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Pomegranate juice, but not an extract, confers a lower glycemic response on a high GI food:
randomized, crossover, controlled trials in healthy subjects

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Abbreviations used: incremental area under the curve (IAUC); sodium dependent glucose
transporter type 1 (SGLT1); glucose transporter type 2 (GLUT2); DMSO, dimethyl
sulfoxide.

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Running header: Pomegranate juice reduces the GI of bread

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numbers NCT02486978, NCT02624609 and NCT03242876.

1 ABSTRACT

2 **Background:** Low glycemic index diets have demonstrated health benefits associated with a
3 reduced risk of developing type 2 diabetes.

4 **Objectives:** We tested whether pomegranate polyphenols could lower the glycemic response
5 of a high glycemic index food when consumed together, and the mechanism by which this
6 might occur.

7 **Design:** We compared the acute effect of a pomegranate juice and a polyphenol-rich extract
8 from pomegranate (supplement) on the bread-derived post-prandial blood glucose
9 concentration in 2 randomized, crossover, controlled studies (double-blinded for the
10 supplements), each on 16 healthy volunteers. An additional randomized, crossover, controlled
11 study on 16 volunteers consuming constituent fruit acids in a pH balanced solution (same pH
12 as pomegranate) and bread was conducted to determine any contributions to post-prandial
13 responses caused by acidic beverages.

14 **Results:** As primary outcome, the incremental area under the curve for bread-derived blood
15 glucose ($-33.1 \pm 18.1\%$, $p = 0.000005$) and peak blood glucose ($25.4 \pm 19.3\%$, $p = 0.0004$)
16 were attenuated by pomegranate juice, compared to a control solution containing equivalent
17 amount of sugars. In contrast, the pomegranate supplement, or a solution containing the malic
18 and citric acid components of the juice, were ineffective. The pomegranate polyphenol
19 punicalagin was a very effective inhibitor of human α -amylase *in vitro*, comparable to the
20 drug acarbose. Neither the pomegranate extract, nor the individual component polyphenols,
21 inhibited ^{14}C -D-glucose transport across differentiated Caco-2-TC7 cell monolayers, but
22 inhibited uptake of ^{14}C -glucose into *Xenopus* oocytes expressing the human sugar transporter
23 GLUT2. Further, some of the predicted pomegranate gut microbiota metabolites modulated
24 ^{14}C -D-glucose and ^{14}C -deoxy-D-glucose uptake into hepatic HepG2 cells.

25 **Conclusions:** These data indicate that pomegranate polyphenols, when present in a beverage,
26 but not in a supplement, can reduce the post-prandial glycemic response of bread, while
27 microbial metabolites from pomegranate polyphenols exhibit the potential to further
28 modulate sugar metabolism much later in the postprandial period.
29

30 INTRODUCTION

31 Post-prandial glycemic control and levels of sugar in the diet are both topics of current
32 concern and controversy. While the glycemic index was introduced many decades ago, its
33 relevance to health maintenance and diseases such as the metabolic syndrome, diabetes and
34 obesity, are only now being fully recognised through reviews and meta-analyses (1-3).

35 Postprandial glucose naturally rises following digestion of rapidly hydrolyzable
36 carbohydrates, such as soluble starch, some forms of cooked starch, or from soluble sugars
37 present in the food such as glucose, fructose and sucrose. After processing by salivary and
38 pancreatic α -amylase, followed by intestinal brush border maltase/glucoamylase and
39 sucrase/isomaltase, the product glucose is rapidly absorbed through intestinal sugar
40 transporters GLUT2 and SGLT1 across the enterocytes, whereas the product fructose is
41 absorbed through GLUT2 and GLUT5.

42 There are several reports of human intervention studies on the effect of pomegranate juice on
43 health biomarkers. Systolic and diastolic blood pressure was reduced in hypertensive patients
44 after consumption for 2 weeks (4), in healthy adults after 4 weeks (5), and in slightly
45 overweight but otherwise healthy adults after 2 and 4 weeks (6). None of these studies
46 however showed a concomitant effect on pulse wave velocity or on flow mediated dilation,
47 while the latter study showed a decrease in fasting plasma insulin. In contrast, in another
48 study on 20 obese adults, pomegranate juice did not modify insulin secretion and sensitivity
49 (7). In hypertensive patients, part of the decrease in blood pressure *in vivo* was ascribed to a
50 decrease in angiotensin converting enzyme (ACE) activity (8).

51 Pomegranate is uniquely rich in punicalagin and punicalin (**Figure 1**), and these polyphenols
52 are responsible for some of the sensory characteristics of the juice. We hypothesized that one
53 possible complementary mechanism to the effects on pomegranate consumption could be on
54 intestinal sugar absorption through inhibition of carbohydrate-digesting enzymes and direct

55 interactions with sugar transporters, ultimately affecting post prandial blood glucose
56 concentrations. The literature in this area is very sparse. In one report, pomegranate juice did
57 not inhibit pig α -amylase nor rat intestinal sucrase (9) while punicalagin, punicalin and
58 ellagic acid weakly inhibited rat intestinal “ α -glucosidase”, but this was only assessed with
59 an artificial *p*-nitrophenol substrate which renders the obtained results irrelevant for
60 interpretation in the *in vivo* setting. As part of the same study, pig α -amylase acting on
61 soluble potato starch was only weakly inhibited (10). In another report on the effect of
62 pomegranate tannins on glucosidase activities, the enzymes used were from *Bacillus*
63 *licheniformis* (α -amylase on potato starch) and from *Aspergillus niger* (glucoamylase on
64 maltose), bearing no similarity or relevance to the human digestive system (11). We have
65 therefore revisited the hypothesis that pomegranate juice may beneficially modulate post-
66 prandial responses through inhibition of carbohydrate digesting enzymes by employing
67 suitable *in vitro* enzyme assays and also assessing the effect on sugar transport.

68

69

70 **SUBJECTS AND METHODS**

71 **Materials**

72 The tested extract (supplement) was prepared from pressed pomegranate (*Punica granatum*
73 var. Mollar) during pomegranate juice processing. It contains husk, internal membranes and
74 remaining seed from which juice has been removed. When given in very high doses to rats,
75 no toxic effects were observed (12), and when given to volunteers for 3 weeks, showed no
76 undesirable side effects and even showed an improvement in various blood cholesterol
77 biomarkers in urolithin-metabotype-B volunteers (13). Pure, 100 % organic pomegranate
78 juice was used for the study ("Biona", Healthy Supplies, UK, www.healthysupplies.co.uk).
79 Sodium bicarbonate used to control the pH of the fruit acid solution was from Health Leads
80 UK (www.healthleadsuk.com). Citric acid was from Minerals-Water, Rainham, UK, and the
81 malic acid was from Bartek Ingredients Inc, Stoney Creek, Canada. Human salivary α -
82 amylase, rat intestine extract (acetone-extracted protein-rich powder), sucrose, maltose and
83 glucose were from Sigma-Aldrich (Dorset, UK). Ellagic acid, punicalagin (isomeric mixture
84 of punicalagin A and B) and punicalin (mixture of A and B) were from Phytolab
85 (Vestenbergsgreuth, Germany). Urolithin metabolites and the corresponding glucuronic acid
86 conjugates were chemically synthesized by Villapharma Research S.L. (Fuente Alamo,
87 Spain) (14). Malic acid and citric acid ion chromatography standards were purchased from
88 Sigma-Aldrich (Dorset, UK).

89

90 **Pomegranate analysis**

91 Juice samples were directly injected after centrifugation and filtration on a reversed phase
92 column on an Agilent 1200 HPLC system equipped with a photodiode array detector and an
93 ion-trap mass spectrometer detector using water-formic acid and acetonitrile as solvents (15).
94 For analysis, pomegranate extract powder from the capsules was first dissolved in methanol-

95 DMSO (1:1; v/v). Punicalin and punicalagin were quantified at 360 nm based on a calibration
96 curve for punicalagin, while their derivatives and other ellagitannins were relative to a
97 calibration curve for ellagic acid. Results are expressed as mean values of three replicates and
98 are shown in Table 1. Sugar quantification was performed using a Shimadzu HPLC
99 instrument equipped with a DGU-20 A5 degasser, a LC-20 AD XR pump system, a SIL-20
100 AC XR auto sampler, column oven, a diode array detector system (SPD-M20A) and a
101 Shimadzu ELSD-LTII low temperature evaporative light scattering detector as described
102 previously (16). Quantitation was carried out based on standard curves with concentrations
103 up to 10 mg/mL ($r^2 > 0.98$). Data from the sugar analysis allowed balancing of the control
104 samples for glucose and fructose naturally present in the pomegranate juice. Fructose and
105 glucose contents were 51.8 ± 0.1 and 54.6 ± 0.1 g/L for the juice, and 0.0066 and 0.0083
106 mg/mg for the extract, respectively. No sucrose was detected in pomegranate juice as
107 expected (17). Analysis of malic and citric acids in pomegranate juice was conducted by ion
108 chromatography on a reagent-free high pressure Ion Chromatography Dionex Integrion
109 system (RFIC, HPIC) equipped with a conductivity detector (ECD), an electrolytic eluent
110 generator to automatically produce an isocratic potassium hydroxide eluent and an AS-AP
111 autosampler (Thermo Scientific, UK). The AS-AP was equipped with an external injection
112 loop (10 μ l) and was run in full-injection-loop mode onto an IonPac AG11-4 μ m guard
113 column (2 x 50 mm) attached to an IonPac AS11-44 μ m analytical column (2 x 250 mm)
114 (Thermo Scientific, UK) at a flow rate of 0.38 ml/min. The column compartment was held at
115 35 C and the ECD compartment at 20 C. The optimized analytical run gradient started at 1
116 mM KOH, was held isocratically for 10.7 min, and then increased linearly to 15 mM from
117 10.7 to 24 min, 30 mM after 13.3 min and 60 mM after a further 13.3 min. An 8 min isocratic
118 60 mM KOH period was included to wash out any strongly adsorbing components, followed

119 by re-equilibration to 1 mM for 7min. Malic acid eluted at 27.96 min and citric acid at 43.57
120 min.

121

122 **Subjects**

123 Healthy individuals free of symptomatic disease, aged between 18 and 75, not diabetic, not
124 pregnant or lactating, not on a special diet (weight loss or fruits supplements) or on long term
125 prescribed medication with fasting blood glucose between 3.9 and 5.9 mmol/L were recruited
126 at the School of Food Science and Nutrition, University of Leeds, UK by means of local
127 poster adverts. Volunteers expressing interest were screened for fasting blood glucose, and
128 upon meeting all eligibility criteria, they provided written informed consent and were
129 allocated codes by the researcher responsible for intervention studies which were used in the
130 allocation of the order of interventions. All meals consisted of 109.0 ± 1.2 g white bread (50
131 g available carbohydrate as analyzed by the method of Englyst (18)). Some individuals could
132 have participated in more than one of the studies reported here.

133

134 **Design and intervention**

135 A total of 16 healthy volunteers were recruited for the pomegranate supplements study from
136 June 2015. The volunteers were aged 26 ± 6 y with BMI of 23 ± 2 kg/m² and fasting blood
137 glucose of 4.7 ± 0.4 mmol/l. The study intervention was randomized, controlled, double-
138 blinded (HNS conducted the intervention and recruited volunteers; blinding of capsules was
139 done by a code given and stored by a third party) and with a crossover design. Each volunteer
140 conducted three visits; receiving bread together with reference (400 mg placebo capsules
141 (cellulose)), test dose 1 (200 mg placebo and 200 mg pomegranate supplement) and test dose
142 2 (400 mg pomegranate supplement) together with 200 ml water. Sugars present in the

143 pomegranate capsules were negligible in terms of human consumption. Bread was consumed
144 5 min after taking the supplements to allow for dissolution in the stomach.

145 The 16 volunteers for the pomegranate juice study were recruited from November 2015 until
146 March 2016 and were aged 31 ± 5 y with BMI of 23 ± 3 kg/m² and fasting blood glucose
147 concentration of 4.7 ± 0.5 mmol/L and were recruited separately based on the same eligibility
148 criteria at a different time. The study protocol was un-blinded due to the nature of the test
149 meal. Each volunteer attended four visits, two where reference meals (200 ml water with
150 balancing sugars and bread) were consumed and another two at which test meals of the same
151 dose (200 ml pure pomegranate juice and bread) were ingested. The reference meal included
152 10.9 g fructose and 10.3 g glucose dissolved in 200 ml water to standardize the amounts of
153 sugars present in the pomegranate juice.

154 The 16 volunteers for testing the acid solution, equivalent to the malic acid, citric acid and
155 potassium content of pomegranate juice, were recruited from October 2016 until August 2017
156 and were aged 33 ± 9 years with BMI of 25.0 ± 3.8 kg/m², fasting blood glucose
157 concentration of 4.9 ± 0.4 mM and were recruited separately based on the same eligibility
158 criteria at a different time. The study protocol was un-blinded due to the nature of the test
159 meal. The volunteers consumed 109 g bread as above, together with 200 ml water containing
160 3.82 g citric acid and 0.118 g malic acid, adjusted to pH 3.2 with sodium bicarbonate,
161 compared to 200 ml tap water as control. Each volunteer attended, in randomized order, for
162 the control and for the test.

163 For all studies, following a baseline glucose measurement, the volunteers consumed the meal.
164 The timer was started upon the first bite or sip and the whole meal was consumed in less than
165 15 min. The primary outcome was blood glucose concentration. Blood glucose measurements
166 were repeated at 15, 30, 45, 60, 90, 120, 150 and 180 min post-consumption and recorded
167 immediately. For all studies, the meals were administered in a randomized pattern and a

168 glucometer was used to instantly measure the blood glucose from a finger-prick for each time
169 point. The glucometer showed excellent agreement (within 0.1 mM) with the glucose
170 hexokinase assay reported previously (19). There was no apparent harm nor side effects
171 incurred during the consumption of the meals, or by the finger prick, and no adverse effects
172 were observed. The study protocols were approved by the University of Leeds, Faculties of
173 Mathematics and Physical Sciences and Engineering Ethics Committee (MEEC 14-029,
174 MEEC 12-037 and MEEC15-044a) and the protocols were registered with
175 ClinicalTrials.gov, ID numbers NCT02486978, NCT02624609 and NCT03242876 for the
176 pomegranate supplements, pomegranate juice and fruit acid studies respectively. All
177 interventions were conducted by the researcher, HNS, responsible for intervention studies.

178

179 **Enzyme inhibition assays *in vitro***

180 The effect of pomegranate extract on α -amylase was tested *in vitro* as previously described
181 using a fully validated and characterised assay procedure using amylose, the naturally-
182 occurring and unbranched component of starch (20). Inhibition of rat intestinal brush border
183 α -glucosidase was determined by measuring the hydrolysis of maltose into glucose using a
184 hexokinase-linked assay (19).

185

186 **Glucose transport**

187 Caco-2/TC7 cells were a kind gift from Prof. Monique Rousset, (INSERM, France). For
188 transport experiments, cells were seeded on 6-well Transwell plates (Corning 3412, Appleton
189 Woods, Birmingham, UK) at a density of 6.43×10^4 cells/cm² until full differentiation of the
190 monolayer (21 - 23 days) in Dulbecco's Modified Eagle's Medium (25 mM, DMEM)
191 supplemented with 20% (v/v) Fetal Bovine Serum (FBS), 100 U/ml penicillin, 0.1 mg/ml
192 streptomycin at 37°C with 10% CO₂ in a humidified atmosphere. All cell culture reagents

193 were from Sigma (Sigma Aldrich, Gillingham, UK). On or after 22 d studies were initiated
194 and cells in both compartments were washed and incubated with transport buffer A (HEPES,
195 20 mM; NaCl, 137 mM; KCl, 4.7 mM; CaCl₂ 1.8 mM, MgSO₄ 1.2 mM; adjusted to pH 7.4
196 using NaOH, 1 M) for 30 min. Transepithelial electrical resistance (TEER) measurements
197 were recorded using a Millicell ERS volt-ohm meter (Millipore Ltd, Watford, UK). The
198 buffer was aspirated and the relevant test solution at pH 7.4 was added apically with 5 mM
199 glucose and 0.1 µCi/ well D-[U-¹⁴C] glucose. The pomegranate extract naturally contained
200 0.46 mM glucose and 0.37 mM fructose when made up in a solution of DMSO; 0.37 mM
201 fructose did not affect glucose transport compared to 5 mM glucose when tested in the same
202 set-up (data not shown). Plates were incubated for 30 min and all solutions were collected
203 and mixed with 5 ml of scintillation liquid (Gold Star, Meridian Biotechnologies, Surrey,
204 UK) for radioactivity measurements with a Packard 1900 TR Liquid Scintillation Analyser.

205

206 **D-[U-¹⁴C]-glucose and [U-¹⁴C]-deoxy-D-glucose uptake by HepG2 cells**

207 Uptake of D-[U-¹⁴C] glucose and deoxy-D-glucose by HepG2 cells was performed as fully
208 described and validated previously (22). Uptake of D-[U-¹⁴C]-glucose into *Xenopus* oocytes
209 expressing human GLUT2 was performed as described and optimized previously (23).

210

211 **Statistical analysis**

212 All three intervention trials were designed to have 90% power to detect a clinical difference
213 of 15% IAUC between the test and reference meal ($\alpha = 0.05$). A total of 15 volunteers were
214 required for the reference and test meals to achieve the above power and clinical difference.
215 Thus a minimum of 15 participants were recruited for each study as each participant was a
216 control of themselves. The trapezoidal rule was used to calculate the incremental area under
217 the glucose curves (IAUC) for each volunteer. Data analysis was performed by the two tailed

218 paired *t*-test and confirmed with the one factor repeated measures analysis of variance
219 (ANOVA) by SPSS v24 (IBM). Comparisons between control and treatment in D-[U-¹⁴C]-
220 glucose and [U-¹⁴C]-deoxy-D-glucose uptake cell experiments was carried out by
221 independent samples two tailed Student's *t*-test between control and treatment and the 2-
222 tailed values were adjusted for multiple comparisons with the Bonferroni correction. D-[U-
223 ¹⁴C] glucose uptake into *Xenopus* oocytes expressing human GLUT2 was normalized against
224 water injected oocytes for each condition. Two-tailed homoscedastic Student's *t*-test was
225 used to assess significance between uptake with and without varying concentrations of
226 pomegranate. For α -amylase assays, all IC₅₀ values are given as mean \pm standard deviation
227 from triplicates of 3 independent assays obtained by regression.
228

229 RESULTS

230 Inhibition of α -amylase and α -glucosidase activities *in vitro*

231 The dissolved pomegranate extract inhibited human α -amylase and rat intestinal brush border
232 maltase/sucrase activities *in vitro* (**Figure 2**, Table 2). Of the individual components,
233 punicalagin was a very effective inhibitor of α -amylase, but ellagic acid and punicalin were
234 much weaker (Table 2). The inhibition of α -amylase by punicalagin exhibited a K_i value of
235 $10.1 \pm 0.6 \mu\text{M}$ with kinetically competitive inhibition.

236

237 Inhibition of D-[U- ^{14}C]-glucose transport across Caco-2 cell monolayers and into

238 *Xenopus* oocytes expressing GLUT2

239 To test if pomegranate extract, juice or its constituent polyphenols have the potential to affect
240 intestinal glucose transport, we employed differentiated Caco-2 cell monolayers which
241 express the relevant transporters involved in glucose transport (24) and have been well
242 characterized and reported to be highly suitable for this purpose (25). All extracts and
243 individual compounds showed no inhibition of D-[U- ^{14}C]-glucose transport (Figure 2) when
244 tested at millimolar concentrations of glucose. At lower concentrations of glucose,
245 pomegranate inhibited the uptake of D-[U- ^{14}C]-glucose by *Xenopus* oocytes expressing
246 GLUT2 (Figure 2).

247

248 Effect of pomegranate juice on bread-derived post-prandial blood glucose

249 Based on the above *in vitro* data, we then tested whether the observed inhibition would be
250 sufficient to affect the post-prandial response of bread as an added cooked starch source. A
251 randomized, controlled, crossover intervention was conducted on 16 healthy volunteers, and
252 the control and treatment were both performed twice on the same volunteers, making a total
253 of 4 visits for each volunteer (**Figure 3**). There was a significant difference for both the

254 IAUC and peak glucose concentration between the reference and test meal (**Figure 4** and
255 Table 3). Pomegranate juice brought about a decrease in the glucose IAUC of $-33.1 \pm 18.1\%$
256 ($p = 0.000005$; $n = 16$) and in peak glucose concentration ($-25.4 \pm 19.3\%$, $p = 0.0004$) When
257 analysed separately, no significant difference was observed between the two control meals,
258 nor between the two test meals ($p > 0.05$).

259

260 **Effect of a pomegranate polyphenol supplement on bread-derived post-prandial blood** 261 **glucose**

262 Since pomegranate juice attenuated post-prandial blood glucose concentrations, we then
263 tested if the constituent extracted polyphenols could also have the same function when given
264 as a supplement in a capsule. Qualitatively, the juice and extract contained the same
265 polyphenols, but the absolute amounts were different. The amount of our proposed most
266 “active” component for digestive enzyme inhibition, punicalagin, was ~4-fold higher in the
267 capsules compared to the juice (Table 1). A randomized, placebo-controlled, double blinded,
268 two-dose, crossover intervention on 16 healthy volunteers was performed (Figure 3). There
269 was no significant difference ($p > 0.05$) between IAUC of the reference, low dose and high
270 dose of the interventions. The peak glucose concentrations were also not significantly
271 different ($p > 0.05$) (Figure 4 and Table 3).

272

273 **Effect of pomegranate fruit acids on bread-derived post-prandial blood glucose**

274 Pomegranate is a somewhat acidic beverage since it contains constituent fruit acids. It has
275 been reported that vinegar, which is highly acidic, reduced the glycemic response of a bagel
276 by 20% (26), malic acid was proposed to reduce glycemic responses when present in various
277 fruit and vegetables (although importantly the polyphenol content was not considered) (27),
278 and addition of organic acids, as in sourdough bread, may somewhat suppress the glycemic

279 response (28). We therefore tested whether any changes in blood glucose could have been
280 augmented by the acidity (due to malic acid and citric acid) of the pomegranate juice. The
281 (measured) pH of the pomegranate juice used was 3.2. Healthy volunteers (n = 16) consumed
282 200 ml of a solution of malic and citric acid (at the same concentration as measured in
283 pomegranate juice) balanced to pH 3.2 with sodium bicarbonate together with bread. There
284 was no significant effect on post-prandial glycemia when compared to a water control
285 consumed with bread (Table 3), indicating that these components were unlikely to contribute
286 to the effect of pomegranate juice on post-prandial glycemia.

287

288 **Further sugar metabolism by hepatic uptake**

289 Since the liver plays a major role in glucose metabolism after uptake by the gut, we also
290 tested whether colonic microbiota metabolites derived from pomegranate polyphenols could
291 affect glucose uptake into HepG2 cells as a model for the post-prandial disposition of glucose
292 into hepatocytes (22). Pomegranate polyphenols are predominantly absorbed in the form of
293 urolithins and ellagic acid after conversion by gut microbiota. Some of the urolithins and
294 their conjugates modulated cellular uptake of D-[U-¹⁴C]-glucose in HepG2 cells (**Figure 5**).
295 UroA inhibited, whereas UroC and UroD stimulated, uptake of the non-metabolizable
296 glucose analog, [U-¹⁴C]-deoxy-D-glucose, indicating an effect on transport. On the other
297 hand, UroB-glucuronide, UroA, UroC and UroD decreased the cellular uptake of D-[U-¹⁴C]-
298 glucose, indicating a potential effect on glucose metabolism.

299

300 Discussion

301

302 The role of supplements and extracts in support of a healthy diet remains controversial, and
303 much of the dietary advice available from government agencies is related to food and diets.
304 Here we show that pomegranate juice, rich in polyphenols, can reduce post-prandial blood
305 glucose spikes when consumed together with bread as a digestible carbohydrate source. The
306 effect is quite substantial, since the area under the glucose curve is reduced by a third, with
307 high significance. Based on *in vitro* data, the mechanism of action is inhibition of α -amylase
308 by the polyphenolic constituent, punicalagin, which is more potent than punicalin and ellagic
309 acid, and possibly inhibition of glucose transport at low glucose concentrations. However, a
310 polyphenol-rich extract from pomegranate, when co-consumed with bread, did not exhibit the
311 same effect. Since these capsules contained ~4-fold higher level of the putative main active
312 component, punicalagin, we propose that the lack of effectiveness could be due to insufficient
313 mixing in the stomach and intestine with the bread, or inefficient solubilization in the
314 stomach and small intestine. The capsule material itself dissolved rapidly in 5 minutes under
315 conditions mimicking the stomach (data not shown).

316

317 The effect of the juice is comparable to the non-absorbed drug acarbose, which is
318 administered to diabetic patients to limit post-prandial glucose excursions. When 50 mg
319 acarbose was given in 3 doses before breakfast, lunch and dinner to healthy volunteers, the
320 average reduction in post-prandial glucose was also about one third (29), comparable to the
321 study on pomegranate juice reported here. Acarbose reduces the risk of cardiovascular
322 disease and hypertension in patients with impaired glucose tolerance and with type 2 diabetes
323 (30, 31). The digestion of bread to glucose and intestinal absorption of glucose require at
324 least 3 biochemical steps (**Figure 6**), the first of which is α -amylase, followed by conversion

325 of the product into glucose by brush border maltase activity. Although pomegranate extract
326 was mildly effective at inhibiting maltase activity, this effect was not due to the constituent
327 polyphenols (punicalagin, punicalin nor ellagic acid, Table 2). The third step is glucose
328 absorption across the intestine which has been modelled using differentiated Caco-2 cell
329 monolayers, and by human GLUT2 expression in *Xenopus* oocytes. Neither pomegranate
330 juice nor its constituent polyphenols were able to affect glucose transport across Caco-2 cells,
331 but could interact with GLUT2 in *Xenopus* oocytes. These lines of evidence point to
332 inhibition of α -amylase as the main mechanism of action, with a potential contribution by
333 interactions with GLUT2. The IC₅₀ value for inhibition of human salivary α -amylase on
334 amylose by punicalagin was measured as 9 μ M, which is comparable to that reported
335 previously for acarbose (3.5 μ M) under the same conditions (23).

336 In other studies, pomegranate consumption was shown to affect sugar metabolism in different
337 ways by alternative mechanisms. In patients with type 2 diabetes, chronic pomegranate juice
338 consumption led to reduced fasting glucose concentrations in those subjects with blood
339 glucose levels between 7.1 and 8.7 mmol/L, compared to patients with higher levels (32).

340 Pomegranate juice consumption also decreased plasma malondialdehyde and carbonyl levels
341 after exercise (33) and plasma malondialdehyde in type 2 diabetes patients (34), but the
342 relevance of these markers for disease risk is controversial, see for example (35). The role of
343 post-prandial glucose in disease risk is becoming appreciated, and in a review of 45 relevant
344 publications, lower glycemic index (GI) diets reduced both fasting blood glucose and
345 glycosylated proteins. These effects were greater in persons with poor fasting blood glucose
346 control (2).

347 The intestinal fate and absorption of pomegranate polyphenols has been described.

348 Ellagitannins such as punicalagin and punicalin in pomegranate are readily hydrolysed to
349 ellagic acid, further converted to urolithins by the gut microbiota, and conjugated by

350 intestinal or hepatic phase II metabolism. The urinary level of urolithin A glucuronide was
351 not significantly different after consumption of pomegranate juice, a pomegranate polyphenol
352 liquid extract and a pomegranate polyphenol powder extract (36), demonstrating that
353 pomegranate polyphenols as supplements are ultimately solubilised in the gastrointestinal
354 tract at least by the time they reach the colon. We show for the first time that these
355 metabolites have the potential ability to further modulate sugar metabolism, as assessed here
356 using the HepG2 cell model, during the late post-prandial period (3-6 h). Urolithins C and D
357 stimulated deoxy-D-glucose uptake and modulated glucose metabolism, urolithin B
358 glucuronide modulated glucose metabolism, and urolithin A inhibited deoxy-D-glucose
359 uptake and modulated glucose metabolism. These data show that pomegranate polyphenols
360 have the potential to further influence glucose metabolism and is a subject worthy of future
361 study. The concentrations used are within the same order of magnitude to those found in
362 some individuals *in vivo*, although the concentrations in plasma and urine are subject to
363 substantial inter-individual variation, where individuals can be classed as producers or non-
364 producers for some types of urolithins (13). These differences arise from the resident
365 microflora of consumers further highlighting underlying potential benefits in different groups
366 (37), and could provide a mechanistic rationale for the chronic effect of pomegranate juice on
367 fasting blood glucose levels seen previously (32).

368

369 In conclusion, we have shown that pomegranate polyphenols, when present in a beverage, but
370 not a supplement, can reduce the acute post-prandial glycemic response of bread, and we
371 propose that this is primarily due to the ability of punicalagin to inhibit α -amylase. Further,
372 pomegranate polyphenol microbial metabolites may modulate sugar metabolism following
373 the acute postprandial period.

374

375

376 **Contributions**

377

378 AK, GW and HN planned and conceived the studies.

379 AK conducted all cell experiments.

380 HN conducted the pomegranate interventions and enzyme inhibition assays.

381 JSG conducted the experiments on *Xenopus* oocytes expressing GLUT2

382 FATB provided samples and conducted HPLC analysis.

383 GW wrote the first version of the manuscript. All authors contributed to writing the

384 manuscript and approved the final version.

385

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387

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395 help in the pomegranate sample preparation and analyses.

396

397 **Conflicts of interest**

398 GW has recently, or currently, received other research funding from Nestle and Florida

399 Department of Citrus, and conducted consultancy for Nutrilite, USA, and Suntory, UK. The

400 other authors declare no conflict of interest.

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TABLE 1.Composition of pomegranate juice and extracts¹.

	Amount in juice ²		Amount in capsules	
	mg/L	Per dose (mg)	mg/g	Per high dose ³ capsule (mg)
Punicalin	357.3 ± 1.1	71.5	6	2.4
Punicalagin	61.9 ± 0.6	12.4	121	48
Ellagic acid hexose	14.2 ± 0.1	2.8	5.9	2.4
Ellagic acid	24.0 ± 0.3	4.8	101	40.4
Malic acid ⁴	595.4 ± 24.5	119.1	0	0
Citric acid ⁴	19095 ± 570	3819	0	0
Glucose	51800 ± 1000	10400	8.3	3.3
Fructose	54700 ± 900	10900	6.6	2.6

¹Juice and extracts were analyzed by HPLC relative to authentic standards.

²For juice (200 ml), mean and standard deviation shown, n = 3.

³The high dose contained 400 mg of extract, double that of the low dose.

⁴Analysis carried out by HPIC (see methods section)

TABLE 2

Inhibition of digestive enzymes by pure pomegranate polyphenols compared to acarbose.

Enzyme	IC ₅₀ ¹ (mg/ml)	IC ₅₀ (μM)		Inhibition (%) at 200 μM	
	Pomegranate extract	Acarbose	Punicalagin	Punicalin ²	Ellagic acid
α-Amylase	0.06 ± 0.01	3.5 ± 0.2	9.0 ± 1.0 ³	29.9 ± 0.9	26.5 ± 0.5
Maltase	1.0 ± 0.1	0.43 ± 0.1	NI ⁴	NI	NI
Sucrase	1.2 ± 0.3	12 ± 2	NI	NI	NI

¹Experimental IC₅₀ values for human salivary α-amylase using amylose as substrate and rat α-glucosidase using maltose and sucrose as substrates for pomegranate extract and its major polyphenols (n = 3).

²A further increase in the concentration of punicalin and ellagic acid to 1000 μM did not significantly increase the inhibition obtained at 200 μM.

³A K_i value of 10.1 ± 0.6 μM was measured for punicalagin on α-amylase at different concentrations of amylose, and calculated according to (19).

⁴NI is no inhibition at 200 μM compared to acarbose as positive control.

TABLE 3.

Post-prandial blood glucose after a single dose of bread together with pomegranate juice, supplements or fruits acids.

Intervention study	Test meal	IAUC ¹ (mmol/L.min)	Peak glucose ¹ (mmol/L)
Bread (109 g) with pomegranate supplements	Placebo	159 ± 57	6.8 ± 1.0
	Capsule (200 mg extract)	183 ± 87	6.8 ± 0.8
	Capsule (400 mg extract)	184 ± 61	6.7 ± 0.9
Bread (109 g) with pomegranate juice	200 ml solution of balancing sugars	199 ± 64 ^a	7.7 ± 0.9 ^c
	200 ml juice	134 ± 62 ^b	6.8 ± 1.0 ^d
Bread (109 g) with malic acid and citric acid pH 3.2	200 ml water	152 ± 56	7.2 ± 0.8
	200 ml of test solution	177 ± 71	7.3 ± 1.0

¹Average IAUC and peak glucose concentrations after consumption of indicated foods and beverages. Bread with pomegranate supplements at 2 doses was compared to placebo capsules. Bread with pomegranate juice was compared to water containing balancing sugars as control. All studies were a crossover design with 16 participants. Values are mean ± standard deviation where different superscript letters indicate significant difference ($p < 0.01$) using student t-test and confirmed by ANOVA (p value).

FIGURE 1. Chemical structures of pomegranate polyphenols

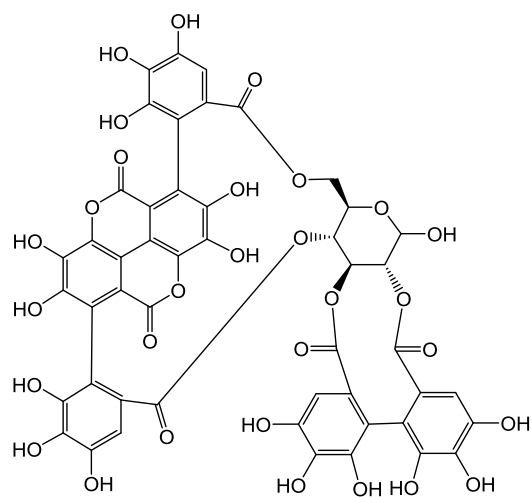
FIGURE 2. Inhibition of sugar transport and digestive enzyme by pomegranate extract and constituent compounds. Inhibition of human salivary α -amylase (■) using amylose as substrate and rat intestinal glucosidase using maltose (●) and sucrose (▲) as substrate by pomegranate extract (panel A). Inhibition of apical to basolateral transport of D-[U-¹⁴C]-glucose across differentiated monolayers of Caco-2 TC7 cells (12-30 replicates per data point \pm SD, panel B) and uptake of D-[U-¹⁴C]-glucose into *Xenopus* oocytes expressing the human glucose transporter GLUT2 (6 replicates of 3 oocytes expressing GLUT2, normalized to water controls, \pm SEM (panel C) by pomegranate extract. Inhibition of apical to basolateral transport of D-[U-¹⁴C]-glucose across differentiated monolayers of Caco-2 TC7 cells by pomegranate polyphenols is shown in Panel D for punicalagin (▲), panel E for punicalin (■) and panel F for ellagic acid (◆) (6 replicates per data point \pm SD, control samples contained the equivalent amount of DMSO). Significant differences to the control are shown (***, $p < 0.001$).

FIGURE 3. Participant flow diagrams for the intervention on pomegranate juice (Panel A), on constituent fruit acids (Panel B) and on pomegranate extract (Panel C). Simple randomization was used to determine the different groups (test or control), and block randomization was used to randomize participants into groups to ensure equal number of participants in each group. The four different sequences obtained were then allocated to participant codes by the principal investigator by pre-assigning the order of the meals to each code, which then determined the order of intervention.

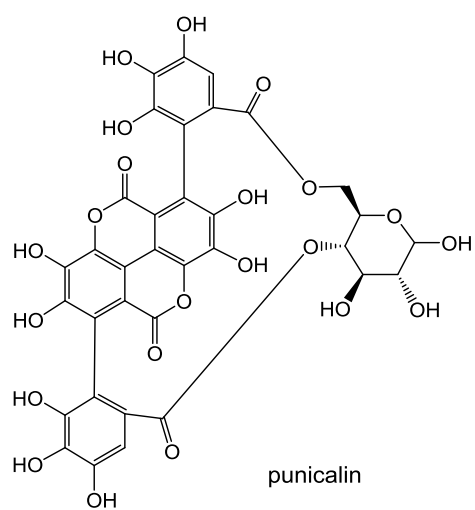
FIGURE 4. Post-prandial blood glucose concentrations after consumption of bread with pomegranate juice or extract. Glucose curves after consumption of control (○, □) and pomegranate juice (■, ●) with bread (16 volunteers) (panel A). Individual changes in the IAUC of reference and test meals are significantly different (**** $p < 0.000005$) (panels C and E, C = control, J = juice). Average glucose curves after consumption of reference (□), pomegranate capsules containing lower dose (●) and pomegranate capsules containing higher dose (▲) meals for 16 volunteers (panel B). There is no significance difference between IAUC of reference meals and test meals at either dose (panels D and F, C = control, D1 = lower dose, D2 = higher dose).

FIGURE 5. Effect of pomegranate polyphenol gut microbiota metabolites on uptake of sugars in human hepatic HepG2 cells. Effect of urolithins and conjugates on uptake of [U-¹⁴C]-deoxy-D-glucose (panel A) and of [U-¹⁴C]-glucose (panel B) by HepG2 cells (n = 12, ± SD). Each treatment was compared to the control: * $p < 0.05$; ** $p < 0.01$ by independent samples Students *t*-test and the 2-tailed values were adjusted for multiple comparisons with the Bonferroni correction. Urolithin A, UroA; Urolithin B, UroB; Urolithin C, UroC; Urolithin D, UroD; Urolithin A glucuronide, UroAglu; Urolithin B glucuronide, UroBglu; all compounds at 5 μM.

FIGURE 6. Proposed mechanisms of action. Black arrow shows strong site of inhibition of bread digestion by punicalagin, which then affects blood glucose postprandially, and light gray arrows show possible but weaker points of interaction. Potential of sites of action of metabolites of pomegranate polyphenols after the post-prandial period are shown by white arrows.



punicalagin



punicalin

Figure 1

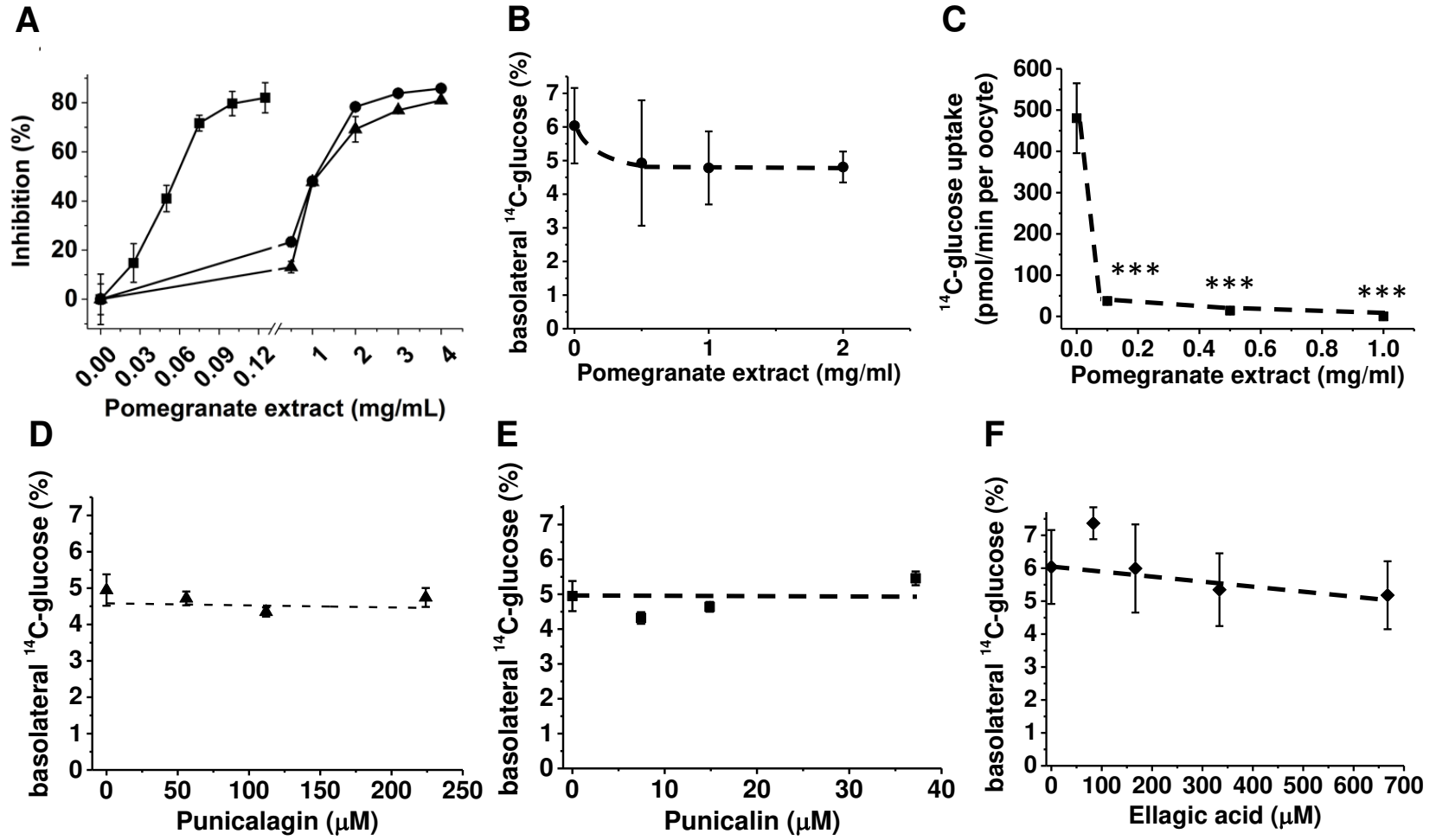


Figure 2

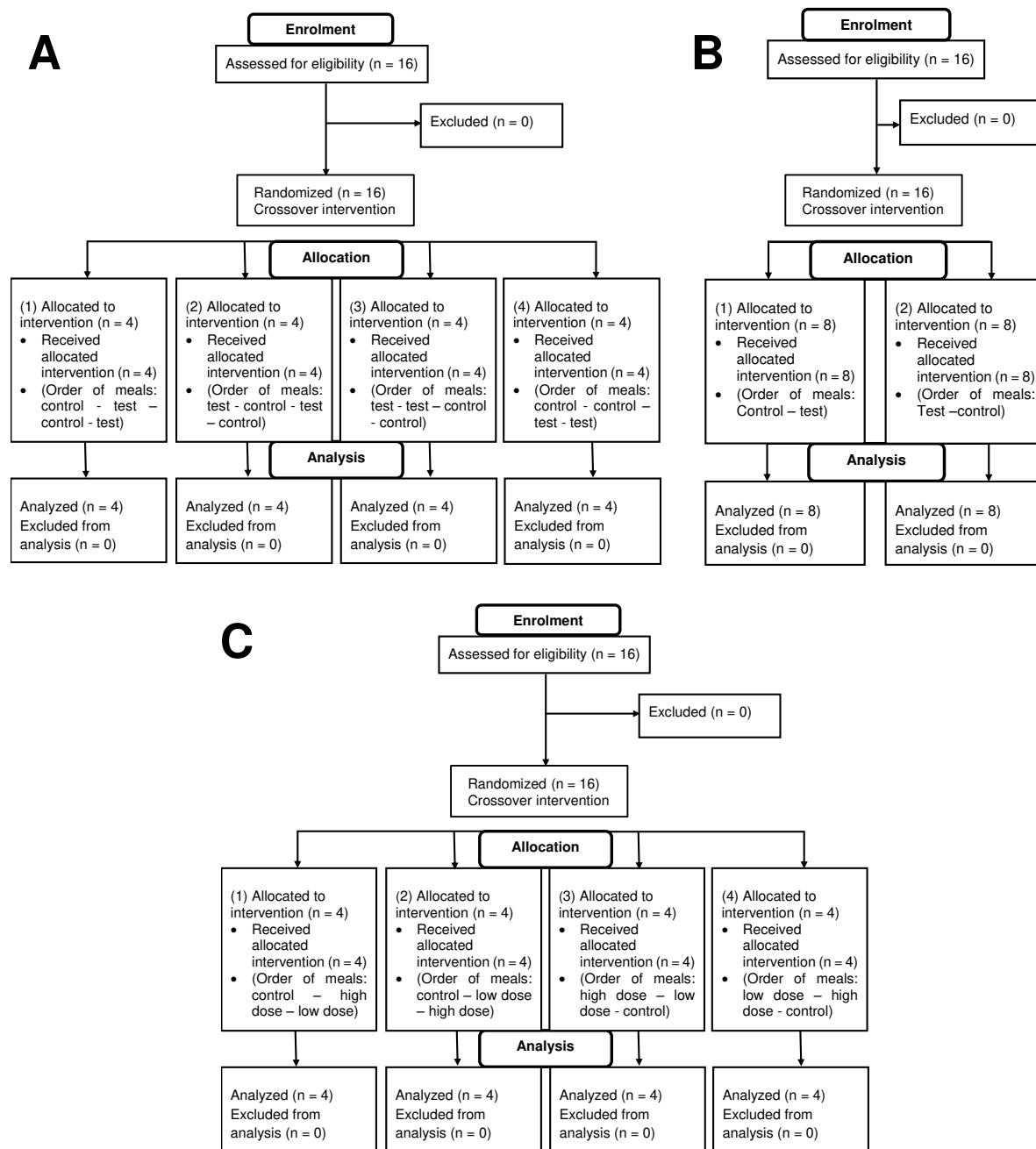


Figure 3

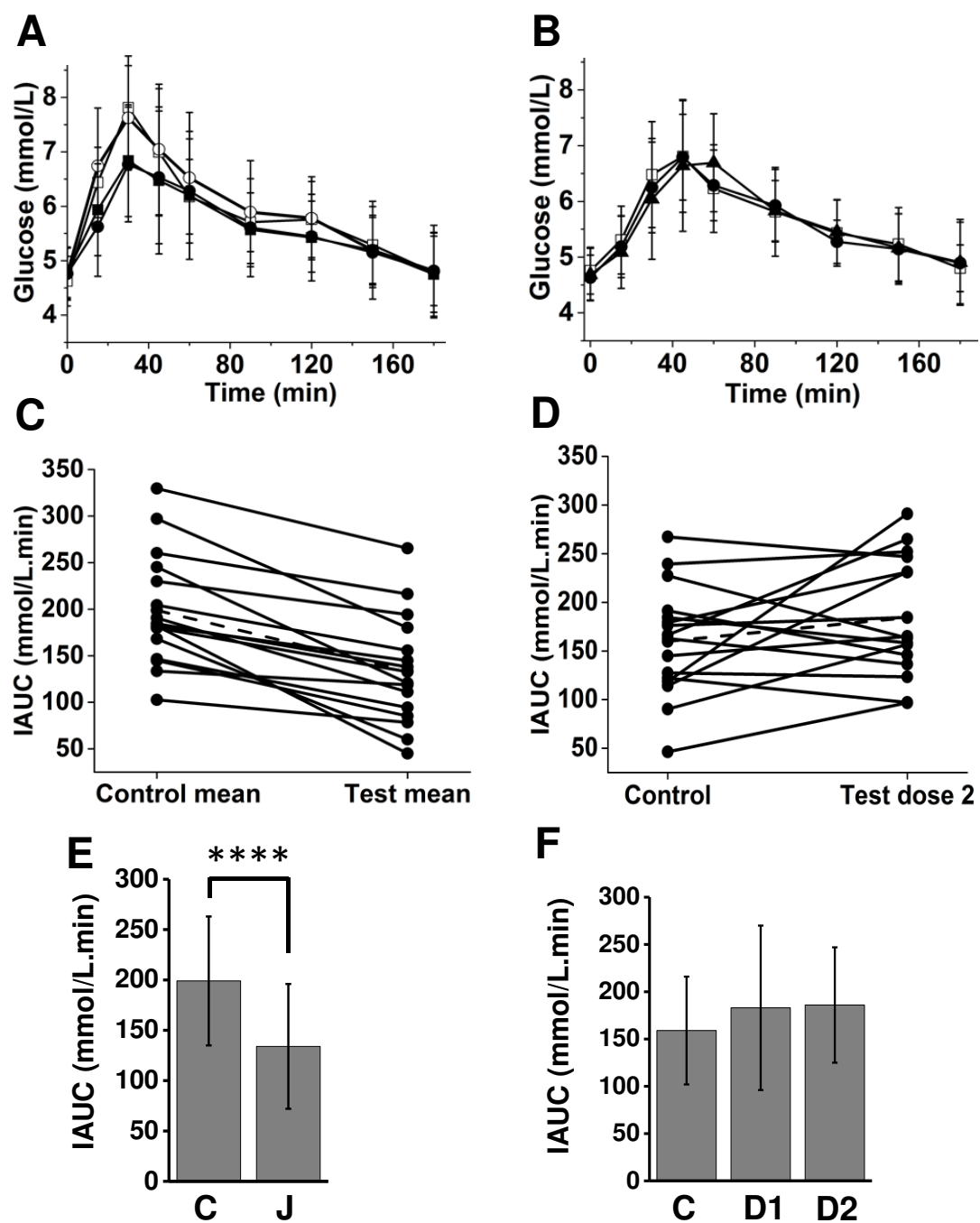


Figure 4

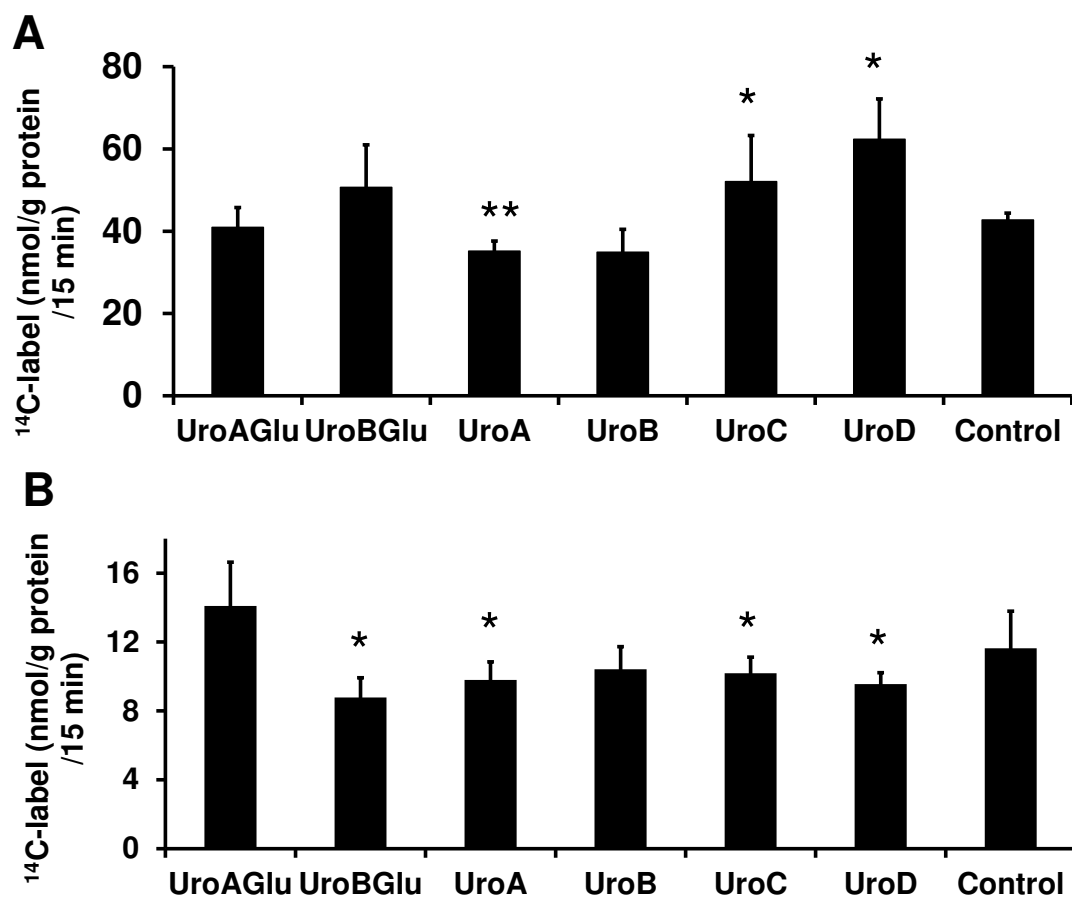


Figure 5

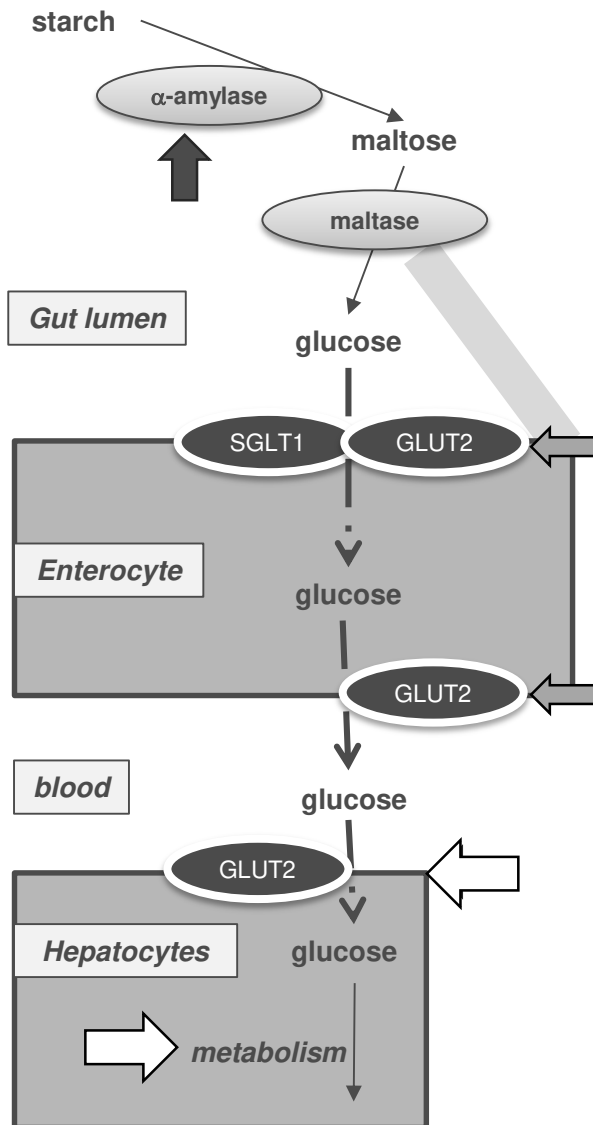


Figure 6.