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Is the timing of caloric intake associated with variation in diet-induced thermogenesis and in the metabolic pattern? A randomized cross-over study

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19 **Is the Timing of Caloric Intake Associated with Variation in Diet-Induced Thermogenesis and in the Metabolic**
20 **Pattern? A Randomized Cross-Over Study.**

21

22 Simona Bo^{1*}, Maurizio Fadda², Anna Castiglione³, Giovannino Ciccone³, Antonella De Francesco², Debora
23 Fedele¹, Alice Guggino¹, Mirko Parasiliti Caprino¹, Silvia Ferrara¹, Marco Vezio Boggio¹, Giulio Mengozzi⁴, Ezio
24 Ghigo¹, Mauro Maccario¹, Fabio Broglio¹

25

26 ¹Department of Medical Sciences, University of Turin, Turin, Italy

27 ²Unit of Clinical Nutrition, “Città della Salute e della Scienza” Hospital of Turin, Turin, Italy

28 ³Unit of Clinical Epidemiology, CPO, “Città della Salute e della Scienza” Hospital of Turin, Turin, Italy

29 ⁴Clinical Biochemistry Laboratory, “Città della Salute e della Scienza” Hospital of Turin, Turin, Italy

30

31 *Corresponding author: Simona Bo, Department of Medical Sciences, University of Turin, Corso Dogliotti 14,
32 10126 Turin, Italy; Telephone +(39)(011)6336036 Fax+(39)(011)6335401 E-mail: simona.bo@unito.it

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42

43 **Abstract**

44 **Background/Objectives:** food-induced thermogenesis is generally reported to be higher in the morning
45 although contrasting results exist due to differences in experimental settings related to the preceding fasting,
46 exercise, sleeping, and dieting. In order to definitively answer to this issue, we compared the calorimetric and
47 metabolic responses to identical meals consumed at 8:00 am and at 8:00 pm by healthy volunteers, after
48 standardized diet, physical activity, duration of fast and resting. **Subjects/Methods:** 20 subjects (age range 20-
49 35 years, BMI 19-26 kg/m²) were enrolled to a randomized cross-over trial. They randomly received the same
50 standard meal in the morning, and 7 days after, in the evening, or vice versa. A 30-min basal calorimetry was
51 performed; a further 60-min calorimetry was done 120-min after the beginning of the meal. Blood samples
52 were drawn every 30-min for 180-min. General linear models (GLMs), adjusted for period and carry-over, were
53 used to evaluate the “morning effect”, i.e. the difference of morning delta (after-meal minus fasting values)
54 minus evening delta (after-meal minus fasting values) of the variables. **Results:** fasting Resting-Metabolic-Rate
55 (RMR) did not change from morning to evening; after-meal RMR values were significantly higher after the
56 morning meal (1916; 95%CI 1792,2041 vs 1756; 1648,1863 kcal; p<0.001). RMR was significantly increased
57 after the morning meal (90.5; 95%CI 40.4,140.6 kcal; p<0.001), while differences in areas-under-the-curve for
58 glucose (-1800; -2564,-1036 mg/dl×h, p<0.001), log-insulin (-0.19; -0.30,-0.07 μU/ml×h; p=0.001) and fatty free
59 acid concentrations (-16.1;-30.0,-2.09 mmol/l×h; p=0.024) were significantly lower. Delayed and larger
60 increases in glucose and insulin concentrations were found after the evening meals.

61 **Conclusions:** the same meal consumed in the evening determined a lower RMR, and increased
62 glycemic/insulinemic responses, suggesting circadian variations in the energy expenditure and metabolic
63 pattern of healthy individuals. The timing of meals should probably be considered when nutritional
64 recommendations are given.

65 **ClinicalTrials.gov identifier:** NCT02343380

66

67 **Introduction**

68 An increasing number of studies have shown that the timing of food intake influences energy regulation, the
69 risk of weight gain and obesity, independent from total daily caloric intake, dietary composition and estimated
70 energy expenditure [1].

71 Early insulin secretion after a meal ingestion is significantly higher in the morning than in the evening, leading
72 to a more rapid glucose clearance from blood [2], whereas insulin sensitivity decreases later during the day [3-
73 4]. Circadian variations in the concentrations of hormones and peptides regulating satiety and appetite, as well
74 as genetic patterns of the circadian clock genes have been implicated in the daily modification of specific
75 metabolic pathways [1]. Additionally, gastric emptying seems to be more rapid at morning than in the evening
76 [5], and an increased efficacy of dietary carbohydrates absorption has been demonstrated under late
77 supertime conditions [6].

78 A few studies evaluated the circadian variation of the energy balance with contrasting results [7-12]. The
79 thermic effect of food, also called diet-induced thermogenesis (DIT), is defined as the increase in resting
80 metabolic rate (RMR) after the ingestion of a meal. This component accounts for a small proportion of the total
81 energy expenditure (about 10%), but it has been reported to be implicated in the development and persistence
82 of obesity [13]. DIT is significantly higher after the consumption of a snack in the morning than after the same
83 snack at night [7], and a reduced evening thermic response may be due to nocturnal insulin resistance [8].

84 Furthermore, habitual nighttime eating or snacking have been associated with reduced fat oxidation,
85 potentially promoting weight gain [14].

86 However, data in the literature often show contrasting results [9-12] with differences in the exercise level,
87 hours of sleeping, antecedent diets, variation in the duration of the fasting state, presence of comorbidity, and
88 low number of the subjects enrolled likely acting as confounding factors [15]. Indeed, the ideal setting to study
89 diurnal variation in energy expenditure is to measure post-absorptive RMR under the same conditions at
90 different times of the day. The aim of our study was therefore to compare the calorimetric and metabolic

91 responses to identical meals (a high-protein, low-carbohydrates meal) consumed in the morning (8:00 am) and
92 in the evening (8:00 pm) by healthy volunteers, after standardizing diet, physical activity level, duration of fast
93 and resting.

94

95 **Subjects and Methods**

96 *Recruitment of participants*

97 Twenty healthy volunteers (ten males and ten females) were enrolled among students and graduates attending
98 the Department of Medical Sciences of Turin (Italy).

99 Inclusion criteria were: age 20-35 years, body mass index (BMI) 19-26 kg/m², habitual moderate exercise level,
100 smoking <10 cigarettes/day. Exclusion criteria were: any acute or chronic diseases, menopause, any drugs or
101 supplementations, any alimentary restrictions or specific diets, being a shift or night workers, unable to give a
102 written informed consent.

103 *Design*

104 This was a randomized cross-over trial.

105 *Outcomes*

106 The primary *outcome* was evaluating changes in RMR after the morning meal consumption compared to
107 changes in RMR after the evening meal. The secondary outcomes were analyzing changes in circulating
108 concentrations of glucose, insulin, free fatty acids (FFA) and triglycerides after the morning and evening meal
109 consumption.

110 *Intervention*

111 Participants were randomly allocated to receive a *standard meal* at 8:00 am and the week after the same
112 *standard meal* at 8:00 pm, or vice versa. Eight hours before the meal (respectively at 12:00 pm or at 12:00 am),
113 participants received the same *standard meal* (without protein supplementation) at their home, and then were
114 asked to spend the following 6-h in bed. Drinking coffee, alcohol or other beverages, and smoking were not

115 permitted. The week preceding each test, participants were instructed not to change their usual diet and to
116 refrain from heavy physical activity. A 24-h urine collection was collected the day before each test in order to
117 determine total urinary nitrogen excretion.

118 The *standard meal* consisted of: 100g white bread, 100g ham, 50g cheese, 125g yogurt, 200ml fruit juice, plus
119 25g protein supplement (Resource Instant Protein, Nestlé, Switzerland). The nutritional composition of the
120 meal was: 30% protein, 31% fat, 39% carbohydrates; total kcal 1168. The participants had to consume each
121 meal in 25-30-min. Participants were taken by car to the laboratory. At 7:00 am (or pm, according to the
122 randomization), the participants underwent to anthropometric measurements and to the insertion of a 16-G
123 indwelling catheter into an antecubital vein of the forearm, subsequently kept patent by the slow infusion of
124 500 ml of saline solution until the end of the testing session. In order to avoid blood drawing related stress, all
125 the blood samples have been withdrawn from an extension line tubing.

126 A 30-min basal calorimetric exam was performed (**Figure 1**). Participants remained in a supine position but
127 awake and motionless on a hospital bed for the whole period, except during the meal, when they could sit to
128 eat and were allowed to void. At 8:00 am (or pm), the participants consumed the meal, and then rested in a
129 supine position for 90-min, followed by a second 60-min calorimetric evaluation. In order to obtain a better
130 compliance to the experiment, the second calorimetric evaluation lasted 60-min (from 120 to 180 min from the
131 beginning of the meal), since we have previously performed a pilot study in six volunteers to evaluate the
132 tolerance to the calorimetric exam, and have found that maintaining immobility while awaking was difficult for
133 more than 1 hour consecutive.

134 Blood samples were drawn every 30-min from the first calorimetric exam (baseline) until the end of the second
135 (post-prandial) (Figure 1). Time 0 was after the first calorimetry and before the meal. Times 30, 60, 90, 120, 150
136 and 180 were referred to the time intervals in minutes from the beginning of the meal. The same time
137 schedule was adopted in the case of the morning meal (at 8:00 am) and the evening meal (at 8:00 pm). The
138 second test was carried out after 7 days from the first.

139 *Sample size*

140 A sample size of 20 subjects (10 in the “morning-first” group and 10 in the “evening-first” group) was required
141 to test a 0.66 effect size with a power equal to 80% and a two-tailed α -value=0.05.

142 *Randomization*

143 The random sequence (morning/evening, evening/morning meal) was computer-generated, using blocks of
144 different lengths in random order.

145 *Measurements*

146 The Minnesota Leisure Time Physical Activity questionnaire [16], previously validated in an European cohort
147 [17], was completed by all the participants together with a 3-day food record, which consisted of a detailed
148 written food diary [18]. Subjects were instructed to record everything they ate or drank during 2 consecutive
149 week days and 1 week-end day. The 3-day food record data were loaded on the Win Food Pro 3 software
150 (Medimatica, Colonnella, Teramo, Italy), and the mean nutritional values for the 3 days were reported.

151 Body weight and height were measured with subjects wearing light clothes using a mechanical column scale
152 (SECA 711, Hamburg, Germany) to the nearest 0.1 kg and 0.1 cm, respectively. Waist circumference was
153 measured by a plastic tape meter at the top of the iliac crest, after a normal expiration. Fat and fat-free mass
154 were determined by single frequency bioelectrical impedance using manufacturer equations (BIA 101, Akern,
155 Italy). Indirect calorimetry by Deltatrac II (DATEX, Division of Instruments Corp. Helsinki, Finland) was used to
156 measure the rate of energy expenditure, by measuring the inspired and expired concentrations of oxygen (VO_2)
157 and carbon dioxide (VCO_2), which reflect nutrient metabolism. Deltatrac II has become one of the reference
158 tools for reliable measurements, validated in vitro and in vivo, and its accuracy has been detailed in several
159 studies [19]. Before measurements, the instrument was warmed-up for 30-min. The canopy was placed over
160 the subjects' face and carefully checked to prevent air leakage. After 5-min of initial calibration, gas sample
161 were continuously analyzed by a paramagnetic and infrared gas chamber for sensing O_2 and CO_2 respectively.
162 During the calorimetric exam, participants were not allowed to talk. The exams were performed in a quiet

163 room with a temperature kept at 23-25° C. The energy expenditure was calculated within 3-h from the
164 beginning of the meal, because it has been reported that the DIT response to meal can be assessed with
165 sufficient precision within 3-h and prevent the movements occurring with more prolonged periods of
166 immobility [15].

167 Blood samples were immediately centrifuged and aliquots of plasma were stored separately at -80° until
168 analysis. The following determinations were performed: glucose, insulin, FFA, triglycerides. Serum glucose was
169 measured by enzymatic colorimetric assay (Menarini Diagnostics, Florence, Italy); serum insulin was
170 determined by immunoradiometric assay (Beckman Coulter, Immunotech, Prague, Czech Republic; intra-assay
171 coefficients of variation $\leq 3.99\%$ and inter-assay coefficients of variation $\leq 4.8\%$). FFA concentrations were
172 measured by a fluorometric assay (Sigma-Aldrich, St. Louis, MO, USA). Plasma triglycerides were assayed by
173 enzymatic colorimetric method (Hitachi, Roche Diagnostics, Mannheim, Germany). Total urinary nitrogen
174 excretion was determined on the 24-h urine collection of the day before each test. Total nitrogen excretion
175 was assessed by a kinetic assay (Hitachi, Roche Diagnostics, Mannheim, Germany).

176 *Definitions*

177 The physical activity level was calculated as the product of the duration and frequency of each activity (in
178 hours/week), weighted by an estimate of the metabolic equivalent of the activity (METS) and summed for the
179 activities performed.

180 Both basal and after-meal RMR were calculated according to the formula of Weir [20]. RMR was calculated also
181 in relation to fat free mass (FFM) determined by bio-impedance as: RMR/FFM and expressed as kcal/kg.

182 DIT was considered as the difference between average after-meal RMR and the basal RMR (after-meal RMR –
183 basal RMR).

184 The Respiratory Quotient (RQ) was the ratio between VCO_2 and VO_2 (VCO_2/VO_2).

185 Glucose and fat oxidation were calculated according to the Ferranini formulas [21]:

186
$$\text{Carbohydrate (CHO) oxidation (g/min)} = 4.55 VCO_2 \text{ (l/min)} - 3.21 VO_2 \text{ (l/min)} - 2.87 N \text{ (g/min)}$$

187 Fat oxidation (g/min) = $1.67 \text{ VO}_2 \text{ (l/min)} - 1.67 \text{ VCO}_2 \text{ (l/min)} - 1.92 \text{ N (g/min)}$

188 VO_2 =oxygen consumption; VCO_2 =carbon dioxide production; N= urinary nitrogen excretion.

189 Rate of N, an index of protein oxidation, was assumed to be constant during the calorimetry.

190 Area-under-the curve (AUC) values for glucose, insulin, FFA and triglycerides were calculated according to the
191 trapezoidal model [22].

192 We defined as Delta the difference between each variable at fasting and after the meal:

193 Delta = variable value after the meal – variable value at fasting

194 In the case of the calorimetric variables, the values at fasting corresponded to the results of the basal
195 calorimetry; delta RMR therefore coincided with DIT. In the case of the laboratory variables, the values at
196 fasting corresponded to the values at time 0.

197 *Blinding*

198 Due to the nature of the intervention, blinding participants and health professionals was not feasible. The
199 laboratory personnel who performed the biochemical analyses was blinded to the group assignment.

200 *Ethics*

201 The study was approved by the ethics committee of “Città della Salute e della Scienza” of Turin; all the
202 procedures conformed to the principles of the Helsinki Declaration. All participants provided written informed
203 consent to take part to the study.

204 *Statistical methods*

205 Clinical and laboratory variables were presented as mean and standard deviation (SD). Triglyceride and insulin
206 values were logarithmically transformed in order to approximate normal distribution. The Student’s *t*-test for
207 paired data was applied to investigate within-subject changes of the variables at morning and at evening.
208 Within-subject changes in the variables after the morning and the evening meal consumption were further
209 analyzed to estimate the “morning effect”, i.e. the difference between the morning delta and the evening
210 delta:

211 “Morning effect” = morning delta – evening delta

212 In the case of the AUCs, the “morning effect” was the difference between morning AUC for the variable minus
213 evening AUC for the variable.

214 General linear models (GLM) with patients as random effects were performed to assess and adjust for the
215 possible period and carry-over effects and to estimate crude and adjusted “morning effects” and 95%
216 confidence intervals (95% CI). In order to make easier the interpretation of log-transformed variables, the
217 adjusted estimates of triglycerides and insulin AUCs were expressed both as differences and as ratios.

218 In an explorative analysis we analyzed by GLM the “morning effect” on glucose, insulin, FFA and triglyceride
219 values at all time-points.

220 The repeated measures from 0 to 180 minutes of glucose, insulin, FFA and triglycerides were graphically
221 reported as means and as means and 95% CIs of variations from time 0.

222 Statistical analyses were performed using STATA 13.1 (StataCorp LP, College Station, Texas).

223

224 **Results**

225 Mean age, weight, height, body mass index (BMI), and waist circumference of the participants were 27.6±3.4
226 years, 67.3±12.5 kg, 1.70±0.01 m, 23.4±3.2 kg/m², and 81.8±8.0 cm, respectively. Fat mass and fat-free mass
227 determined by bioelectrical impedance analyses were 14.5±6.0 kg and 53.5±11.3 kg.

228 Participants were moderately active: their median METs h/week were 46.3, and usually consumed a high-lipid
229 low-fiber diet (total kcal 1989.9±523.0; fat 39.9±15.7 % total kcal; saturated fatty acids 11.1±2.6 % total kcal;
230 monounsaturated fatty acids 14.9±4.2 % total kcal; carbohydrates 46.6±6.6 % total kcal; fiber 11.9±3.5 g/day).

231 There were no meaningful differences between the two groups of randomization (morning-first *versus* evening-
232 first) for anthropometric and fasting RMR values (**Table 1SI, Supplementary Information**).

233 In **Table 1**, the morning and evening calorimetric variables at fasting and after-meal in the 20 participants are
234 reported. Fasting RMR was slightly lower at evening. After-meal RMR and DIT values were significantly higher

235 after the morning meal. Fasting and after-meal RQs at morning were significantly higher than the
236 corresponding RQs at evening. Both fasting and after-meal CHO oxidation values were significantly higher and
237 fasting and after-meal fat oxidation significantly lower in the morning in comparison with the evening values.
238 Period and carry-over effects were tested by using GLM and resulted not statistically significant for all
239 variables. The crude and un-adjusted effects of the “morning effects” did not differ; therefore, only the
240 adjusted effects were reported. The difference in delta RMR values, i.e. the difference in DIT, indicated a higher
241 DIT increase after the morning than after the evening meal (**Table 2**). On the other hand, the difference in delta
242 RQ values, and the differences between morning and evening AUCs for glucose, insulin and FFA concentrations
243 were negative, indicating a significantly higher effect of the evening meal on these variables than the morning
244 meal.

245 Adjusted estimates of the “morning effects” tested by GLM showed significantly lower values of glucose at
246 time 60, 90, 120, 150; lower values of insulin at time 90, 120, 150; lower values of FFA at time 120, 150, 180,
247 and lower values of triglycerides at time 60 (**Table 2SI, Supplementary Information**).

248 Basal values of glucose, insulin, FFA and triglycerides did not differ from morning to evening. In **Figures 2-5**
249 (panels A) the mean values according to the different time points of glucose, insulin, FFA and triglycerides were
250 reported. In the panels B of the Figures 2-5, the mean and 95%CI variations from time 0 of the same variables
251 were presented.

252

253 **Discussion**

254 The results of the present paper indicate that the time of food intake per se affects both the thermogenic and
255 the metabolic responses to meals. This could have practical implications since the time to eat should probably
256 be considered when planning a healthy diet.

257

258 *Energy expenditure and metabolic responses to meals*

259 Our data showed that the meal-induced DIT increase after the morning meal was higher than that observed at
260 dinner, while basal RMRs were only slightly lower at evening, consistent with data available in the literature
261 [7,15]. Furthermore, glucose, insulin and FFA AUCs values resulted significantly higher after the evening meal
262 rather than at morning.

263 A circadian pattern in the thermic and metabolic responses to nutrients could be therefore hypothesized.
264 DIT consists of two components: an obligatory component, linked to the energy expenditure by digestion,
265 absorption and metabolism of nutrients, and an additional facultative component, likely mediated by the
266 sympathetic nervous system [23-24]. A circadian rhythmicity of circulating norepinephrine and epinephrine has
267 been found, with increased values in the morning [25]. Epinephrine increases metabolic rate, RQ and glucose
268 oxidation [26]. Other explanations for the reduced evening post-prandial thermic and metabolic answers may
269 be the slower evening gastric emptying, with increased efficacy of dietary carbohydrates absorption [5-6], and
270 the reported decrement in insulin sensitivity as evening progresses [3]. Possible contributors to the daily
271 variations in insulin secretion, with higher morning beta-cell function and action, could be the circadian
272 fluctuation in the concentrations of cortisol, ACTH, glucagon-like peptide 1, glucose-dependent insulinotropic
273 polypeptide, as well as in the meal-induced glucagon responses, showing a delayed peak after evening meals
274 [2-3,27-29]. Insulin resistance may determine a decreased thermic effect of glucose because of the diminished
275 insulin-mediated glucose uptake and metabolism by skeletal muscle that results in diminished glucose-induced
276 thermogenesis [8,30]. Furthermore, sympathetically mediated thermogenesis is decreased in the presence of
277 hyperinsulinemia or insulin resistance [26,30]. Therefore, both the circadian variation in catecholamine
278 concentrations and in insulin sensitivity might contribute to the evening reduction of DIT. Consistent with the
279 reported reduced insulin sensitivity at evening, we found lower glucose/insulin AUCs in the morning and
280 delayed and larger increases in the concentrations of glucose and insulin in the evening (Figures 2-3, Table 2SI,
281 Supplementary Information).

282 A circadian control of FFA metabolism has been described, with highest levels around noon [31] or in the
283 afternoon [2], and an increased activity of lipoprotein lipase during the daytime [27]. The circadian activity of
284 clock target genes in adipocytes may impact on lipid breakdown and adipokine function, by regulating FFA
285 mobilization from adipose tissue, thus acting on energy homeostasis and metabolic processes [32-33]. It is
286 difficult to establish with our data if the higher increase in FFA AUC after the evening meal was the
287 consequence of insulin resistance or rather the cause of the impaired insulin sensitivity, because of the known
288 effects of FFA on the reduction in glucose uptake and phosphorylation in skeletal muscles and on the
289 impairment in insulin signaling and action [34-35].

290

291 Not all the authors found a lower DIT after the evening meal [9-12]. Westrate failed to find a significant
292 difference between morning and afternoon DIT in 10 normal weight young men, but the fasting period was
293 shorter in the afternoon than in the morning and the metabolic conditions of the participants to this study
294 were not the same [9]. Nevertheless, in the morning, DIT was slightly higher, energy balance (the difference
295 between energy intake and expenditure) lower, and early post-meal values of RQs and CHO oxidation
296 significantly increased, in line with our results [9]. Another study found a higher energy expenditure at night
297 during enteral nutrition, but it involved older patients hospitalized in a Neurologic unit on artificial nutrition,
298 therefore these results are difficult to be compared with those in our healthy individuals [11]. Sato found no
299 differences in 24-h energy expenditure in a room-size respiratory chamber between normal (7:00 pm) or late
300 (10:30 pm) evening meals in 10 young Japanese, but the measure of DIT was not available, the times of the two
301 experimental meals were not so different (approximately 3-h), and, consistent with our results, postprandial
302 glucose AUC values were significantly increased after the late meal [10]. Similarly, delaying the time of an
303 identical meal of about 3-h for a week did not change postprandial energy expenditure, but decreased CHO
304 oxidation and glucose tolerance [12].

305 In our study protocol, we used extreme conditions (meals at 12-h distance) and a high-protein content, which is
306 known to exert a greater effect on energy expenditure [36-37]. It is possible that with a mixed meal we could
307 have found lower values of DIT and lower differences between the morning and evening tests.
308 Nevertheless, the circadian variation in DIT might explain at least in part the previously found large intra-
309 individual variability in DIT and CHO and fat oxidation, which could not be accounted by variation in the
310 antecedent diets or in the method of measurement [7,15]. Intriguingly, the significant difference in morning-
311 evening DIT in our sample persisted even when energy expenditure was calculated in relation to the FFM
312 (Table 2).

313

314 *Respiratory Quotients*

315 RQ values generally range within 0.70-1.00, with 100% lipid oxidation at 0.70, and 100% carbohydrate
316 oxidation at 1.00 [19]. We found reduced CHO oxidation and RQ values and increased fat oxidation (Table 1) in
317 the evening, suggesting a modification of the metabolic pathways toward a higher utilization of lipid substrates
318 in the evening, supported by the faster reduction of FFA levels after the evening meal (Figure 3, panel B),
319 although starting from higher FFA values (Figure 3, panel A).

320 After the experimental infusion of glucose/insulin [23] as well as after a meal [38], both an increase in CHO
321 oxidation and a decreased lipid oxidation have been reported, in line with our data. Other studies have found a
322 higher RQ in the morning than in the evening [7,9,12,15,39]. A significantly higher lipid oxidation and a lower
323 CHO oxidation were described with meals at 6:00 pm when compared with meals at 10:00 am in obese
324 subjects both after a short (3 days) and a long (18 days) term protocol, and the morning has been considered
325 the time of the day in which anabolic functions take place [40], in line with the circadian rhythm of the
326 glycogen synthesis [41]. Even if delta RQs were slightly higher after the evening meal (Table 2), the increased
327 evening fat oxidation and FFA AUCs in our sample suggested, in line with the higher insulin resistance at that
328 clock time, a preferential use of lipid substrates in our sample.

329

330 *Clinical perspectives*

331 Human studies have shown that both in adolescents and in adults, consuming more of the daily energy intake
332 at evening is associated with an increased risk of obesity, hyperglycemia, lipid abnormalities, metabolic
333 syndrome, non-alcoholic fatty liver diseases, and cardiovascular diseases [1,4,12,14,28,42-45]. Circadian
334 misalignment has adverse metabolic and cardiovascular consequences [4,46]. Examples of chronic conditions
335 of this phenomenon are shift work and sleep deprivation, both conditions being associated with an increased
336 risk of obesity, metabolic syndrome and cardiovascular diseases [27,31,33].

337 The timing of meals also influences the success of weight loss strategies: late-lunch eaters lose less weight than
338 early eaters [47]; overweight/obese women lose significantly more weight after a low-calorie dinner weight-
339 loss program than after an isocaloric high-calorie dinner program [48]. Therefore, dietary recommendations
340 should probably include indications on the time-of-day for food consumption, besides advice on food quality
341 and quantity.

342

343 *Limitations*

344 First of all, caution is needed when trying to link results from short-term studies to long-term effects.

345 We did not evaluate the energy expenditure longer than 3-h after the beginning of the meal, according to some
346 [7], but not other authors who recommended >5-h measurements [13]. However, our experiment was
347 consistent with studies showing that energy expenditure is lower after 3-h and that DIT response to meals can
348 be assessed with sufficient accuracy for the comparison across subjects within this time interval [7,15,49].

349 Furthermore, most of the differences in the metabolic patterns we found were present in the first 2-h after
350 meal. We used the glucose equation to calculate CHO oxidation [21], even if after an overnight fast most of
351 plasma glucose turnover is derived from liver glycogenolysis, but comparisons were performed within-

352 individuals under the same conditions and, as Ferrarini stated [21], assuming exclusive glucose oxidation does
353 not introduce a major error.

354

355 *Conclusions*

356 Consuming a high-protein, low-carbohydrates meal at evening seems energetically and metabolically
357 unfavorable with respect to the consumption of the same meal at morning. Energy expenditure and
358 metabolism may be tightly linked to circadian rhythms; gaining further insights into these processes may be
359 useful to curb the current increasing rate of metabolic disorders.

360

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365

366 **Conflicts of interest:** The authors report no conflict of interest.

367

368 Supplementary information is available at journal's website.

369

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Figure Legends

Figure 1

The arrows (↓) indicate the time when the blood samples were drawn. During the visit, participants were submitted to anthropometric measures and a venous catheter was peripherally inserted. “Basal” corresponded to the time before the first calorimetric examination was performed. The time 0 was after the first calorimetry and before the meal. The times 30, 60, 90, 120, 150 and 180 were referred to the time interval in minutes from the **beginning** of the meal.

The same time schedule was adopted in the case of the morning meal (at 8:00 am) and the evening meal (at 8:00 pm).

Figure 2

Mean glucose values at the different time points (panel A). Variation of glucose from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 3

Mean insulin values at the different time points (panel A). Variation of insulin from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

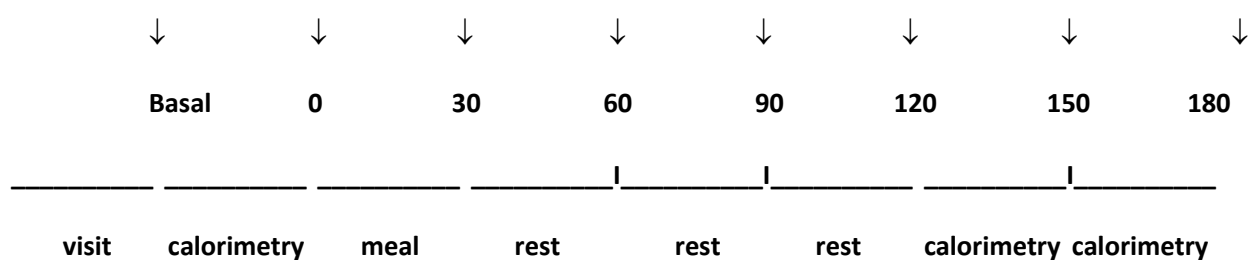
Figure 4

Mean FFA values at the different time points (panel A). Variation of FFA from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 5

Mean triglyceride values at the different time points (panel A). Variation of triglycerides from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

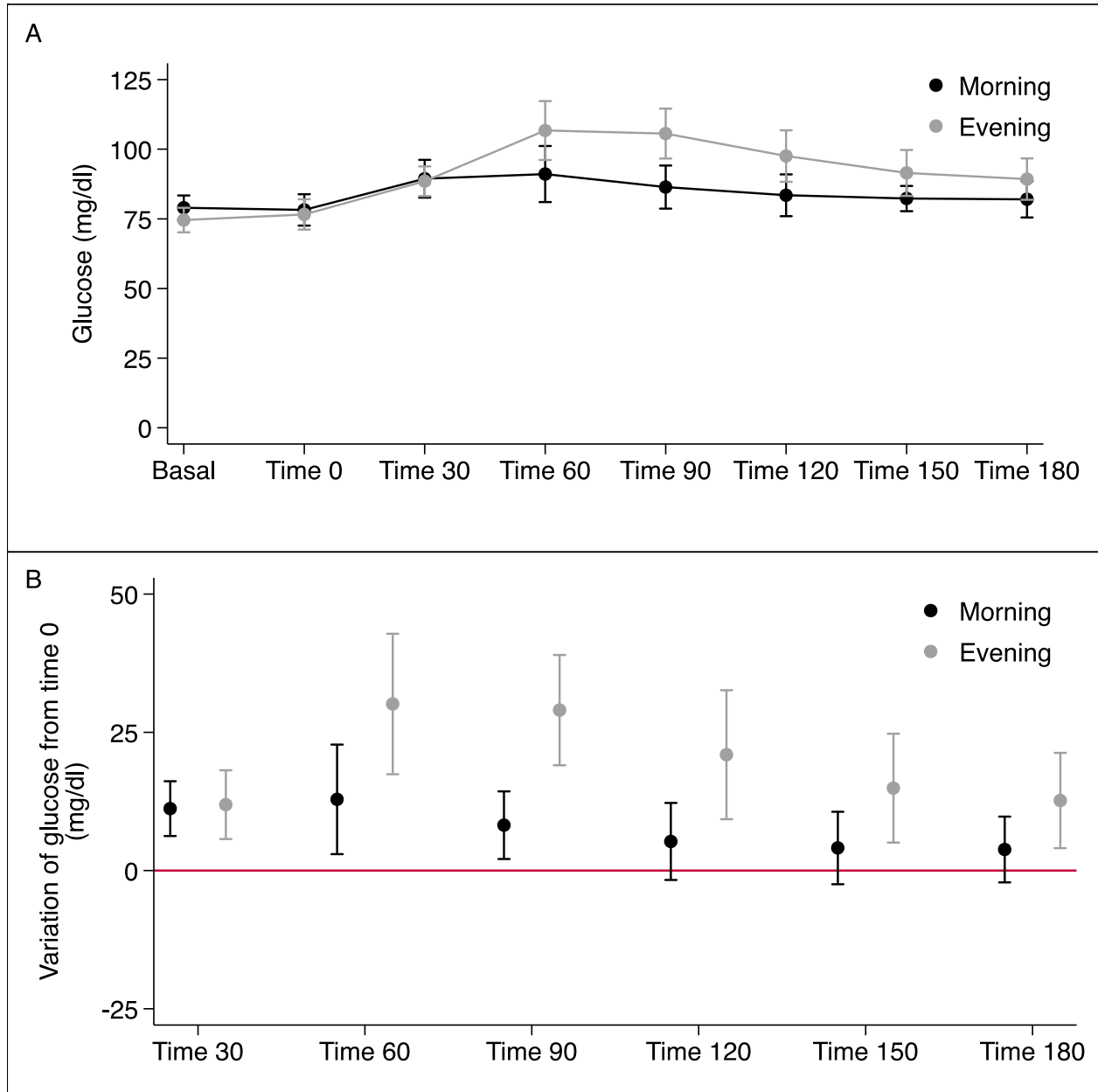
Figure 1. Time schedule of the study



The arrows (↓) indicate the time when the blood samples were drawn. During the visit, participants were submitted to anthropometric measures and a venous catheter was peripherally inserted. “Basal” corresponded to the time before the first calorimetric examination was performed. The time 0 was after the first calorimetry and before the meal. The times 30, 60, 90, 120, 150 and 180 were referred to the time interval in minutes from the **beginning** of the meal.

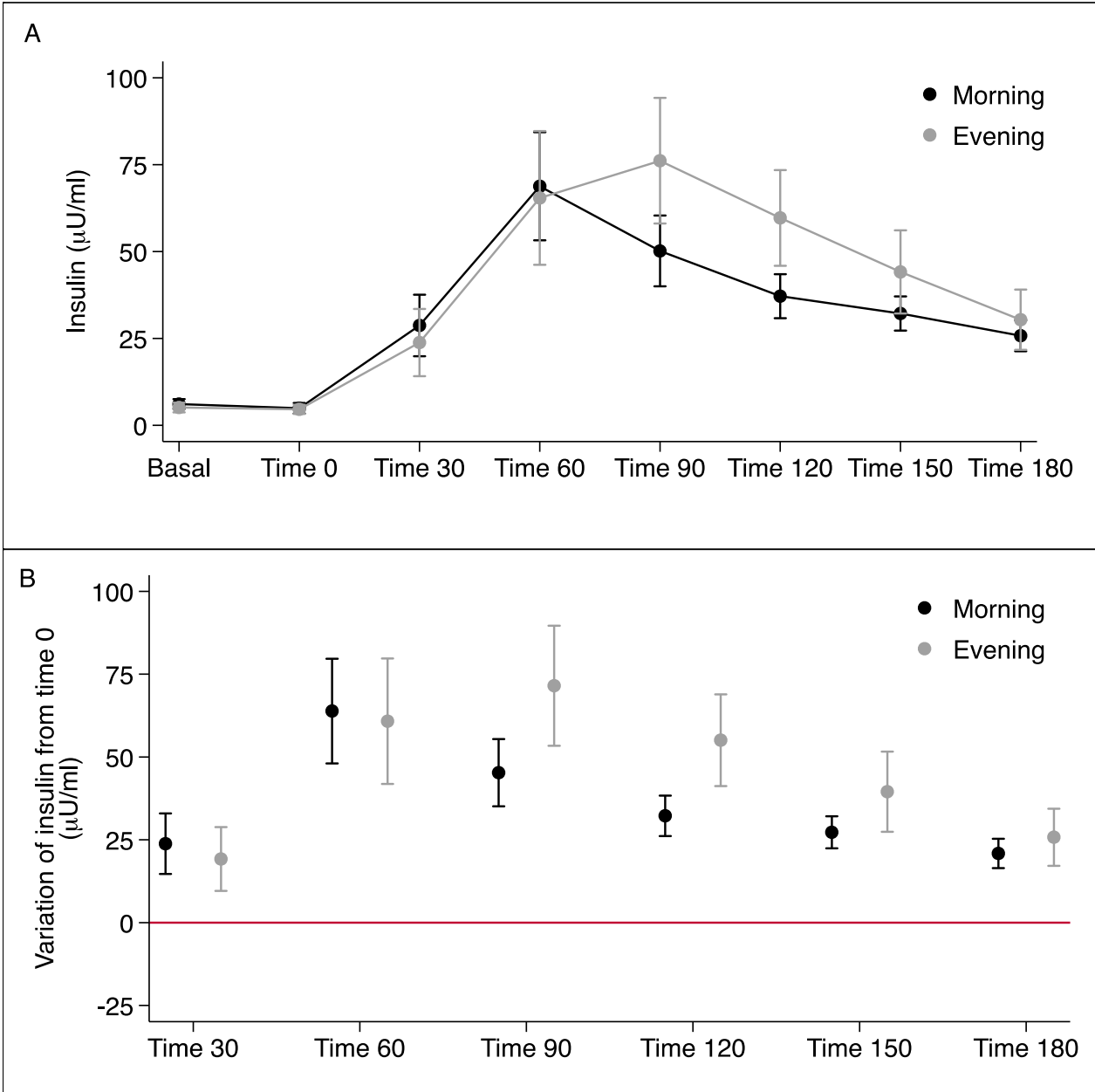
The same time schedule was adopted in the case of the morning meal (at 8:00 am) and the evening meal (at 8:00 pm).

Figure 2. Mean glucose values at the different time points (panel A) and variation of glucose from time 0 (panel B)



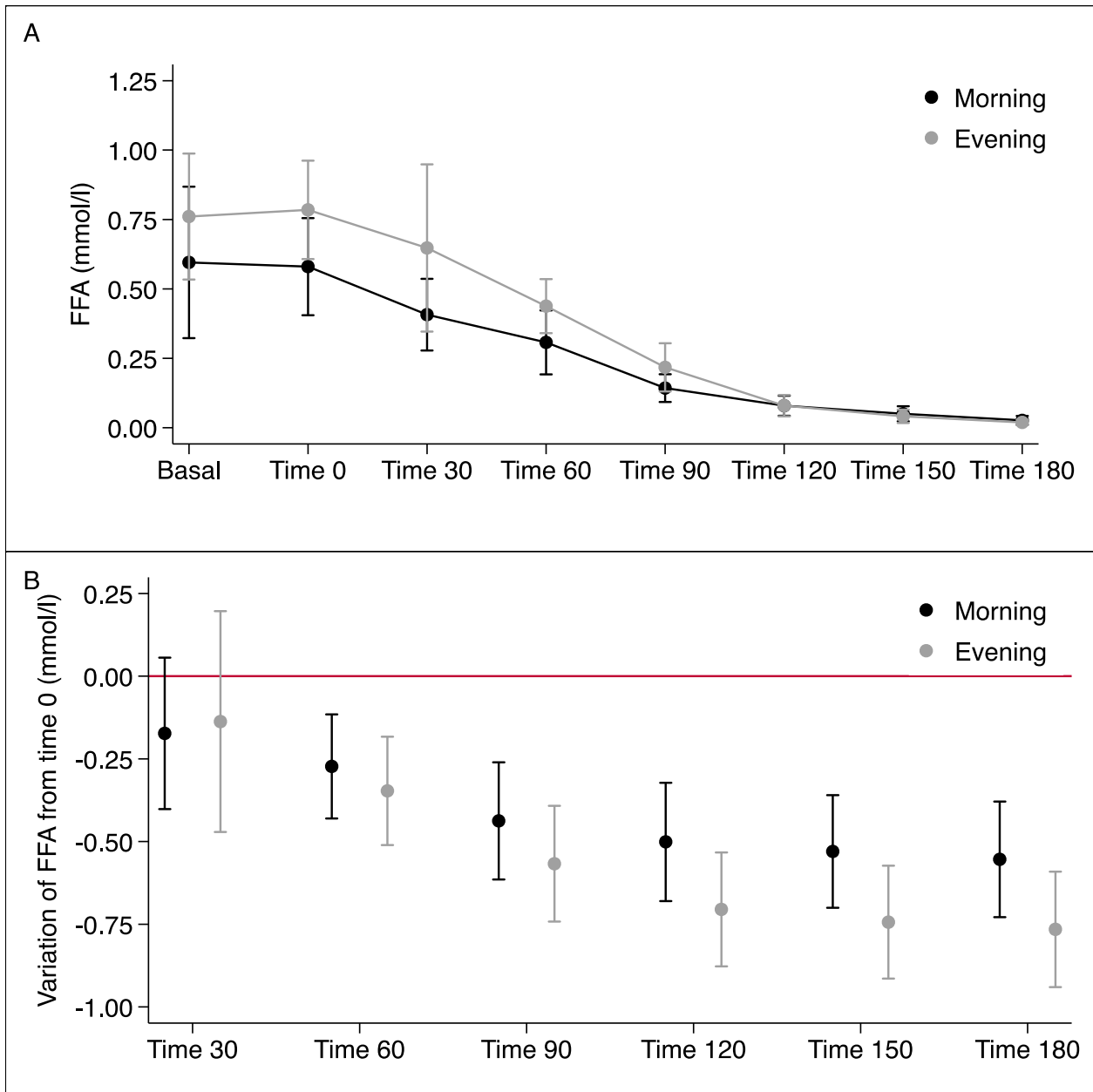
Mean glucose values at the different time points (panel A). Variation of glucose from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 3. Mean insulin values at the different time points (panel A) and variation of insulin from time 0 (panel B)



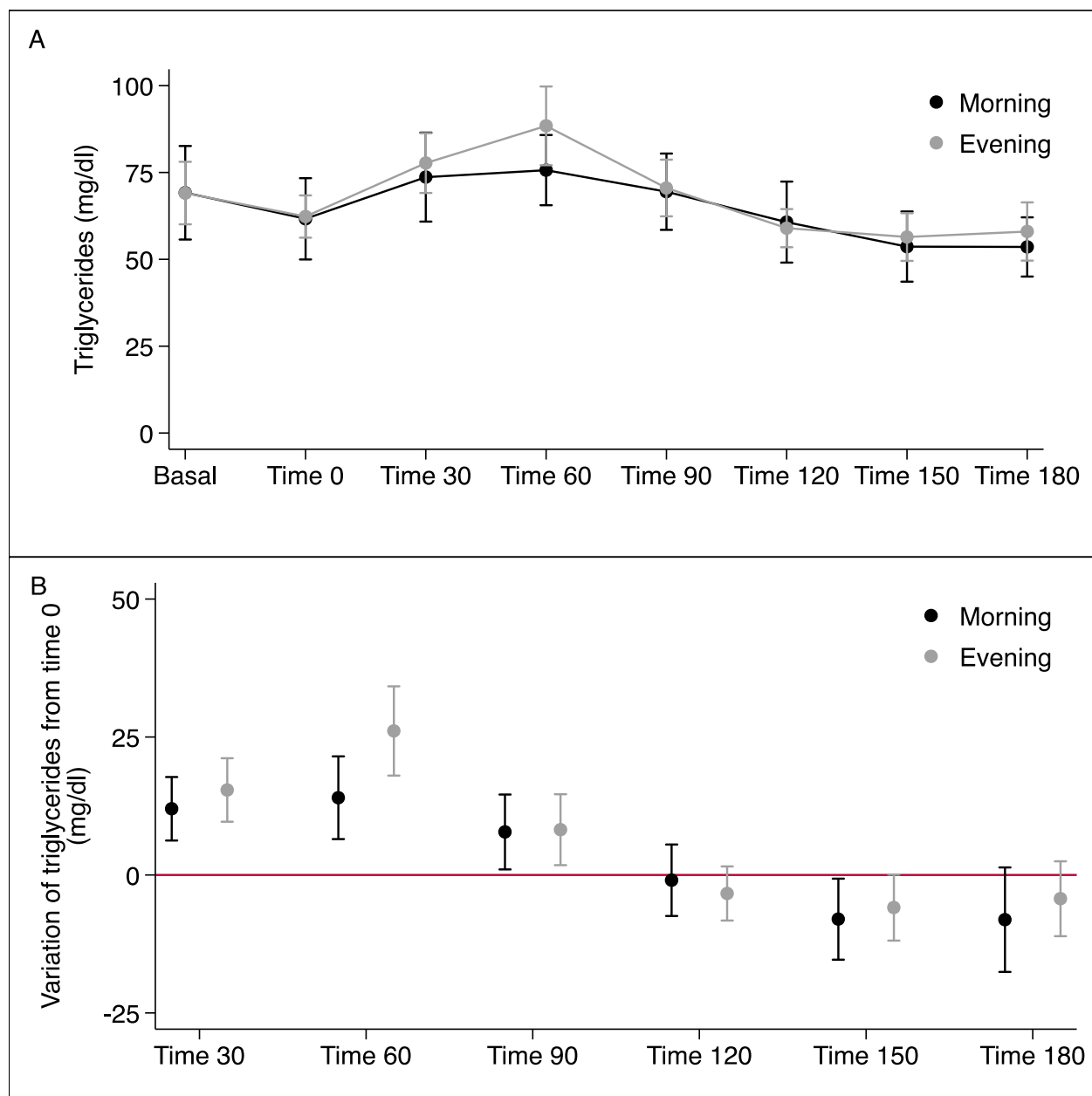
Mean insulin values at the different time points (panel A). Variation of insulin from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 4. Mean FFA values at the different time points (panel A) and variation of FFA from time 0 (panel B)



Mean FFA values at the different time points (panel A). Variation of FFA from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Figure 5. Mean triglyceride values at the different time points (panel A) and variation of triglycerides from time 0 (panel B)



Mean triglyceride values at the different time points (panel A). Variation of triglycerides from time 0: morning mean changes from the value at time 0 (black dot), evening mean changes from the value at time 0 (grey dot). The whiskers indicate the 95% CIs (panels A and B).

Table 1. Calorimetric variables before and after morning and evening meals

	Morning		Evening		p-value
Number	20		20		
Fasting RMR (kcal)	1588.5	[1464.9;1712.1]	1518.5	[1407.7;1629.3]	0.098
After-meal RMR (kcal)	1916.0	[1791.5;2040.5]	1755.5	[1648.0;1863.0]	<0.001
DIT (kcal)	327.5	[279.0;376.0]	237.0	[195.1;278.9]	0.003
Fasting RMR ¹ (kcal/kg FFM)	30.2	[27.8;31.7]	29.1	[27.0;31.2]	0.180
After-meal RMR ¹ (kcal/kg FFM)	36.7	[34.6;38.8]	33.7	[31.4;36.0]	<0.001
DIT ¹ (kcal/kg FFM)	6.46	[5.16;7.76]	4.62	[3.55;5.70]	0.003
Fasting RQ	0.87	[0.85;0.89]	0.80	[0.78;0.82]	<0.001
After-meal RQ	0.90	[0.89;0.92]	0.85	[0.82;0.88]	0.002
RQ Difference	0.03	[0.01;0.05]	0.05	[0.02;0.08]	0.055
Fasting CHO oxidation (g/min)	0.13	[0.10;0.15]	0.05	[0.02;0.07]	<0.001
After-meal CHO oxidation (g/min)	0.20	[0.18;0.22]	0.12	[0.08;0.16]	<0.001
CHO oxidation difference	0.07	[0.05;0.09]	0.08	[0.05;0.10]	0.856
Fasting fat oxidation (g/min)	0.01	[0.01;0.02]	0.04	[0.03;0.05]	<0.001
After-meal fat oxidation (g/min)	0.01	[0.00;0.02]	0.03	[0.02;0.04]	0.006
Fat oxidation difference	-0.01	[-0.01;0.00]	-0.01	[-0.03;0.00]	0.116

Mean [95%CI]; p-values calculated by the t-test for paired data

RMR = Resting Metabolic Rate; DIT = Diet Induced Thermogenesis; RQ = Respiratory Quotient; CHO = carbohydrates

¹energy expenditure calculated in relation to fat free mass

Table 2. Estimates of “morning effect” adjusted for period and carry over effects by General Linear Models**(GLMs)**

	Effects	95% CI	p-value
RMR ¹ (kcal)	90.5	[40.4,140.6]	<0.001
RMR ^{1,2} (kcal/kg FFM)	1.84	[0.81,2.87]	<0.001
RQ ³	-0.02	[-0.04,-0.001]	0.035
CHO oxidation ³ (g/min)	0.00	[-0.02,0.02]	0.848
Fat oxidation ³ (g/min)	0.01	[-0.00,0.02]	0.089
Glucose AUC ⁴ (mg/dl×h)	-1800.1	[-2564.1,-1036.0]	<0.001
Log-insulin AUC ^{4,5} (μU/ml×h)	-0.19	[-0.30,-0.07]	0.001
Log-insulin AUC ^{4,6}	0.83	[0.74,0.93]	0.001
FFA AUC ⁴ (mmol/l×h)	-16.1	[-30.0,-2.09]	0.024
Log-triglycerides AUC ^{4,5} (mg/dl×h)	-0.08	[-0.21,0.05]	0.230
Log-triglycerides AUC ^{4,6}	0.92	[0.81,1.05]	0.230

RMR = Resting Metabolic Rate; RQ = Respiratory Quotient; CHO = carbohydrates; AUC = Area-under-the curve;

FFA = free fatty acids

¹morning Diet-Induced-Thermogenesis (DIT) – evening DIT

²RMR calculated in relation to fat free mass

³Morning delta minus evening delta

⁴Morning AUC – evening AUC

⁵Estimated effects expressed as difference in log-terms

⁶Estimated effects expressed as ratio.