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Apple polyphenol-rich drinks dose-dependently decrease early-phase postprandial glucose concentrations following a high-carbohydrate meal: a randomized controlled trial in healthy adults and *in vitro* studies.

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Key words

Randomised controlled trial, Postprandial glycemia, Polyphenols, Phlorizin, Apples, Glucose transport.

Abstract

Background: Previous research demonstrated that a high dose of phlorizin-rich apple extract (AE) can markedly inhibit early-phase postprandial glycemia, but efficacy of lower doses of the AE is unclear.

Objective: To determine whether lower AE doses reduce early-phase postprandial glycemia in healthy adults and investigate mechanisms.

Design: In a randomized, controlled, double-blinded, cross-over acute trial, drinks containing 1.8 g (HIGH), 1.35 g (MED), 0.9 g (LOW), or 0 g (CON) of a phlorizin-rich AE were consumed before 75 g starch/sucrose meal. Postprandial blood glucose, insulin, C-peptide, glucose-dependent insulinotropic polypeptide (GIP) and polyphenol metabolites concentrations were measured 0-240 min, acetaminophen concentrations to assess gastric emptying rate, and 24 h urinary glucose excretion. Effects of AE on intestinal glucose transport were investigated in Caco-2/TC7 cells.

Results: AE significantly reduced plasma glucose iAUC 0-30 min at all doses: mean differences (95% CI) relative to CON were -15.6 (-23.3, -7.9), -11.3 (-19.6, -3.0) and -8.99 (-17.3, -0.7) mmol/L/min for HIGH, MEDIUM and LOW respectively, delayed T_{max} (HIGH, MEDIUM and LOW 45 min vs. CON 30 min), but did not lower C_{max}. Similar dose-dependent treatment effects were observed for insulin, C-peptide, and GIP. Gastric emptying rates and urinary glucose excretion did not differ. Serum phloretin, quercetin and epicatechin metabolites were detected postprandially. A HIGH physiological AE dose equivalent decreased total glucose uptake by 48 % in Caco-2/TC7 cells.

Conclusions: Phlorizin-rich AE, even at a low dose, can slightly delay early-phase glycaemia without affecting peak and total glycaemic response.

Keywords: Randomized controlled trial, Postprandial glycemia, Polyphenols, Phlorizin, Apples, Glucose transport.

1. Introduction

Food and beverages high in available carbohydrates elicit a marked glycemic and insulinemic response. Lowering the glycemic index/load of diets may improve glycemic control, particularly in individuals with raised fasting blood glucose [1,2] and reduces the risk of developing chronic diseases such as type 2 diabetes (T2D) [3]. Chronic exposure to exaggerated postprandial glucose excursions can lead to adverse modifications to functional proteins, oxidative stress and pancreatic beta cell dysfunction [4]. Intervention studies have shown that prescription of acarbose, a potent inhibitor of carbohydrate digestive enzymes, is a promising metabolic modifier that can reduce the risk of T2D. A 6 % reduction in diabetes incidence was observed in high-risk patients over 3 years [5]. Given the widespread availability of refined, high-carbohydrate foods in industrialised countries, dietary strategies that may moderate postprandial glycemia is a vital area of research for prevention of cardiometabolic diseases [6].

Glycemic responses to foods are determined by a range of factors relating to the individual's metabolic health status, recent physical activity [7], dietary and alcohol intakes [8,9], and sleep patterns [10], as well as characteristics of the meal: amount, type and digestibility of starch; non-nutrient components that may interact (e.g. α-amylase inhibitors, phytates and polyphenols); amount and type of fat, sugar, and dietary fibres; food matrix structure;

viscosity within the digestive tract [11]. Polyphenols are a large and heterogeneous group of phytochemicals containing 1 or more phenol ring [6], with the main classes being flavonoids, stilbenes, lignans and phenolic acids. Polyphenols may contribute to the cardiometabolic protective effects of fruits, vegetables and wholegrains [6,12–14]. Polyphenol intake among a large, health-conscious US cohort was estimated at 0.8 g/day using food frequency questionnaires, with coffee, fruits (citrus, apples and pears, and purple/red fruits) and fruit juices as the main food contributors [15]. In the UK, the adult average polyphenol intake has been estimated at 1-1.6 g/day using 4-day food diaries and 24 h recalls [16–18], with apples and pears being the biggest fruit contributors [16]. Data extracted from the UK National Diet and Nutrition Survey (NDNS) rolling programme years 1-4 (2008/12) showed 37 % of respondents (age ≥19 y old, n=3450) reported eating whole apples (with or without skin) during a 4 day period; median intake in consumers was 38 g/day (IQR 36) (approximately equivalent to consuming 2-3 apples per week) [19]. Apples contain a complex profile of polyphenols; quantitatively (by weight), the most representative are hydroxycinnamic acids, flavanols/procyanidins, flavonols and dihydrochalcones [20].

Growing evidence using apple juices and extracts suggests constituents of apples inhibit the rate of glucose absorption in the intestine [21–23]. Apple polyphenols may inhibit glucose absorption by inhibiting intestinal enzymes α-amylases and α-glucosidases [24–26], slowing down the breakdown of starch and sucrose, and by inhibiting the intestinal glucose transporters, SGLT1 and GLUT2 [22,27]. A considerable body of evidence has highlighted the mechanistic importance of the dihydrochalcone polyphenol, phlorizin [22,27–29] which is found in uniquely high concentrations in apples [20]. Phlorizin is poorly absorbed in the small intestine; approximately 80 % of phlorizin is hydrolysed to phloretin prior to absorption [30]. Phlorizin competitively inhibits the sodium-dependent glucose transporter SGLT1, which is

predominantly distributed in the intestine but also the bile duct, lung, heart and straight (S3 segment) of the proximal tubules of the kidney. Renal SGLT1 is expressed to a lesser extent than SGLT2, which is distributed in the convoluted (S1 and S2 segments) of the renal proximal tubules, and is also inhibited by phlorizin [31–33]. Polyphenols may also interact with chemoreceptors in the stomach or duodenum, for example, bitter taste receptors [34,35], to trigger a delay in gastric emptying rate by an, as yet, unexplained mechanism such as vagal afferent signalling, gut hormone release, or direct inhibition of smooth muscle contractility via ion channels [34,36,37].

Our group recently reported that 1.8 g AE, containing approximately 1.2 g total phenolics, markedly reduced plasma glucose concentrations up to 30 min following a high starch and sucrose meal [23]. However, this level of intake of polyphenols would be difficult to achieve by consuming fresh apples alone, since this amount equates to over two thirds of average daily polyphenol intake in adults and roughly 4-6 average-sized eating apples [16–18]. The efficacy of lower doses that would be more feasible to use in the formulation of functional foods or beverages, in terms of overall organoleptic properties as well as cost, is unclear. Decreasing the rate of glucose absorption from carbohydrate in foods would lead to a more sustained delivery of glucose to the tissues which could be of benefit to individuals with insulin resistance, and also to endurance athletes who need a more prolonged glycemia without the reduction in the amount of available carbohydrate and demands on digestive processes that might occur following whole-food, high-fibre foods. The present study tests the hypothesis that drinks containing phlorizin-rich AE will dose-dependently decrease the rate of glucose absorption, assessed by measuring early-phase postprandial glucose concentrations (incremental area under the curve (iAUC) 0-30 min) as the primary outcome variable, following a high-starch/high sucrose meal. The primary outcome was iAUC (0-30 min), intended to capture the time period when the rate of intestinal glucose absorption is the main

determinant of plasma glucose concentrations and the rate of liver glucose uptake on first pass metabolism is not yet a major factor. After 30-45 min (the approximate time of peak glycemia) blood glucose concentrations also reflect other factors that affect the rate of disappearance, primarily the amount of insulin secreted, the degree of muscle and adipose tissue insulin sensitivity, and the level of suppression of hepatic glucose output postprandially. Secondary *in vivo* outcomes include: total glycemic response (iAUC 0-120); a prolonged period of glycemic monitoring (iAUC 0-240 min); peak glucose concentrations (C_{max}); time of peak glucose concentrations (T_{max}); postprandial parameters of insulin secretion (insulin, C-peptide); postprandial incretin response indicated by glucose-dependent insulinotropic polypeptide (GIP); novel data on postprandial apple polyphenol metabolites; gastric emptying rates by the acetaminophen absorption test in a sub-group; 24 h renal glucose output as an indicator of phlorizin-induced inhibition of renal glucose reabsorption. To identify potential underpinning mechanisms of action that exist *in vivo*, the capacity of the same AE to inhibit glucose transport was studied using Caco-2/TC7 cells as an *in vitro* model of the human enterocyte.

2. Materials and Methods

2.1 Human study

2.1.1 Participants

Ethical approval was received from King's College London (KCL) ethics committee (REC reference: BDM 16/17-3762). The trial was carried out in accordance with the principles outlined in the Declaration of Helsinki of 1975 as revised in 2013 and registered at ClinicalTrials.gov (NCT02940249). Healthy participants were recruited from KCL and the general public in London and surrounding counties. An advertisement was placed in the London Metro/Evening Standard, emails circulated within KCL, postings on social

networking websites, and poster advertising at KCL. Using data from our previous study [23], it was calculated that a sample size of 30 subjects has 80 % power to detect a difference between average mean iAUC values (0-30 min) of 10 mmol glucose /L.min between test drinks with a significance level of α=0.01 (two-tailed), calculated from a 16 mmol/L.min standard deviation (SD) of the mean difference (Castro-Acosta et al., 2017). The previous study by our group [23] found a mean difference between 1.8 g AE and control (no AE) of 25 mmol glucose/L.min; our study was statistically powered to detect any differences following lower doses that are 40 % of effect size. For the acetaminophen sub-group, a sample size of 6 had 80 % power to detect a difference between the C_{max} of test drinks of 0.054 mmol/L with a significance level of α=0.05 (two-tailed), calculated from 0.048 mmol/L SD and correlation between paired observations of 0.6.

Inclusion criteria were: healthy men and women aged 18-70 y; BMI 18-35 kg/m^2; able to understand the information sheet; willing to comply with study protocol; able to give informed written consent. Exclusion criteria were: phenylketonuria; allergy, hypersensitivity or intolerance to any foods/food ingredients and/or acetaminophen; participation in another clinical trial; those with full blood counts and liver function tests outside of the normal range; current smokers or those who gave up smoking within the last 6 months; reported history of cardiovascular disease, cancer, liver, kidney or bowel disease; fasting glucose ≥7.1 mmol/L or uncontrolled T2D; presence of gastrointestinal disorder or use of drug which is likely to alter gastrointestinal function; history of substance abuse or alcoholism; unwilling to restrict consumption of specified high polyphenol foods for 48 h before the study; weight change >3 kg in preceding 2 months and body mass index <18 or >35 kg/m2; cholesterol ≥7.5 mmol/L; fasting TAG ≥5 mmol/L; blood pressure ≥160/100 mmHg; current use of medications that may interfere with the study such as α-glucosidase inhibitors (e.g. acarbose), insulin-

sensitising drugs (e.g. metformin, thiazolidinediones), sulfonylureas, and lipid-lowering drugs; medications that may react with acetaminophen (e.g. ketoconazole, metoclopramide); current use of nutritional supplements that may interfere with the study such as higher dose vitamins/minerals (more than double the UK Reference Nutrient Intake (RNI)/US Daily Reference Intake (DRI)), B vitamins, Vitamin C, calcium, copper, chromium, iodine, iron, magnesium, manganese, phosphorus, potassium and zinc. Subjects already taking vitamin or minerals at a dose 100 % or less up to 200 % of the UK RNI, or evening primrose/algal/fish oil supplements were asked to maintain habitual intake patterns and advised not to stop taking or begin new supplements during the study.

Interested volunteers were provided with a participant information sheet. Respondents were initially interviewed over the telephone. Eligible participants were invited to a screening visit at the Metabolic Research Unit in the Diabetes & Nutritional Sciences Division, KCL. Participants arrived following an overnight fast for measurement of body mass index (BMI: weight, kg / height, m^2), waist and hip circumferences (mid-point between the lowest rib and iliac crest and widest gluteal girth, respectively), body fat percentage (Tanita™ Body Composition Analyser), supine blood pressure, liver function tests, hematology, plasma glucose and lipid profile.

Subjects who met all inclusion and exclusion criteria were randomized according to the randomization schedule created using Research Randomizer software (https://www.randomizer.org). The allocation of treatment sequence was blinded from the investigators, technicians performing analysis of blood samples and participants by a laboratory technician. Investigators and participants remained blinded until completion of the study and data analysis.

2.1.2 Study design

A randomized, controlled, double-blind, cross-over design study was used to compare 4 test drinks consumed by participants in random order, at 4 separate study visits and with at least 7 days wash-out between each visit. Test drinks consisted of a "no added sugar" fruit drink concentrate, diluted with water and with increasing doses of AE added, providing 81 kJ (19 kcal), 4 g carbohydrate and <0.1 g protein, fat and fibre (**Table 1**). Drinks contained either no AE (CON), 1.8 g AE (HIGH), 1.35 g AE (MED) or 0.9 g AE (LOW). Test drinks were formulated by the researchers. The extract was dispersed in 40 ml of a very low-polyphenol double concentrate apple and blackcurrant squash (Robinson's, United Kingdom) mixed with water. Sucrose (3 g) was added to disguise any difference in taste between drinks. Test drinks (200 ml) were matched for macronutrient and energy content; **Table 1** provides the polyphenol content of the test drinks. A gastric emptying sub-study was conducted (n=6), whereby 1.5 g crushed acetaminophen (Sainsbury's Paracetamol Caplets) was dispersed into the drink. By measuring blood acetaminophen concentrations at sequential time points, the rate of absorption of acetaminophen from the duodenum is assumed to reflect the rate of gastric emptying.

196 **Table 1.** Polyphenol composition of the test drinks

Mg per 200 ml[1]	**HIGH (SD)**	**MED**	**LOW**	**CON**[2]	**Molar %**
Individual polyphenols[3]					
Flavonols					
Quercetin	7.4 (0.59)	5.5	3.7	N	3.1
Q-3-*O*-diglucoside	0.2 (0.01)	0.1	0.1	N	0.0
Q-3-*O*-Rhamnoside	24.7 (1.33)	18.5	12.3	N	7.0
Q-3-*O*-Glucoside	50.6 (2.27)	37.9	25.3	N	14.0
Dihydrochalcones					
Phlorizin (phloretin 2-O-β-glucoside)	142.9 (6.64)	107.2	71.5	N	41.9
Phloretin	1.3 (0.08)	0.9	0.6	N	0.6
Phenolic acids					
Caffeic acid	0.9 (0.09)	0.7	0.5	N	0.7
Chlorogenic acid	17.8 (0.99)	13.4	8.9	N	6.5
Cinnamic acid	0.0 (0.00)	0.0	0.0	N	0.0
Procyanidin oligomers[4]					
Dp2	17.2 (1.67)	12.9	8.6	N	3.8
Dp3	5.8 (0.37)	4.3	2.9	N	0.8
Dp4	4.9 (0.25)	3.7	2.5	N	0.5
Dp5	2.5 (0.20)	1.9	1.3	N	0.3
Dp6	1.7 (0.31)	1.3	0.8		0.1
Epicatechin	46.9 (3.25)	35.2	23.5	N	20.7
Sum of individual analysed polyphenols	324.7	243.6	162.4	-	
Total phenolics (catechin equivalents) [5]	936	702	468	<5	

197 HIGH; 1800 mg of apple extract, MED; 1350 mg of apple extract, LOW; 900 mg of apple
198 extract, CON; 0 mg of apple extract. SD; standard deviation. N; negligible.
199 [1] All drinks contained 80.6 kJ (19 kcal), 4.1 g carbohydrate (of which 4.1 g sugars, containing
200 3 g sucrose). Per gram apple extract contained: 5 kJ (1 kcal), 0.02 g protein, 0.06 g fat, 0.17
201 g carbohydrate, 0.01 g dietary fibre, 3 mg sodium, 0.2 mg chloride, 0.27 mg calcium, 0.04
202 mg iron. Analysed at King's College London, UK.
203 [2] Estimated from Hollands *et al.* [38] for apple and blackcurrant juices from concentrate.
204 [3] Estimated from analysis of raw extract by mass spectrophotometer and HPLC methods.
205 [4] Molecular weights estimated from Ye *et al.* [39].
206 [5] Estimated from analysis of raw extract by the Folin-Ciocalteu method, not by direct
207 analysis of the drinks.

All test drinks were dispensed into identical black opaque bottles and labelled with codes by a laboratory technician; study investigators and participants were blind to the contents in each bottle. Drinks were consumed through a straw within 2 minutes. Once drunk, all bottles were shaken with an additional 100 ml of water and swallowed to ensure all contents was consumed. The first mouthful of the test drink marked T+0 (time zero). The drink was immediately followed by consumption of a mixed high-carbohydrate meal consisting of 100 g of thick slice white bread (Hovis, London, UK) spread with 43 g of apricot jam (Hartley's, Hain Daniels Group, Leeds, UK). Both high-carbohydrate foods are low in phenolics (<6 mg/100 g fresh weight). The meal was consumed within 5 minutes and provided 986 kJ (233 kcal) containing 70.9 g carbohydrate (29.8 g sugars and 41.1 g starch). The drink and meal provided a total of 75 g carbohydrate and were consumed within 7 minutes.

For premenopausal female participants, in order to avoid any influence on outcomes of cyclical reproductive hormones, study visits were not scheduled on the week before and during menses (targeting the period between follicular and luteal phases). Before the first study visit, participants completed a 7-day food diary for subsequent analysis of baseline food (Nutritics 4.3) and polyphenol (Polyphenol Explorer and USDA Databases) intakes. Two days prior to each study visit, participants were asked to avoid certain high-polyphenol foods from a list provided (Appendix A). High-fat foods (from a list provided), caffeine and alcohol along with strenuous exercise were avoided 24 h before a study visit to limit potential effects on insulin sensitivity (Appendix A). Participants were asked to consume a standard low-fat meal the evening before each visit and arrived following a 12 hour fast.

Upon arrival, participants emptied their bladder, were weighed and then rested in a supine position for 10 minutes. Blood pressure was recorded in duplicate using a calibrated automated blood pressure monitor (Omron M3, Omron Healthcare Europe B.V). A venous cannula was inserted in a vein in the antecubital fossa or a forearm vein by a trained

phlebotomist. Baseline blood samples (T-5 min) for analysis of glucose, insulin, C-peptide, NEFA, acetaminophen and GIP were collected. Glucose baseline samples were taken in duplicate (at T -10 and -5 min). The drink and test meal were consumed at T+0 min. Blood samples were taken at frequent intervals up to 4 h post-test meal (**Figure 1**). Participants were kept hydrated with water (100 ml/hour). Twenty-four hour urine samples were collected (over 2 g boric acid as a preservative), and fractionated into different collection periods of T+ 0-2.5 h, 2.5-5h, 5-12 h and 12-24 h. Collection containers were kept in opaque cool bags until sampled. **Figure 1** outlines the study visit protocol.

2.1.3. Apple extract composition

Powdered AE (Appl'In™ by DIANA Food Ltd, Antrain, France) was produced from the juice, flesh, seeds, skin and core of cider apples, with the aim of ensuring a higher phlorizin content (>5% w/w) than if the extract was derived from the juice and pulp alone. The doses of AE used in this trial was determined by matching the total amount provided in the HIGH treatment with that used in the previous study by Castro-Acosta *et al.*[23] with a 2-fold reduction for the lowest dose. **Table 1** outlines the full details of the AE polyphenol composition.

2.1.4 Blood sample analysis

Blood samples were collected at T+ 10, 20, 30, 45, 60, 75, 90, 120, 150, 180, 240 min into: fluoride/oxalate tubes (BD Vacutainer® Cat No. 368921) for glucose analysis; SST™ serum tubes (BD Vacutainer® Cat No. 367956; 367954; 366882) for insulin, C-peptide, polyphenols (T+ 120, 240 min only), acetaminophen, and NEFA analysis; and at T+ 30, 60, 90, 120, 180, 240 min into K2 EDTA tubes for GIP analysis (BD Vacutainer® Cat No. 367838). All samples were centrifuged at 1300 g, 4 °C for 15 min and plasma/serum aliquots were stored at -80 °C until biochemical analysis. Enzymatic assays on ILab 650 chemistry analyser

(Instrumentation Laboratory™, Werfen Company, UK) were used to analyse glucose (Werfen Cat No. 00018250840) and NEFA (Randox Cat No. FA115). Insulin, C-peptide, (Siemens Healthcare Diagnostics Ltd, Frimley, Surrey, UK) and GIP concentrations (Merck Millipore Corporation MA., USA) were analysed by ELISA. Acetaminophen concentrations were measured colorimetrically (Randox Laboratories Ltd., London, UK).

2.1.5. Serum polyphenol metabolites analysis

The serum polyphenol metabolites of 10 randomly selected participants were analysed. Taxifolin internal standard added (10µl of 1 mg/ml solution in methanol) was added to 250 ul serum sample. Three volumes (750 µl) of methanol was added and the sample agitated for 10 minutes at room temperature. After 2 min, the sample was centrifuged for 10 min at 15000 rpm. The supernatant (400 µl) was removed and filtered (0.2 µm) into an autosampler vial to be passed for LC-MS analysis. LC-MS analysis used targeted MRM detection for native compounds and their associated sulphate and glucuronide conjugates. Standards included phloretin, phlorizin, epicatechin, quercetin, isorhamnetin, chlorogenic acid, caffeic acid, ferulic acid and trans-cinnamic acid and were purchased from Sigma-Aldrich. Standards of quercetin 3-O-glucuronide, quercetin-3-O-sulfate and isorhamnetin-3-O-glucuronide were synthesised in-house (Quadram Institute Bioscience). MRM tracking of parent sulphates and glucuronides of phlorizin, phloretin, isorhamnetin and epicatechin (and methylated derivatives) were achieved by calculating the addition of mass of a sulphate, glucuronide and/or methyl group to the native compound. Standards were prepared using blank serum over a range of 100 to 25000 nM alongside the samples. LC-MS was performed using an Agilent 1290 UPLC coupled to a 6490 triple quadrupole mass spectrometer operated in electrospray mode. Chromatographic separation was achieved using a Waters HSS T3 100 x 2.1mm, 1.7µm column at 35°C. Flow rate was 400 µl/min and injection volume was 2 µl. The

binary gradient used solvent A (Water + 0.1% v/v formic acid) and solvent B (Acetonitrile + 0.1% v/v formic acid) with a gradient of 25% B at injection to 0.3 min, 30% B at 2 min, then 95% B at 8 min before returning to initial conditions for 3 min with an overall run time of 11 min.

2.1.6. Urine sample analysis

Urine samples were collected over 0.2 g, 0.2 g, 0.6 g and 1 g boric acid (Sigma-Aldrich) for collection periods 1 (0-2.5 h), 2 (2.5-5 h), 3 (5-12 h) and 4 (12-24 h), respectively. Urine volumes were measured and all samples were centrifuged at 350 *g*, 4 °C for 15 min and supernatant stored at -80 °C until analysis. Urinary glucose and creatinine concentrations were analysed on ILab 650 chemistry analyser (Instrumentation Laboratory, Warrington, UK) using enzymatic assays (Werfen cat no. 00018250840 and 00018255540, respectively.).

2.2. *In vitro* studies

2.2.1. Caco-2 cells

The human intestinal Caco-2 cell line (TC7 subclone) was cultured as previously described ([21]). Cells were cultured at 37 °C in a humidified incubator (BIOHIT, HealthCare, UK) in a 5% CO2–95% air atmosphere in high-D-glucose (25 mM) with glutamine Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich), containing 10% fetal bovine serum (Sigma-Aldrich), 50 units penicillin and 50 µg streptomycin (Sigma-Aldrich), 0.1 mM MEM non-essential amino acids (Life Technologies), additional 2 mM L-Glutamine (Life Technologies) and 5 µg/ml Plasmocin (InvivoGen). For glucose transport assays using Caco-2 monolayers, Krebs buffer solution (KBS) containing 30 mM HEPES (Sigma Aldrich), 130 mM NaCl, 4 mM KH2PO4, 1 mM MgSO4·7H20, 1 mM CaCl2, dH2O, and adjusted pH 7.4, was used as wash media. KBS supplemented with 0.2% bovine serum albumin (Sigma

Aldrich) was used as pre-incubation and uptake media. Uptake media contained 10 mM D-glucose (BDH Laboratory Supplies) or 10 mM L-glucose (Santa Cruz Biotechnology) and 0.1 μCi/ml Glucose D-[14C(U)] or 0.1 μCi/ml Glucose L-[1-14C] (Perkin Elmer) as tracer. Times for pre-incubation and uptake in all experiments were 15 and 10 min, respectively. Cells were seeded at 8000 cell/cm^2 density in 24-well plates and cultured for 21 d; cells were used between passages 45–53 in all experiments. Before uptake started, DMEM was removed and cells were washed once with room temperature KBS. Cells were pre-incubated for 15 min with pre-incubation media; uptake was initiated by replacing pre-incubation media with uptake media. Uptake media contained, except for controls, increasing concentrations of the same AE used in the dietary intervention trial at concentrations in the physiological range estimated to be present in the small intestine after ingestion of the test drinks in human studies (Minekus et al., 2014) allowing for dilution by gastrointestinal fluids, in addition to higher and lower concentrations (concentration range 0.28-4.5 mg AE/ml). The AE was dissolved in KBS and filtered through 0.45 μm syringe filter before it was added to uptake media. After 10 min the uptake media was aspirated and the transport process was stopped by washing each well 3 times with ice cold KBS, cells were then processed for radioactivity count on a Liquid Scintillation Counter (Beckman Coulter, LS6500).

2.3. Statistical analysis

Incremental AUC was calculated by subtracting baseline values from all subsequent time-point values [40]. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) v.22 (IBM, UK). Natural logarithmic transformation was used where data were not normally distributed. Each iAUC, C_{max} and main effects of drink and drink × time interactions for the change from baseline at each time point were analysed with linear mixed effect modelling for each analyte. The models included subject as a factor (a random effect)

and fixed factors were drink (and time and drink × time interaction where appropriate) and study period. Baseline values (mean of -10 and -5 min values for plasma glucose concentrations) were used as a covariate and Bonferroni post hoc test was selected for identifying significance between two treatments. However, Dunnett's post hoc procedure was used for pairwise comparisons of treatment doses against Control in GraphPad Prism version 7.00 (GraphPad software, CA, USA) for drink and drink x time effects on change from baseline data (since SPSS does not provide Dunnett's multiple testing adjustment in the linear mixed model facility with repeated measures). The assumption of normality and homogeneity of variance was investigated. Differences in T_{max} and polyphenol metabolites between treatments were analysed by Friedman's non-parametric test with Wilcoxon post hoc test. Baseline HOMA-IR (fasting insulin (microU/L) x fasting glucose (nmol/L)/22.5) and R-QUICKI (1/(log glucose (mg/dL) + log Insulin (μU/mL) + log NEFA (mmol/L)) were calculated from mean fasting values over the four study visits. All data are represented as mean ± 95 % confidence intervals (CIs) or standard error of the mean (SEM). *In vitro* data were analysed using the software GraphPad Prism 7 (GraphPad software, CA, USA). Data were analysed by one-way analysis of variance with Dunnett's multiple comparison post hoc test and IC50 was estimated using SigmaPlot 14 for Windows (Systat Software Inc. CA. USA).

3. Results

3.1. Human study

A total of 64 healthy men and women aged 18 to 68 years attended screening sessions; 55 met all inclusion criteria. Of the 34 participants randomized to treatment, 30 completed the study. All completing participants were fully compliant with the study protocol. Details of flow of participants through study stages are shown in a Consort diagram (**Figure 2**). Baseline characteristics of participants who completed the study are shown in **Table 2**.

Table 2. Baseline characteristics of the study population

Variable[1, 2]	
Age (y)	33.9 (14.5); 18-68
Males (n=11)	29.6 (11.8); 18-52
Females (premenopausal; n=13)	27.2 (7.7); 18-43
Females (postmenopausal; n=6)	56.2 (7.1); 46-68
Sex (male to female ratio)	11:19
Body Mass Index (kg/m^2)	22.3 (3.0); 18.3-30.2
Systolic blood pressure (mmHg)	112.9 (13.4); 93-138
Diastolic blood pressure (mmHg)	72.4 (9.2); 58-96
Waist circumference (cm)	
Males	86.9 (9.0); 77.5-10.1
Females (premenopausal)	74.5 (7.5); 66.5-88
Females (postmenopausal)	80.6 (10.5); 68-94
Body fat (%)	
Males	17.8 (5.4); 11.2-28
Females (premenopausal)	25.6 (5.7); 17.2-37.5
Females (postmenopausal)	32.4 (6.1); 22.5-39.5
Fasting plasma glucose (mmol/L)[3]	4.9 (0.35); 4.2-5.7
Fasting plasma triacylglycerol (mmol/L)[4]	0.8 (0.4); 0.4-2.2
Fasting plasma total cholesterol (mmol/L)[5]	4.7 (1.0); 3.2-6.7
HOMA-IR [6]	1.2 (0.5); 0.4-2.4
R-QUICKI [6]	0.12 (0.003); 0.11-0.13
Total:HDL cholesterol[7]	3.0 (0.8); 1.8-4.6
Total energy intake (kJ/day, kcal/d)[8]	7360 (1278), 1759 (413); 4778-13464, 1142-3218
Total polyphenol intake (mg/d)[8]	755 (491); 115-1763

[1]Values are means (standard deviation); minimum-maximum. N=30.
[2]Women aged 45 y or older who reported not having had a period for 12 months or longer were defined as postmenopausal.
[3]Values are: 5.1 (0.3), 4.7 (0.3), 5.0 (0.4) mmol/L for males, premenopausal females and postmenopausal females, respectively.
[4]Values are: 1.2 (0.5), 0.6 (0.1), 0.8 (0.2) mmol/L for males, premenopausal females and postmenopausal females, respectively.

[5]Values are: 4.6 (1.0), 4.3 (0.7), 5.6 (1.1) mmol/L for males, premenopausal females and postmenopausal females, respectively.
[6]Values are means from baseline glucose, insulin and NEFA concentrations over 4 study visits.
[7]Values are: 3.6 (0.8), 2.5 (0.5), 2.9 (0.6) for males, premenopausal females and postmenopausal females, respectively.
[8]Calculated from 7-day food diary completed before first study visit.

3.1.1. Postprandial glycemia

A significant treatment x time effect (P <0.0001; **Figure 3A**) was observed for plasma glucose concentrations 0-240 min; Dunnett's post hoc comparison showed significantly lower glucose concentrations following all AE doses (HIGH, MED, LOW) compared with CON at 20 and 30 min post-drink (**Figure 3A**). All AE doses (HIGH, MED, LOW) significantly lowered plasma glucose concentrations in the early postprandial period (0-30 min) compared with CON (iAUC 0-30 min P <0.00001; **Supplementary Table 1**; **Figure 3B**) but no differences were observed over longer postprandial periods (iAUC0-120 min and iAUC0-240 min). There were significantly higher glucose concentrations following MED dose compared with CON at 45 min, and at 60 min there were significantly higher glucose concentrations following all AE doses (**Figure 3A**). The time of peak concentration was delayed following all doses (T_{max} 45 min) relative to CON (T_{max} 30 min) (P = 0.0356). None of the AE doses lowered maximal plasma glucose concentration ($C_{max;}$ **Supplementary Table 1**).

3.1.2. Postprandial insulinemia and incretin secretion

All AE drinks significantly lowered postprandial insulin concentrations compared with CON in the early postprandial period (iAUC 0–30 min; **Figure 4A** and **Supplementary Table 1**). HIGH and MED drinks significantly lowered early postprandial C-peptide concentrations (iAUC 0-30 min) compared to CON and there was a trend towards decreased iAUC0-30 min concentrations with the LOW v CON drinks (P = 0.054), as shown in **Figure 4B** and **Supplementary Table 1**. Significant

396 treatment x time effects (P = 0.001) were observed for insulin and C-peptide concentrations
397 0-240 min. Dunnett's post hoc test analysis on change from baseline data showed
398 significantly lower insulin and C-peptide responses following all AE doses compared with
399 CON at 20 min (Insulin: HIGH, MED P = 0.0001; LOW P = 0.0006; C-peptide: HIGH P =
400 0.0001; MED P = 0.0002; LOW P = 0.02) and 30 min (Insulin all P = 0.0001; C-peptide all P
401 = 0.0001) post-drink (**Figure 4**). The ratios of insulin and C-peptide iAUC 0-30 min: iAUC
402 30-90 min were significantly lower for all AE drinks compared to CON (P <0.00001). The
403 time of peak insulin concentration was delayed following all doses (T_{max} 45 min) relative to
404 CON (T_{max} 30 min) (P = 0.0001). Maximum insulin and C-peptide concentrations (C_{max}),
405 iAUC 0-120 min and iAUC 0-240 min did not differ between drinks (**Supplementary Table**
406 **1**).
407
408 The HIGH drink significantly lowered GIP concentrations compared with CON in the early
409 postprandial period (iAUC 0-30 min P = 0.018; **Figure 4C**; **Supplementary Table 1**). A
410 treatment x time effect was observed over 240 min (P = 0.037) and Dunnett's post hoc
411 pairwise comparison procedure showed the HIGH and MED drinks produced significantly
412 lower GIP concentrations at 30 and 60 min post-meal compared with CON (**Figure 4C**), but
413 C_{max} or T_{max} were not significantly different (**Supplementary Table 1**). There was no effect
414 of drink on plasma NEFA concentrations (**Supplementary Figure 1**).
415
416 **3.1.3. Gastric emptying rate by acetaminophen test**
417 There was no effect of drink on acetaminophen concentrations **(Figure 4D**; **Supplementary**
418 **Table 1**). Tests drinks did not produce differences in iAUC 0-30, 0-120, 0-240 min
419 acetaminophen concentrations and there was no treatment x time interaction observed. The
420 C_{max} and T_{max} concentrations were the same for CON and all test drinks.

421

422 **3.1.4. Postprandial serum polyphenol metabolites**

423 Five compounds were detected in serum: a phloretin glucuronide, a phloretin sulphate, an
424 epicatechin sulphate, quercetin 3-O-glucuronide and quercetin-3-O-sulfate (**Figure 5**;
425 **Supplementary Figures 2, 3**). All other target compounds were not detected or were below
426 the 100 nM threshold of detection. We anticipated the presence of (epi)-catechin glucuronides
427 in serum samples, but these were not detected, and in the absence of authentic standards of
428 epicatechin-3'-O-glucuronide and 4'-O-glucuronide we did not explore this further.
429 Significant dose-response treatment effects were observed for all detected metabolites. All
430 polyphenol metabolites were detected at higher concentrations at 120 min compared to 240
431 min, and were highly variable, with medians (IQR) after consumption of the HIGH drink of:
432 epicatechin sulfate 3172 nmol/L (2022), total phloretin 2871 nmol/L (1413) and total
433 quercetin 1313 nmol/L (1480). It is typical to see high inter-individual variation in the
434 appearance of polyphenol conjugates in human serum [37, 62]. **Figure 5** shows total
435 phloretin, total quercetin and epicatechin-sulphate plasma concentrations; at 120 min
436 phloretin glucuronide contributed 97 % to total phloretin (**Supplementary Figure 2**) whilst
437 quercetin-3-O-sulfate accounted for 98 % of total quercetin measured (**Supplementary**
438 **Figure 3**).

439

440 **3.1.5. Urinary Glucose**

441 There was no significant effect of treatment on total 24 h renal glucose output, nor during
442 defined time periods over the 24 h period, as outlined in **Figure 6** (and **Supplementary**
443 **Table 2**).

444

3.2. Caco-2 Cells

3.2.1. Caco 2 results

Dose-response assays in the presence of different concentrations of AE showed a significant inhibition of total glucose uptake down to a concentration of 1.12 mg AE per ml of uptake media ($P < 0.05$; **Figure 7**). The concentration of AE necessary to inhibit total glucose uptake by half (IC50) relative to control (no AE) was 1.19 (± 0.35) mg/ml. Doses that were estimated to correspond to physiological doses in the human study, HIGH (1.8 g AE in the 200 ml drink, 1.12 mg AE/ml) and LOW (0.9 g AE in the 200 ml drink, 0.56 mg AE/ml), inhibited total glucose uptake by 48 % and 30 %, respectively.

4. Discussion

This study aimed to establish the minimum dose of a polyphenol-rich AE delivered in a fruit drink that could significantly inhibit the rate of glucose absorption following a high-carbohydrate meal in a representative healthy population. The predominant polyphenols in the AE were the dihydrochalcone, phlorizin, followed by flavonoids such as quercetin and epicatechin. Based on published literature, phlorizin is expected to lower the rate of intestinal glucose absorption by inhibition of SGLT1 [22], phloretin and quercetin are expected to inhibit GLUT2 [41], and apple procyanidins are likely to inhibit digestive enzyme activity (e.g. pancreatic α-amylase and disaccharidases) [25,26]. In agreement with the hypothesis, all AE doses inhibited the increase in postprandial glucose, insulin, C-peptide and GIP concentrations in the first 30 minutes (iAUC 0-30 min; **Figures 3, 4; Supplementary Table 1**), clearly indicating a reduced rate of intestinal glucose absorption, with lower incretin and insulin responses in the early postprandial phase as a result. The delayed T_{max} and relatively higher plasma glucose concentrations from 45-60 min demonstrates that digestion/intestinal

absorption of starch and sucrose was deferred but not slowed, since there were no differences in C_{max}, nor total glycemic response.

A limited number of human studies [22,28,29] have shown that consuming polyphenol-rich AE alongside a sugary drink (either glucose only or mixed sugars) can attenuate early postprandial glucose response. One of these showed that ingestion of 2.8 g AE (approximately 1200 mg polyphenols) reduced the iAUC within the first 45 minutes following an oral glucose tolerance test (OGTT) relative to control [22]. It is relevant to note that intervention studies in this area have used glucose or high-glucose (glucose, sucrose and fructose) drinks for the carbohydrate challenge, which overlooks the potential inhibitory effects of apple polyphenols on digestive enzymes that break down starch and sucrose, the main forms of dietary carbohydrate in terms of contribution to energy intakes. We previously demonstrated that 1.8 g AE, consumed immediately prior to a mixed starch and sucrose meal, lowered C_{max} and inhibited the average iAUC (0-30 min) by 54 %, relative to matched placebo [23]. The present study has extended this finding by demonstrating a 34 % reduction in iAUC (0-30 min) at half this dose. Given that the lowest AE dose of total polyphenols was equivalent to consuming approximately 1-2 whole eating apples this amount could be achievable on a regular basis for most individuals. However, whole apples do not contain such a high proportion of phlorizin and it would require consumption of 14 apples to consume the amount of phlorizin provided by the lowest AE dose [42,43]. Furthermore, whole apples contain the soluble fibre pectin so direct comparisons of amounts of total polyphenols delivered are not very meaningful.

None of the doses exerted any effect on total glycemic responses over 2 or 4 h. The similarity in the magnitude of total postprandial glycemia between AE doses and control drinks despite

early phase reductions is explained by the higher glucose concentrations at 45-60 min following AE. This was not offset by a proportionate increase in insulin secretion at 45-60 min, as evidenced by the lack of significant difference in C-peptide concentrations between treatments. Previous research has shown that GIP secretion is dose-dependently related to the rate of duodenal glucose perfusion [44], and may be a more sensitive indicator of glucose absorption rates than plasma glucose concentrations [45], which are the sum effect of absorption, tissue uptake and hepatic output. Previous studies have consistently shown that GIP is reduced by polyphenol consumption during a carbohydrate challenge [23,28,46,47]. Hence, lower plasma GIP concentrations 30-60 min may be linked to the lack of augmentation in insulin secretion (C-peptide concentrations) in response to later peak glucose concentrations after AE treatment. Despite the lack of effect of the AE on total glycemic response (0-2 h and 0-4 h), the slower rate of glucose absorption and consequently insulin concentrations in the early postprandial phase could suggest a potential physiological benefit in at-risk individuals such as prediabetecs, due to a reduced first-phase insulin secretory demand on beta-cells. However, this would need to be confirmed by future robust RCTs.

This study was not designed to investigate potential effects of polyphenols on beta cell function, but it is worth noting that there is another plausible mechanism whereby AE might have acute effects on insulin secretory responses, other than directly through reducing the rate of intestinal glucose absorption. Phlorizin is mostly hydrolysed to its aglycone form, phloretin, in the intestinal lumen, which is partially absorbed along with phloretin conjugates [48–50]. Our bioavailability data showed that after consumption of the AE drinks there were circulating concentrations of phloretin glucuronide and phloretin sulphate, but no detection of phlorizin. Phloretin, at non-physiological doses (24-40 µM), inhibits GLUT transporters that mediate glucose transport into pancreatic β-cells *in vitro,* and reduces glucose-dependent

insulin secretion [51]. However, in the current study, if there were any inhibitory effects of circulating phloretin metabolites (maximum serum concentration was ~6 µmol/L total phloretin) on GLUT-mediated glucose transport, then they were clearly minor effects, without any clinically meaningful effect on either circulating insulin concentrations, nor systemic release of reabsorbed glucose in the kidney as discussed further on.

The lack of inhibitory effect of the HIGH AE dose on glucose C_{max} was unexpected as this contradicts our previous findings at an equivalent dose [23]. Although the age range was similar between these RCTs, premenopausal women were included in the current study but not the previous one [22], which may partially account for this inconsistency since premenopausal women have a greater degree of insulin sensitivity than similar aged men [52] and postmenopausal women [53]. The AE administered in the previous study was from a batch derived from the same geographical sources and technology, although there may be differences in composition due to seasonal variations. Cmax is of relevance for the health effects of postprandial glycemic response as frequent large swings in glucose levels, oscillating from high peaks to low nadirs, may be deleterious to vascular and pancreatic beta-cell function leading to increased risk of T2D and cardiovascular disease [3,54–56], and risk of complications in T2D patients [57,58]. Marked upsurges in postprandial glucose concentrations, can increase oxidative stress in normoglycemic populations [59]. Hyperglycemic induction of excessive oxidative stress is a key factor in the increased risk of disease progression [60], and risk of complications in T2D patients [57]. In this healthy cohort, all individual C_{max} results fall within the normal range (< 7.8 mmol/L; WHO, 2006), thus there was little scope for lowering C_{max} by acute dietary modification. These findings cannot be extrapolated to at-risk groups and it is important to repeat the current study design in a sample population with impaired glucose tolerance, in order to determine whether low doses of AE can reduce C_{max} if postprandial hyperglycemia is present.

Inhibition of carbohydrate digestive enzymes (e.g. alpha-amylase, alpha-glucosidase) and glucose transporters (e.g. SGLT1/GLUT2) are the most likely mechanisms to explain the glucoregulatory properties of fruit polyphenols. Our *in vitro* data supports the theory that the AE in the present human study may have exerted an inhibitory effect on small intestinal glucose transport, since the same AE dose-dependently inhibited total glucose uptake into Caco-2 cells, a well-established model of the human enterocyte. The IC50 (i.e. the AE concentration necessary to inhibit total glucose uptake by half) was calculated to be 1.2 mg/ml, demonstrating the inhibitory potency of the AE. This is approximately equivalent to the estimated physiological HIGH dose used in the human study which inhibited iAUC 0-30 min plasma glucose by 58 % compared to CON. Furthermore, a dose corresponding to the estimated physiological LOW dose that lowered iAUC 0-30 min by 34% in the human study, inhibited total glucose uptake in Caco-2 cells by approximately 30 % relative to control. The similarity in the magnitude of inhibition suggests that modulation of glucose transporters are likely to be a key mechanism for the glucoregulatory effects of this apple extract. However, caution is needed when directly comparing the size of effect between an intestinal cell model and the intact human gastrointestinal tract, particularly as *in vivo* glucose has to be liberated from starch and sucrose by digestive enzymes, which can also be inhibited by polyphenols found in apples [26,61–64].

A further plausible mechanism to explain the glucoregulatory effects of apple polyphenols lies in the modulation of gastric motility. We have presented novel data that suggests that the effects of apple polyphenols on postprandial glycemia cannot be explained by delayed gastric emptying, since there were no differences in acetaminophen kinetics following the test drinks containing acetaminophen, a simple but effective gastric emptying test when using low-fat

meals in liquid form. However, more robust methodology using MRI or ultrasound technology is required to confirm these results.

Previously, Schulze *et al*. [22] reported that 2.8 g of apple extract containing 448 mg phlorizin increased urinary glucose excretion in the first 3 h after administration. This dose of phlorizin was just over 3 times higher than the amount administered in the HIGH dose in the current study. The limited bioavailability of phlorizin prevents its use therapeutically as an SGLT2 inhibitor drug for inhibiting renal glucose reabsorption and lowering blood glucose concentrations in the treatment of T2D [30]. We have provided original data on detectable apple polyphenol metabolites after the consumption of a cider apple-derived AE. The polyphenol profile of the AE was reflected in the metabolites detected in serum following consumption, although phlorizin and surprisingly epicatechin glucuronides were not detected as previously reported [22,65]. It is possible for a very small proportion of intact phlorizin to be absorbed through the intestine [30], and therefore consumption of high doses could lead to higher circulating concentrations which may potentially inhibit SGLT1 and SGLT2 in the proximal tubule [66,67]. Increases in circulating phloretin might also inhibit GLUT2-mediated glucose transport in the kidney. However, we have shown that doses of phlorizin up to 143 mg did not significantly increase urinary glucose excretion at any time over 24 h post-administration in our study population.

Strengths of this study include the robust trial design with strict adherence to double blinding along with the control drink being fully matched to the test drinks for taste, appearance and nutrient composition. The mixed-carbohydrate test meal allowed us to examine the effects of AE on commonly consumed high-glycemic foods in the diet, delivering doses of polyphenols that could be achievable through diet. Furthermore, the trial was carried out in a broad cross-

section of healthy men and women, including premenopausal females, and the results are therefore applicable to a large section of the general population. However, the disadvantage of selecting a broad sample population is that it introduces a greater degree of heterogeneity in glycemic responses to AE; a larger sample size would have allowed further analysis by sub-populations (e.g. age or sex) to explore determinants of inhibition of postprandial glycemia. A final strength of the study is that the baseline serum polyphenol metabolite data showed very good subject compliance with the low- polyphenol dietary advice prior to study days. We used a polyphenol-rich extract, as opposed to whole fruit, thereby avoiding confounding effects of other apple constituents such as pectin. However, this approach is limited by restricted translatability to real foods, i.e. when polyphenols are encapsulated within plant cell walls and consumed in combination with other bioactive components. Indeed, due to analytical constraints it is very difficult to quantify all components of fruit polyphenol extracts and there may be other bioactive components that were unaccounted for. Therefore, it is important to recognise that research needs to characterize the behaviour of polyphenols within the food matrix before we can extrapolate findings to whole fruits. In fact, the efficacy of polyphenol-rich extracts, such as the AE, when consumed as part of a regular mixed-macronutrient meal should also be investigated, as both fat and protein can delay gastric emptying and interact with other food constituents, which may influence the outcome.

In conclusion, a low dose of AE moderately delayed postprandial glucose absorption in a predominantly normoglycemic study population. However, there were no differences in maximal glucose concentrations nor the total glycemic response. Furthermore, it has been demonstrated that gastric emptying rate was unaffected by addition of polyphenols to a drink, and there were no significant effects on urinary glucose excretion. Since this study and

previous work has presented strong evidence that addition of polyphenols to a drink can delay the glycemic response to a starch and sucrose meal, further clinical investigation trials are justified to determine whether low doses of AE can lower total postprandial glycemia in populations with impaired glucose tolerence/prediabetes. Preventing sharp exaggerated glucose peaks via consumption of fruit polyphenols could help reduce progression to T2D and cardiovascular injury. Lastly, these results demonstrate that an apple extract has mild glucoregulatory properties, providing further support for dietary guidelines that encourage consumption of fruits and vegetables, alongside wholegrains, nuts and seeds as other rich sources of polyphenols and fibre. Since apples and pears contribute 4-12 % and 5% of total polyphenol intake in Europe [16] and USA populations [15] respectively, then the public health impact of encouraging apple consumption in at-risk populations could be significant.

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Author contributions

EJP was involved in the design of all experiments, conducted the clinical trial and in vitro research, collected and analysed data, performed the statistical analysis and wrote the manuscript. WLH was involved in the design of the clinical trial, participated in the

interpretation of data and writing and revision of the manuscript. CPC was involved in the design of in vitro experiments, analysis and provided writing advice. BA, BB, EL, PP, YH performed the clinical trial research, collected and analyzed data. PK and MP were responsible for polyphenol metabolite and extract polyphenol analysis and edited the manuscript. All authors read and approved the final draft. All of the authors declared they had no conflicts of interest.

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Conflict of interest

None declared.

Appendix A.

Supplementary data.

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Figure 1. Study visit protocol

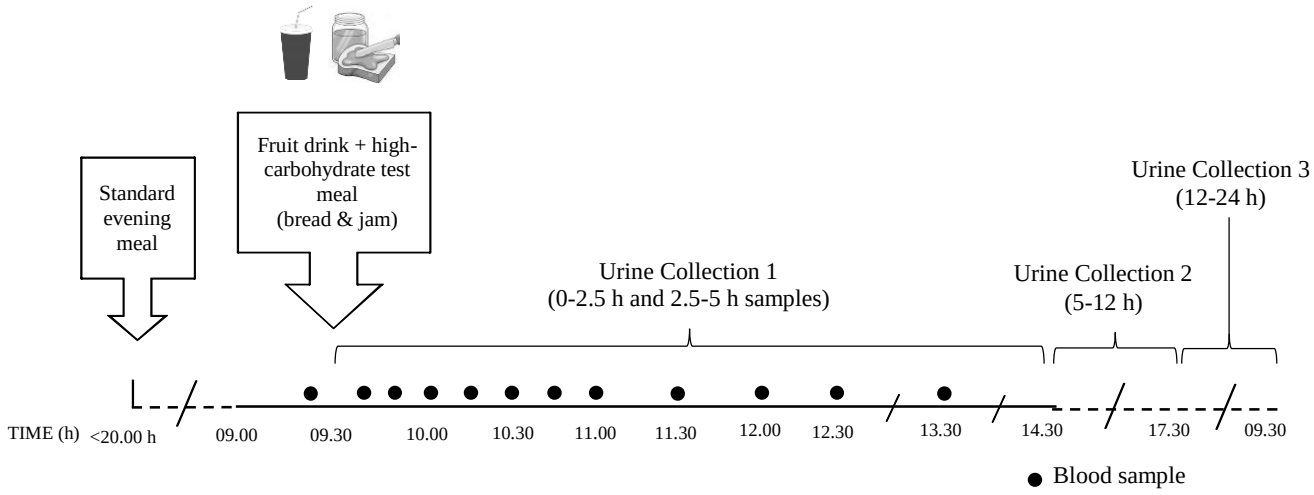

Figure 1. Study visit protocol.

Figure 2. Consort diagram

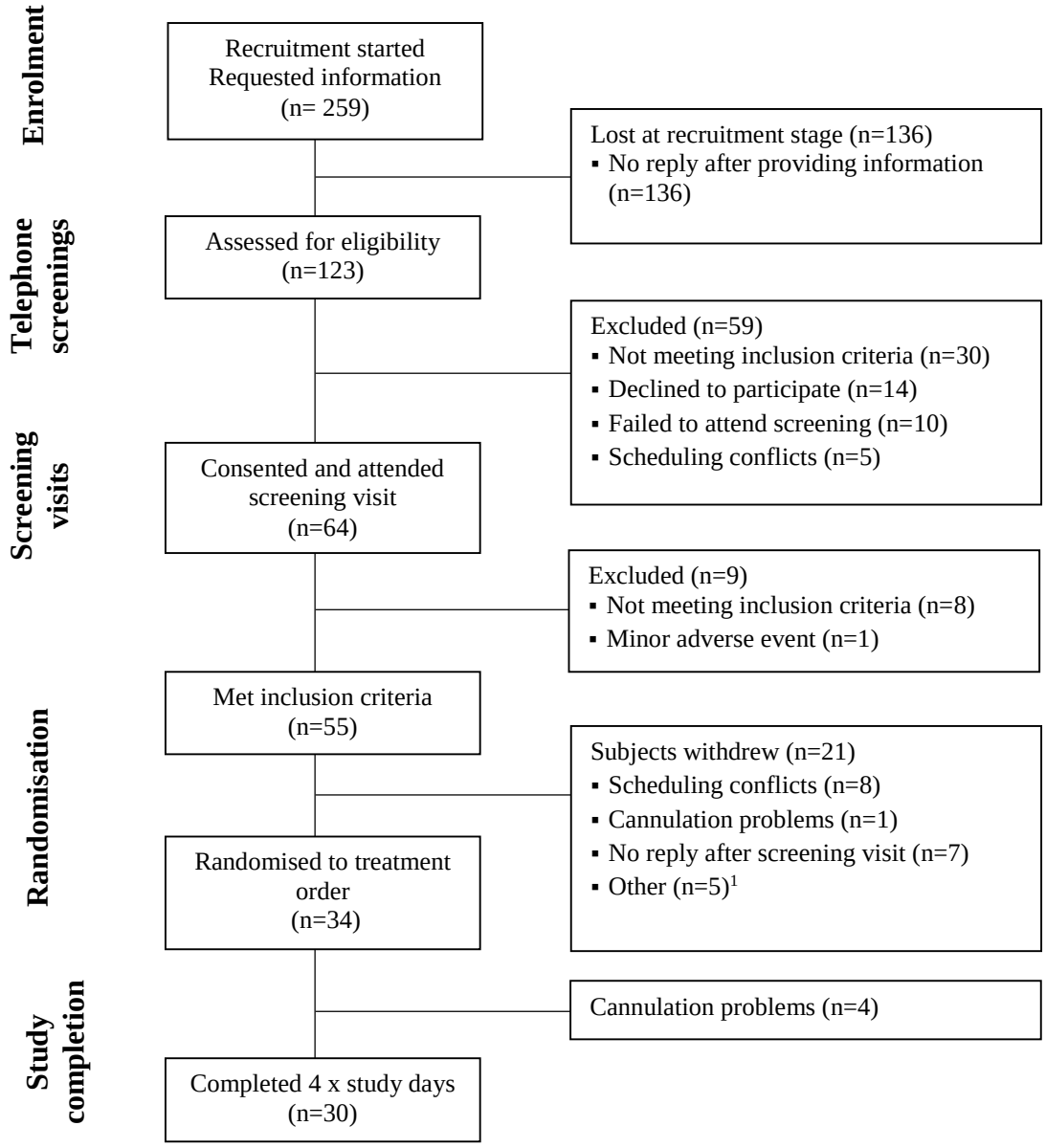

Figure 2. Consort diagram.

[1]Failed to attend first visit (n=3); BMI fallen below inclusion criteria (n=1); insufficient remuneration (n=1).

Figure 3. Plasma glucose concentrations

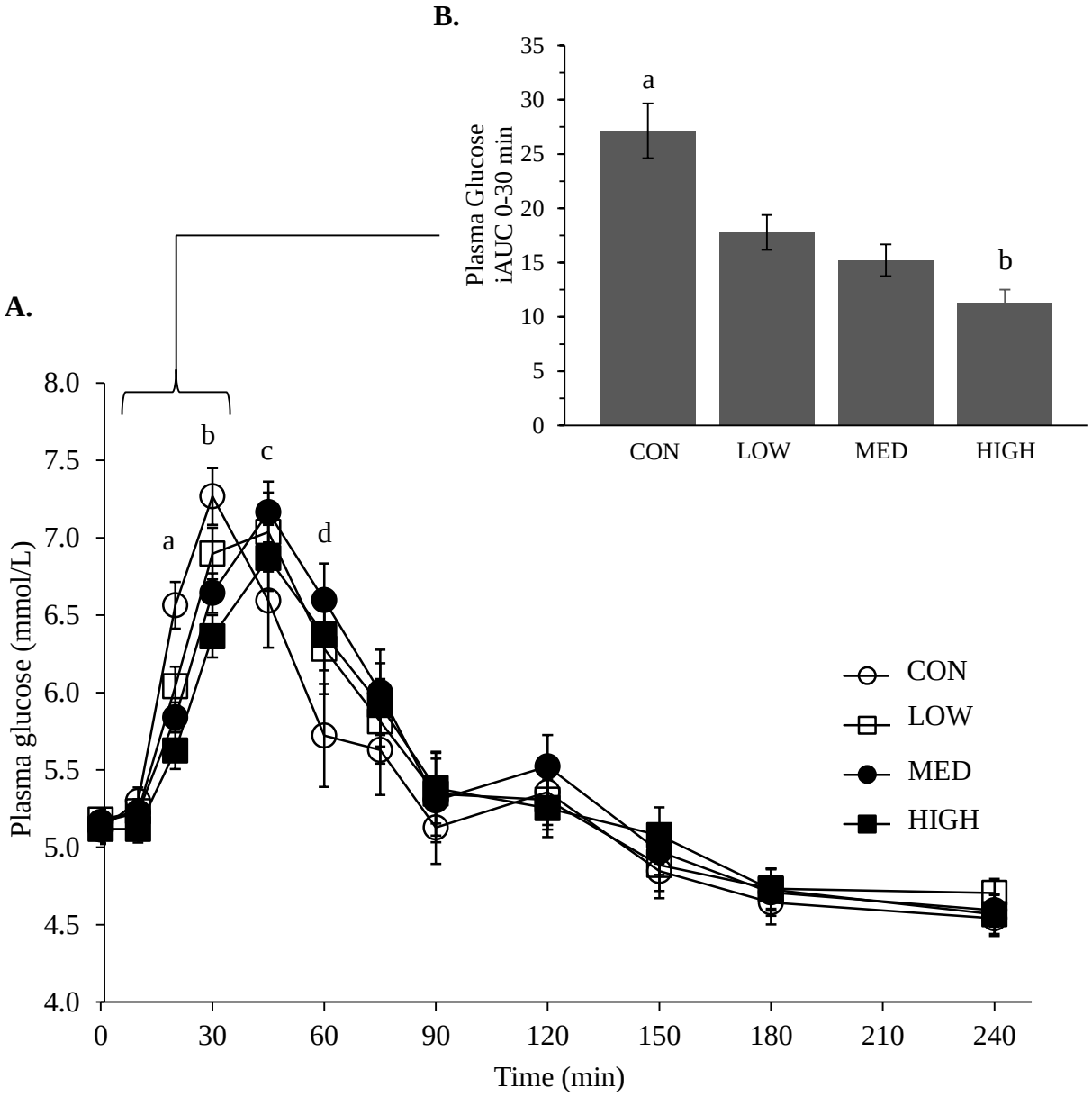

Figure 3. A). Plasma glucose concentrations after consumption of high (1.8 g), medium (1.2 g), low (0.9 g) doses of apple extract (AE) and control drinks from 0-240 min in the study population. Linear mixed modelling analysis: P <0.0005 for an overall treatment effect on glucose concentrations 10-240 min, adjusted for baseline concentrations. Post hoc analysis of timepoint differences in change from baseline in glucose compared to CON with Dunnett's adjustment: [a] P <0.0005 for difference between HIGH and CON, and MED and CON, and P <0.005 for difference between LOW and CON; [b] P <0.0005 for difference between HIGH

and CON, and MED and CON, and P <0.05 for difference between LOW and CON; [c] P <0.0005 for difference between MED and CON; [d] P <0.0005, P <0.0005 and P = 0.06 for difference between HIGH and CON, MED and CON, and LOW and CON, respectively **B).** Incremental area under the curve 0-30 min plasma glucose following consumption of high (1.8 g), medium (1.2 g), low (0.9 g) doses of apple extract and control drinks in the study population. Linear mixed model analysis: P <0.000005 for difference in iAUC 0-30 min between apple extract drinks and CON; [a] Bonferroni post hoc test for difference in iAUC compared to CON: P <0.00005 (HIGH), P <0.005 (MED), P <0.005 (LOW). [b] Bonferroni post hoc test for difference between HIGH and LOW drinks: P <0.05. N=30. Mean ± SEM.

Figure 4. Insulin C-peptide GIP and acetaminophen

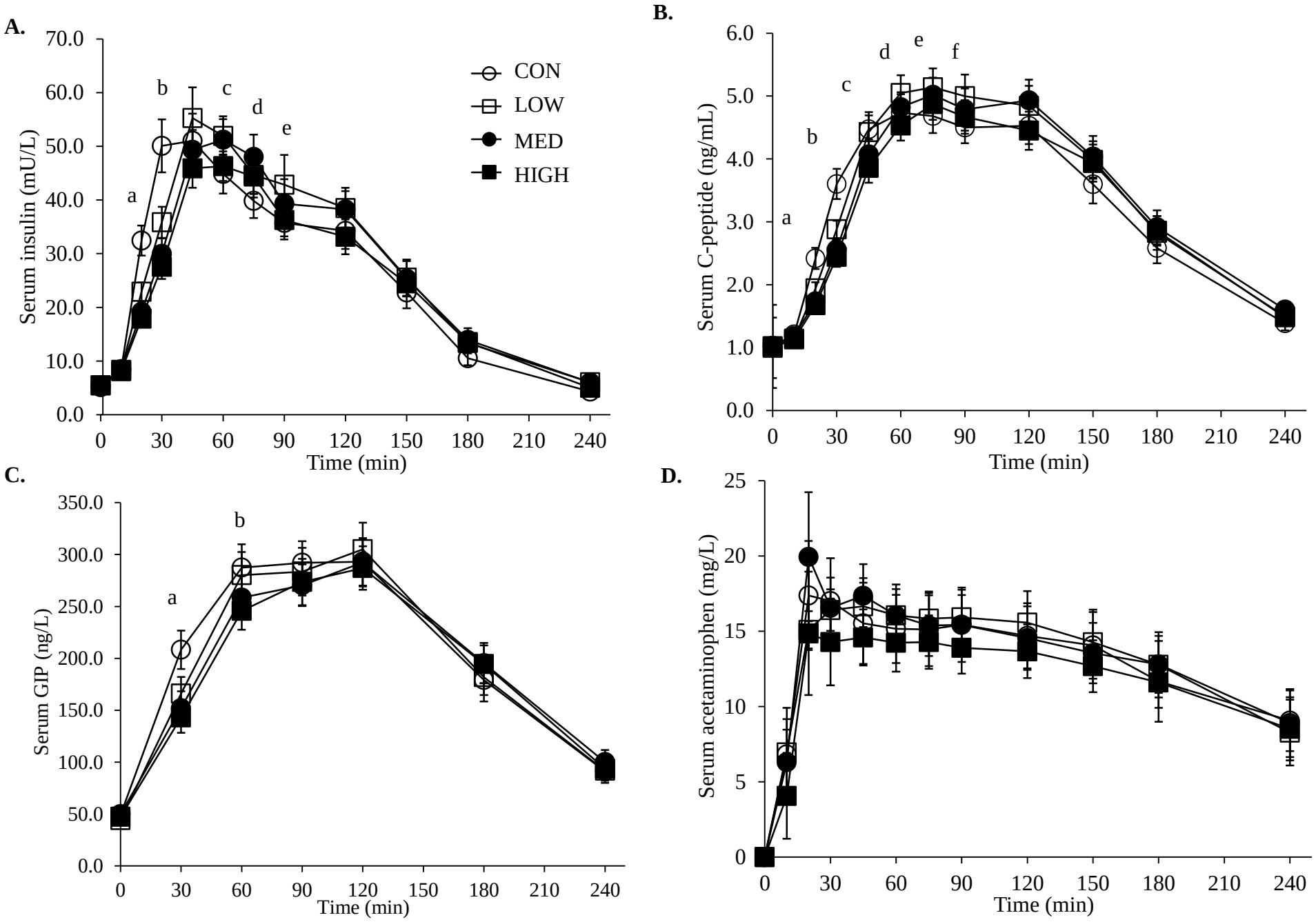

Figure 4. Plasma concentrations of **A).** insulin **B).** C-peptide **C).** Glucose-dependent insulinotropic polypeptide (GIP) **D).** acetaminophen 0-240 min following consumption of high (1.8 g), medium (1.2 g), low (0.9 g) doses of apple extract (AE) and control drinks. Linear mixed modelling analysis: P <0.0001 (**A+B**), P <0.05 (**C**) for an overall drink effect on raw values from baseline to 240 min. Post hoc analysis of timepoint differences in change from baseline compared to CON with Dunnett's adjustment: (A) : [a] P <0.0005 for difference with HIGH and MED; P <0.01 for difference with LOW. [b] P <0.0005 for difference with all drinks. [c] P <0.05 for difference with LOW. [d] P <0.005 for difference with MED; (B) [a] P <0.0005, P <0.005 and P <0.05 for difference with HIGH, MED and LOW, respectively. [b] P <0.0005 for difference with all drinks. [c] P <0.005 for difference with HIGH; P <0.05 for difference with MED. [d] P <0.05 for difference with LOW. [e] P <0.01 for difference with LOW. [f] P <0.005 for difference with MED; P <0.05 for difference with LOW; (C) [a] P <0.0005 for difference with HIGH and MED; P <0.005 for difference with LOW. [b] P <0.0005 for difference with HIGH; P <0.05 for difference with MED. N=30. Mean ± SEM.

Figure 5. Serum polyphenols V2

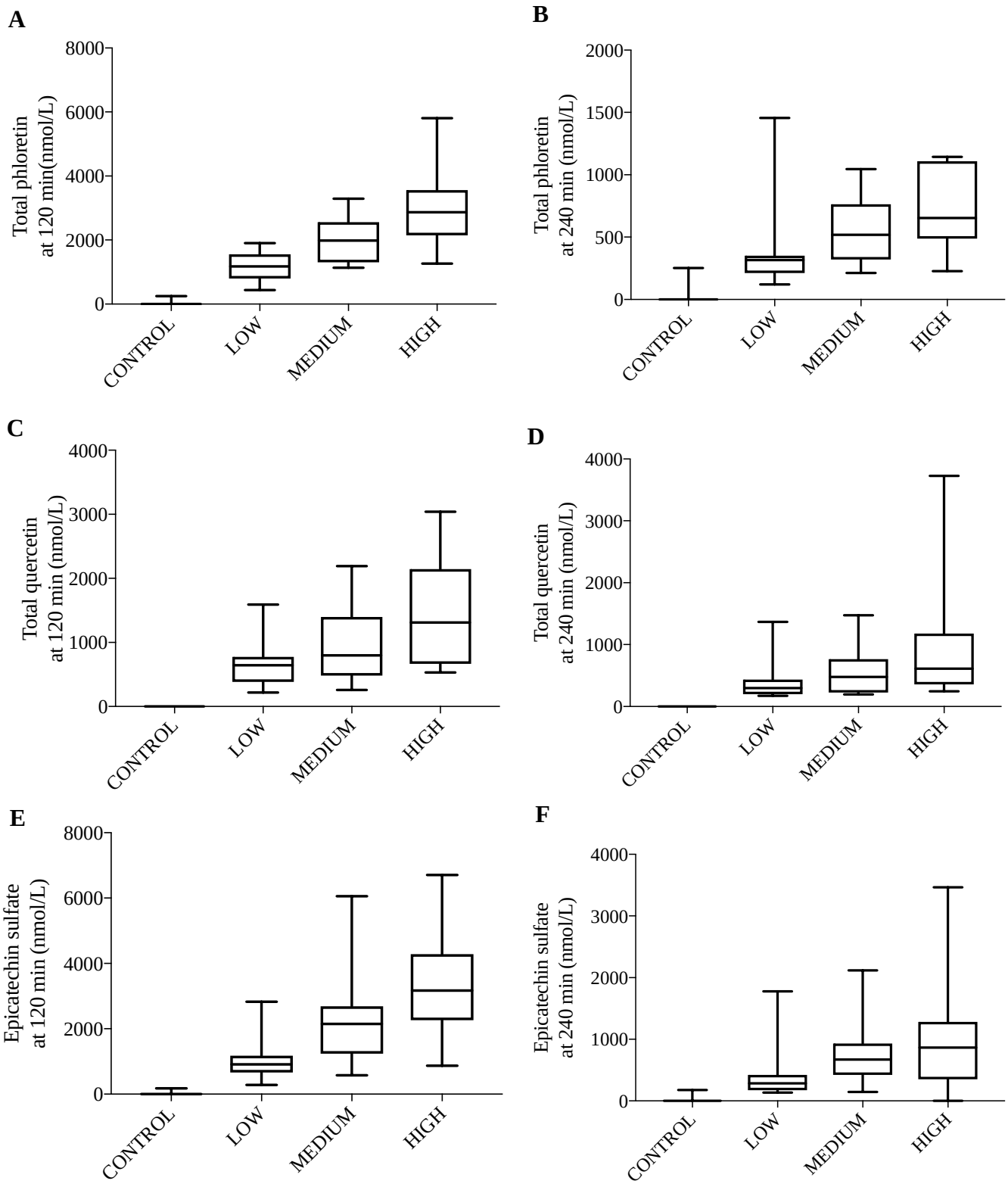

Figure 5. Serum total phloretin (sum of phloretin glucuronide and phloretin sulfate metabolites), total quercetin (sum of quercetin glucuronide and quercetin sulfate metabolites) and epicatechin sulfate metabolites concentrations after consumption of High, Medium, Low doses of apple extract and Control drinks at 120 min and 240 min in a randomly selected sub-group of the study population. N=10. High, 1800 mg apple extract. MED, 1200 mg apple extract. LOW, 900 mg apple extract. Boxes represent median (centre line) and interquartile range. Whiskers are maximum and minimum values. Serum total phloretin concentrations at 120 min (**A**), 240 min (**B**). Serum total quercetin concentrations at 120 min (**C**), 240 min (**D**). Serum epicatechin sulfate concentrations at 120 min (**E**), 240 min (**F**). Significant treatment effects were observed on serum concentrations of total phloretin, total quercetin and epicatechin sulfate at 120 and 240 min by Friedman's non-parametric test (all P <0.0001).

Figure 6. Urinary glucose

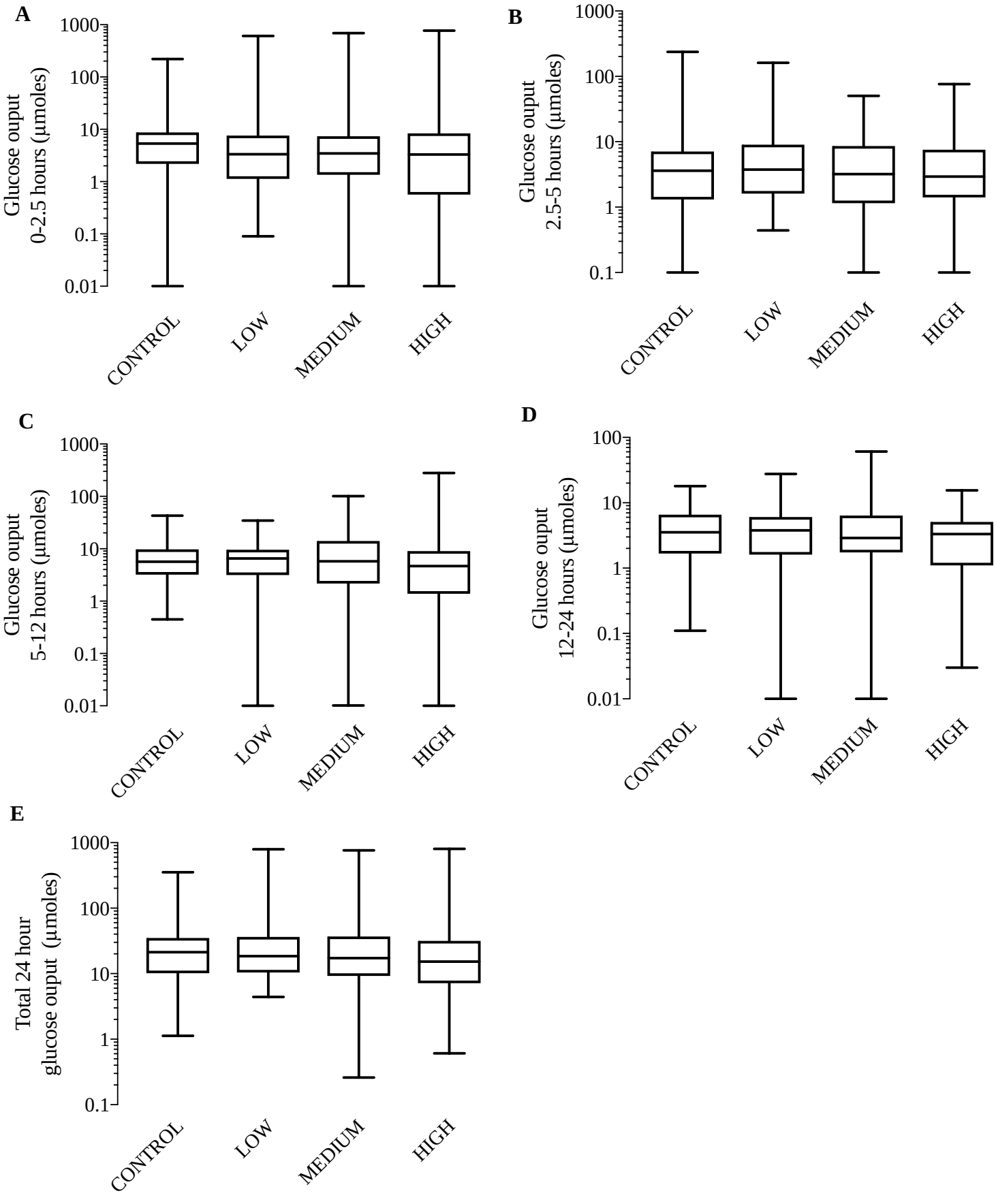

Figure 6. Urinary glucose output after consumption of high (1.8 g), medium (1.2 g), low (0.9 g) doses of apple extract (AE) and control drinks from 0-2.5 hours (**A**), 2.5-5 hours (**B**), 5-12 hours (**C**), 12-24 hours (**D**) and 0-24 hours (**E**). Y axis is on a Log10 scale. Boxes represent median and interquartile range. Whiskers are minimum and maximum values. N=30. Statistics calculated using linear mixed effect modelling with natural log data. No effect of drink on urinary glucose output.

Figure 7. Caco2 glucose uptake

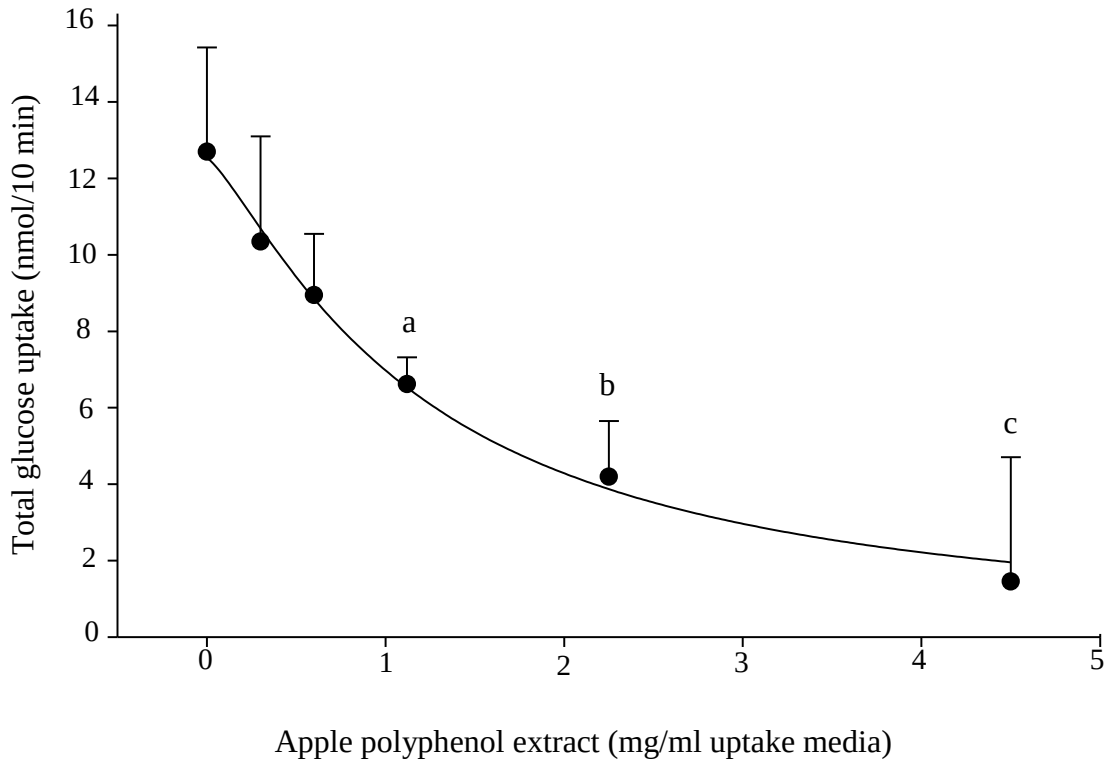

Figure 7. Acute effects of increasing concentrations of Apple Extract on total glucose uptake. Caco-2/TC7 cells were treated with increasing concentrations of apple extract contained in uptake media, except for Control (0 mg/ml). Values were corrected for simple diffusion by subtracting L-glucose uptake. Data are presented as mean + SEM (N=6). **A)** Total glucose uptake: one-way analysis of variance followed by multiple comparisons against Control (0 mg/ml) with Dunnett's adjustment: $P = 0.0006$ for overall treatment effect; [a] $P = 0.0252$ v 1.12 mg/ml; [b] $P = 0.0026$ v 2.25 mg/ml; [c] $P = 0.0003$ v 4.5 mg/ml. IC50 (1.19 ± 0.348 mg/ml apple extract): concentration of AE necessary to inhibit total glucose uptake by half relative to control, estimated using SigmaPlot 14 for Windows (Systat Software Inc. CA. USA).

Emily Prpa: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization. ***Wendy Hall***: Conceptualization, Methodology, Formal analysis, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. **Christopher Corpe**: Methodology, Resources, Writing – Review & Editing, Supervision. **Ben Atkinson**: Investigation, Writing – Review & Editing. **Brittany Blackstone**: Investigation, Writing – Review & Editing. **Elizabeth Leftley**: Investigation, Writing – Review & Editing. **Priya Parekh**: Investigation, Writing – Review & Editing. **Paul Kroon**: Methodology, Resources, Writing – Review & Editing. **Mark Philo:** Methodology, Investigation, Writing – Review & Editing.

Online supplementary material