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Title: Calcium co-ingestion augments postprandial glucose-dependent insulinotropic peptide₁₋₄₂, glucagon-like peptide-1 and insulin concentrations in humans.

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Running title: Calcium co-ingestion: impact on GIP, GLP-1 and insulin.

1 ABSTRACT

2 *Purpose* This study determined whether calcium co-ingestion potentiates postprandial GIP₁₋₄₂ and GLP-1
3 concentrations in humans, and the concomitant impact on insulin, appetite sensations and substrate
4 metabolism.

5 *Methods* Ten healthy males consumed two energy- and macronutrient-matched meals in a double-blind,
6 randomized, crossover design. The calcium content of the control meal was 3 mg/kg body mass, which
7 was increased to 15 mg/kg body mass with calcium co-ingestion. Circulating concentrations of GIP₁₋₄₂,
8 GLP-1 and insulin were determined over a 180-min postprandial period, followed by 60 min of exercise.
9 Visual analogue scales were used to determine subjective appetite sensations. Rates of energy
10 expenditure and substrate (lipid and carbohydrate) oxidation were estimated using indirect calorimetry.

11 *Results* Calcium co-ingestion resulted in a 47% increase in GIP₁₋₄₂, a 22% increase in GLP-1 and a 19%
12 increase in insulin areas under the curve for the 120 min following consumption (all $P < 0.05$).
13 Furthermore, appetite sensations were suppressed by calcium co-ingestion by 12% ($P = 0.034$). No
14 differences, however, were observed in substrate metabolism ($P > 0.05$).

15 *Conclusion* Ingestion of a high-calcium meal potentiates postprandial GIP₁₋₄₂, GLP-1 and insulin
16 concentrations in humans. Subjective appetite is also temporarily suppressed, although substrate
17 metabolism is unaffected.
18

19 **Keywords:** Dairy, GIP, GLP-1, Incretin, Appetite, Lipid oxidation, Exercise
20

21 **Abbreviations:** GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide 1; DPP-IV,
22 dipetidyl-peptidase IV; NEFA, non-esterified fatty acid; VAS,
23 visual analogue scale; CHO, carbohydrate; CON, control trial; CAL,
24 high-calcium trial; AUC, area under the curve; RER, respiratory
25 exchange ratio; VO₂, rate of oxygen consumption; VCO₂, rate of
26 carbon dioxide production.
27
28

29 INTRODUCTION

30 Habitual calcium intake is inversely associated with obesity[1] and type 2 diabetes[2], and calcium and
31 vitamin D supplementation can augment fat loss under energy restriction[3]. Currently, potential explanations
32 for the protective effect of higher-calcium intake include, improvements in appetite regulation[4], increases in
33 lipid oxidation[5] (which may be greater during exercise/energy deficit[6]) and/or reductions in dietary fat
34 absorption[7].

35 Of these putative mechanisms, the least well studied is that of calcium intake and appetite. Some have
36 shown a reduction in 24-h energy intake following a high-calcium meal [4], whilst others have found no
37 difference in energy intake, appetite ratings, or postprandial concentrations of appetite-related hormones such as

38 insulin and glucagon-like peptide-1 (GLP-1)[8].

39 Following food consumption, the gastrointestinal peptides, glucose-dependent insulintropic peptide₁₋₄₂
40 (GIP₁₋₄₂) and glucagon-like peptide-1₇₋₃₆ (GLP-1₇₋₃₆), are secreted by K-cells and L-cells of the intestine [for a
41 review see Holst[9]]. These peptides potentiate insulin secretion by direct action on β -cells[10,11], but GLP-1₇₋
42 ₃₆ also acts via the nervous system[12,9] providing an anorectic component[13]. Due to their unique properties,
43 therapies for obesity[14] and diabetes are currently being developed based on these peptides, with promising
44 efficacy[15] thereby highlighting their metabolic importance. Further physiological effects of these peptides are
45 currently being uncovered, including lipolysis[16] and substrate metabolism[17-20].

46 Both GIP₁₋₄₂ and GLP-1₇₋₃₆ provide a substrate for dipeptidyl-petidase IV (DPP-IV) in the N-terminal
47 regions, thought to be crucial for receptor activation[21,22]. Consequently, following cleavage, the remaining
48 peptides GIP₃₋₄₂ and GLP-1₉₋₃₆ are thought to be principally inactive.

49 It is known that major macronutrients (fat and carbohydrate) stimulate GIP and GLP-1 secretion by
50 direct contact with K- and L-cells[23]. Some recent evidence suggests that calcium could also play a crucial
51 function. When isolated rodent intestine was perfused with increasing luminal concentrations of calcium [at
52 humanly physiological concentrations[24]], total GIP (the accumulation of active and inactive forms) and GLP-
53 ₁₋₃₆ secretion was stimulated[25]. The stimulation of GIP and GLP-1 by calcium was greater in the presence of
54 the amino acid L-phenylalanine indicating a synergistic effect. It is not currently known whether oral ingestion
55 of calcium can augment circulating GIP and GLP-1 concentrations in humans. Due to the significance of GIP
56 and GLP-1 in metabolic disease (whereby alterations in postprandial GIP and GLP-1 profiles are seen in type 2
57 diabetes[26] and improvements in glycemic control following bariatric surgery parallel changes in gut
58 peptides[27]), they may help to explain the relationship between calcium intake, obesity and type 2 diabetes.

59 Accordingly the primary aim of the present study was to explore whether a co-ingestion of calcium
60 with a meal augments gastrointestinal peptide concentrations in humans. Given the well-documented impact of
61 these peptides on insulin secretion[10] and appetite[13], and some evidence that calcium can reduce energy
62 intake[4], a secondary aim was to examine the effect of a high-calcium meal on insulinemia and appetite during
63 the postprandial state. As both gastrointestinal peptides and calcium intake have been implicated in
64 lipolysis[4,16] and substrate metabolism, particularly during exercise[6,20], the third objective was to assess the
65 concomitant impact on substrate metabolism at rest and during exercise.

66

67 **SUBJECTS AND METHODS**

68 **Participants**

69 Young, healthy, recreationally active males were recruited between April-August 2012 from the
70 student and staff population at Northumbria University. Participants provided written informed consent prior to
71 the study. Eligibility criteria included, young (< 35 y), non-obese (BMI < 30 kg/m²), self-reported physically
72 active (>30 min of structured exercise, 5 times/week) and no known metabolic or gastrointestinal diseases or
73 food allergies. The protocols were approved by the Faculty of Health and Life Sciences Ethics Committee at
74 Northumbria University and are therefore in accordance with the Declaration of Helsinki.

76 **Preliminary measurements**

77 Prior to main trials, participants undertook preliminary tests to establish 1) the relationship between
78 oxygen uptake and running speed on a flat treadmill using a 16-min test, and 2) their VO_{2peak} using an
79 incremental treadmill test whereby the gradient was increased by 1%/min to exhaustion as previously described
80 in full detail[28]. On the same day, participants were familiarized with the visual analogue scales (VAS) to later
81 assess subjective appetite sensations in main trials. A food frequency questionnaire, previously validated and
82 used in similar populations[29,30], was completed to estimate habitual calcium intake.

84 **Experimental design**

85 Participants completed two trials in a randomized (randomization performed by J.T.G. using an
86 electronic statistical package), double-blind (J.T.G. and the participants were blinded), crossover design
87 separated by 7 d, which consisted of a control (CON) and high-calcium (CAL) trial (Figure 1).

88 All trials were performed under similar laboratory conditions (mean ± SEM; Temperature: 21.9 ± 0.7
89 and 21.9 ± 0.7 °C; Humidity: 39 ± 1 and 41 ± 2 %; Pressure: 1006 ± 2 and 1006 ± 4 mbar for CON and CAL
90 trials, respectively; all *P* > 0.05).

91 Food and fluid diaries were kept for the day preceding the first trial and participants were instructed to
92 replicate this for all subsequent trials. Participants were asked to avoid all foods containing dairy in the final
93 meal prior to trials and to abstain from alcohol, caffeine and vigorous activity (defined as any structured

94 exercise) for 24 h prior to trials. Participants arrived at the laboratory at Northumbria University at 0730 after a
95 12 h fast.

96 Arterialized venous blood samples were obtained by catheterization of a pre-heated dorsal hand vein as
97 previously described[31]. Following baseline VAS and blood samples, breakfast was consumed and consisted
98 of, instant oats (Oatso Simple Golden Syrup, Quaker Oats, Reading, UK), whole milk (Cravendale, Arla Foods,
99 Denmark) and 100 ml water. This was cooked in a microwave at 1000W for 2 min to produce a porridge of
100 semi-solid consistency. This provided 0.5 g carbohydrate/kg body mass. (energy: 1258 ± 33 kJ; 299 ± 8 kcal,
101 protein: 11 ± 0 g, carbohydrate: 41 ± 1 g and fat: 10 ± 0 g). We chose a mixed-macronutrient meal to exploit the
102 synergistic dose-response between amino acids and calcium on GIP and GLP-1 secretion observed in rodent
103 intestine[25], and the glucose-dependent insulin secretions of GIP and GLP-1[10].

104 The CON breakfast contained 3 mg calcium/kg body mass (248 ± 7 mg). The calcium content was
105 increased for CAL trials to 15 mg calcium/kg body mass in (1239 ± 33 mg) by using a milk-extracted calcium
106 powder (Capolac®, Arla Foods Ingredients amba, Denmark) and was therefore a dairy source rather than
107 supplemental calcium carbonate. The source of calcium is an important consideration regarding the
108 physiological response[8]. The calcium powder was completely soluble in milk and the quantities used to
109 increase the calcium content of the meal resulted in negligible increases in protein, carbohydrate and fat (all <
110 0.5 g) and sodium, magnesium, chloride and potassium (all < 90 mg). As our working hypothesis suggests that
111 dietary calcium influences gastrointestinal peptide secretion at the level of the gut, calcium absorption was not
112 pertinent. The dose of calcium in CAL was chosen as an attempt to maximize the difference in the luminal
113 concentration of calcium between CON and CAL (as a proof-of-concept), without exceeding the upper tolerable
114 limit for adults[32].

115 Water consumption was *ad libitum* during the postprandial period in the first trial and replicated for the
116 subsequent trial. A 180-min postprandial period started upon consumption of the first mouthful of breakfast,
117 which was consumed within 5 min. Following this, participants ran on a treadmill for 60 min at a speed
118 designed to elicit 60% of peak oxygen uptake, considered moderate-intensity[33].

119

120 **Anthropometric measurements**

121 Body mass was determined to the nearest 0.1 kg using balance scales (Seca, Birmingham, UK) upon
122 arrival to the laboratory, where participants wore only light clothing. Height was measured to the nearest 0.1 cm
123 using a stadiometer (Seca, Birmingham, UK).

124

125 **Blood sampling and analysis**

126 Blood samples were collected at baseline, and at 15, 30, 45, 60, 90, 120, 180 min following breakfast
127 consumption. Additional blood was sampled at 20, 25, 35 and 40 min (when it was expected that
128 gastrointestinal peptide and insulin concentrations would peak) in order to increase resolution of the
129 postprandial AUC. Samples were obtained whilst participants were supine to control for posture-induced
130 changes in plasma volume. A 20 µl capillary tube was filled with whole blood to determine glucose and lactate
131 concentrations immediately using a glucose/lactate analyzer (Biosen C_line, EKF Diagnostics, Magdeberg,
132 Germany). 10 ml of whole blood was allowed to stand for 30 min in a non-anticoagulant tube before being
133 centrifuged at 3000 g and 4°C for 10 min. Aliquots of serum were stored at -80°C for later determination of
134 insulin (IBL International, Hamburg, Germany) and NEFA (WAKO Diagnostics, Richmond, VA)
135 concentrations in duplicate. Intra-assay coefficients of variation were 3.7 % and 5.7 % for insulin and NEFA
136 assays, respectively.

137 Further, 4 ml EDTA tubes were filled, containing 200 kIU of aprotinin per ml of whole blood and were
138 centrifuged immediately at 3000 g and 4°C. The supernatant was stored immediately at -80°C for later
139 determination of GIP₁₋₄₂ (Immuno-Biological Laboratories Co., Ltd, Japan) and total GLP-1 concentrations
140 (Epitepe Diagnostics, San Diego, CA).

141

142 **Subjective ratings**

143 Paper based, 100 mm VAS were completed at baseline, immediately following breakfast and every 30
144 min thereafter. Questions asked were used to determine hunger, fullness, satisfaction and prospective food
145 consumption at all time points. These were also combined to give a combined-appetite score[34] where:

146

147 Combined-appetite score = [hunger + prospective food consumption + (100 – fullness) +
148 (100 – satisfaction)] / 4

149

150 The individual components (hunger, fullness, prospective consumption and satisfaction) were still presented
151 alongside the combined-appetite score, in order to discern the aspects of appetite that may have different
152 determinants. Fullness, for instance, may be more closely associated with peripheral physiological changes than
153 other aspects[35,36], which may additionally be influenced by emotional and environmental cues.

154 Immediately following breakfast consumption a further VAS was completed, whereby questions asked
155 were used to determine meal palatability, visual appeal, smell and taste. At the end of the trial participants were
156 also asked to indicate whether they believed they had consumed the CON or CAL meal to assess whether the
157 calcium could be detected.

158

159 **Energy expenditure and substrate oxidation**

160 Substrate metabolism was estimated with rates of oxygen consumption (VO_2) and carbon dioxide
161 production (VCO_2) using stoichiometric equations, and was adjusted during exercise to account for the
162 contribution of glycogen to metabolism[33]:

163

164 Rate of lipid oxidation at rest and during exercise (g/min) = $(1.695 \times VO_2) - (1.701 \times$
165 $VCO_2)$

166

167 Rate of carbohydrate oxidation at rest (g/min) = $(4.585 \times VCO_2) - (3.226 \times VO_2)$

168

169 Rate of carbohydrate oxidation during exercise (g/min) = $(4.585 \times VCO_2) - (2.962 \times$
170 $VO_2)$

171

172 (VO_2 and VCO_2 are L/min)

173

174 Energy expenditure was calculated based on lipids, glucose and glycogen providing 40.81, 15.64 and
175 17.36 kJ/g, respectively. At rest, calculations were based on glucose providing all the carbohydrate for

176 metabolism, whereas during moderate intensity exercise carbohydrate oxidation is met by glucose and glycogen
177 providing 20 and 80% contributions, respectively[33].

178 Expired gas samples were collected using a breath-by-breath system (Metalyzer 3B, Cortex, Germany)
179 calibrated using gases of known concentration and a 3 L syringe. For resting samples, participants wore a
180 facemask and lay supine and after a 5-min stabilization phase, 10-min samples were obtained and averaged at
181 baseline, and every 60 min after breakfast consumption in accordance with best practice methods[37]. Expired
182 gas was continuously sampled throughout exercise and averaged over each 5-min period ignoring the first 5 min
183 to allow for VO_2 and VCO_2 to reach a steady-state.

184

185 **Statistical analysis**

186 As we could not find data pertaining to either the expected effect size or the typical error of
187 measurement for our primary outcomes of postprandial GIP_{1-42} or $GLP-1$ responses, we based our sample size
188 on the insulin response. Using pilot data, a high-calcium meal resulted in a 12.9% increase in insulinemia
189 (unpublished observation. Gonzalez JT, Rumbold PL, Stevenson EJ. 2012). With a typical error of 8.4% for
190 postprandial insulinemia[38], 10 subjects should provide statistical power above 80% with an alpha level of
191 0.05.

192 Subjective appetite ratings and blood analyte concentrations were converted into time-averaged area
193 under the curve (AUC) using the trapezoidal rule. As the time points after ingestion may influence the effect of
194 a particular satiety related component (hormonal, metabolic, physical or cognitive[39]) the postprandial period
195 was split into 0-60, 0-120 and 0-180 min.

196 Data were tested for normal distribution using the Anderson-Darling normality test and data not
197 displaying normal distribution were log-transformed prior to statistical analysis.

198 Paired t-tests were used to determine differences at baseline, and differences in postprandial AUCs between
199 trials.

200 To determine whether habitual calcium intake influenced the postprandial responses to calcium co-
201 ingestion, pearson product-moment correlation coefficients were used to determine relationships between
202 habitual calcium intake and the change in postprandial AUC of each of the variables.

203 Statistical significance was set at $P < 0.05$. All results are presented as mean \pm SEM unless stated
204 otherwise.

205

206 **RESULTS**

207 All participants completed all trials ($n = 10$), however due to difficulties with blood collection in one
208 participant, data for GIP₁₋₄₂, GLP-1 and NEFA are presented from 9 participants. The participants age, height,
209 body mass, BMI, peak oxygen uptake and habitual calcium intake were (mean \pm SD) 25 ± 3 y, 178.3 ± 4.9 cm,
210 82.6 ± 6.9 kg, 26.0 ± 2.1 kg/m², 53.1 ± 4.1 ml/kg/min and 1084 ± 544 mg/d.
211

212 **Plasma GIP₁₋₄₂ and GLP-1 and serum insulin**

213 No significant differences were observed for plasma GIP₁₋₄₂ or GLP-1, or serum insulin concentrations
214 at baseline ($P > 0.05$).

215 Postprandial GIP₁₋₄₂ rose to a peak concentration of 27.5 ± 7.0 pmol/L in the CON trial and a
216 significantly greater 47.7 ± 7.0 pmol/L in the CAL trial ($P = 0.028$; Figure 2A). The GIP₁₋₄₂ postprandial AUC
217 for 60, 120 and 180 min were 60, 47 and 43 % greater in the CAL trial, compared to CON, respectively (Table
218 1; all $P < 0.05$).

219 GLP-1 rose following breakfast consumption to peak concentrations of 5.2 ± 1.3 and 5.9 ± 1.3 pmol/L
220 in CON and CAL trials, respectively ($P > 0.05$; Figure 2B). The GLP-1 AUC for 120 min post-breakfast was 22
221 % greater in CAL vs. CON (Table 1; $P = 0.047$).

222 Peak insulin concentrations tended to be greater following CAL vs. CON (445 ± 59 vs. 547 ± 72
223 pmol/L; $P = 0.063$; Figure 2C). The insulin AUC for the 120 min following breakfast consumption was 19%
224 greater with CAL vs. CON ($P = 0.03$; Table 1).

225

226 **Glucose, lactate and NEFA**

227 There was no significant difference between trials in glucose or lactate or NEFA concentrations at
228 baseline ($P > 0.05$).

229 No significant differences were detected for the glucose AUC (Figure 3A; Table 1), however, the
230 lactate AUC for the first 60 min after meal consumption was significantly greater in CAL vs. CON (Figure 3B;
231 Table 1; $P < 0.036$).

232 The AUC for NEFA were not significantly different between trials (Table 1). NEFA concentrations
233 were maximally suppressed at ~30 min following breakfast consumption to 0.08 ± 0.01 and 0.08 ± 0.00 mmol/L
234 before rising to 0.29 ± 0.05 and 0.33 ± 0.06 mmol/L at the end of the postprandial period in the CON and CAL
235 trials, respectively. As such NEFA concentrations were virtually back to baseline values at the onset of exercise
236 (Figure 3C).

237

238 **Subjective ratings**

239 The visual appeal (CON: 54 ± 7 , CAL: 59 ± 7), smell (CON: 71 ± 5 , CAL: 76 ± 3), taste (CON: 66 ± 6 ,
240 CAL: 66 ± 7), and palatability (CON: 65 ± 7 , CAL: 71 ± 7) of the breakfasts were not significantly different (all
241 $P > 0.05$). Additionally, participants guessed the correct breakfast administered on 7 out of 20 occasions, which
242 is below the 50% considered as random chance.

243 There were no significant differences in any appetite ratings at baseline (all $P > 0.05$). The satisfaction
244 AUC for the first hour following breakfast consumption was 5 mm (10%) greater in the CAL trial compared to
245 CON ($P = 0.036$).

246 The combined-appetite AUC for the first hour following consumption was 5 mm (12%) lower in the
247 CAL trial compared to CON ($P = 0.034$; Table 2; Figure 4).

248

249 **Energy expenditure and substrate utilisation**

250 There was no difference in lipid oxidation, carbohydrate oxidation or energy expenditure at baseline
251 (all $P > 0.05$). There was also no interaction effect for either lipid oxidation, carbohydrate oxidation or energy
252 expenditure (all $P > 0.05$). Neither postprandial nor exercise substrate oxidation differed between trials (Table 3;
253 all $P > 0.05$).

254

255 **Correlations between variables**

256 There were no significant relationships between habitual calcium intake and the change in the
257 postprandial AUC of GIP₁₋₄₂, GLP-1, insulin or subjective appetite sensations in response to calcium co-
258 ingestion (all $P > 0.05$).

259

260 **DISCUSSION**

261 The primary finding from this study is that calcium co-ingestion potentiates postprandial plasma
262 glucose-dependent insulintropic peptide₁₋₄₂ and glucagon-like peptide-1 concentrations in humans.
263 Postprandial insulinemia and satiety were also increased with high-calcium ingestion. Substrate metabolism on
264 the other hand, was unaffected by the calcium content of the meal.

265 High luminal calcium concentrations stimulate the secretion of GIP and GLP-1₇₋₃₆ by isolated rat
266 intestine[25], probably acting via the calcium sensing receptor. Thus, we hypothesized that an increase in the
267 calcium content of a meal would increase the calcium concentration that the K- and L-cells are exposed to, and
268 thus potentiate postprandial GIP and GLP-1 concentrations. This is the first study in humans to show that
269 increasing the calcium content of a meal (from ~250 mg to ~1240 mg) amplifies the 2 h postprandial responses
270 of GIP₁₋₄₂ and total GLP-1 (by 47% and 22%, respectively). Although we could not confirm that we were able to
271 increase the calcium concentration in the intestine, this is a likely mechanism for the responses observed.

272 Lending support to this thesis, GIP₁₋₄₂ concentrations were potentiated to a greater extent, and the
273 enhancement was initiated more rapidly, than GLP-1 concentrations [GIP is secreted primarily from the more
274 proximal duodenum[40], compared to the more distal jejunum and ileum for GLP-1[40] and postprandially, the
275 duodenum is exposed to a higher concentration of calcium than the ileum[24]].

276 A second potential mechanism could be that decreased dietary fat absorption[7] resulted in a greater
277 luminal fat content in the distal small intestine, thereby stimulating GLP-1 secretion in the later postprandial
278 period, though this does not explain the rapid changes in GIP₁₋₄₂ concentrations. Calcium can also delay
279 gastric emptying[41], and delayed gastric emptying can influence the gut peptide response[42]. However, it is
280 just as plausible that the calcium induced increase in GLP-1, could drive the gastric emptying response[17] and
281 hence, the direction of causality is currently unclear.

282 To the best of our knowledge, only one other study has determined GLP-1 responses to acute calcium
283 ingestion[8], and found no significant effect on the postprandial AUC (GLP-1 was however, higher at 60 min
284 following consumption of calcium carbonate). Although the large energy load (50% of total daily energy intake)
285 provided, could have produced such a large hormonal perturbation that more subtle effects were masked.
286 Others, using a smaller energy load determined insulin concentrations and appetite sensations following high-
287 calcium meals[4,43], although by sampling at an hourly rate, the transient effects that are reported in the present
288 study would have been missed.

289 CAL also increased insulinemia and influenced subjective appetite ratings in the postprandial period. It
290 may be that increased GIP₁₋₄₂ and GLP-1 concentrations are, in part, responsible for this. Both peptides potently
291 stimulate insulin secretion when blood glucose concentrations are elevated[10]. The transient period with which
292 insulin concentrations were potentiated imply that this is a glucose-dependent effect, as the amplification is only
293 present when blood glucose concentrations were also above fasting values.

294 The lower combined-appetite score with CAL was a relatively small effect and short-lived. Moreover,
295 the impact of these findings on subsequent energy intake should be interpreted with caution, as energy intake is
296 determined primarily by the portion size served[39] in the “real-world” setting (ie. individuals consume most of
297 what they are served, most of the time, regardless of appetite sensations or hormonal profile). Nonetheless, self-
298 served portion size is influenced by the previous satiety experienced by food consumption[44]. Hence, an
299 increase in satiety (decreased appetite) could have a modest effect on long-term energy balance. Moreover, a
300 ~16% reduction in 24-h energy intake has been reported after a high-calcium meal[4].

301 It cannot be proved that the transient reduction in combined-appetite sensations with CAL was due to
302 the changes in GLP-1 and insulin reported, particularly as other hormones, known to be influenced by calcium
303 ingestion (such as calcitonin), are anorectic[45]. However, as serum calcitonin peaks at ~180 min following
304 calcium ingestion[46], at which point appetite was similar between trials, it is unlikely to be a factor in the
305 present study. In contrast, the time-course of gastrointestinal peptide, insulin and appetite responses all appear to
306 be similar.

307 Acute insulin administration suppresses food intake in rodents[47], and is strongly associated with
308 postprandial fullness sensations in humans[48,35]. In addition, GLP-1₇₋₃₆ infusion dose-dependently reduces
309 appetite and food intake in humans[13]. Hence, insulin and GLP-1 both affect appetite directly (with GIP
310 providing an indirect influence through insulin). The simultaneous effects, combined with the evidence from

311 infusion studies makes it tempting to speculate that these responses are linked, but this needs clarifying with
312 future work.

313 We chose to measure total GLP-1 rather than the “active” GLP-1₇₋₃₆. In humans, all the GLP-1 secreted
314 is in the 7-36 form[49]. Before entering the systemic circulation, approximately 50% has been cleaved [9]
315 producing GLP-1₉₋₃₆, believed to be physiologically inactive [but may act through receptors other than the
316 classical GLP-1 receptor[50]]. However it is thought that GLP-1 can act centrally [acting on the hypothalamus
317 via sensory afferent neurons and subsequently neurons of the solitary tract nucleus [51]] prior to entering the
318 circulation, and potentiate insulinemia through neural and direct pancreatic β -cell stimulation[12,52]. Thus,
319 measuring the active form and its metabolite provides an indication of total GLP-1 secretion, which is likely to
320 have physiological effects before entering the circulation[9].

321 Contrary to GLP-1, GIP is thought to act only through its receptor as GIP₁₋₄₂[50] and as such, we chose
322 to measure GIP₁₋₄₂ to reflect the tissue exposure to the active peptide. GIP₁₋₄₂ is not believed to have a direct
323 impact on appetite, but could have an influence through GLP-1 and insulin secretion.

324 In spite of a 20% increase in insulinemia, we saw no difference in glycemia or serum NEFA
325 availability between trials. The reason for this is likely due to the glucagonotropic effect of GIP[10,53,54],
326 thereby maintaining the glucagon/insulin ratio. Furthermore, the greater insulinemia was transitory in nature
327 (with the greatest differences seen from 20-60 min postprandially), and this not of sufficient duration to
328 influence glucose uptake.

329 The similar lipid oxidation between trials support the findings of some[55,56] but not others[4,57].
330 When lipid oxidation has been stimulated by a high-calcium meal, this has occurred with simultaneous
331 enhanced NEFA availability[57,4]. We did not observe a difference in NEFA availability, and NEFA
332 concentrations had returned to almost fasting values prior to exercise. Therefore the meal-induced suppression
333 of NEFA concentrations[58] was not overriding the ability to detect a difference in either NEFA availability or
334 lipid oxidation. The discrepancy between studies may, in part, be accounted for by the populations used (healthy
335 BMI vs. overweight/obese) as metabolic functions of GLP-1, are more apparent in obesity[17,18].

336 Blood lactate concentrations were elevated with calcium co-ingestion for the first 60 min following
337 ingestion, which could indicate a greater reliance on carbohydrate oxidation. An explanation for this is not
338 readily forthcoming, particularly given that DPP-IV inhibition (which elevates GIP₁₋₄₂ and GLP-1₇₋₃₆
339 concentrations) decreases muscle dialysate lactate concentration[19], although the 120 and 180 min postprandial

340 AUC were similar between trials.

341 The dose of calcium in the CAL trial was within the upper tolerable limit for adults[32], although
342 would unlikely be consumed in a single-meal under normal-circumstances (this dose equates to almost 1L of
343 whole milk). This may in part, explain the discrepancy between the present study and that of Lorenzen *et al.*[8]
344 as the high calcium dose with a small meal in the present study would result in a higher concentration of
345 calcium in the gastrointestinal tract, compared to a similar or smaller dose of calcium diluted in a large meal[8].
346 Furthermore the findings of this study should be placed into the context of clinical relevance. On average, the
347 participants had an adequate habitual calcium intake and a normal BMI. Calcium supplementation at high levels
348 has been implicated in renal stone formation[59] and cardiovascular disease mortality[60]. Although neither of
349 these are without controversy[61,62]. Insulin *per se* can cause atherogenesis and insulin resistance, which in
350 turn are strongly associated with cardiovascular disease (reviewed in[63]). Thus raising the question of the
351 cost/benefit of the insulin and gut peptide responses seen in the present study, particularly if this is also seen in
352 high-risk populations.

353 A limitation with this study is the relatively small sample size, and thus the relevance to a wider
354 population needs evaluating. This study does however, provide a proof-of-principle that calcium ingestion
355 influences postprandial insulin and gastrointestinal peptide concentrations and appetite sensations, and further
356 work should aim to establish the dose-response of this relationship.

357 It is noteworthy that apart from the calcium content, the test meals were identical in nutritional
358 composition and similar in palatability, visual appeal, taste and smell. Participants were also unable to detect a
359 difference between the test meals and as such the impact of palatability on postprandial appetite[64] or
360 insulinemia[65] can be eliminated.

361 In conclusion, increasing the calcium content of a meal augments postprandial circulating GIP₁₋₄₂ and
362 total GLP-1 concentrations in humans. The high-calcium meals also resulted in greater insulinemia and satiety.
363 Substrate metabolism, however, was not affect by calcium co-ingestion.

364

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TABLE 1

Postprandial responses of circulating parameters to calcium co-ingestion in young healthy males

Variable	Time-averaged postprandial area under the curve					
	0-60 min		0-120 min		0-180 min	
	CON	CAL	CON	CAL	CON	CAL
Plasma GIP ₁₋₄₂ (pmol/L)	21 ± 6	33* ± 6	18 ± 5	25* ± 5	13 ± 3	18* ± 3
Plasma GLP-1 (pmol/L)	3.18 ± 0.75	3.96 ± 1.17	2.72 ± 0.62	3.71* ± 1.26	2.43 ± 0.56	3.41 ± 1.24
Serum insulin (pmol/L)	235 ± 24	278 ± 27	161 ± 12	192* ± 15	133 ± 10	161 ± 13
Blood glucose (mmol/L)	5.6 ± 0.2	5.7 ± 0.2	5.1 ± 0.1	5.2 ± 0.1	4.9 ± 0.1	5.0 ± 0.1
Blood lactate (mmol/L)	0.73 ± 0.05	0.90* ± 0.06	0.75 ± 0.05	0.86 ± 0.07	0.73 ± 0.06	0.79 ± 0.06
Serum NEFA (mmol/L)	0.18 ± 0.02	0.21 ± 0.03	0.15 ± 0.02	0.16 ± 0.02	0.17 ± 0.02	0.19 ± 0.02

Data are expressed as means ± SEM. Data not normally distributed as assessed by the Anderson-Darling normality test were log-transformed for statistical analysis. CON, control; CAL, calcium co-ingestion; GIP₁₋₄₂, glucose-dependent insulinotropic peptide₁₋₄₂; GLP-1, glucagon-like peptide-1; NEFA, non-esterified fatty acid. *Significant difference between CAL vs. CON, $P < 0.05$ assessed by paired t-tests.

TABLE 2

Postprandial subjective appetite responses to calcium co-ingestion in young healthy males

Variable	Time-averaged postprandial area under the curve					
	0-60 min		0-120 min		0-180 min	
	CON	CAL	CON	CAL	CON	CAL
Hunger	40 ± 6	36 ± 6	43 ± 6	41 ± 6	47 ± 6	46 ± 5
Fullness	56 ± 7	60 ± 7	51 ± 6	53 ± 6	45 ± 6	49 ± 6
Satisfaction	54 ± 6	59* ± 6	50 ± 6	52 ± 6	45 ± 6	47 ± 6
Prospective consumption	47 ± 7	40 ± 7	50 ± 7	47 ± 6	54 ± 6	52 ± 6
Combined-appetite	44 ± 6	39* ± 6	48 ± 6	46 ± 6	53 ± 6	51 ± 6

Data are expressed as means ± SEM. CON, control; CAL, calcium co-ingestion. *Significant difference between CAL vs. CON, $P < 0.05$ assessed by paired t-tests.

TABLE 3

Substrate oxidation and energy expenditure during the postprandial and exercise periods

Variable	Postprandial period (0-180 min)		Exercise period (180-240 min)	
	CON	CAL	CON	CAL
Lipid oxidation (g)	12.2 ± 0.6	13.1 ± 0.5	27.3 ± 1.1	29.1 ± 1.0
CHO oxidation (g)	42.7 ± 1.5	38.6 ± 1.3	129.0 ± 3.4	121.2 ± 4.0
Energy expenditure (kJ)	1168 ± 17	1139 ± 20	3135 ± 34	3085 ± 33
RER (au)	0.870 ± 0.01	0.856 ± 0.004	0.894 ± 0.004	0.883 ± 0.005

Data are expressed as means ± SEM. CON, control; CAL, calcium co-ingestion; CHO, carbohydrate; RER, respiratory exchange ratio. No significant differences were detected between trials.

Figure Legends:

FIGURE 1. Schematic representation of the main trials.

FIGURE 2. Plasma GIP₁₋₄₂ (A) and GLP-1 (B), and serum insulin (C) concentrations following consumption of a control (CON; 3 mg calcium/kg body mass) or high-calcium (CAL; 15 mg calcium/kg body mass) meal. Data are means \pm SEM, $n = 9$. GIP₁₋₄₂, glucose-dependent insulinotropic peptide₁₋₄₂; GLP-1, glucagon-like peptide-1; grey rectangle represents the exercise period.

FIGURE 3. Blood glucose (A), lactate (B), and serum NEFA (C) concentrations following consumption of a control (CON; 3 mg calcium/kg body mass) or high-calcium (CAL; 15 mg calcium/kg body mass) meal. Data are means \pm SEM, $n = 10$ for glucose and lactate and $n = 9$ for NEFA. Grey rectangle represents the exercise period.

FIGURE 4. Combined-appetite scores following consumption of a control (CON; 3 mg calcium/kg body mass) or high-calcium (CAL; 15 mg calcium/kg body mass) meal. Data are mean \pm SEM, $n = 10$. Grey rectangle represents the exercise period.