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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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ABSTRACT

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

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

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

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

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

INTRODUCTION

Post-prandial glycemic control and levels of sugar in the diet are both topics of current concern and controversy. While the glycemic index was introduced many decades ago, its relevance to health maintenance and diseases such as the metabolic syndrome, diabetes and obesity, are only now being fully recognised through reviews and meta-analyses (1-3). Postprandial glucose naturally rises following digestion of rapidly hydrolyzable carbohydrates, such as soluble starch, some forms of cooked starch, or from soluble sugars present in the food such as glucose, fructose and sucrose. After processing by salivary and pancreatic α-amylase, followed by intestinal brush border maltase/glucoamylase and sucrase/isomaltase, the product glucose is rapidly absorbed through intestinal sugar transporters GLUT2 and SGLT1 across the enterocytes, whereas the product fructose is absorbed through GLUT2 and GLUT5.

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

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

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

SUBJECTS AND METHODS

Materials

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

Pomegranate analysis

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

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

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

Subjects

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

Design and intervention

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

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

The 16 volunteers for the pomegranate juice study were recruited from November 2015 until March 2016 and were aged 31 ± 5 y with BMI of 23 ± 3 kg/m^2 and fasting blood glucose concentration of 4.7 ± 0.5 mmol/L and were recruited separately based on the same eligibility criteria at a different time. The study protocol was un-blinded due to the nature of the test meal. Each volunteer attended four visits, two where reference meals (200 ml water with balancing sugars and bread) were consumed and another two at which test meals of the same dose (200 ml pure pomegranate juice and bread) were ingested. The reference meal included 10.9 g fructose and 10.3 g glucose dissolved in 200 ml water to standardize the amounts of sugars present in the pomegranate juice.

The 16 volunteers for testing the acid solution, equivalent to the malic acid, citric acid and potassium content of pomegranate juice, were recruited from October 2016 until August 2017 and were aged 33 ± 9 years with BMI of 25.0 ± 3.8 kg/m^2, fasting blood glucose concentration of 4.9 ± 0.4 mM and were recruited separately based on the same eligibility criteria at a different time. The study protocol was un-blinded due to the nature of the test meal. The volunteers consumed 109 g bread as above, together with 200 ml water containing 3.82 g citric acid and 0.118 g malic acid, adjusted to pH 3.2 with sodium bicarbonate, compared to 200 ml tap water as control. Each volunteer attended, in randomized order, for the control and for the test.

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

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

Enzyme inhibition assays in vitro

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

Glucose transport

Caco-2/TC7 cells were a kind gift from Prof. Monique Rousset, (INSERM, France). For transport experiments, cells were seeded on 6-well Transwell plates (Corning 3412, Appleton Woods, Birmingham, UK) at a density of 6.43 x 10^4 cells/cm^2 until full differentiation of the monolayer (21 - 23 days) in Dulbecco's Modified Eagle's Medium (25 mM, DMEM) supplemented with 20% (v/v) Fetal Bovine Serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin at 37°C with 10% CO_2 in a humidified atmosphere. All cell culture reagents

were from Sigma (Sigma Aldrich, Gillingham, UK). On or after 22 d studies were initiated and cells in both compartments were washed and incubated with transport buffer A (HEPES, 20 mM; NaCl, 137 mM; KCl, 4.7 mM; $CaCl_2$ 1.8 mM, $MgSO_4$ 1.2 mM; adjusted to pH 7.4 using NaOH, 1 M) for 30 min. Transepithelial electrical resistance (TEER) measurements were recorded using a Millicell ERS volt-ohm meter (Millipore Ltd, Watford, UK). The buffer was aspirated and the relevant test solution at pH 7.4 was added apically with 5 mM glucose and 0.1 µCi/ well D-[U-^{14}C] glucose. The pomegranate extract naturally contained 0.46 mM glucose and 0.37 mM fructose when made up in a solution of DMSO; 0.37 mM fructose did not affect glucose transport compared to 5 mM glucose when tested in the same set-up (data not shown). Plates were incubated for 30 min and all solutions were collected and mixed with 5 ml of scintillation liquid (Gold Star, Meridian Biotechnologies, Surrey, UK) for radioactivity measurements with a Packard 1900 TR Liquid Scintillation Analyser.

D-[U-^{14}C]-glucose and [U-^{14}C]-deoxy-D-glucose uptake by HepG2 cells

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

Statistical analysis

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

paired t-test and confirmed with the one factor repeated measures analysis of variance (ANOVA) by SPSS v24 (IBM). Comparisons between control and treatment in D-[U-^{14}C]-glucose and [U-^{14}C]-deoxy-D-glucose uptake cell experiments was carried out by independent samples two tailed Student's t-test between control and treatment and the 2-tailed values were adjusted for multiple comparisons with the Bonferroni correction. D-[U-^{14}C] glucose uptake into Xenopus oocytes expressing human GLUT2 was normalized against water injected oocytes for each condition. Two-tailed homoscedastic Student's t-test was used to assess significance between uptake with and without varying concentrations of pomegranate. For α-amylase assays, all IC_{50} values are given as mean ± standard deviation from triplicates of 3 independent assays obtained by regression.

RESULTS

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

The dissolved pomegranate extract inhibited human α-amylase and rat intestinal brush border maltase/sucrase activities in vitro (**Figure 2**, Table 2). Of the individual components, punicalagin was a very effective inhibitor of α-amylase, but ellagic acid and punicalin were much weaker (Table 2). The inhibition of α-amylase by punicalagin exhibited a K_i value of 10.1 ± 0.6 μM with kinetically competitive inhibition.

Inhibition of D-[U-^{14}C]-glucose transport across Caco-2 cell monolayers and into Xenopus oocytes expressing GLUT2

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

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

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

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

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

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

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

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

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

Further sugar metabolism by hepatic uptake

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

Discussion

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

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

of the product into glucose by brush border maltase activity. Although pomegranate extract was mildly effective at inhibiting maltase activity, this effect was not due to the constituent polyphenols (punicalagin, punicalin nor ellagic acid, Table 2). The third step is glucose absorption across the intestine which has been modelled using differentiated Caco-2 cell monolayers, and by human GLUT2 expression in Xenopus oocytes. Neither pomegranate juice nor its constituent polyphenols were able to affect glucose transport across Caco-2 cells, but could interact with GLUT2 in Xenopus oocytes. These lines of evidence point to inhibition of α-amylase as the main mechanism of action, with a potential contribution by interactions with GLUT2. The IC_{50} value for inhibition of human salivary α-amylase on amylose by punicalagin was measured as 9 μM, which is comparable to that reported previously for acarbose (3.5 μM) under the same conditions (23).

In other studies, pomegranate consumption was shown to affect sugar metabolism in different ways by alternative mechanisms. In patients with type 2 diabetes, chronic pomegranate juice consumption led to reduced fasting glucose concentrations in those subjects with blood glucose levels between 7.1 and 8.7 mmol/L, compared to patients with higher levels (32). Pomegranate juice consumption also decreased plasma malondialdehyde and carbonyl levels after exercise (33) and plasma malondialdehyde in type 2 diabetes patients (34), but the relevance of these markers for disease risk is controversial, see for example (35). The role of post-prandial glucose in disease risk is becoming appreciated, and in a review of 45 relevant publications, lower glycemic index (GI) diets reduced both fasting blood glucose and glycated proteins. These effects were greater in persons with poor fasting blood glucose control (2).

The intestinal fate and absorption of pomegranate polyphenols has been described. Ellagitannins such as punicalagin and punicalin in pomegranate are readily hydrolysed to ellagic acid, further converted to urolithins by the gut microbiota, and conjugated by

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

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

Contributions

AK, GW and HN planned and conceived the studies.
AK conducted all cell experiments.
HN conducted the pomegranate interventions and enzyme inhibition assays.
JSG conducted the experiments on Xenopus oocytes expressing GLUT2
FATB provided samples and conducted HPLC analysis.
GW wrote the first version of the manuscript. All authors contributed to writing the manuscript and approved the final version.

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Conflicts of interest

GW has recently, or currently, received other research funding from Nestle and Florida Department of Citrus, and conducted consultancy for Nutrilite, USA, and Suntory, UK. The other authors declare no conflict of interest.

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TABLE 1.

Composition of pomegranate juice and extracts[1].

	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

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

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

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

[4]Analysis carried out by HPIC (see methods section)

TABLE 2

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

	IC_{50}[1] (mg/ml)	IC_{50} (μM)		Inhibition (%) at 200 μM	
Enzyme	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

[1]Experimental IC_{50} 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).

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

[3]A 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).

[4]NI 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

[1]Average 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

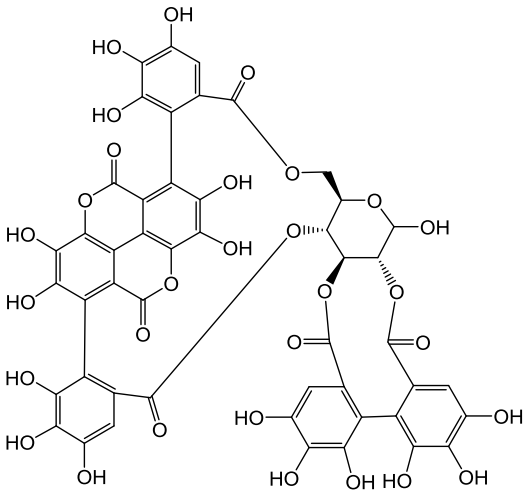
FIGURE 2. Inhibition of sugar transport and digestive enzyme by pomegranate extract and constituent compounds. Inhibition of human salivary α-amylase (■) using amylose as substrate and rat intestinal glucosidase using maltose (●) and sucrose (▲) as substrate by pomegranate extract (panel A). Inhibition of apical to basolateral transport of D-[U-^{14}C-]-glucose across differentiated monolayers of Caco-2 TC7 cells (12-30 replicates per data point ± SD, panel B) and uptake of D-[U-^{14}C-]-glucose into Xenopus oocytes expressing the human glucose transporter GLUT2 (6 replicates of 3 oocytes expressing GLUT2, normalized to water controls, ± SEM (panel C) by pomegranate extract. Inhibition of apical to basolateral transport of D-[U-^{14}C-]-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 ± 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.

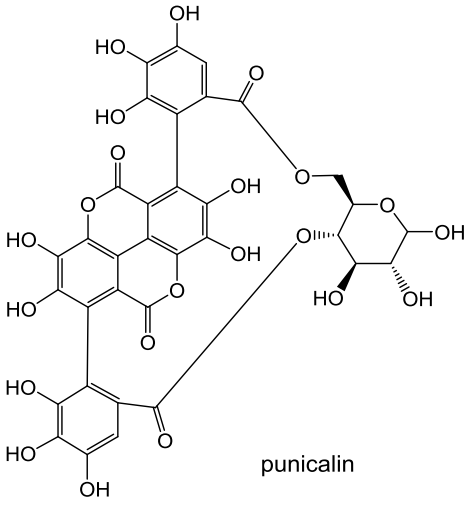
FIGURE 4. Post-prandial blood glucose concentrations after consumption of bread with pomegranate juice or extract. Glucose curves after consumption of control (○, □) and pomegranate juice (■, ●) with bread (16 volunteers) (panel A). Individual changes in the IAUC of reference and test meals are significantly 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-^{14}C]-deoxy-D-glucose (panel A) and of [U-^{14}C]-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

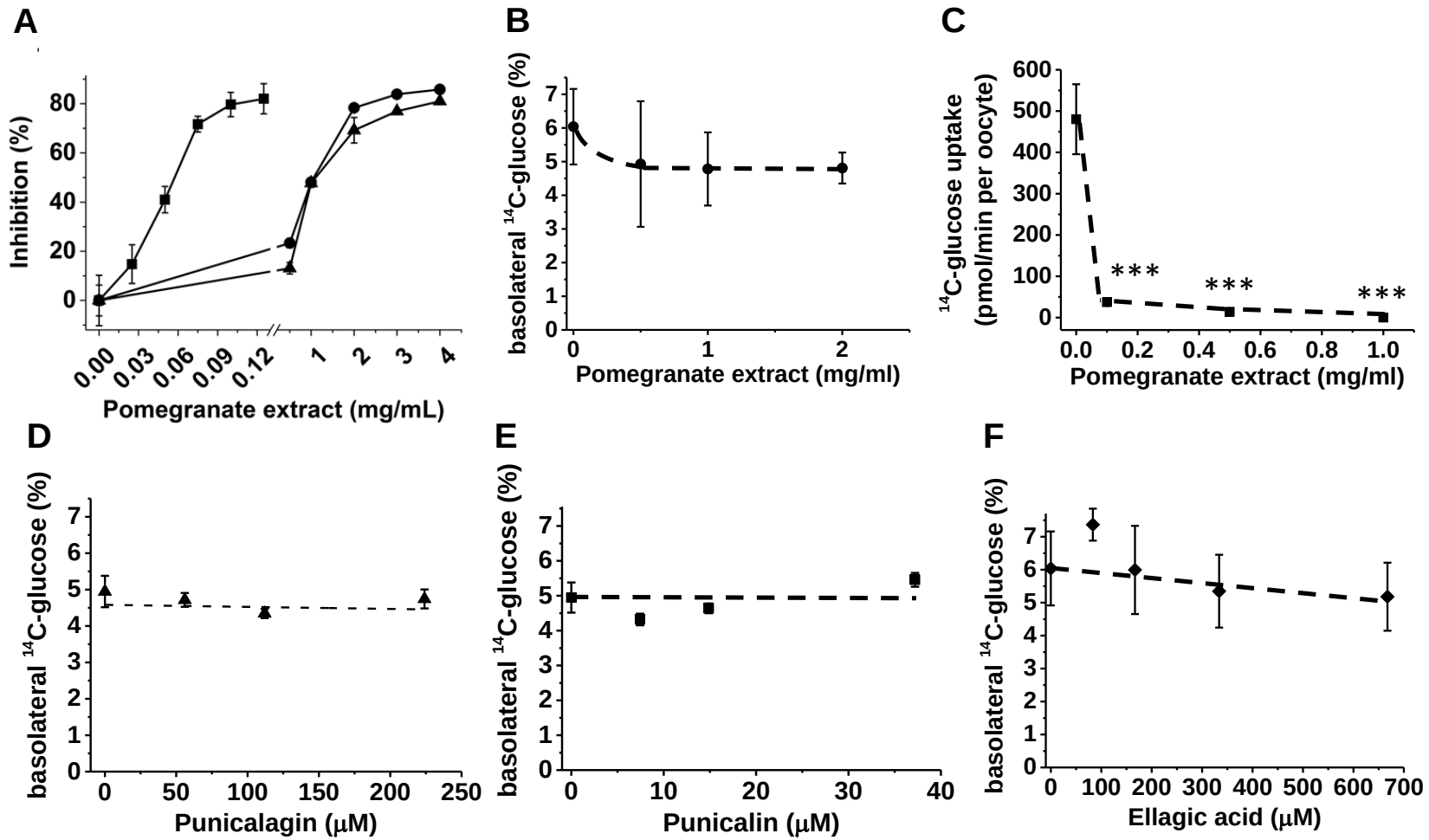

A
Inhibition (%)
0
20
40
60
80
0.00
0.03
0.06
0.09
0.12
1
2
3
4
Pomegranate extract (mg/mL)
B
basolateral ^{14}C-glucose (%)
0
1
2
3
4
5
6
7
0
1
2
Pomegranate extract (mg/ml)
C
^{14}C-glucose uptake (pmol/min per oocyte)
0
100
200
300
400
500
600
***
***
***
0.0
0.2
0.4
0.6
0.8
1.0
Pomegranate extract (mg/ml)
D
basolateral ^{14}C-glucose (%)
0
1
2
3
4
5
6
7
0
50
100
150
200
250
Punicalagin (μM)
E
basolateral ^{14}C-glucose (%)
0
1
2
3
4
5
6
7
0
10
20
30
40
Punicalin (μM)
F
basolateral ^{14}C-glucose (%)
0
1
2
3
4
5
6
7
0
100
200
300
400
500
600
700
Ellagic acid (μM)

Figure 2

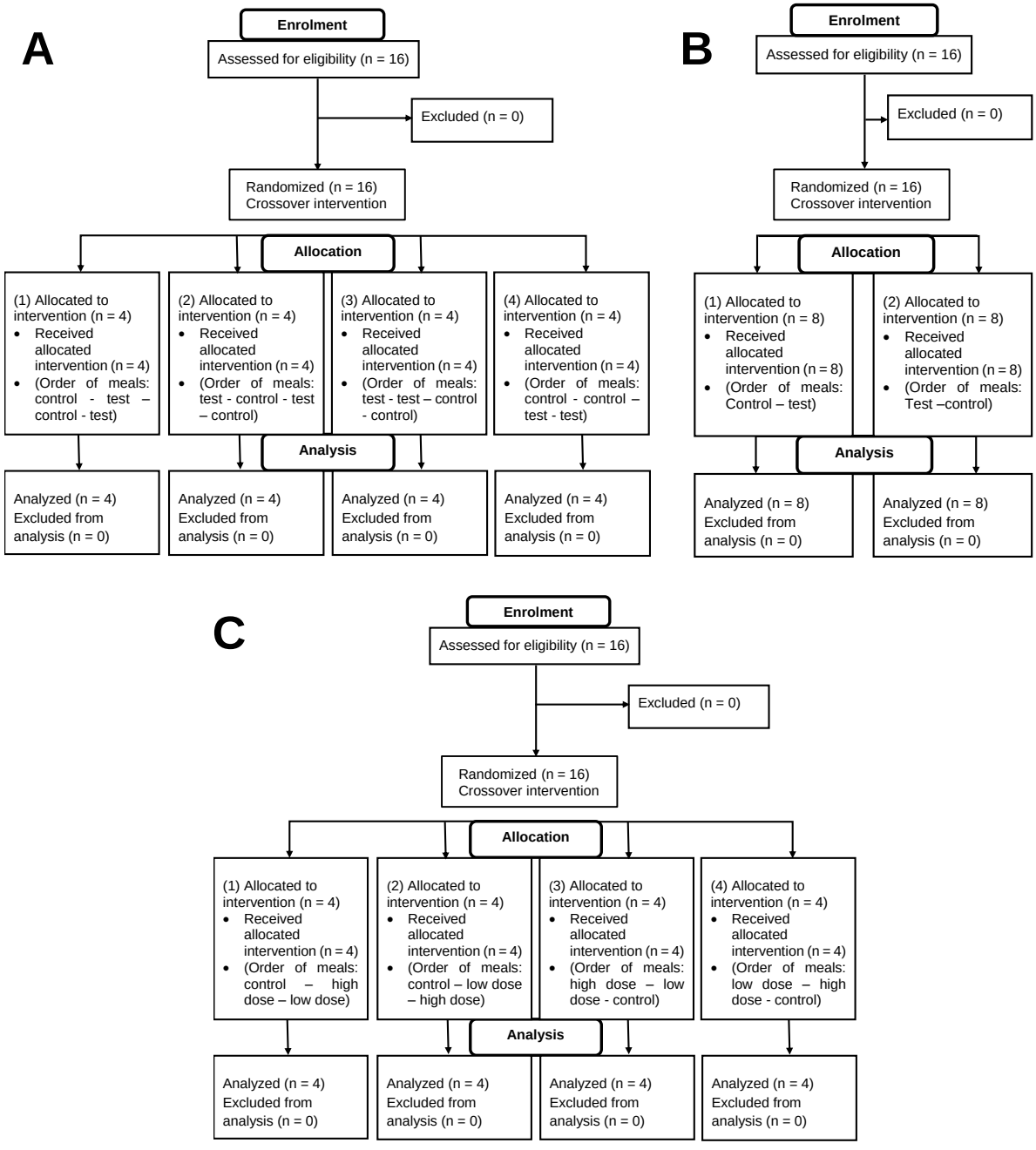

A
Enrolment
Assessed for eligibility (n = 16)
Excluded (n = 0)
Randomized (n = 16)
Crossover intervention
Allocation
(1) Allocated to intervention (n = 4)
• Received allocated intervention (n = 4)
• (Order of meals: control - test – control - test)
(2) Allocated to intervention (n = 4)
• Received allocated intervention (n = 4)
• (Order of meals: test - control - test – control)
(3) Allocated to intervention (n = 4)
• Received allocated intervention (n = 4)
• (Order of meals: test - test – control - control)
(4) Allocated to intervention (n = 4)
• Received allocated intervention (n = 4)
• (Order of meals: control - control – test - test)
Analysis
Analyzed (n = 4) Excluded from analysis (n = 0)
Analyzed (n = 4) Excluded from analysis (n = 0)
Analyzed (n = 4) Excluded from analysis (n = 0)
Analyzed (n = 4) Excluded from analysis (n = 0)
B
Enrolment
Assessed for eligibility (n = 16)
Excluded (n = 0)
Randomized (n = 16)
Crossover intervention
Allocation
(1) Allocated to intervention (n = 8)
• Received allocated intervention (n = 8)
• (Order of meals: Control – test)
(2) Allocated to intervention (n = 8)
• Received allocated intervention (n = 8)
• (Order of meals: Test –control)
Analysis
Analyzed (n = 8) Excluded from analysis (n = 0)
Analyzed (n = 8) Excluded from analysis (n = 0)
C
Enrolment
Assessed for eligibility (n = 16)
Excluded (n = 0)
Randomized (n = 16)
Crossover intervention
Allocation
(1) Allocated to intervention (n = 4)
• Received allocated intervention (n = 4)
• (Order of meals: control – high dose – low dose)
(2) Allocated to intervention (n = 4)
• Received allocated intervention (n = 4)
• (Order of meals: control – low dose – high dose)
(3) Allocated to intervention (n = 4)
• Received allocated intervention (n = 4)
• (Order of meals: high dose – low dose - control)
(4) Allocated to intervention (n = 4)
• Received allocated intervention (n = 4)
• (Order of meals: low dose – high dose - control)
Analysis
Analyzed (n = 4) Excluded from analysis (n = 0)
Analyzed (n = 4) Excluded from analysis (n = 0)
Analyzed (n = 4) Excluded from analysis (n = 0)
Analyzed (n = 4) Excluded from analysis (n = 0)

Figure 3

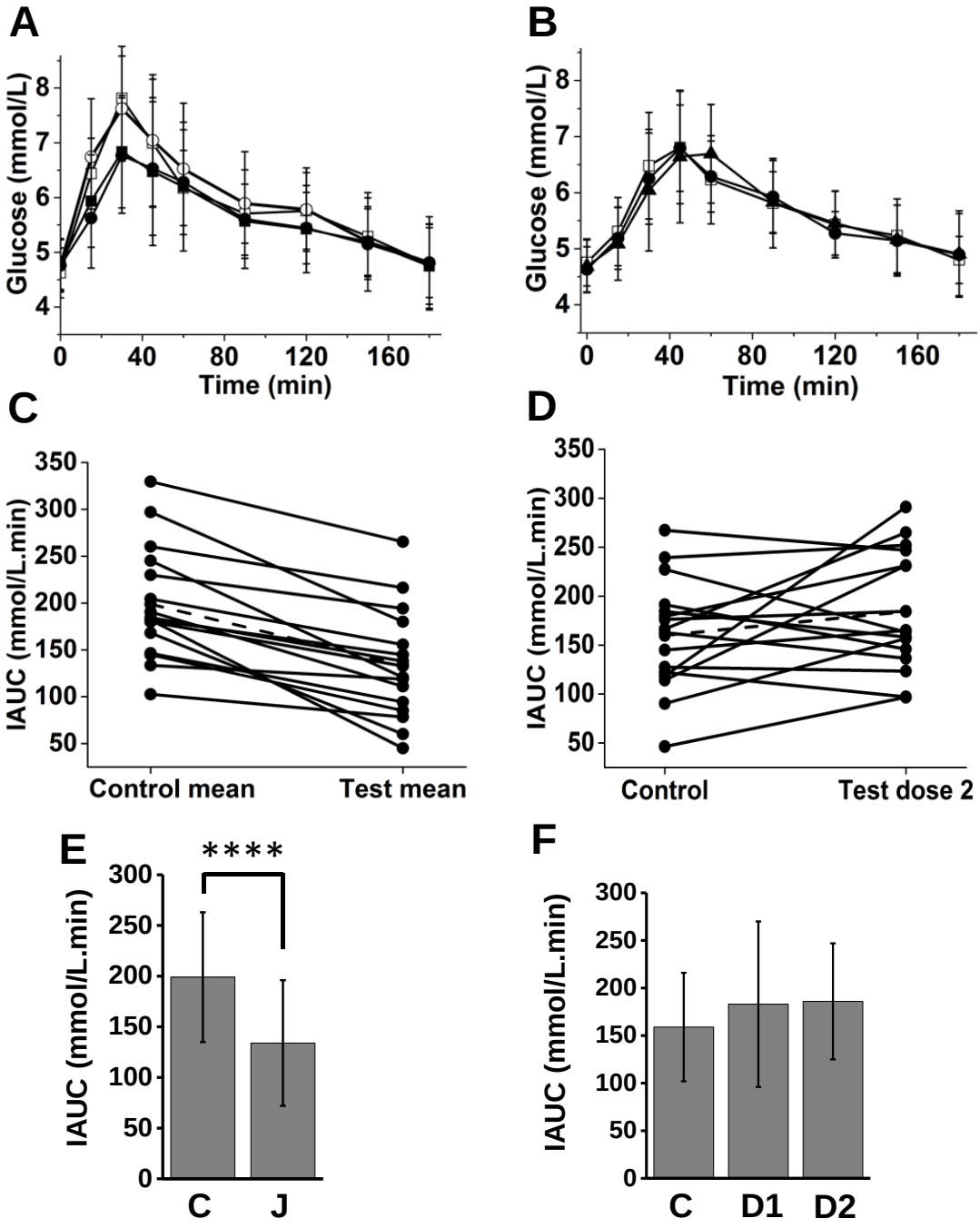

A
Glucose (mmol/L)
Time (min)
B
Glucose (mmol/L)
Time (min)
C
IAUC (mmol/L.min)
Control mean
Test mean
D
IAUC (mmol/L.min)
Control
Test dose 2
E
****
IAUC (mmol/L.min)
C
J
F
IAUC (mmol/L.min)
C
D1
D2

Figure 4

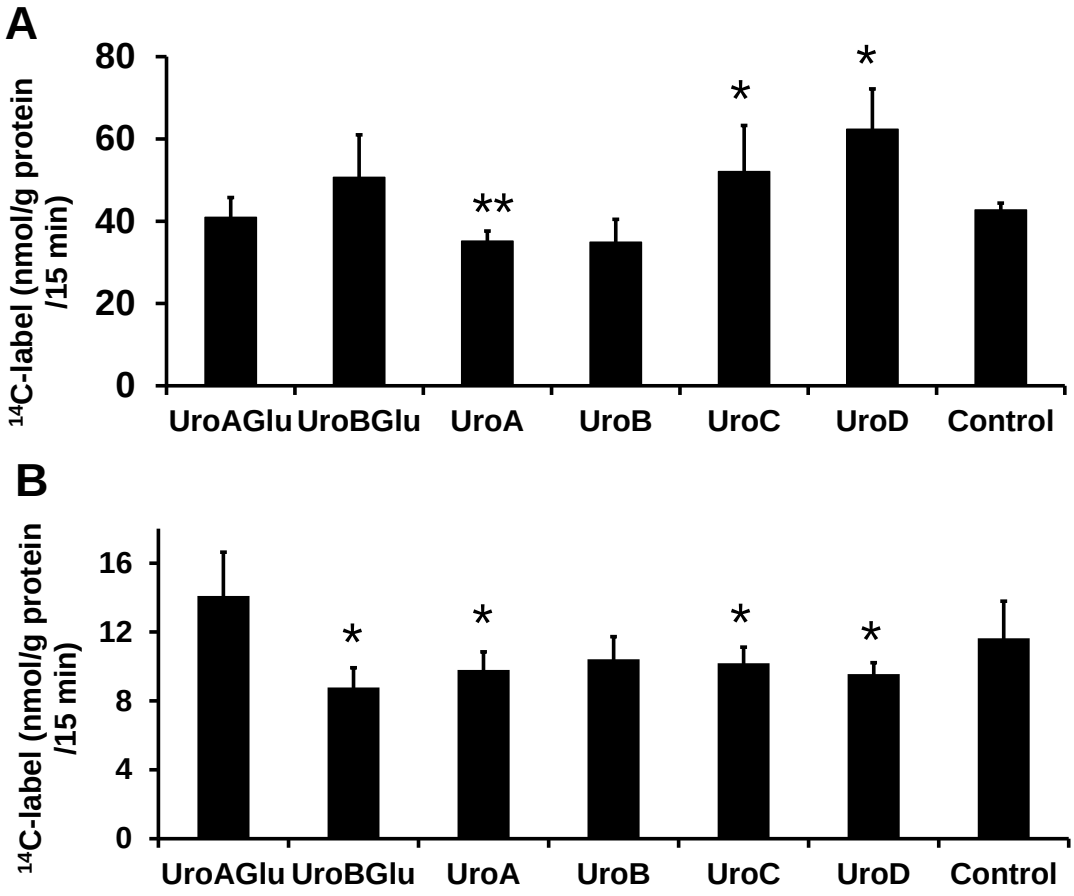

A
80
60
40
20
0
^{14}C-label (nmol/g protein /15 min)
**
*
*
UroAGlu
UroBGlu
UroA
UroB
UroC
UroD
Control
B
16
12
8
4
0
^{14}C-label (nmol/g protein /15 min)
*
*
*
*
UroAGlu
UroBGlu
UroA
UroB
UroC
UroD
Control

Figure 5

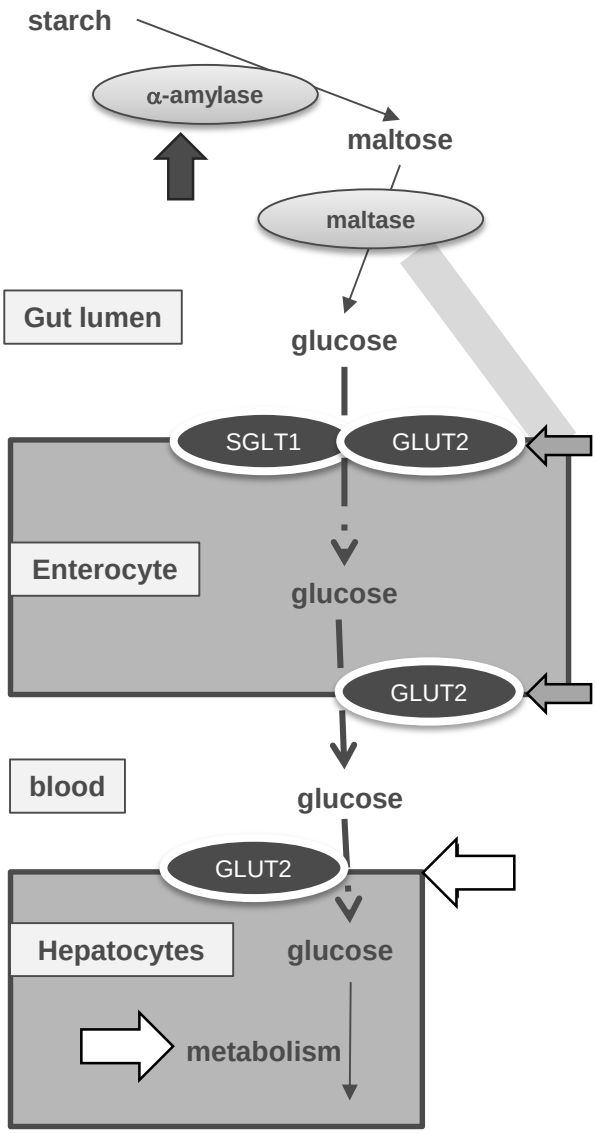

starch
α-amylase
maltose
maltase
Gut lumen
glucose
SGLT1
GLUT2
Enterocyte
glucose
GLUT2
blood
glucose
GLUT2
Hepatocytes
glucose
metabolism

Figure 6.