301 higher at 4h when compared with control ($P<0.01$), whereas a smaller but significant increase from
302 baseline was also observed in the control trial at 4h ($P<0.01$). However, the suppression of the FFA
303 observed in both trials following ingestion of the milk protein and dextrose solution at 4h was
304 attenuated in the lipid trial such that values were higher ($P<0.001$) for the next 90 min (between 4h-
305 5.5h) when compared with control (**Figure 1D**).

306 **RER and whole-body substrate oxidation rates.** Non-protein RER tended to be lower (treatment
307 effect $P=0.06$) in the lipid trial when compared to control both 4h after ingestion of the test meal (0.75
308 ± 0.01 and 0.80 ± 0.02 , respectively) and 3h after ingestion of the milk protein plus dextrose solution
309 (0.79 ± 0.01 and 0.83 ± 0.02 , respectively) (**Figure 1E**). As a result, fat oxidation rates were higher
310 (0.11 ± 0.01 versus 0.06 ± 0.02 g/min, $P<0.05$) in the lipid trial when compared to control.

311 **Forearm glucose uptake.** There was no effect of treatment (Control vs. Lipid) on postprandial
312 concentrations of glucose in arterialised or deep venous blood in response to feeding of the milk
313 protein plus dextrose solution, and thus no significant differences between trials were found in glucose
314 uptake across the forearm tissue (**Figure 1F**).

315 **Plasma amino acids.** Two-way ANOVA revealed a significant treatment effect for plasma leucine
316 ($P<0.001$) and a trend for phenylalanine ($P=0.054$) concentrations. Both amino acids were unchanged
317 during the first 4h following ingestion of the test meal in the control and lipid trials (**Figure 2A and**
318 **2B**) but, following the ingestion of the milk protein plus dextrose solution, the increase in their levels
319 was attenuated in the lipid trial. On the other hand, two-way ANOVA revealed a trend for an
320 interaction effect ($P=0.058$) in plasma tyrosine levels, which declined at 3.5h and 4h after ingestion of
321 the lipid but not control test meal. Following the ingestion of the milk protein plus dextrose solution,
322 plasma tyrosine levels increased in both trials but this effect was attenuated in the lipid trial (**Figure**
323 **2C**).

324 **Plasma enrichment of [$1-^{13}\text{C}$]phenylalanine and incorporation into muscle protein.** Plasma [$1-$
325 ^{13}C]phenylalanine enrichment (MPE) was negligible at baseline and during the first 4h after ingestion
326 of the test meal but increased in both trials following the ingestion of the intrinsically labelled milk
327 protein (time effect $P<0.0001$) and reached a plateau after 90 min. However, there was no difference
328 in the enrichment between trials (**Figure 2D**). Skeletal muscle [$1-^{13}\text{C}$]phenylalanine tracer to tracee
329 ratio (TTR), reflecting the incorporation of dietary protein derived amino acids into the skeletal muscle
330 myofibrillar protein pool, was 2-fold higher ($P<0.05$) in the lipid trial when compared to control
331 (**Figure 2E**).

332 **Muscle BCAA, keto acid and short-chain acylcarnitine content.** There was a strong trend for
333 intramuscular leucine (interaction effect $P=0.06$), isoleucine (interaction effect $P=0.08$) and valine
334 (interaction effect $P=0.06$) content and their sum (interaction effect $P=0.06$) to be lower in the lipid
335 trial (**Figure 3A-D**). Accordingly, their respective keto acids KIC (treatment effect $P<0.01$), KMV
336 (treatment effect $P<0.01$) and KIV (interaction effect $P<0.05$) content and their sum (interaction effect

337 $P < 0.01$) was also lower in the lipid trial when compared with control (**Figure 3E-H**). Furthermore, the
338 sum of BCAA-derived short-chain acylcarnitines (C3, C5 and ISOC5), which provides an index of
339 BCCA oxidative flux, tended to be lower (interaction effect $P = 0.069$; $n = 7$) in the lipid trial (0 min:
340 $20.3 \pm 3.5 \mu\text{mol/kg dm}$; 180 min: $14.8 \pm 1.9 \mu\text{mol/kg dm}$) when compared to control (0 min: $16.8 \pm$
341 $2.1 \mu\text{mol/kg dm}$; 180 min: $21.6 \pm 4.4 \mu\text{mol/kg dm}$).

342 **Muscle IMCL content.** The IMCL content (% area of muscle fibre analysed) was similar between
343 trials and remained unchanged in response to the ingestion of test meal and the intrinsically labelled
344 milk protein plus dextrose solution (**Figure 2F**).

345 **Muscle PDC and signalling proteins.** Muscle PDC activity was unchanged in response to the
346 ingestion of test meal and the intrinsically labelled milk protein plus dextrose solution, with no
347 differences observed between trials (**Figure 4A**). Muscle Akt phosphorylation at serine⁴⁷³ was similar
348 between trials 4h after ingestion of the test meal and increased to a similar degree after the ingestion of
349 the milk protein plus dextrose solution (time effect $P < 0.01$) (**Figure 4B**). Muscle mTOR
350 phosphorylation at serine²⁴⁴⁸ (**Figure 4C**) also increased after the ingestion of the milk protein plus
351 dextrose solution although there was a trend for it to be higher during the lipid trial when compared
352 with control (time effect $P < 0.05$ and treatment effect $P = 0.10$). However, muscle p4EBP1
353 phosphorylation at threonine^{37/46} was unchanged in response to the ingestion of test meal and the
354 intrinsically labelled milk protein plus dextrose solution, with no differences observed between trials
355 (**Figure 4D**).

356 **Short-term (fat overfeeding) Study.**

357 **Body mass and BMI.** The overfeeding period increased body mass (from 97.0 ± 3.3 to 97.7 ± 3.1 kg,
358 $P < 0.01$) and BMI (from 30.1 ± 1.1 to 30.4 ± 1.1 kg/m², $P < 0.05$).

359 **Changes in fasting markers of insulin sensitivity and lipid markers.** There was no effect of
360 overfeeding on fasting levels of blood glucose, insulin or HOMA-IR (all markers of fasting insulin
361 sensitivity) following two weeks of overfeeding (**Table 1**). There was also no effect of overfeeding on
362 fasting FFA, total cholesterol, HDL, LDL or TAG concentrations (**Table 1**).

363

364 **IMCL accumulation.** Two weeks of fat overfeeding did not affect IMCL content in mixed skeletal
365 muscle. In particular, there was no difference between isocaloric and fat overfeeding diets in percent
366 total coverage of lipid droplets or lipid present in the subsarcolemmal and intermyofibrillar regions
367 (**Table 2**). Furthermore, there was no effect of fat overfeeding in lipid droplet size or the number of
368 lipid droplets per fibre.

369

370 **Muscle DAGs and ceramides.** Following the 2-week fat overfeeding period, there was a trend for an
371 increase in C16:0 DAG ($P = 0.06$), although there were no changes in either C16:0/C18:1 or C18:1
372 DAG (**Figure 5A-C**). However, there was a significant increase in C16:0 ($P < 0.01$) but not C18:0 or
373 C24:0 ceramide species (**Figure 5D-F**).

374 **Insulin sensitivity and muscle protein synthesis.** When comparing the steady state phase between
375 120-270 min, the 2-week fat overfeeding period resulted in similar glucose rate of disappearance (Rd)
376 during the insulin clamp prior to protein ingestion. The consumption of protein caused an increase in
377 glucose Rd both after the isocaloric and the fat overfeeding periods (time effect $P < 0.001$). (**Figure**
378 **6A**). There was no difference in serum insulin concentrations during the steady-state phase of the
379 insulin clamp before protein feeding between trials (**Figure 6B**). Serum insulin concentrations
380 increased following the ingestion of protein (time effect $P < 0.0001$) but there was no difference
381 between the isocaloric and fat overfeeding periods (**Figure 6B**).

382 Mixed-muscle FSR in response to the protein drink (270-360min) was not affected by the overfeeding
383 intervention (**Figure 6C**). Similarly, there was no significant difference in FSR during the
384 hyperinsulinaemic clamp before the ingestion of the protein drink following the 2-week fat
385 overfeeding period (**Figure 6C**).

386 **Plasma phenylalanine.** There was no significant difference in fasting plasma phenylalanine levels
387 after fat overfeeding in comparison to the isocaloric condition (50.7 ± 4.2 vs. 52.7 ± 3.3 $\mu\text{mol/l}$,
388 respectively). Plasma phenylalanine levels increased after protein ingestion after both the isocaloric
389 and fat overfeeding periods (time effect $P < 0.0001$) (**Figure 6D**), peaking at 330 min before decreasing

390 at 360 min. Moreover, when plasma phenylalanine was calculated as molar percent excess (MPE)
391 there was no significant difference between the isocaloric and overfeeding conditions (area under the
392 curve: 2548 ± 82 vs. 2509 ± 75 , respectively).

393 **Muscle p4E-BP1.** There was no significant difference in p4E-BP1 (threonine^{37/46}) phosphorylation
394 before or after protein ingestion. However, p4E-BP1 (threonine^{37/46}) significantly increased following
395 overfeeding after protein ingestion when compared with the isocaloric diet (overfeeding effect $P <$
396 0.05) (**Figure 7**).

397

398

399 Discussion

400 The main finding from these studies was that neither acute nor short-term dietary fat overload with
401 primarily saturated fatty acids in middle-aged overweight/obese individuals had a detrimental effect on
402 skeletal muscle protein synthetic response to dietary protein ingestion. Surprisingly, acute lipid
403 feeding resulted in a two-fold increase in the incorporation of dietary [1-¹³C]phenylalanine into the
404 muscle myofibrillar protein pool, representing the uptake and incorporation of dietary protein derived
405 amino acids in the myofibrillar protein pool.

406 This increase in muscle protein synthesis occurred in the absence of lipid-induced changes in muscle
407 CHO metabolism and insulin sensitivity as indicated by similar muscle glucose uptake, PDC activity
408 and Akt phosphorylation responses to the intrinsically labelled milk protein and dextrose solution
409 between the acute control and lipid trials. This finding supports our previous premise that insulin
410 resistance may be required to induce anabolic resistance under conditions of acute lipid overload in
411 humans (39). In the latter study, the inability of skeletal muscle to increase protein synthesis in
412 response to insulin and amino acid administration under conditions of elevated lipid availability
413 appeared to be mediated, at least in part, via the repression of translation initiation at the level of 4E-
414 BP1 but was independent of the phosphorylation of mTOR. The sensitivity of 4E-BP1 to elevated
415 lipid availability was corroborated in a recent study that showed an attenuated phosphorylation of 4E-
416 BP1 in response to 5 hours infusion of a lipid emulsion (intralipid) that elevated circulating FFA levels
417 by ~4-fold in middle-aged, overweight individuals (38). In contrast, in the present study there was no
418 difference in muscle p4EBP1 phosphorylation at threonine^{37/46} between trials, although muscle mTOR
419 phosphorylation at serine²⁴⁴⁸ tended to be higher during the lipid trial when compared with control,
420 indicating that lipid-induced activation of mTOR might be involved in the increase in muscle protein
421 synthesis following acute lipid feeding. Amino acids and insulin promote the activation of the mTOR
422 pathway leading to increased protein synthesis through the regulation of mRNA translation (3). In
423 C2C12 myotubes, palmitate has been shown to induce the mTORC1/p70S6K pathway through a
424 decrease in raptor (regulatory-associated protein of mammalian target of mTOR) phosphorylation
425 associated with inhibition of AMPK (30). However, the mTOR complex 1 (mTORC1) signalling

426 pathway is also involved in SFA-induced insulin resistance, and chronically activated or aberrant
427 mTORC1 signalling in obese skeletal muscle attenuates its response to insulin and amino acids (17).

428 In the present study, the attenuated levels of plasma AA (leucine, tyrosine and phenylalanine) in the
429 lipid trial may suggest a delayed appearance in the circulation. Indeed, carbohydrate (a mixture of
430 dextrose and maltodextrin) coingestion with protein was previously shown to delay dietary protein
431 digestion and absorption (as indicated by delayed appearance of exogenous labelled phenylalanine in
432 the circulation) without affecting postprandial muscle protein synthesis (22). The fact that plasma
433 enrichment of [1-¹³C]phenylalanine was similar between trials in the present study, suggests that
434 ingestion of dietary lipids several hours before the coingestion of protein and dextrose may enhance
435 the uptake of AA into skeletal muscle of overweight/obese individuals (rather than delay their
436 appearance in the circulation) and hence facilitate their subsequent availability for augmentation of
437 protein synthesis. The fact that muscle BCAA (leucine, isoleucine and valine) concentrations, their
438 respective ketoacids and short-chain acylcarnitines, an index of BCCA oxidative flux, were also lower
439 in the lipid trial, when compared with control, further supports the notion of enhanced redirection of
440 intramuscular free AA towards incorporation into the muscle myofibrillar protein pool (as indicated by
441 increased FSR in the present study) rather than oxidative catabolism. Indeed, it has previously been
442 shown in animal models that elevated FFA availability via infusion of triglycerides results in protein
443 sparing via reductions in whole-body leucine flux and oxidation (41). Although muscle protein
444 breakdown was not assessed in the present study, in postabsorptive humans elevated levels of
445 circulating FFA via infusion of lipid emulsions were previously shown to inhibit release of muscle AA
446 and muscle protein breakdown (23, 45, 48).

447 Although our findings are in agreement with the study by Katsanos et al (28), they are in contrast to a
448 recent study that showed an attenuated muscle protein synthetic response to whey protein ingestion
449 during a 5-hour infusion of a lipid emulsion (intralipid) that elevated circulating FFA levels by ~4-
450 fold, when compared with saline infusion, in middle-aged overweight individuals (38). Although there
451 was no concurrent infusion of insulin or coingestion with CHO (which precluded assessment of
452 insulin sensitivity in the latter study), the high levels of lipid infusion used and the resulting

453 supraphysiological circulating concentrations of FFA have previously been associated with profound
454 development of both insulin resistance (40, 42) and anabolic resistance (39). However, in the current
455 study we used dietary fat overload to acutely elevate circulating FFA to more modest levels, typically
456 observed during the postprandial period in humans, and coingested milk protein with dextrose to
457 maximise the insulin response and provide a real-life metabolic milieu for our observations. These
458 methodological differences may explain the apparent discrepancies in muscle protein synthetic responses
459 to dietary amino acids between those studies under conditions of acutely elevated fat availability in
460 humans.

461 Since neither insulin resistance nor accumulation of intramuscular lipids was observed in the acute
462 lipid overload study, we tested the hypothesis that more prolonged dietary lipid overload may be
463 required to induce accumulation of intramuscular lipids and attenuate FSR in overweight/obese
464 individuals. Although two weeks of overfeeding with a diet rich in SFA successfully increased body
465 mass by around 1kg, it did not affect total IMCL content in mixed skeletal muscle. However,
466 overfeeding increased skeletal muscle DAG levels along with its ceramide species. In contrast to that
467 reported previously (1, 12, 26), the increase in muscle DAG and ceramide levels were not associated
468 with the development of insulin resistance, as indicated by unchanged fasting HOMA-IR and glucose
469 Rd during the hyperinsulinaemic euglycaemic clamp both in the absence and presence of oral protein
470 ingestion. Interestingly, although the subjects in the overfeeding study had lower fasting HOMA
471 values than the subjects in the acute study, their relatively low insulin-stimulated glucose disposal
472 rates during the insulin clamp indicate some degree of muscle insulin resistance prior to the
473 intervention (14). Perhaps longer high fat feeding periods may have been required to induce further
474 skeletal muscle insulin resistance at the level of glucose disposal in humans, which is preceded by
475 altered intracellular partitioning of glucose metabolism leading to decreased oxidation and increased
476 non-oxidative glucose disposal (6, 13, 16). Interestingly, fat overfeeding did not attenuate mixed-
477 muscle FSR in response to the oral protein drink, which suggests that accumulation of intramuscular
478 lipids is not the driving force for the attenuated FSR observed in previous studies under conditions of
479 lipid-induced insulin resistance (39). This is in contrast to animal studies demonstrating that the time

480 course of chronic high-fat overfeeding induced obesity and anabolic resistance coincides with muscle
481 lipid accumulation (32), but supported by studies showing that diet-induced obesity via high fat
482 feeding in mice can induce insulin resistance after 3 weeks (34) and impair the activation of skeletal
483 muscle protein synthesis in response to feeding of a mixed meal after 9 weeks (2). Moreover, in
484 contrast to other studies where lipid induced anabolic resistance was associated with suppressed muscle
485 4E-BP1 phosphorylation (38, 39), two weeks of high fat overfeeding increased p4E-BP1 in the present
486 study, perhaps as a result of increased energy content of the diet *per se*. Taken together, it would
487 appear that overt insulin resistance must be present to observe a blunted anabolic response to protein
488 ingestion in obese individuals. Interestingly, physical inactivity or disuse also impairs the anabolic
489 response to protein ingestion (10, 46), and can induce severe insulin resistance (18). Therefore,
490 differences in habitual physical activity levels may, at least in part, explain the apparent discrepancy in
491 the published literature regarding the impact of obesity on postprandial anabolic resistance (4, 29).

492
493 In conclusion, neither acute nor short-term dietary fat overload with primarily saturated fatty acids in
494 middle-aged overweight/obese individuals have a detrimental effect on skeletal muscle protein
495 synthetic response to dietary protein ingestion. In the absence of insulin resistance, dietary-induced
496 accumulation of intramuscular lipids is not associated with anabolic resistance to dietary protein
497 ingestion *per se*, and it appears that FFA availability may retain its protein sparing ability in middle-
498 aged obese individuals. These findings have important implications for maintenance of muscle mass
499 and its relationship to lipid-induced insulin resistance in obesity and ageing as alterations in muscle
500 protein turnover play an important role in age-related decline in skeletal muscle mass (sarcopenia),
501 with insulin resistant obese type 2 diabetes patients being particularly susceptible to it.

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510

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513

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515

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517 DHK provided liquid chromatography and reversed phase chromatography coupled to high resolution
518 mass spectrometry reagents and materials. KT, FBS, LvL, CG and CC analyzed data and performed
519 statistical analysis. KT, FBS and LvL designed the studies. KT, FBS, CG and LvL wrote the
520 manuscript. All authors revised the manuscript.

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668 Figure legends

669 **Figure 1.** Effect of an oral test drink consisting of either a fat emulsion (lipid trial) or water (control
670 trial), ingested 4 hours before the ingestion of a bolus of CHO (dextrose) and intrinsically labelled
671 milk protein drink, on (A) arterialized blood glucose concentration (mmol/l), (B) arterialized serum
672 insulin concentration (mU/l), (C) arterialized plasma FFA concentration (mmol/l), (D) arterialized
673 serum TAG concentration (mmol/l), (E) non-protein RER, and (F) forearm glucose uptake
674 ($\mu\text{mol}/\text{min}$), before (0 min), during and 180 min after the ingestion of the CHO and milk protein drink.
675 Data are means \pm SEM; n = 8 for all variables except for glucose uptake (n=5).

676 **Figure 2.** Effect of an oral test drink consisting of either a fat emulsion (lipid trial) or water (control
677 trial), ingested 4 hours before the ingestion of a bolus of CHO (dextrose) and intrinsically labelled
678 milk protein drink, on (A) arterialized plasma leucine concentration ($\mu\text{mol}/\text{l}$), (B) arterialized plasma
679 phenylalanine concentration ($\mu\text{mol}/\text{l}$), (C) arterialized plasma tyrosine concentration ($\mu\text{mol}/\text{l}$), (D)
680 arterialized plasma [$1\text{-}^{13}\text{C}$]phenylalanine enrichment (MPE), (E) skeletal muscle [$1\text{-}^{13}\text{C}$]phenylalanine
681 TTR, and (F) IMCL content (% area of muscle fibre analysed), before (0 min), during and 180 min
682 after the ingestion of the CHO and milk protein drink. Data are means \pm SEM; n=8 for all variables
683 except for IMCL (n=5); *p < 0.05 from Control.

684 **Figure 3.** Effect of an oral test drink, consisting of either a fat emulsion (lipid trial) or water (control
685 trial), ingested 4 hours before the ingestion of a bolus of CHO (dextrose) and intrinsically labelled
686 milk protein drink, on (A) muscle leucine concentration ($\mu\text{mol}/\text{kg dm}$), (B) muscle isoleucine
687 concentration ($\mu\text{mol}/\text{kg dm}$), (C) muscle valine concentration ($\mu\text{mol}/\text{kg dm}$), (D) sum of muscle
688 BCCA (leucine, isoleucine and valine) concentrations ($\mu\text{mol}/\text{kg dm}$), (E) muscle KIC concentration
689 ($\mu\text{mol}/\text{kg dm}$), (F) muscle KMV concentration ($\mu\text{mol}/\text{kg dm}$), (G) muscle KIV concentration
690 ($\mu\text{mol}/\text{kg dm}$), and (H) sum of muscle ketoacid (KIC, KMV and KIV) concentrations ($\mu\text{mol}/\text{kg dm}$),
691 before (0 min) and 180 min after the ingestion of the CHO and milk protein drink. Data are means \pm
692 SEM; n=7 for all variables; *p < 0.05 from Control; **p < 0.01 from Control (based on post hoc
693 analysis).

694 **Figure 4.** Effect of an oral test drink consisting of either a fat emulsion (lipid trial) or water (control
695 trial), ingested 4 hours before the ingestion of a bolus of CHO (dextrose) and intrinsically labelled
696 milk protein drink, on (A) muscle PDC activity (mmol acetylCoA/kg dm/min), (B) muscle Akt
697 phosphorylation at serine⁴⁷³ (normalized to α -actin content), (C) muscle mTOR phsosphorylation at
698 serine²⁴⁴⁸ (normalized to α -actin content), and (D) muscle 4E-BP1 phsosphorylation at threonine^{37/46}
699 (normalized to α -actin content), before (0 min) and 180 min after the ingestion of the CHO and milk
700 protein drink. Data are means \pm SEM; n=8 for all variables.

701 **Figure 5.** Effect of fat overfeeding or isocaloric diet on overnight fasted values of (A) muscle DAG
702 C16:0 concentration (μ mol/kg dm), (B) muscle DAG C16:0/C18:1 concentration (μ mol/kg dm), (C)
703 muscle DAG C18:1 concentration (μ mol/kg dm), (D) muscle ceramide C16:0 concentration (μ mol/kg
704 dm), (E) muscle ceramide C18:0 concentration (μ mol/kg dm), and (F) muscle ceramide C24:0
705 concentration (μ mol/kg dm). Data are means \pm SEM; n=7 for all variables; **p<0.01 from isocaloric
706 diet.

707 **Figure 6.** Effect of fat overfeeding or isocaloric diet on (A) glucose rate of disappearance (Rd)
708 (mg/kg/min), (B) serum insulin concentration (mU/l), (C) muscle FSR (%/h), and (D) plasma
709 phenylalanine concentration (μ mol/l), during an insulin clamp before (Insulin) and after the ingestion
710 of an oral protein drink (Insulin+ Protein). Data are means \pm SEM; n=9 for glucose Rd and serum
711 insulin; n=8 for muscle FSR and plasma phenylalanine; ***p<0.001 from respective Insulin,
712 ****p<0.0001 from respective Basal (based on post hoc analysis).

713 **Figure 7.** Effect of fat overfeeding or isocaloric diet on muscle 4E-BP1 phsosphorylation at
714 threonine^{37/46} (normalized to α -actin content). Data are means \pm SEM; n=6.

715

Table 1. Fasting insulin sensitivity and lipid makers after isocaloric and fat overfeeding diets.

	Isocaloric	Fat overfeeding
Fasting glucose (mmol/l)	4.8 ± 0.2	4.7 ± 0.2
Fasting serum insulin (mU/l)	10.1 ± 1.9	12.5 ± 3.2
HOMA-IR	2.2 ± 0.4	2.7 ± 0.7
TAG (mmol/l)	1.42 ± 0.24	1.47 ± 0.17
FFA (mmol/l)	0.43 ± 0.05	0.44 ± 0.04
Cholesterol (mmol/l)	5.17 ± 0.38	5.48 ± 0.44
HDL (mmol/l)	1.03 ± 0.04	1.09 ± 0.08
LDL (mmol/l)	3.47 ± 0.36	3.72 ± 0.40

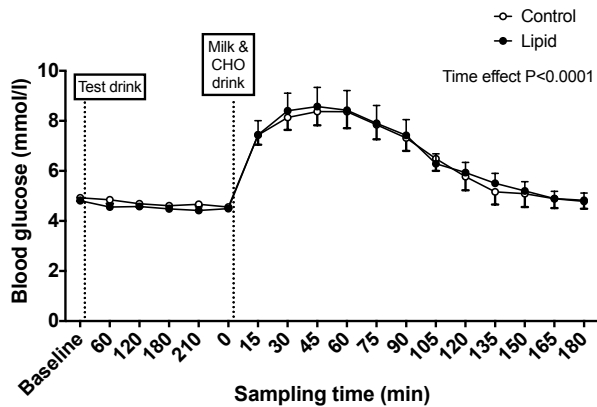
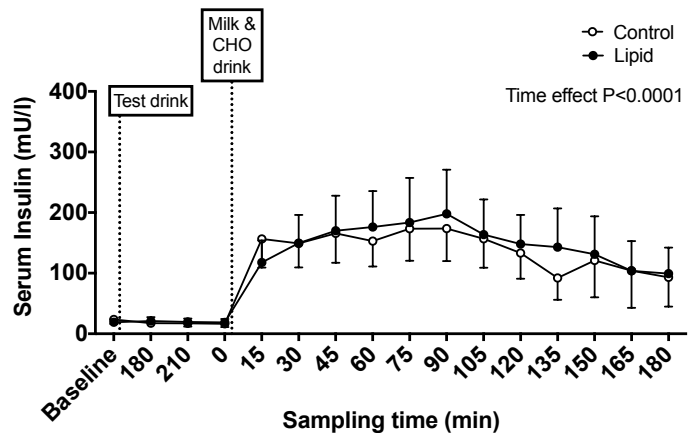
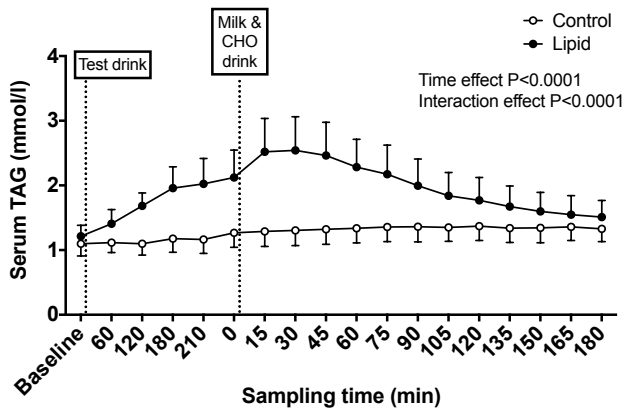
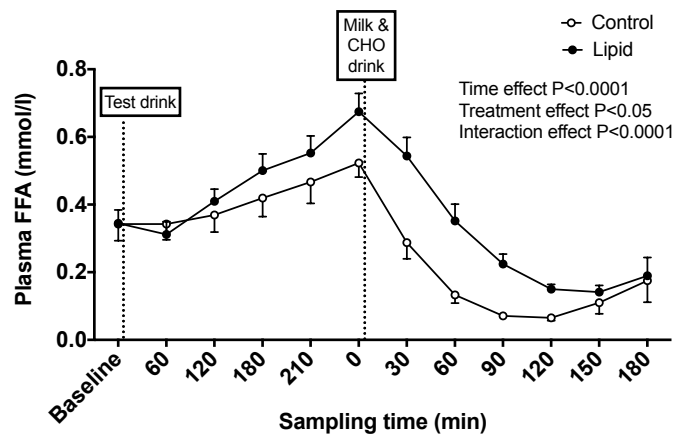
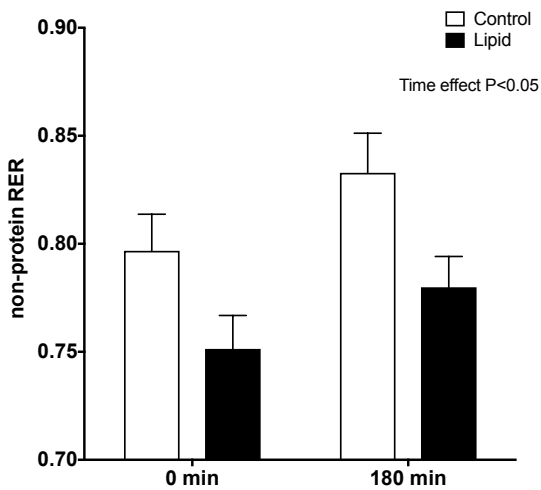
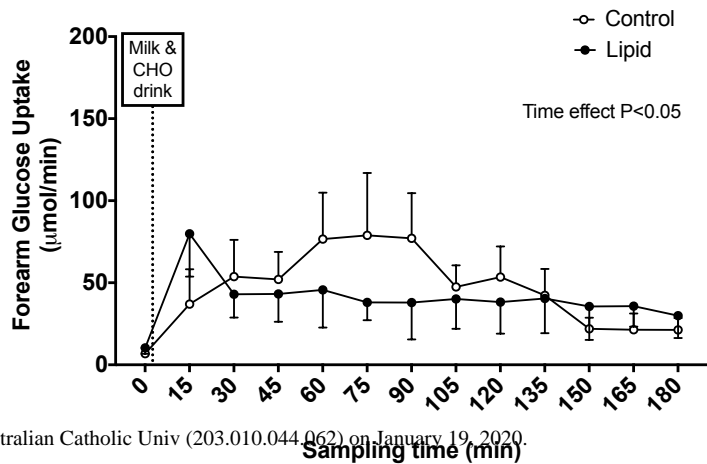
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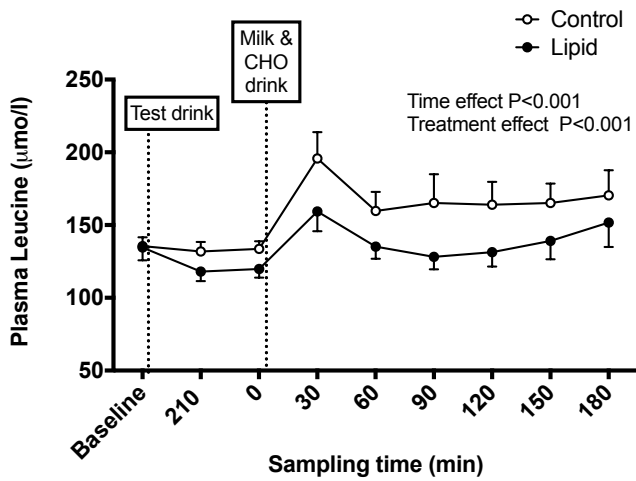
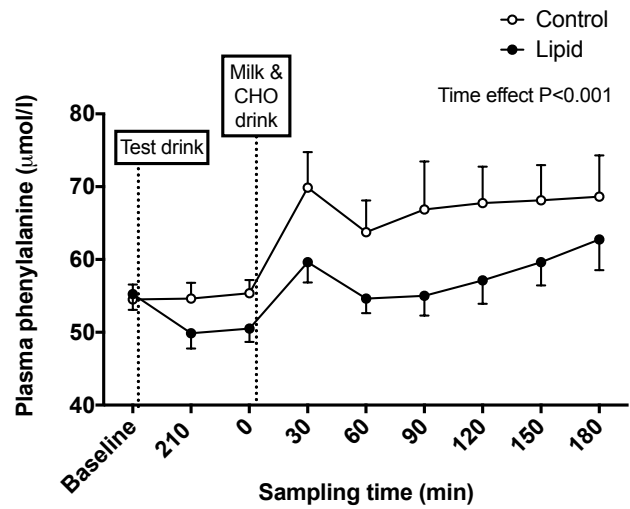
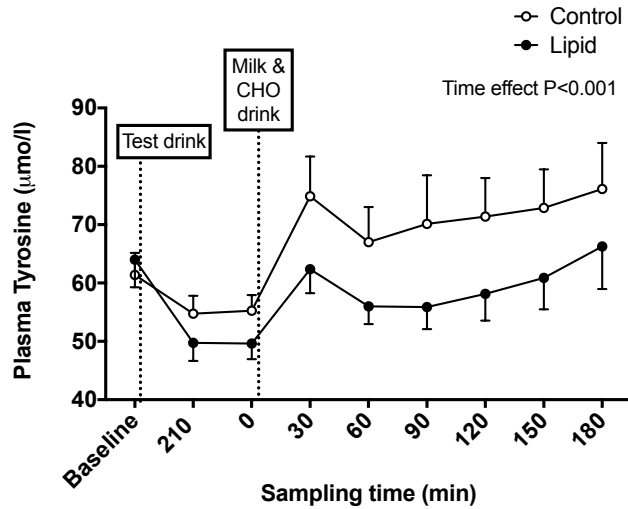
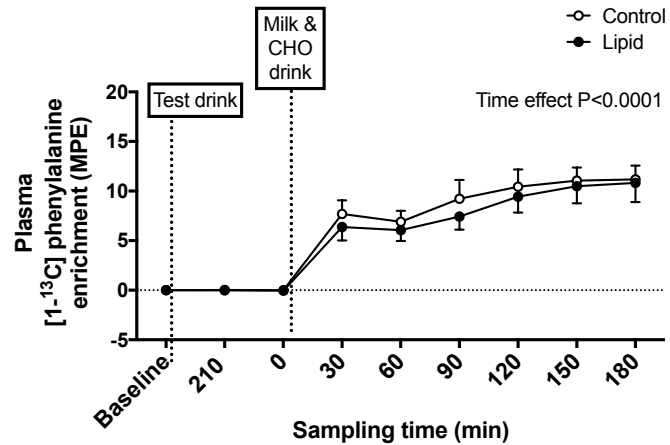
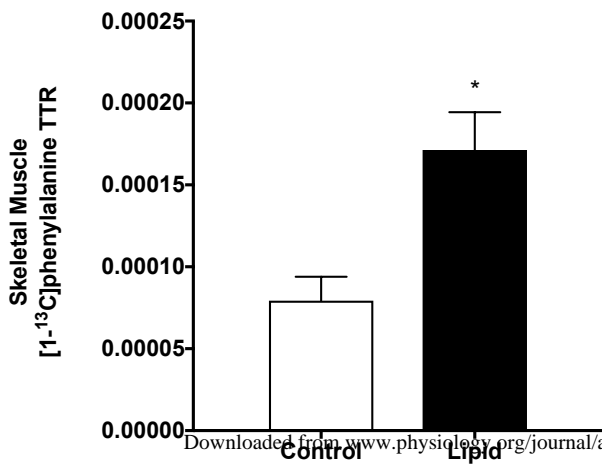
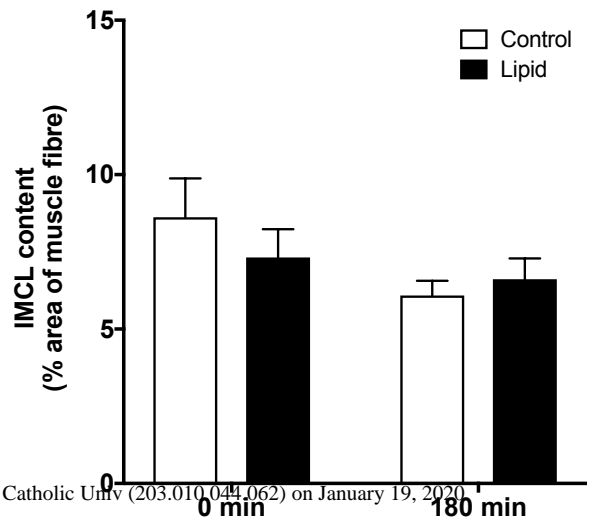
Table 2. IMCL after isocaloric and fat overfeeding diets.

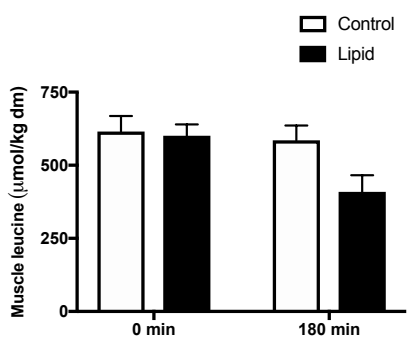
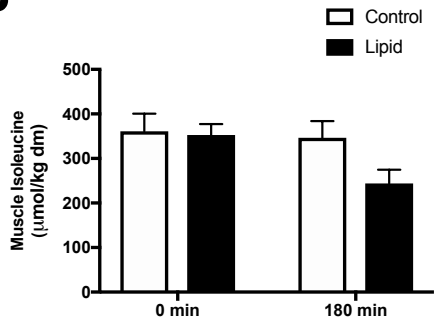
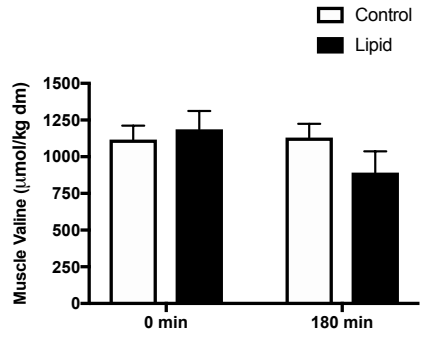
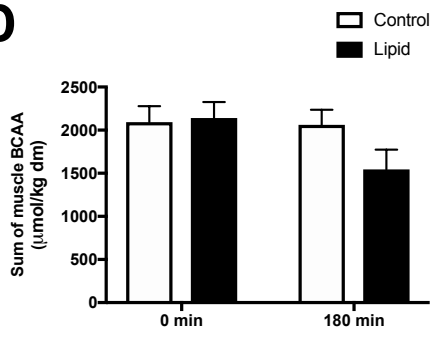
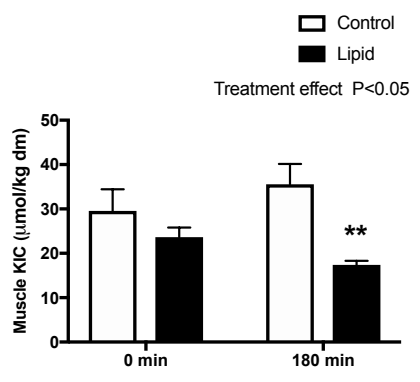
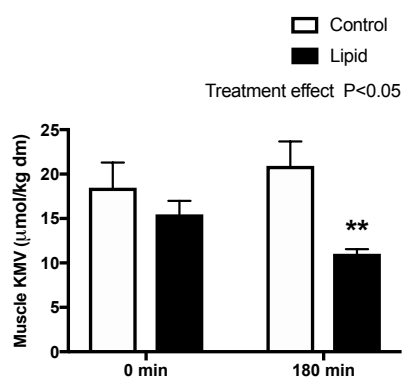
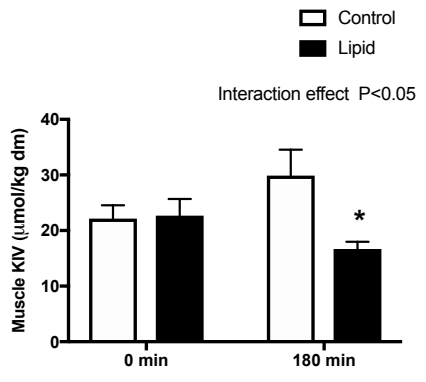
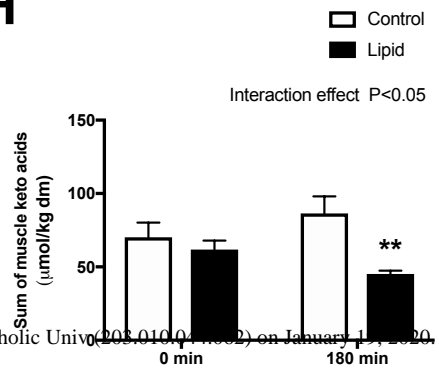
	Isocaloric	Fat overfeeding
% Lipid coverage	3.16 ± 0.27	3.21 ± 0.27
SSL LD (% fibre area)	2.61 ± 0.25	2.69 ± 0.24
IMF LD (% fibre area)	0.55 ± 0.07	0.53 ± 0.06
LD per fibre	56.3 ± 8.1	45.3 ± 5.9
LD size (µm ²)	0.46 ± 0.01	0.47 ± 0.01

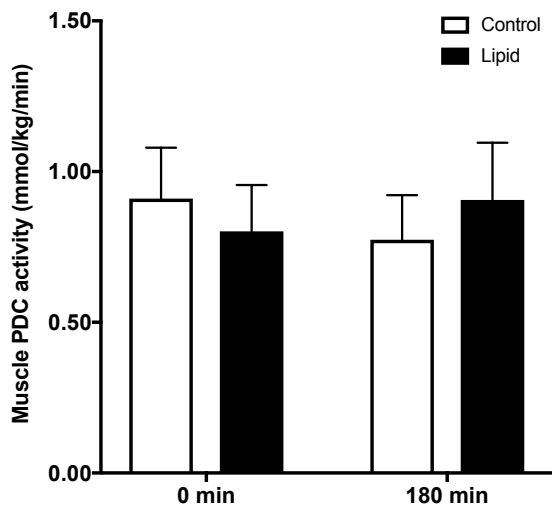
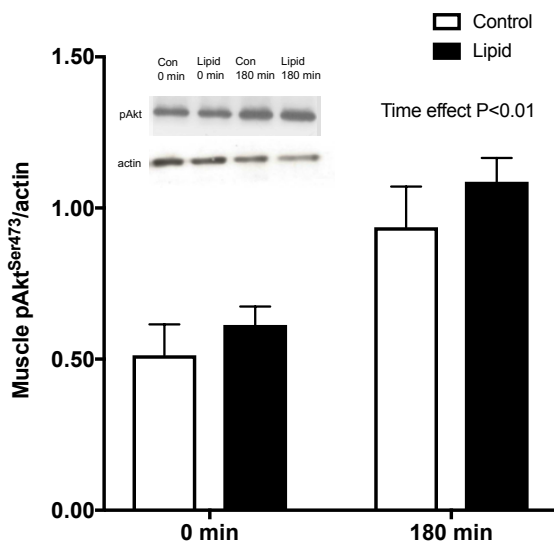
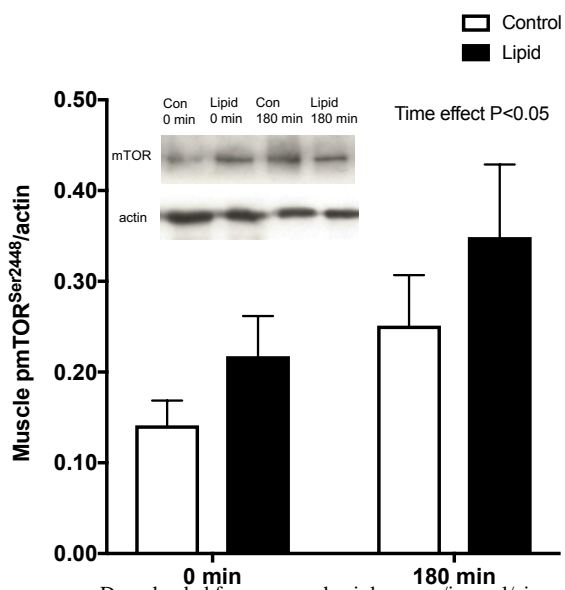
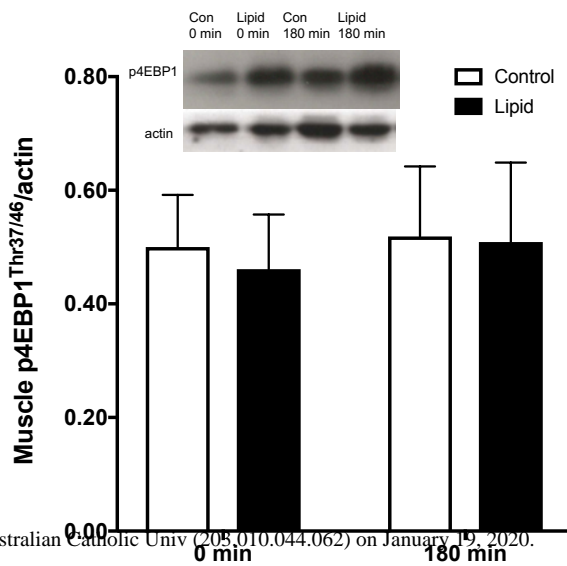
All values are mean ± SEM; n = 9. Abbreviations:

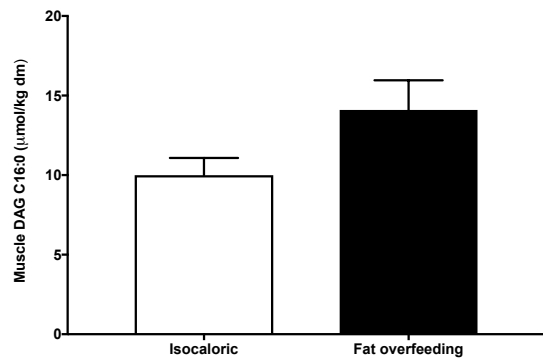
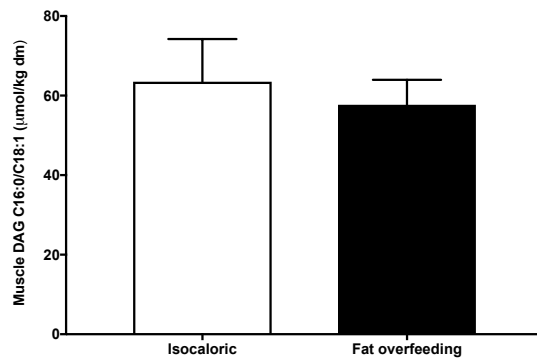
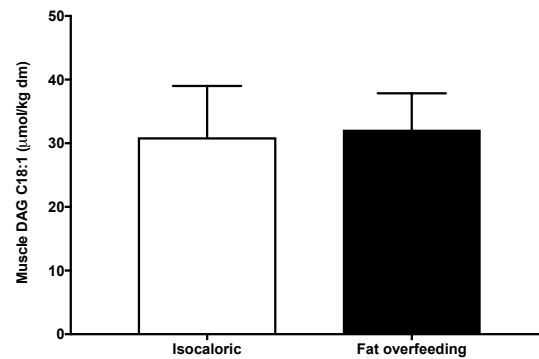
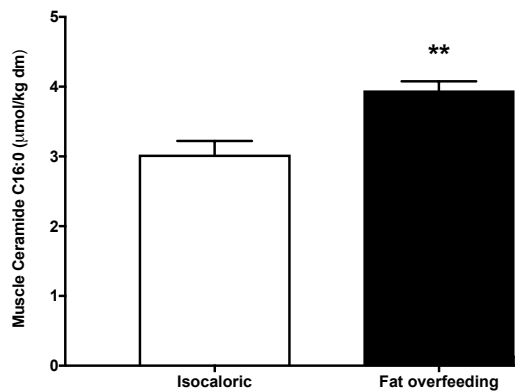
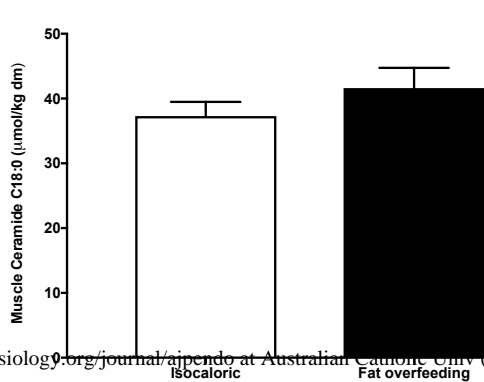
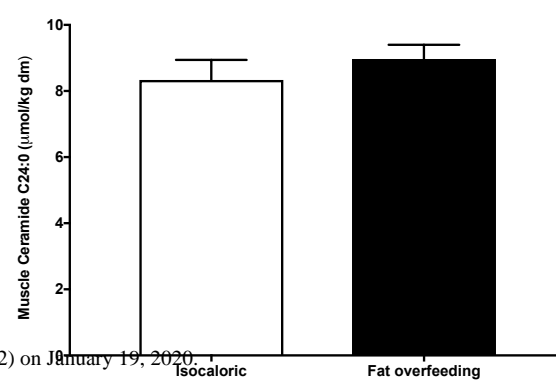
Lipid droplet (LD); Subsarcolemmal (SSL), and Intermyo-fibrillar (IMF).

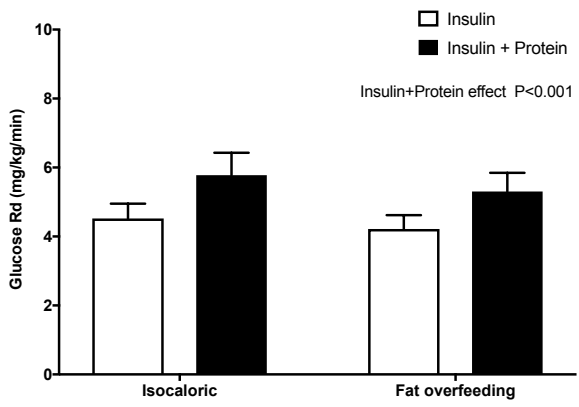
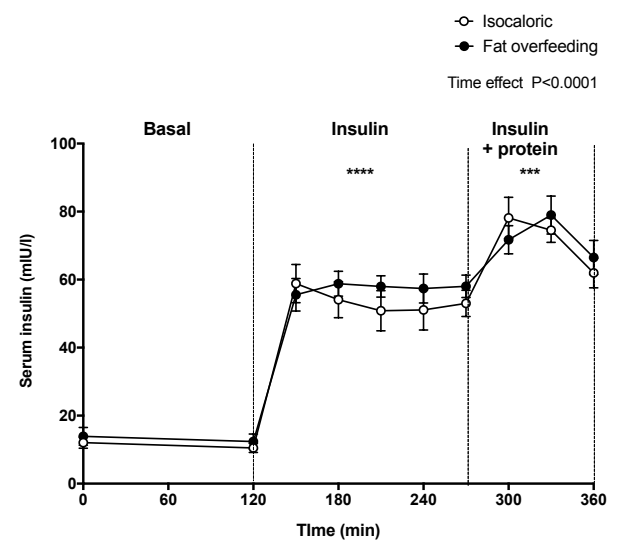
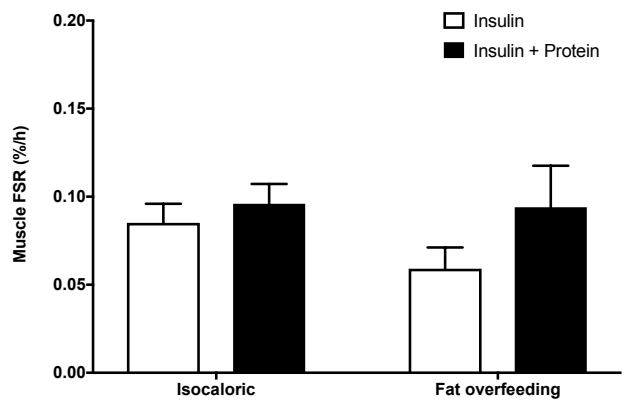
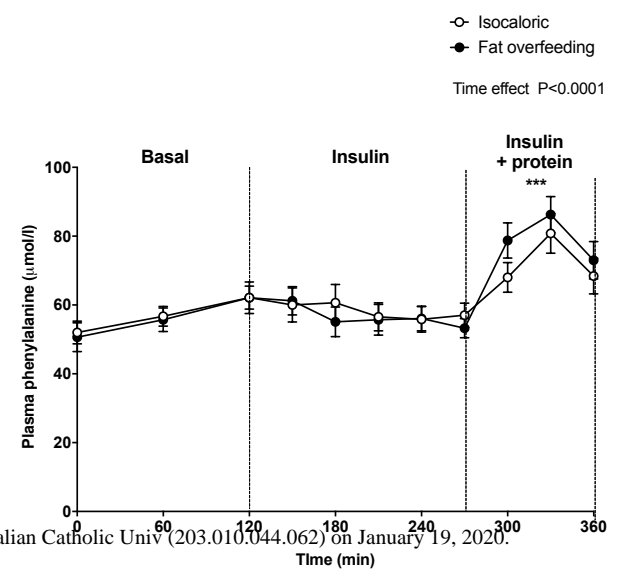
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Iso Iso Fat OF Fat OF
Ins Ins+Pr Ins Ins+Prot

□ Insulin

■ Insulin + Protein

P4EBP1

actin

Overfeeding effect $P < 0.05$

