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Effect of age and sex on the urinary elimination of a single dose of mixed flavonoids: results from a single-arm intervention in healthy UK adults

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1 **Effect of age and sex on the urinary elimination of a single dose of mixed flavonoids:**  
2 **results from a single-arm intervention in healthy UK adults**

3

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27 **Running Head:** Age, sex and flavonoid absorption and metabolism

28

29 **Abbreviations:** absorption, distribution, metabolism, and elimination (ADME); maximum  
30 urine excretion ( $C_{max}$ ); randomized control trials (RCTs); time of maximum urine excretion  
31 ( $T_{max}$ ); total amount excreted in urine over 24 h ( $A_{eu}$  0-24)

32

### 33 **ABSTRACT**

34 **Background:** Nutrition intervention trials demonstrate that increased flavonoid intake can  
35 have clinically meaningful impacts on disease outcomes/biomarkers; however, high  
36 variability in absorption and metabolism and large heterogeneity in biochemical and  
37 physiological responses are observed. The etiology of this variability is poorly understood.

38 **Objective:** To explore the relationships between sex, age, and microbiota speciation on  
39 mixed flavonoid elimination over 24 hour (h).

40 **Methods:** Healthy males and **females** (n=163) prospectively recruited on the basis of age  
41 (18-30y or 65-77 y) and sex, consumed a standardized flavonoid-rich test meal providing  
42 640mg of cocoa/chocolate flavan-3-ols, 340mg of citrus flavanones, and 390mg of  
43 blackberry anthocyanins. Urinary samples collected at baseline (-24 h to 0 h), 0-3.5 h, >3.5h-  
44 7 h and >7-24 h were analysed for flavonoids and their metabolites by Ultra high-  
45 performance liquid chromatography-mass spectrometry (UPLC-MS/MS). Stool microbiome  
46 speciation was determined via Illumina sequencing. Linear mixed-effect models were used to  
47 assess differences in cumulative excretion across age and sex with time-by-group interaction  
48 taken as the principal analysis of effect.

49 **Results:** There were no group (older females, older males, younger females, younger males)  
50 differences in total 24 h urinary metabolite recovery, but there was a trend towards a higher  
51 rate of cumulative recovery in older males at 24h ( $\beta$  (95% CI) -61.4 (-107, -16.0 younger  
52 males compare to older males; P-group at 24hr=0.06). Of 76 metabolites, 20 had  
53 significantly different Tmax by age and 9 by sex, with a later mean Tmax observed for older  
54 participants (92% of instances). Associations with age were not mediated by BMI or  
55 microbiome speciation. Significant differences in Cmax by sex were observed for only 6  
56 metabolites and differences by age for 5 metabolites.

57 **Conclusion:** Total elimination recovery of (poly)phenols was relatively consistent across age  
58 and sex groups, while elimination kinetics differed substantially; possibly resulting from  
59 differences in intestinal transit time or kidney clearance. Assuming (poly)phenol metabolites  
60 have varying biological activities, establishing dose response relationships, and defining  
61 metabolite profiles in population subgroups is required to inform the future development of  
62 dietary flavonoid/(poly)phenol recommendations.

63 **Clinical Trial Registry:** NCT01922869

64 **Keywords:** absorption, metabolism, excretion, flavonoids, polyphenols, genes, gut-  
65 microflora

## 67 INTRODUCTION

68 Flavonoids are a diverse group of dietary phytochemicals which are structurally subclassified  
69 into anthocyanins, flavonols, flavones, flavanones, flavan-3-ols, and isoflavones, and  
70 includes their oligomeric (i.e., flavonoids) and polymeric (i.e., proanthocyanidins) forms(1,  
71 2). Their bioactivity is in part responsible for the health benefits of fruits and vegetables, and  
72 other plant-based foods, such as tea, fruit juice, wine, herbs and spices(3, 4). In prospective  
73 cohort studies, a high flavonoid intake has been associated with a 10-40% reduction in all-

74 cause mortality, dementia incidence and cognitive decline, cardiovascular events, and risk of  
75 specific cancers and a lower body weight(5-9). An accumulating number of randomised  
76 control trials (RCTs) are demonstrating, at a group level, clinically meaningful impacts of  
77 increased flavonoid intake on chronic disease biomarkers, providing insight into the  
78 underlying mechanisms of action at play(5, 6, 8, 10). The dose-response relationships  
79 between flavonoid consumption and phenotype are however, highly variable, with large  
80 heterogeneity in the concentration of flavonoid metabolites in biological samples following  
81 intake of a given dose, as well as the biochemical and physiological responses observed(1, 8,  
82 10-13). The molecular and physiological basis for this heterogeneity in dietary flavonoid  
83 metabolism and responsiveness observed across studies is poorly understood.

84 As reviewed, although a small number of existing studies have investigated potential  
85 modulators of (poly)phenol metabolism and elimination, most have had a limited number of  
86 participants, with heterogeneity in response a secondary *post hoc* rather *a priori* aim, which  
87 often results in a poorly powered analysis(14, 15).

88 Once ingested, the absorption, distribution, metabolism, elimination (ADME) of flavonoids is  
89 influenced by human intestinal, liver and kidney functions, including phase 1 and phase 2  
90 metabolising enzymes, many of which are also important in the metabolism of xenobiotics  
91 and drugs(1, 4). Although not well characterised for flavonoid metabolism, there is emerging  
92 evidence to suggest that the functionality of these enzymes may be influenced by such  
93 physiological variables as sex and age(16-18). Furthermore, a substantial fraction of  
94 flavonoid ADME is impacted by gut microbial metabolism, and flavonoid intake has also  
95 been shown to influence microbiota speciation(19, 20). However, the role of gut microbiota  
96 functional and metabolic diversity in flavonoid metabolism and bioavailability is still poorly  
97 understood(1, 21).

98

99 Here, using a prospective recruitment approach we conducted the first systematic  
100 investigation of the impact of sex, age and microbiota speciation on flavonoid elimination  
101 over 24 h following the consumption of a standardized cocoa/chocolate flavan-3-ol, citrus  
102 flavanone, and blackberry anthocyanin rich test meal. Such establishment of metabolism and  
103 elimination kinetics in population subgroups are needed to inform and refine the development  
104 of dietary flavonoid recommendations(22).

105

## 106 **METHODS**

107 Study participants. The acute chocolate, orange/citrus, blackberry intervention study (COB  
108 study) recruited prospectively on the basis of sex and age and aimed to include equal  
109 numbers of males and females and those aged between 18-30y or 65-77y who were generally  
110 healthy. Exclusion criteria were: BMI < 18.5 kg/m<sup>2</sup> or > 30 kg/m<sup>2</sup>; smokers or nicotine users;  
111 hypertension with either a systolic blood pressure >140mmHg or a diastolic blood pressure  
112 >90mmHg; a medical condition or significant past medical history likely to affect study  
113 measurements e.g., diagnosed type 2 diabetes, cardiovascular, renal, liver, thyroid or  
114 gastrointestinal diseases; vaccinations or antibiotics use in the previous 3 months, (as these  
115 will impact on the gut microflora composition and metabolism and also phase 1 and 2  
116 metabolism); blood biochemistry (including alanine aminotransferase (ALT; >0-40IU/L),  
117 alkaline phosphatase (ALP; >30-130 IU/L), bilirubin, albumin, urea and creatinine) outside  
118 the normal ranges; prescribed medication use that could interact with the enzymes involved  
119 in the metabolism of flavonoids (Supplementary Table S1); taking flavonoid containing  
120 supplements or other dietary supplements for one month prior to the study; known allergies to  
121 the intervention foods; consume more than 21 alcohol unit/week for males, or 14 units/week  
122 for females; pregnant, lactating, or planning a pregnancy (or having fertility treatment);  
123 unable to provide informed consent to participate in the study.

124 Since excessively high intake of flavonoids has been shown to alter human metabolic  
125 pathways(23, 24), individuals who are atypically high consumers of flavonoids as part of  
126 their regular diet were precluded from participating. At the time of the COB trial delivery,  
127 UK adults were reported to consume on average 10 servings of flavonoid-rich foods  
128 (including tea, coffee, chocolate, fruit, vegetables and other plant-based products) per day  
129 (National Diet and Nutrition Survey (NDNS; 2011)(25). Individuals were excluded if they  
130 consumed more than 15 servings of flavonoid-rich foods per day. Flavonoid intake was  
131 assessed using the EPIC food frequency questionnaire(26).

132

133 Study Design. During the dietary run-in period and acute test meal protocol, participants were  
134 asked to adhere to a restricted diet to minimise the intake of (poly)phenols (including  
135 flavonoids and phenolics) by avoiding the consumption of fruits, vegetables, chocolate,  
136 spices, high-fibre products, tea, coffees, fruit juices and alcoholic beverages (Supplementary  
137 Table S2) for 48 h before the study day and for the 24 h after the consumption of the  
138 flavonoid-rich test meal (**Figure 1**). In addition to the medications listed in Supplementary  
139 Table S1, participants were asked to avoid acetaminophen (paracetamol) or other  
140 nonsteroidal anti-inflammatory drugs for the 10 h (fasting period) prior to the acute study day  
141 and for the duration of the sample collection, unless it was a medical requirement.

142 Participants were provided with a standardized low-flavonoid ready meal, consisting of either  
143 a vegetable-free fish pie or macaroni cheese, along with a bread roll and a portion of fruit-  
144 free sponge cake to consume the evening prior to attending the clinic visit in order to control  
145 for any inter-individual variability in background dietary (poly)phenols from their previous  
146 meals, and then undergo an overnight fast (only water allowed during fasting period). The  
147 participants attended the clinical trial unit (University of East Anglia, Norwich), after an  
148 overnight fast (only water allowed) for the acute test-meal session, containing mixed-

149 flavonoids. The standardized mixed flavonoid-rich test meal consisted of a bar of dark  
150 chocolate (Barry Callebaut, Lebbeke, Belgium, 50g), and a freeze-dried orange extract  
151 (Monteloeder, Alicante, Spain, 362mg) and blackberry (Nutra Ingredients Ltd, Brighton, UK,  
152 37.5g) powder in water, known as the COB mixture, with the composition of the chocolate  
153 provide by the manufacturer (Barry Callebaut, Belgium) and the flavonoid composition of the  
154 blackberry powder and citrus extracts established using an internal UHPLC-MS/MS based  
155 analysis(10, 27, 28). This provided 640 mg of flavan-3-ols (~30% monomers, ~180mg (-)-  
156 epicatechin), 340 mg of flavanones (89.3% hesperitin, 3.4% narirutin) and 390 mg of  
157 anthocyanins (99+% cyanidin-3-*O*-glucoside). Urine samples were collected at baseline (0 h  
158 a 24 h before the test meal), 0 – 3.5 h, 3.5 - 7 h, 7 - 24 h. Participants were free to leave the  
159 clinical trial unit at 7 h and return at the 24 h points for the relevant urine sampling. During  
160 the day of the intervention, two standardized low flavonoid meals (lunch and dinner), were  
161 provided along with low-flavonoid snacks (Figure 1).

162 The study was approved by the National Health Service (NHS) Health Research Authority  
163 (IRAS Project ID 1251207), followed the principles of the Declaration of Helsinki and was  
164 conducted at the Clinical Research Facility, University of East Anglia. Informed consent was  
165 obtained from all participants before study commencement. Recruitment occurred between  
166 October 2013 and March 2015, with follow-up completed by April 2015. Clinical Trials  
167 Registration # NCT01922869.

### 168 **Dietary assessment**

169 Participants completed a 131-item validated (26) FFQ which captured dietary habits over the  
170 previous 12 months, from which nutrient intakes were determined using McCance and  
171 Widdowson Food Tables(29, 30) and flavonoid intakes were calculated using the updated  
172 USDA databases for the flavonoid and proanthocyanin content of food, as previously  
173 described(31). If no values were available in the USDA database (USDA Database for the

174 Flavonoid Content of Selected Foods Release 3.1) for foods in the FFQ, available data from  
175 the phenol explorer database (www.phenol-explorer.eu)(32, 33) were included. Flavonoid  
176 intakes were derived for the six main flavonoid subclasses habitually consumed: flavanones  
177 (eriodictyol, hesperetin, and naringenin); anthocyanins (cyanidin, delphinidin, malvidin,  
178 pelargonidin, petunidin, and peonidin); flavan-3-ols (catechins and epicatechins); flavonols  
179 (quercetin, kaempferol, myricetin, and isorhamnetin); flavones (luteolin and apigenin); and  
180 polymers (including proanthocyanidins, theaflavins, and thearubigins). Total flavonoid  
181 intakes were estimated by summing the six component subclasses.

182

### 183 **Analytical methods**

184 Total urine voids were collected into light-protected collection bottles containing 1 g ascorbic  
185 acid. Aliquots were stored at -80 °C until analysis. Urinary metabolites were purified from 50  
186 µL human urine (1mL aliquots acidified with 40 µL formic acid) samples by 96-well plate  
187 solid phase extraction (SPE; Strata™-X Polymeric Reversed Phase, microelution 2 mg/well).  
188 Taxifolin was spiked into the urine and used as a SPE recovery reference standard and  
189 scopoletin post SPE as a chromatography internal standard. The solid phase extraction treated  
190 samples were chromatographically separated on an Exion ultra-high performance UHPLC  
191 coupled to a SCIEX QTRAP 3200+ triple quadrupole mass spectrometer (MS/MS; SCIEX,  
192 Framingham, MA, USA) with electrospray ionization source, as previously reported(10, 27,  
193 28). The samples were injected into Kinetex® (Phenomenex®) 2.6 µm PFP 100 Å, LC  
194 Column 100 x 4.6 mm (Part Number: 00D-4477-E0) with SecurityGuard™ ULTRA  
195 cartridges for PFP, with oven temperature maintained at 37°C. Mobile phase A and B  
196 consisted of 0.1% v.v. formic acid in water and 0.1% v.v. formic acid in acetonitrile  
197 respectively, with a binary gradient ranging from 1% B to 90% B over 28min and flow rate at  
198 1.5 mL/min. MS/MS scanning was accomplished using a targeted advanced scheduled MRM

199 (ADsMRM) assay using polarity switching between positive and negative ionisation mode in  
200 Analyst (v.1.6.3, SCIEX) and with quantitation conducted using MultiQuant (v.3.0.2,  
201 SCIEX) software platforms. If two metabolite isomers could not be resolved, they were  
202 quantified relative to a single species. Finally, metabolites previously reported in nutrition  
203 intervention studies feeding (poly)phenol-rich foods(27, 28, 34-45) were confirmed on the  
204 basis of established retention times (using authentic and synthesised standards; Supplement  
205 Table S3) and three or more precursor-to-product transition ions. In total, 82 (poly)phenol  
206 were quantified via UPLC-MS/MS; 6 metabolites were below the limit of quantitation in all  
207 samples, with statistical analysis completed for 76 analytes. All the metabolites were  
208 quantified relative to their reference standard, with the exception of cyanidin-diglucuronide  
209 which was quantified using the cyanidin-3-*O*-glucoside reference standard. Matrix matched  
210 standard curves were prepared for quantification ranging from 0-10 $\mu$ M. Reference standards  
211 scopoletin, taxifolin and phloridzin were used as quality controls and for internal standard  
212 adjustment in the urine samples, with system blanks monitored for carryover effects and pre-  
213 extracted urine as a reference blank.

214

215 Stool microbiome speciation. Stool specimens were collected on the day prior to the study  
216 day using EasySampler® kits (GP Medical Devices, Nupark, Holstebro, Denmark),  
217 transferred into sterile containers (Sarstedt, Leicester, UK), transported in cooled bags  
218 provided by the research team and immediately transferred to a -20 °C freezer upon arrival.  
219 Prior to DNA extraction, 200-300mg of faeces was transferred to a 2 mL safe lock tube  
220 (Eppendorf, city, country) containing a 5mm steel ball bearing and was subjected to  
221 mechanical disruption in 1.2 mL sterile PBS using a tissue lyser (Qiagen Tissue Lyser II, 30  
222 Hz for 4 min at 4°C). Samples were centrifuged to remove insoluble material (1 min, 10,000  
223 g at 4°C). DNA was extracted from 800  $\mu$ l of the supernatant using a QIA Symphony SP

224 automated platform (Qiagen) using the QIA Symphony DSP virus/bacteria midi kit and the  
225 Complex 400 V6 DSP program. DNA was eluted in 110  $\mu$ l buffer AVE containing carrier  
226 RNA as provided in the kit. The DNA was quantitated to confirm a concentration between  
227 5–20 ng/ $\mu$ l, and stored at -20°C until analysis. The region of the 16S gene was sequenced at  
228 APHA, Surrey using paired-end Illumina MiSeq short read sequencing. The Qiime(46) 1.9.0  
229 illumina workflow was used for joining the reads, demultiplex and filter samples and pick  
230 operational taxonomic units (OTUs). Read counts varied across the samples from 70 in a  
231 control sample to 112944 from sample COB354. Sequencing control samples were two  
232 separate PBS samples and one sample with no template. Two samples were excluded based  
233 on having a sequencing depth below the threshold. In addition, Qiime excluded two more  
234 samples according to the quality control filter. This filter is described in Bokulich et al  
235 (2013)(47). Qiime alpha diversity analyses (secondary analysis) involved testing a range of  
236 multiple rarefactions run in parallel. The resulting graph from these technical analyses did not  
237 indicate large-scale differences from using data at different sequencing depth levels (data not  
238 shown).

239

#### 240 **Statistical analysis**

241 Given the absence of established effect size estimates at the time the intervention was  
242 designed, we justified our sample size based on prior empirical data from Czank et al.  
243 2013(11), where significant changes in polyphenol metabolites were observed in 8  
244 participants. Based on the observed confidence intervals in that study, we estimated that  
245 group sizes of 30–40 participants would be sufficient for age and sex subgroup analyses. In  
246 the analysis of metabolites, values above the signal to noise (S/N) but below the limit of  
247 detection (LOD) were set as zero in the statistical analysis. If metabolites had the majority of  
248 their values at 0, they were excluded from the analysis. Calibration curves were established

249 between 1nM and 10uM. Values below the lower limit of quantitation (LLOQ) but above the  
250 LOD were reported as a “near 0” value (0.0001) in the statistical analysis. Linear mixed-  
251 effect models were used to assess differences in cumulative excretion by age (younger  
252 compared to older), sex (males compared to females) and combined age by sex (younger  
253 females, younger males, older females, older males) groups. Models included cumulative  
254 excretion as the dependent variable, “participant” as a random effect, time (0 min, 210 min,  
255 420 min and 1440 min) and groups as predictors, with the time x group interaction taken as  
256 the principal analysis of effect. The linear combinations of coefficients between groups were  
257 explored at the 24 h timepoint. Total flavonoid intake, as calculated from the FFQ, was  
258 included as a covariate on a continuous scale (where indicated) to explore if habitual diet  
259 markedly changed the results. Data were not transformed as generalized linear models have  
260 shown to be tolerant of distribution assumptions and can provide valid inference regardless of  
261 the distribution of the data(48). Total recovery of the metabolite mass in a urine sample is  
262 calculated by converting its molar concentration (moles per volume) to mass (e.g., ng) using  
263 the molecular weight of the metabolite, then multiplying by the total urine volume excreted  
264 : ((Metabolite value [nmol/l] \* molecular weight [ng/nmol] /1000000)\*(urine volume  
265 [L]))/time [h]. Where indicated in data tables, low concentrations are presented as  $10^2$  (for  
266 example  $0.01 \times 10^{-2} = 0.0001$  ng). The UPLC-MS/MS standard mix contained 82  
267 (poly)phenols (i.e., comprising both precursor polyphenols and phenolic metabolites;  
268 Supplement S3), and 76 were above the lower limit of quantitation in the COB urine samples.  
269 Six metabolites were found below the limit of detection in the majority of samples and were  
270 therefore not included in the final statistical analysis. Elimination kinetic parameters were  
271 calculated for each metabolite, C<sub>max</sub> (maximum urine concentration), T<sub>max</sub> (time of  
272 maximum concentration, 0 h, 3.5 h, 7 h or 24 h), and total amount excreted in urine over 24 h  
273 (A<sub>eu</sub> 0-24). Differences in C<sub>max</sub> and T<sub>max</sub> were compared between groups using a linear

274 regression model with the elimination parameter as the dependent variable, group as the  
275 predictor and habitual total flavonoid intake on a continuous scale as a covariate (where  
276 indicated). If metabolite data were missing at individual time points cumulative excretion was  
277 calculated up to the missing data point. Cmax and Tmax were calculated if data were  
278 available for at least one time-point. P-values <0.05 were considered statistically significant.  
279 The Benjamini-Hochberg method for false discovery rate (using a false discovery rate Q-  
280 value <0.20) was also used to explore the possible impact of multiple testing on observed age  
281 and sex affects. Statistical analyses were performed with Stata statistical software version 16  
282 (StataCorp, Texas, USA).

283

284 Linear Discriminant Analysis (LDA) was used to identify which combination of metabolites  
285 separated the participants by group and how effective these metabolites were as predictive  
286 discriminators of age. Metabolites included in the model were those with a significant time-  
287 by-age group interaction in the primary analysis.

288 Differences in relative abundance of taxa at the Family level and microbial alpha diversity  
289 (Shannon Index) were compared across age (younger compared to older), sex (males  
290 compared to females) and combined age by sex (younger females, younger males, older  
291 females, older males) groups using linear regression with group as the predictor. We  
292 excluded taxa where relative abundance was < 0.01% in at least 10% of samples leaving 38  
293 taxa in the final analysis.

294 Hierarchical regression analysis (using metabolite concentration as the dependent variable)  
295 was used to examine if models including sex and microbial composition improved prediction  
296 of metabolite concentrations over models including age. For microbial composition we  
297 combined the taxa significantly associated with age using principal component analysis,  
298 considering the first component defined. The metabolites included in the model were those

299 with a significant time-by-group interaction in the primary analysis.

300

## 301 **RESULTS**

302 A total of 186 participants completed the COB protocol (**Figure 2**). Metabolite  
303 concentrations in urine were quantified in 163 participants [44 older females, 46 older males,  
304 42 younger females, 31 younger males] for 24 h and data on habitual diet was available for  
305 161 participants (**Table 1**). Older males had the highest caloric intake (age and sex effect;  
306  $p < 0.04$  and  $0.03$  respectively) and BMI was higher in older participants and highest in older  
307 males ( $P < 0.01$ ). Older participants generally consumed considerably more flavonoids  
308 ( $P < 0.01$ ), with nearly three times higher habitual intakes than younger individuals, with  
309 highest intakes reported in older females. Habitual intakes of flavonoids and flavonoid sub-  
310 classes also differed by age, with lower intake reported by younger participants compared to  
311 older participants, which was reflected in lower total fruit and vegetable and tea intakes. No  
312 notable differences in flavonoid intakes were observed by sex, while females reported lower  
313 total energy and carbohydrate intakes and a higher fruit and vegetable intake.

314 **Cumulative Excretion – sum of all (poly)phenols and metabolites.** There was a trend for  
315 age-by-sex differences in cumulative (all urine “time bins”) 24 h urinary metabolite recovery  
316 ( $P = 0.06$ ; treatment effect, all metabolites and parent (poly)phenols; Supplement Table 4);  
317 however, no significant time-by-group interactions were observed ( $p = 0.30$ ); with mean total  
318 urinary recovery of  $14.59 \pm 2.22$  ng at baseline,  $56.02 \pm 7.72$  ng at 3.5h,  $97.80 \pm 20.73$  ng at 7 h;  
319 and  $122.32 \pm 25.34$  ng at 24 h (mean  $\pm$  SD; data not shown). There were also no significant  
320 group differences (mean, 95% CI) for maximum total excretion ( $C_{max}$ ,  $p = 0.20$ ) [(older  
321 females 505 ng (333, 678), older males 670 ng (502, 839), younger females 452 ng (276,  
322 629), younger males 384 ng (179, 590); however, there was significant group differences in  
323  $T_{max}$  ( $P = 0.04$ ) [older females 15.8 h (12.4, 19.2), older males 15.2 h (11.9, 18.5), younger

324 females 10.6 h (7.1, 14.1), and younger males 12.0 h (7.9, 16.0); (mean, 95% CI)].

325 **Individual (Poly)phenols and Metabolite Excretion.** Differences in cumulative 24 h  
326 excretion were observed for 12 individual metabolites by age (time-by-treatment interaction,  
327  $P < 0.05$ ; 10  $Q < 0.2$ ) and 5 metabolites by sex (1  $Q < 0.2$ ) (**Table 2**; *Complete dataset found in*  
328 *Supplemental Table 4*). Here the metabolites primarily comprised of small molecule  
329 microbial metabolites of (poly)phenols, where younger participants, mainly males, generally  
330 displayed higher cumulative excretion. Adjustment for habitual total flavonoid intake did not  
331 materially change the age differences observed in urinary excretion over time or cumulative  
332 24 h urinary recovery (*Supplemental Table 5*).

### 333 **Elimination Kinetics**

334 Differences in  $C_{max}$  were observed for 7% of measured metabolites by age and 8% by sex,  
335 while differences in  $T_{max}$  were observed for 28% by age and 11% of metabolites by sex  
336 (*Supplement Table S6*; 36  $P < 0.05$ ; 14  $Q < 0.2$ ), with the majority of differences seen as age  
337 effects on  $T_{max}$ .

338 Group differences in  $C_{max}$  were observed for 4 metabolites ( $P < 0.05$ ; 2  $Q < 0.2$ ), with the  
339 highest  $C_{max}$  observed most frequently in older males (**Table 3**; *Complete dataset found in*  
340 *Supplemental Table 6*). Age differences were observed for 5 phenolic metabolites (0  $Q < 0.2$ )  
341 and sex differences for 6 metabolites (0  $Q < 0.2$ ). Metabolites recorded in highest  
342 concentrations [( $C_{max}$ ; mean (95% CI)] having age or sex differences were 3-  
343 hydroxyhippuric acid 22.0  $\mu\text{M}$  (14.7, 29.3) benzoic acid-4-sulfate 17.3  $\mu\text{M}$  (12.7, 21.8), 3-  
344 methoxybenzoic acid-4-sulfate 4.4  $\mu\text{M}$  (3.1, 5.8), 2,5-dihydroxybenzoic acid 0.85  $\mu\text{M}$  (0.53,  
345 1.2), 3-hydroxy-4-methoxybenzoic acid 0.60  $\mu\text{M}$  (0.46, 0.74), hydroxy-methoxybenzoic acid  
346 0.42  $\mu\text{M}$  (0.32, 0.52), and 4-methylhippuric acid 0.03  $\mu\text{M}$  (0.01, 0.04). Adjusting  $C_{max}$  for total  
347 flavonoid intake by age group had limited impact on the statistical output (**Supplemental**  
348 **table 7**).

349

350 The time to maximum urinary concentration (T<sub>max</sub>) varied considerably across metabolites  
351 displaying group, age or sex differences, from 1h and 24 h, with a mean T<sub>max</sub> of 9.1±4.1h  
352 (mean (95% CI); **Table 4**). Group differences were observed for 15 metabolites (4 Q>0.2).  
353 Twenty-two of the metabolites displayed significant T<sub>max</sub> differences by age (14 Q<0.2) and  
354 8 by sex (0 Q<0.2), with 92% of these metabolites reflecting significantly later T<sub>max</sub> in older  
355 participants. Only two analytes, 4-hydroxybenzyl alcohol and 3-methylhippuric acid, had  
356 greater T<sub>max</sub> in younger participants (*all analyte data provided in Supplement Table 6*).  
357 Adjusting T<sub>max</sub> for total flavonoid intake by age group had limited impact on the statistical  
358 output (**Supplemental table 7**).

359

360 **Group Prediction.** In LDA analysis 77% of younger participants and 62% of older  
361 participants were classified into the correct age groups based on their scores on the  
362 discriminant dimensions (**Table 5**). Hydroxy-methoxybenzoic acid, 2-hydroxycinnamic acid,  
363 4-methoxybenzaldehyde (positive correlations), benzoic acid-4-*O*-glucuronide, and 3-  
364 methylhippuric acid (negative correlations) were the metabolites with the largest beta  
365 coefficients and therefore most likely to classify participants in the correct age group.

366 **Gut microbiome composition by age and sex.** Five taxa at the Family level -

367 *Bacteroidaceae*, *Christensenellaceae*, *Clostridiales (unclassified)*, *Dehalobacteriaceae* and  
368 *Rikenellaceae* displayed significant differences by age group (Q <0.20, **Supplemental Table**  
369 **8**). Relative abundance of all taxa was higher in younger participants with the exception of  
370 *Bacteroidaceae* which was higher in older participants. Relative abundance of  
371 *Barnesiellaceae* differed according to sex, with higher abundance in males. There were no  
372 significant differences in alpha diversity (Shannon Index) between older and younger  
373 individuals (P= 0.12) or males and females (P= 0.84) (data not shown). Hierarchical

374 regression analysis revealed that the addition of sex and microbial composition to the model  
375 did not improve the prediction of metabolite concentrations over age (**Table 6**)

376

## 377 **DISCUSSION**

378 Overall, we observed no impact of age or sex on total cumulative 24 h urinary elimination of  
379 (poly)phenols. This lack of effect of age is consistent with a smaller previous study (n=40)  
380 which looked at flavan-3-ol metabolism in men only and observed no difference in total  
381 ‘structurally related epicatechin metabolites’ in plasma 0-6h or urine 0-24h. However, we did  
382 observe that overall age was the main determinant of flavonoid metabolite excretion kinetics  
383 over 24 h. Interestingly, adjustment for BMI and the gut microbial taxa had little effect on the  
384 significance of the observed relationships, indicating that any age associated effects were not  
385 mediated by differences in gut microbial speciation or BMI. Older participants consumed  
386 considerably more flavonoids in their habitual diet, with nearly three times higher intakes  
387 compared to younger individuals, and with the highest intakes reported in older females;  
388 however adjusting for habitual intake only moderately reduced the number of metabolites  
389 showing age effects (**Supplemental Table 5**).

390 The C<sub>max</sub> for individual microbial metabolites of (poly)phenols differed by either age or sex  
391 for 9 metabolites, however, there were no overall differences in cumulative excretion or  
392 C<sub>max</sub> for all polyphenol metabolites combined (i.e., total collective of (poly)phenol  
393 metabolites). Differences in T<sub>max</sub> were more pronounced for many metabolites, including  
394 both precursor (poly)phenols (hesperetin, naringenin, epicatechin, catechin and cyanidin  
395 glucoside) and their microbial metabolites.

396 Metabolites displaying significant group, age or sex effects for T<sub>max</sub> included  
397 hydroxybenzoic acids, 3-(phenyl)propionic acids, 5-(Hydroxyphenyl)-gamma-  
398 valerolactones and hippuric acids. Hydroxybenzoic acids, 3-(phenyl)propionic acids, and

399 hippuric acids are reported to be derived from multiple (poly)phenol-rich food sources; while  
400 the 5-(hydroxyphenyl)-gamma-valerolactones were most likely derived from cocoa flavan-3-  
401 ols and procyanidins in cocoa and blackcurrant, hesperetin-3'-*O*-glucuronide from orange,  
402 and ellagic acid from blackcurrant(2, 4, 21, 36). Interestingly, there were no clear patterns in  
403 elimination kinetics ( $C_{max}$  or  $T_{max}$ ) for precursor flavonoids relative to their conjugated  
404 metabolites (i.e., methyl, sulfate, glucuronide conjugation) between age or sex groups.

405 A substantial fraction of flavonoid intake is subjected to gut microbial metabolism,  
406 structurally altering the precursor flavonoids found in the diet, forming smaller molecules  
407 such as phenolic and aromatic acids, which are more bioavailable and may be highly  
408 bioactive(1, 4). Although flavonoid intake has been shown to influence microbiota  
409 speciation(19, 20), the role of microbiota functional and metabolic diversity in flavonoid  
410 bioavailability is poorly understood. In the present investigation five taxa at the family level  
411 displayed age differences, however hierarchical regression revealed no effects of microbial  
412 composition or sex on metabolite concentration. Within our microbial composition dataset  
413 alpha diversity was not significantly higher in older than younger individuals, however, other  
414 studies have presented a mixed picture of aging versus gut and microbial alpha diversity (49),  
415 and whilst this finding needs to be confirmed in future studies it is noteworthy that we  
416 observed higher habitual flavonoid intakes in older individuals at baseline.

417  $T_{max}$  differed by as much as 3-5h between the age groups and was typically later in older  
418 individuals. This finding highlights the need for future studies to consider longer sampling  
419 strategies when recruiting wide age ranges for exploring metabolite bioactivity and  
420 attempting to establish correlations between peak blood concentration of flavonoid  
421 metabolites and health/disease status biomarkers (e.g., glucose or lipoprotein homeostasis).  
422 The etiology of differences in  $T_{max}$  between older and younger individuals is unknown and  
423 were not explained by differences in BMI or microbiome speciation. They may derive from

424 differences in glomerular filtration rates, liver metabolism, or gastric and intestinal transit  
425 time (50, 51) affecting food digestibility and absorption and elimination of microbial  
426 metabolites, variables which should be captured where possible in future studies.

427 Precursor flavonoids from citrus, cocoa, and berries (naringenin, epicatechin, cyanidin) and  
428 their previously reported metabolites, including microbial metabolites (hippuric acids,  
429 benzoic acids, and valerolactones)(2, 4, 21, 36) were graphically depicted (**Supplement**  
430 **Figures 1-13**) to visualize if differences in rates of elimination of precursor flavonoid across  
431 age or sex groups differed or were predictive of their metabolite elimination. Rates of  
432 elimination (i.e., slopes of cumulative elimination curves) appeared consistent across age and  
433 sex, with older individuals generally having higher C<sub>max</sub> and later T<sub>max</sub> than younger  
434 individuals for these perceived biomarkers of flavonoid intake. This evidence indicates that  
435 monitoring a limited number of perceived intake biomarkers in nutrition interventions is  
436 likely to poorly reflect actual shifts in the dietary metabolomes of individuals and highlights  
437 the importance of using more global metabolomics approaches in future interventions.

438 In the present study both standard ( $P < 0.05$ ) and conservative statistical approaches ( $Q < 0.20$ ;  
439 Benjamini-Hochberg method for false discovery) were utilized to emphasise the potential  
440 impact of multiple testing when characterizing large numbers of metabolites. Moving  
441 forward, polyphenol interventions using untargeted or quantitative metabolomics approaches  
442 will require considerations and consensus on the most appropriate statistical practices.

443 The present study design was relatively unique for flavonoid (and (poly)phenols in general)  
444 interventions as participants were prospectively recruited by age and sex, allowing for the  
445 first comprehensive investigation of the impact of sex, age and microbiota speciation on acute  
446 flavonoid absorption, metabolism and elimination following the consumption of a flavonoid  
447 enriched test meal. The elimination of flavonoids from the background diet 48 h prior to the  
448 intervention was an additional strength as it standardized and minimized the contribution of

449 background diet derived metabolites detected in urine post test meal. The present study was  
450 however, unable to pinpoint a possible mechanism behind the variability in elimination. The  
451 lack of characterization of additional possible mediators, such as gastric emptying, intestinal  
452 transit, liver and kidney function (glomerular filtration rate) and genetic polymorphisms in  
453 phase 1 and 2 metabolism, is identified as a study limitation, along with a lack of capture of  
454 metabolite concentrations beyond 24h, which would have resulted in gut derived metabolites  
455 not being fully recovered. However, it was decided during the trial design stage to limit  
456 further participant burden caused by the restricted diet and repeated sampling over an  
457 additional 24h. Many phytochemical phase II metabolites are/were not commercially  
458 available as references standards for use in quantitative analysis (e.g., glucuronide or sulfate  
459 conjugates of flavan-3-ols, valerolactones, phenylvaleric acids, etc.) and therefore the  
460 present total recovery is likely to be an underestimation of amount and diversity of  
461 polyphenol metabolites excreted. Further, there is likely to be some minimal contribution of  
462 endogenous and dietary aromatic amino acids to the phenolic acid metabolite pools  
463 quantified in the urine in the present study, such as hippuric acids; however, all participants  
464 were on the same intervention meals and the exclusion diet was essentially void of  
465 (poly)phenols, minimizing this possible confounding of endogenous or dietary substrates.  
466 Finally, the research was conducted in a healthy population, with >90% of our study  
467 population being white British, and extrapolation of findings to clinical groups with  
468 significant disease pathology and medication use, or other ethnic groups, should be done with  
469 caution.

#### 470 **Conclusion.**

471 Our study provides evidence of a large impact of age on elimination kinetics of (poly)phenol  
472 metabolites, particularly  $T_{max}$ , which occurs much later in older individuals. The age effects  
473 observed on individual metabolite recovery does not appear to be substantially driven by

474 differences in background diet, BMI and microbiota speciation. Our results indicate that  
475 evaluating blood or urine signatures of (poly)phenol metabolites at a single time point is  
476 unlikely to capture the true absorption and elimination kinetics across age and sex groups.

477

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482

483 **Declaration of interest:** The authors report no conflict of interest.

484

485 **Authors' responsibilities** were as follows: AMM and AC designed the study. SH, NT, BCD,  
486 DB and SL were responsible for participant recruitment, delivery of the intervention protocol  
487 and the collection, processing and storage of biological samples. NT, BCD and CDK  
488 designed, delivered and interpreted the urinary flavonoid analysis. LCC and DM were  
489 responsible for preparation of the faecal samples for microbiome speciation and the  
490 interpretation of the data. AJ conducted all statistical analysis. CDK, AJ and AMM drafted  
491 the paper, with all authors contributing to and approving the final manuscript.

492

493 **Data sharing plan:** Data described in the manuscript, including urine concentration, analytical  
494 methodologies, and statistical analysis data files will be made available upon request and  
495 approval from the corresponding author.

496

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648

649 **TABLES**

Journal Pre-proof

650 **Table 1:** Participant characteristics Mean (95% CI) and habitual dietary intake stratified by age and sex group in 163 participants from the COB  
 651 study.

|                          | <b>Older females</b><br><b>(n=44)</b> | <b>Older males</b><br><b>(n=46)</b> | <b>Younger females</b><br><b>(n=42)</b> | <b>Younger males</b><br><b>(n=31)</b> |
|--------------------------|---------------------------------------|-------------------------------------|---|---------------------------------------|
| Age (y)                  | 66.9 (65.5, 68.2)                     | 67.8 (66.5, 69.1)                   | 22.6 (21.2, 24.0)                       | 22.4 (20.8, 24.0)                     |
| BMI (kg/m <sup>2</sup> ) | 24.2 (23.4, 25.0)                     | 25.4 (24.6, 26.3)                   | 23.4 (22.6, 24.3)                       | 23.0 (22.0, 24.0)                     |
| Energy (kcal)            | 2063 (1861, 2265)                     | 2229 (2036, 2422)                   | 1811 (1609, 2013)                       | 2079 (1844, 2315)                     |
| Protein (g)              | 89.7 (81.7, 97.7)                     | 89.9 (82.3, 97.6)                   | 78.2 (70.3, 86.2)                       | 88.9 (79.6, 98.2)                     |
| Fat (g)                  | 74.5 (65.9, 83.0)                     | 78.5 (70.3, 86.7)                   | 62.6 (54.1, 71.2)                       | 75.5 (65.5, 85.5)                     |
| Carbohydrate (g)         | 258 (229, 286)                        | 287 (259, 314)                      | 241 (212, 269)                          | 269 (236, 303)                        |
| Fibre (g)                | 25.5 (22.8, 28.3)                     | 25.4 (22.8, 28.0)                   | 22.9 (20.1, 25.6)                       | 22.7 (19.5, 25.9)                     |
| Total flavonoids (mg/d)  | 1298 (1150, 1446)                     | 1237 (1096, 1378)                   | 509 (361, 657)                          | 478 (306, 650)                        |
| Flavanones (mg/d)        | 41.1 (31.8, 50.4)                     | 30.8 (22.0, 39.7)                   | 23.4 (14.1, 32.7)                       | 22.1 (11.3, 33.0)                     |
| Anthocyanins (mg/d)      | 34.7 (29.1, 40.3)                     | 29.5 (24.2, 34.9)                   | 19.2 (13.6, 24.8)                       | 11.5 (5.0, 18.0)                      |
| Flavan-3-ols (mg/d)      | 254 (220, 289)                        | 255 (222, 288)                      | 102 (67.6, 136)                         | 84.8 (44.8, 125)                      |
| Flavonols (mg/d)         | 57.1 (51.1, 63.0)                     | 53.2 (47.5, 58.9)                   | 29.7 (23.7, 35.6)                       | 24.6 (17.7, 31.6)                     |

|                            |                 |                |                 |                 |
|----------------------------|-----------------|----------------|-----------------|-----------------|
| Flavones (mg/d)            | 1.9 (1.6, 2.2)  | 1.7 (1.4, 1.9) | 1.6 (1.3, 1.8)  | 1.2 (0.89, 1.5) |
| Polymers (mg/d)            | 909 (801, 1017) | 866 (763, 969) | 333 (225, 441)  | 334 (208, 459)  |
| Fruit and vegetables (g/d) | 794 (696, 891)  | 653 (559, 746) | 583 (486, 681)  | 481 (367, 594)  |
| Tea (g/d)                  | 763 (650, 877)  | 759 (650, 869) | 204 (88.9, 319) | 217 (76.1, 358) |

652 Values are mean (95% CI). n=163 (data missing for n=2 participants for dietary data).

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**Table 2:** Cumulative excretion (ng) by age and sex group at 24h.

| Metabolite (ng)  | Older females |                      | Older males |                      | Younger females |                      | Younger males |                       | p-group            | p-age              | p-sex              |
|--|---------------|----------------------|-------------|----------------------|-----------------|----------------------|---------------|-----------------------|--------------------|--------------------|--------------------|
|  | n=            | Mean (95% CI)        | n=          | Mean (95% CI)        | n=              | Mean (95% CI)        | n=            | Mean (95% CI)         |                    |                    |                    |
| 3-(dihydroxyphenyl)propionic acid*                         | 42            | 0.043 (0.034, 0.053) | 45          | 0.035 (0.025, 0.044) | 42              | 0.056 (0.046, 0.065) | 28            | 0.046 (0.035, 0.058)  | 0.02 <sup>a</sup>  | 0.01 <sup>a</sup>  | 0.05               |
| 4-methylhippuric acid (x 10 <sup>2</sup> )                 | 42            | 0.297 (0.173, 0.421) | 45          | 0.629 (0.509, 0.749) | 42              | 0.296 (0.171, 0.421) | 29            | 0.412 (0.263, 0.562)  | <0.01 <sup>a</sup> | 0.07               | <0.01 <sup>a</sup> |
| 3-methoxycinnamic acid                                     | 42            | 0.033 (0.009, 0.056) | 45          | 0.079 (0.056, 0.101) | 42              | 0.049 (0.025, 0.072) | 29            | 0.024 (-0.004, 0.052) | <0.01 <sup>a</sup> | 0.15               | 0.18               |
| 6-methoxysalicylic acid (x 10 <sup>2</sup> )               | 42            | 0.091 (0.031, 0.150) | 45          | 0.225 (0.166, 0.283) | 41              | 0.147 (0.086, 0.208) | 29            | 0.057 (-0.016, 0.129) | <0.01 <sup>a</sup> | 0.12               | 0.22               |
| 4-hydroxy-3-methoxyacetophenone                            | 42            | 0.020 (0.013, 0.028) | 45          | 0.029 (0.022, 0.037) | 42              | 0.036 (0.028, 0.044) | 29            | 0.037 (0.028, 0.046)  | 0.01 <sup>a</sup>  | <0.01 <sup>a</sup> | 0.28               |
| Benzoylglutamic acid                                       | 42            | 0.012 (0.008, 0.016) | 45          | 0.021 (0.017, 0.025) | 42              | 0.013 (0.009, 0.018) | 29            | 0.013 (0.007, 0.018)  | 0.01 <sup>a</sup>  | 0.11               | 0.03               |
| 5-(Hydroxyphenyl)-gamma-valerolactone-sulfate              | 42            | 0.313 (0.192, 0.434) | 45          | 0.585 (0.468, 0.703) | 42              | 0.506 (0.385, 0.627) | 29            | 0.186 (0.040, 0.331)  | <0.01 <sup>a</sup> | 0.23               | 0.77               |
| 5-(hydroxyphenyl)-gamma-valerolactone (x 10 <sup>2</sup> ) | 42            | 2.4 (1.4, 3.4)       | 44          | 2.2 (1.3, 3.2)       | 42              | 4.1 (3.1, 5.0)       | 28            | 1.4 (0.207, 2.6)      | <0.01 <sup>a</sup> | 0.19               | 0.01 <sup>a</sup>  |
| naringenin-7- <i>O</i> -glucuronide (x 10 <sup>2</sup> )   | 42            | 3.4 (2.5, 4.4)       | 45          | 4.2 (3.3, 5.1)       | 42              | 4.9 (4.0, 5.9)       | 29            | 2.4 (1.2, 3.5)        | <0.01 <sup>a</sup> | 0.88               | 0.17               |
| 2,3-dihydroxybenzoic acid                                  | 42            | 0.059 (0.038, 0.080) | 45          | 0.101 (0.081, 0.121) | 42              | 0.093 (0.072, 0.114) | 29            | 0.067 (0.042, 0.092)  | 0.01 <sup>a</sup>  | 0.86               | 0.29               |
| 3-methylhippuric acid                                      | 42            | 0.006 (0.004, 0.009) | 45          | 0.014 (0.012, 0.016) | 42              | 0.006 (0.004, 0.009) | 29            | 0.005 (0.002, 0.008)  | <0.01 <sup>a</sup> | <0.01 <sup>a</sup> | <0.01 <sup>a</sup> |
| 3-methoxybenzoic acid-4-sulfate                            | 41            | 0.616 (0.441, 0.791) | 42          | 0.870 (0.697, 1.0)   | 41              | 0.525 (0.348, 0.701) | 29            | 0.900 (0.691, 1.1)    | <0.01 <sup>a</sup> | 0.50               | <0.01 <sup>a</sup> |
| Benzoic acid-4- <i>O</i> -glucuronide                      | 42            | 0.034 (0.029, 0.040) | 45          | 0.028 (0.023, 0.033) | 42              | 0.028 (0.022, 0.033) | 29            | 0.017 (0.011, 0.024)  | <0.01 <sup>a</sup> | 0.01 <sup>a</sup>  | 0.02 <sup>a</sup>  |
| 2-hydroxycinnamic acid (x 10 <sup>2</sup> )                | 42            | 0.021 (0.007, 0.035) | 46          | 0.018 (0.005, 0.032) | 41              | 0.022 (0.008, 0.036) | 30            | 0.065 (0.049, 0.082)  | <0.01 <sup>a</sup> | <0.01 <sup>a</sup> | 0.04               |
| 3-(4-hydroxy-3-methoxyphenyl)propionic acid                | 42            | 0.051 (0.026, 0.075) | 46          | 0.081 (0.058, 0.105) | 41              | 0.106 (0.081, 0.130) | 30            | 0.106 (0.077, 0.135)  | <0.01 <sup>a</sup> | <0.01 <sup>a</sup> | 0.31               |
| hesperetin-3'- <i>O</i> -glucuronide                       | 42            | 0.517 (0.346, 0.689) | 46          | 0.772 (0.607, 0.937) | 41              | 0.567 (0.393, 0.741) | 30            | 0.356 (0.152, 0.559)  | 0.02 <sup>a</sup>  | 0.06               | 0.48               |
| 3-methoxy-4-hydroxyphenylacetic acid                       | 42            | 0.922 (0.530, 1.3)   | 46          | 1.7 (1.3, 2.1)       | 41              | 1.0 (0.605, 1.4)     | 30            | 0.842 (0.378, 1.3)    | <0.01 <sup>a</sup> | 0.06               | 0.06               |
| 3-methoxybenzoic acid-4- <i>O</i> -glucuronide             | 42            | 0.394 (0.236, 0.552) | 46          | 0.625 (0.473, 0.777) | 41              | 0.462 (0.302, 0.623) | 30            | 0.285 (0.098, 0.471)  | 0.04 <sup>a</sup>  | 0.13               | 0.46               |
| hydroxy-methoxybenzoic acid*                               | 42            | 0.065 (0.045, 0.085) | 46          | 0.075 (0.056, 0.095) | 41              | 0.090 (0.069, 0.110) | 30            | 0.133 (0.109, 0.157)  | <0.01 <sup>a</sup> | <0.01 <sup>a</sup> | 0.06               |
| Hydroxybenzoic acid*                                       | 42            | 0.010 (0.007, 0.013) | 46          | 0.010 (0.008, 0.013) | 40              | 0.013 (0.010, 0.016) | 30            | 0.005 (0.002, 0.009)  | <0.01 <sup>a</sup> | 0.78               | 0.05               |
| 3-hydroxyhippuric acid                                     | 42            | 1.8 (1.3, 2.2)       | 46          | 2.5 (2.0, 2.9)       | 41              | 1.5 (0.986, 1.9)     | 30            | 1.6 (1.1, 2.2)        | 0.02 <sup>a</sup>  | 0.02 <sup>a</sup>  | 0.04               |

|                                 |    |                       |    |                       |    |                      |    |                      |                    |                    |                    |
|---------------------------------|----|-----------------------|----|-----------------------|----|----------------------|----|----------------------|--------------------|--------------------|--------------------|
| 4-methoxybenzaldehyde           | 41 | 0.007 (0.005, 0.009)  | 46 | 0.005 (0.003, 0.007)  | 41 | 0.008 (0.007, 0.010) | 29 | 0.011 (0.008, 0.013) | <0.01 <sup>a</sup> | <0.01 <sup>a</sup> | 0.59               |
| 3-hydroxy-4-methoxybenzoic acid | 42 | 0.096 (0.074, 0.119)  | 46 | 0.082 (0.061, 0.104)  | 41 | 0.140 (0.117, 0.163) | 29 | 0.166 (0.139, 0.193) | <0.01 <sup>a</sup> | <0.01 <sup>a</sup> | 0.82               |
| Hydroxybenzoic acid-sulfate*    | 39 | 0.318 (0.188, 0.447)  | 44 | 0.544 (0.421, 0.667)  | 39 | 0.303 (0.173, 0.433) | 26 | 0.404 (0.245, 0.563) | 0.03 <sup>a</sup>  | 0.18               | <0.01 <sup>a</sup> |
| Hippuric acid                   | 34 | 58.1 (44.9, 71.3)     | 37 | 77.8 (65.1, 90.5)     | 34 | 58.7 (45.4, 72.0)    | 22 | 45.6 (29.2, 61.9)    | 0.02 <sup>a</sup>  | 0.04               | 0.31               |
| 3-(phenyl)propionic acid        | 34 | 0.019 (-0.020, 0.058) | 38 | 0.009 (-0.028, 0.046) | 34 | 0.047 (0.008, 0.086) | 22 | 0.092 (0.044, 0.140) | 0.04 <sup>a</sup>  | 0.01 <sup>a</sup>  | 0.75               |

655 Values are mean (95% CI). Metabolites shown are those with a significant group, age or sex effect. P values are for group comparisons at 24

656 hours and calculated from linear mixed-effect models. <sup>a</sup> false discovery rate adjusted p values <0.2. *Full dataset providing p values for all*

657 *analytes is found in Supplement Table 4.* \*Metabolite isomers which could not be resolved effectively by HPLC were quantified according to

658 one of the structural isomers. All urine metabolites values are presented as: ((Metabolite value [nmol/l] \* molecular weight [ng/nmol]

659 /1000000)\*(urine volume [L])/time [h]. Where indicated in data tables, low concentrations are presented as 10<sup>-2</sup> (for example 0.01x10<sup>-2</sup> =

660 0.0001 ng).

**Table 3:** Maximum urinary elimination (C<sub>max</sub>) by age and sex group.

| Metabolite (ng)                 | Older females |                   | Older males |                   | Younger females |                    | Younger males |                   | p-group | p-age | p-sex  |
|---------------------------------|---------------|-------------------|-------------|-------------------|-----------------|--------------------|---------------|-------------------|---------|-------|--------|
|                                 | n             | Mean (95% CI)     | n=          | Mean (95% CI)     | n=              | Mean (95% CI)      | n=            | Mean (95% CI)     |         |       |        |
| 2,5-dihydroxybenzoic acid       | 44            | 0.52 (0.26, 0.79) | 46          | 0.83 (0.58, 1.1)  | 42              | 0.54 (0.27, 0.81)  | 31            | 0.85 (0.53, 1.2)  | 0.34    | 0.95  | 0.03   |
| 4-methylhippuric acid           | 44            | 0.02 (0.01, 0.03) | 46          | 0.05 (0.04, 0.06) | 42              | 0.02 (0.003, 0.03) | 31            | 0.03 (0.01, 0.04) | 0.53    | 0.03  | <0.01a |
| 3-methylhippuric acid           | 44            | 0.05 (0.02, 0.07) | 46          | 0.11 (0.08, 0.13) | 42              | 0.04 (0.01, 0.07)  | 31            | 0.04 (0.01, 0.07) | 0.29    | 0.02  | 0.02   |
| 3-methoxybenzoic acid-4-sulfate | 44            | 1.8 (0.68, 2.9)   | 46          | 2.7 (1.6, 3.8)    | 42              | 1.4 (0.29, 2.6)    | 31            | 4.4 (3.1, 5.8)    | 0.04    | 0.45  | <0.01a |
| benzoic acid-4-O-glucuronide    | 44            | 0.16 (0.13, 0.20) | 46          | 0.14 (0.10, 0.17) | 42              | 0.13 (0.09, 0.17)  | 31            | 0.09 (0.05, 0.14) | 0.02    | 0.07  | 0.13   |
| benzoic acid-4-sulfate          | 44            | 9.7 (5.0, 14.3)   | 46          | 17.3 (12.7, 21.8) | 41              | 8.1 (3.3, 12.9)    | 31            | 10.8 (5.3, 16.4)  | 0.62    | 0.09  | 0.02   |
| hydroxy-methoxybenzoic acid*    | 44            | 0.17 (0.09, 0.26) | 46          | 0.25 (0.17, 0.33) | 41              | 0.26 (0.18, 0.35)  | 31            | 0.42 (0.32, 0.52) | <0.01a  | 0.01  | 0.02   |
| 3-hydroxyhippuric acid          | 44            | 17.5 (10.0, 25.0) | 46          | 22.0 (14.7, 29.3) | 41              | 10.5 (2.7, 18.2)   | 31            | 12.6 (3.6, 21.5)  | 0.14    | 0.03  | 0.30   |
| 3-hydroxy-4-methoxybenzoic acid | 44            | 0.28 (0.16, 0.39) | 46          | 0.29 (0.18, 0.41) | 42              | 0.37 (0.25, 0.48)  | 31            | 0.60 (0.46, 0.74) | <0.01a  | <0.01 | 0.12   |

Values are mean (95% CI). Metabolites shown are those with a significant group, age or sex effect; P values calculated from linear regression. <sup>a</sup> false

recovery rate adjusted p values <0.2. Full dataset providing p values for all analytes is found in Supplement Table 6. \*Metabolite isomers which could

be resolved effectively by HPLC were quantified according to one of the structural isomers. All urine metabolites values are presented as:

Metabolite value [nmol/l] \* molecular weight [ng/nmol] /1000000)\*(urine volume [L])/time [h].

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**Table 4: Time (hours) of Maximal urinary elimination (T<sub>max</sub>) by age and sex group.**

| Metabolite                                     | Older females |                   | Older males |                   | Younger females |                   | Younger males |                   | p-group | p-age  | p-sex |
|--|---------------|-------------------|-------------|-------------------|-----------------|-------------------|---------------|-------------------|---------|--------|-------|
|  | n=            | Mean (95% CI)     | n=          | Mean (95% CI)     | n=              | Mean (95% CI)     | n=            | Mean (95% CI)     |         |        |       |
| 3,4-dihydroxyphenylacetic acid                 | 44            | 14.7 (11.4, 17.9) | 46          | 15.7 (12.5, 18.8) | 42              | 16.3 (12.9, 19.6) | 31            | 20.9 (17.1, 24.8) | 0.02    | 0.08   | 0.18  |
| 3-methoxycinnamic acid                         | 44            | 8.1 (4.8, 11.4)   | 46          | 13.3 (10.0, 16.5) | 42              | 6.9 (3.5, 10.3)   | 31            | 9.5 (5.6, 13.5)   | 0.76    | 0.12   | 0.02  |
| 4-hydroxybenzaldehyde                          | 44            | 11.3 (8.0, 14.6)  | 46          | 13.5 (10.3, 16.7) | 42              | 5.5 (2.1, 8.9)    | 31            | 9.3 (5.4, 13.2)   | 0.06    | <0.01a | 0.06  |
| 4-hydroxybenzyl alcohol                        | 44            | 12.0 (8.6, 15.4)  | 46          | 11.2 (7.9, 14.5)  | 42              | 13.6 (10.1, 17.1) | 31            | 17.5 (13.4, 21.5) | 0.04    | 0.05a  | 0.60  |
| 3-caffeoylquinic acid                          | 44            | 14.0 (10.7, 17.2) | 46          | 16.8 (13.6, 20.0) | 42              | 13.1 (9.7, 16.4)  | 31            | 17.7 (13.8, 21.6) | 0.44    | 0.82   | 0.03  |
| hesperetin                                     | 44            | 11.8 (9.0, 14.5)  | 46          | 9.7 (7.0, 12.4)   | 42              | 11.1 (8.2, 13.9)  | 31            | 5.7 (2.4, 9.0)    | 0.03    | 0.20   | 0.02  |
| hesperetin-7'-sulfate                          | 44            | 5.9 (3.4, 8.4)    | 46          | 5.5 (3.1, 8.0)    | 42              | 2.2 (-0.3, 4.8)   | 31            | 3.1 (0.15, 6.1)   | 0.04    | 0.02a  | 0.74  |
| 3,4-dihydroxybenzoic acid methyl ester         | 44            | 5.5 (2.8, 8.2)    | 46          | 8.3 (5.7, 10.9)   | 42              | 2.6 (-0.1, 5.4)   | 31            | 6.0 (2.8, 9.2)    | 0.45    | 0.05a  | 0.02  |
| naringenin-7- <i>O</i> -glucuronide            | 44            | 11.1 (8.5, 13.8)  | 46          | 13.3 (10.7, 15.9) | 42              | 8.5 (5.7, 11.2)   | 31            | 10.2 (7.1, 13.4)  | 0.22    | 0.03a  | 0.12  |
| naringenin-7- <i>O</i> -glucuronide            | 44            | 11.1 (8.5, 13.8)  | 46          | 13.3 (10.7, 15.9) | 42              | 8.5 (5.7, 11.2)   | 31            | 10.2 (7.1, 13.4)  | 0.22    | 0.03a  | 0.12  |
| 3,4-dihydroxybenzoic acid                      | 44            | 12.4 (9.4, 15.3)  | 46          | 12.4 (9.5, 15.3)  | 42              | 9.4 (6.3, 12.4)   | 31            | 7.7 (4.2, 11.2)   | 0.02    | 0.02a  | 0.81  |
| 4-hydroxycinnamic acid                         | 44            | 6.4 (3.9, 8.9)    | 46          | 9.7 (7.3, 12.2)   | 42              | 5.4 (2.9, 8.0)    | 31            | 7.7 (4.7, 10.7)   | 0.90    | 0.20   | 0.02  |
| trihydroxybenzaldehyde*                        | 44            | 6.9 (5.1, 8.8)    | 46          | 7.9 (6.1, 9.7)    | 42              | 4.4 (2.5, 6.3)    | 31            | 4.8 (2.6, 7.1)    | 0.03    | <0.01a | 0.34  |
| 3-methylhippuric acid                          | 44            | 4.4 (1.3, 7.4)    | 46          | 4.2 (1.2, 7.2)    | 42              | 7.1 (4.0, 10.2)   | 31            | 9.3 (5.7, 12.9)   | 0.02    | 0.02a  | 0.74  |
| 2-hydroxy-4-methoxybenzoic acid                | 44            | 5.9 (3.3, 8.4)    | 46          | 5.5 (3.0, 8.0)    | 42              | 2.3 (-0.3, 4.9)   | 31            | 2.3 (-0.7, 5.4)   | 0.02    | 0.01a  | 0.93  |
| 3-(3-hydroxyphenyl)propionic acid              | 44            | 21.3 (18.5, 24.0) | 46          | 19.6 (16.9, 22.3) | 42              | 20.6 (17.8, 23.4) | 31            | 14.0 (10.8, 17.3) | 0.01a   | 0.08   | 0.02  |
| (-)-epicatechin                                | 44            | 9.6 (6.8, 12.3)   | 46          | 9.7 (6.9, 12.4)   | 42              | 6.9 (4.1, 9.7)    | 31            | 5.8 (2.5, 9.1)    | 0.04    | 0.03a  | 0.92  |
| 4-hydroxybenzoic acid-3- <i>O</i> -glucuronide | 44            | 5.9 (3.8, 8.0)    | 46          | 8.7 (6.6, 10.8)   | 42              | 4.2 (2.1, 6.4)    | 31            | 7.6 (5.0, 10.1)   | 0.94    | 0.14   | 0.01  |
| hydroxy-methoxybenzoic acid*                   | 44            | 8.6 (5.9, 11.2)   | 46          | 12.1 (9.5, 14.7)  | 42              | 6.3 (3.6, 9.1)    | 31            | 5.9 (2.7, 9.1)    | 0.04    | <0.01a | 0.14  |
| hydroxybenzoic acid*                           | 44            | 15.4 (12.0, 18.7) | 46          | 13.8 (10.6, 17.1) | 42              | 11.3 (7.9, 14.7)  | 31            | 10.3 (6.3, 14.3)  | 0.03    | 0.04a  | 0.58  |

|  |    |                   |    |                   |    |                  |    |                   |        |        |      |
|--|----|-------------------|----|-------------------|----|------------------|----|-------------------|--------|--------|------|
| 3-hydroxybenzoic acid-4- <i>O</i> -glucuronide | 44 | 9.6 (7.1, 12.0)   | 46 | 11.5 (9.0, 13.9)  | 42 | 6.3 (3.8, 8.8)   | 31 | 4.9 (2.0, 7.9)    | <0.01a | <0.01a | 0.52 |
| cyanidin-3- <i>O</i> -glucoside                | 44 | 6.6 (4.5, 8.7)    | 46 | 6.7 (4.6, 8.7)    | 42 | 2.8 (0.60, 4.9)  | 31 | 6.4 (3.9, 8.9)    | 0.25   | 0.04a  | 0.10 |
| ellagic acid                                   | 44 | 15.6 (12.7, 18.4) | 46 | 15.1 (12.3, 17.9) | 42 | 10.0 (7.1, 12.9) | 31 | 13.4 (10.1, 16.8) | 0.06   | 0.01a  | 0.29 |
| 3-hydroxy-4-methoxybenzoic acid                | 44 | 9.8 (6.8, 12.8)   | 46 | 11.4 (8.5, 14.3)  | 42 | 7.5 (4.4, 10.5)  | 31 | 7.1 (3.6, 10.7)   | 0.11   | 0.04a  | 0.51 |
| catechin*                                      | 44 | 10.1 (7.0, 13.3)  | 46 | 12.3 (9.2, 15.4)  | 42 | 7.2 (4.0, 10.5)  | 31 | 8.6 (4.9, 12.4)   | 0.20   | 0.04a  | 0.21 |
| hydroxybenzoic acid-sulfate*                   | 44 | 9.6 (7.0, 12.1)   | 46 | 14.0 (11.5, 16.5) | 42 | 5.6 (3.0, 8.3)   | 31 | 6.3 (3.2, 9.3)    | 0.01a  | <0.01a | 0.02 |
| 4-hydroxy-3-methoxybenzoic acid methyl ester   | 44 | 13.0 (9.8, 16.3)  | 46 | 9.3 (6.1, 12.4)   | 42 | 6.4 (3.1, 9.7)   | 31 | 6.2 (2.3, 10.1)   | <0.01a | 0.01a  | 0.32 |
| 3-methoxybenzoic acid                          | 44 | 3.5 (1.0, 6.0)    | 46 | 3.9 (1.4, 6.3)    | 42 | 3.3 (0.71, 5.8)  | 31 | 4.7 (1.7, 7.6)    | 0.69   | 0.90   | 0.54 |
| hippuric acid                                  | 44 | 11.8 (8.3, 15.3)  | 46 | 12.7 (9.3, 16.1)  | 42 | 7.6 (4.0, 11.2)  | 31 | 8.5 (4.4, 12.7)   | 0.07   | 0.02a  | 0.49 |

669 Values are mean (95% CI). Metabolites shown are those with a significant group, age or sex effect; P values calculated from linear regression. <sup>a</sup>

670 false discovery rate adjusted p values <0.2. *Full dataset providing p values for all analytes is found in Supplement Table 6.* \*Metabolite isomers

671 which could not be resolved effectively by HPLC were quantified according to one of the structural isomers.

**Table 5: Classification tables obtained from linear discriminant analysis in 163 participants from the COB study**

| Metabolite                                  | Coefficient <sup>1</sup> | P=   |
|---|--------------------------|------|
| hydroxy-methoxybenzoic acid*                | 0.43                     | 0.08 |
| 3-methylhippuric acid                       | -0.44                    | 0.03 |
| 4-methoxybenzaldehyde                       | 0.28                     | 0.02 |
| 2-hydroxycinnamic acid                      | 0.36                     | 0.06 |
| 3-hydroxy-4-methoxybenzoic acid             | 0.17                     | 0.01 |
| 3-(4-hydroxy-3-methoxyphenyl)propionic acid | 0.27                     | 0.13 |
| 4-Hydroxy-3-methoxyacetophenone             | 0.23                     | 0.17 |
| 3-(phenyl)propionic acid                    | 0.18                     | 0.08 |
| 3-(dihydroxyphenyl)propionic acid*          | 0.15                     | 0.22 |
| benzoic acid-4- <i>O</i> -glucuronide       | -0.50                    | 0.13 |

<sup>1</sup> Standardized canonical discriminant function coefficients representing the effect of a 1 SD increase on metabolite concentration on the SD increase in the predicted values on the discriminant function. These values indicate the predictive ability of these metabolites to classify participants into the correct age group. P= calculated using ANOVA. \*Metabolites without numbered nomenclature are isomers of unknown hydroxy, methoxy, sulfate or glucuronide structural orientation (no physical references standards were available for retention time conformation (but were matched for mass and MS/MS spectra).

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1 **Table 6: Hierarchical regression analysis of predictors of metabolite concentrations in COB participants**

| <b>Metabolite</b>   | <b>n=</b> | <b>M1</b>                 | <b>M2</b> | <b>M3</b> |
|---|-----------|---------------------------|-----------|-----------|
|   |           | <b>β (95% CI) for age</b> | <b>P=</b> | <b>P=</b> |
| hydroxy-methoxybenzoic acid*                              | 156       | 0.04 (0.01, 0.07)         | 0.06      | 0.67      |
| 3-methylhippuric acid(x 10 <sup>2</sup> )                 | 155       | -0.42 (-0.84, 0.00)       | 0.05      | 0.74      |
| 4-methoxybenzaldehyde                                     | 154       | 0.00 (0.05, 0.01)         | 0.94      | 0.97      |
| 2-hydroxycinnamic acid(x 10 <sup>2</sup> )                | 156       | 0.02 (0.00, 0.05)         | 0.10      | 0.58      |
| 3-hydroxy-4-methoxybenzoic acid                           | 155       | 0.06 (0.02, 0.09)         | 0.69      | 0.56      |
| 3-(4-hydroxy-3-methoxyphenyl)propionic acid               | 156       | 0.04 (-0.15, 0.08)        | 0.36      | 0.73      |
| 4-Hydroxy-3-methoxyacetophenone                           | 155       | 0.01 (-0.13, 0.02)        | 0.36      | 0.55      |
| 3-(phenyl)propionic acid                                  | 125       | 0.05 (-0.66, 0.11)        | 0.64      | 0.94      |
| 3-(dihydroxyphenyl)propionic acid*                        | 154       | 0.01 (-0.75, 0.02)        | 0.39      | 0.58      |
| benzoic acid-4- <i>O</i> -glucuronide(x 10 <sup>2</sup> ) | 155       | -0.74 (-1.62, 0.00)       | 0.06      | 0.43      |

2 M1 = age; M2 = age and sex; M3 = age and PCA1. β (95% CI) for age were calculated from hierarchical regression with metabolite

3 concentration as the dependent variable and age, sex and PCA1 as predictors. P= p-value of the F-statistic indicating if the subsequent model

4 offered any significant improvement over M1. M= model; PCA1 = A linear combination of the gut microbiome variables associated with age

- 5 (first principal component; 41 % of the variance). \*Metabolite isomers which could not be resolved effectively by HPLC were quantified
- 6 according to one of the structural isomers.

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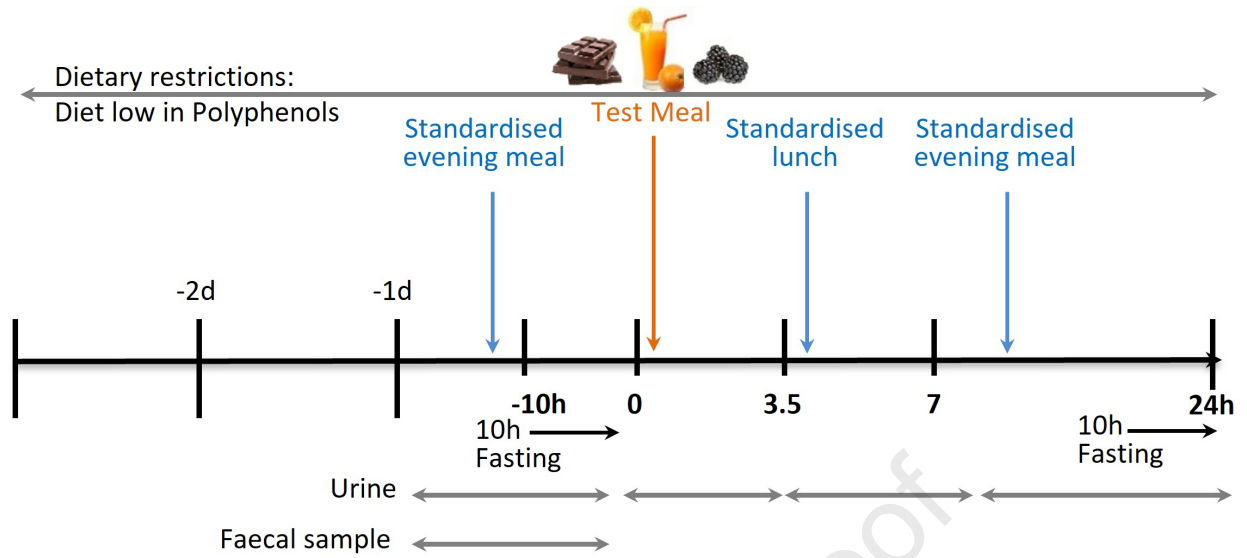
8 **FIGURE LEGENDS**

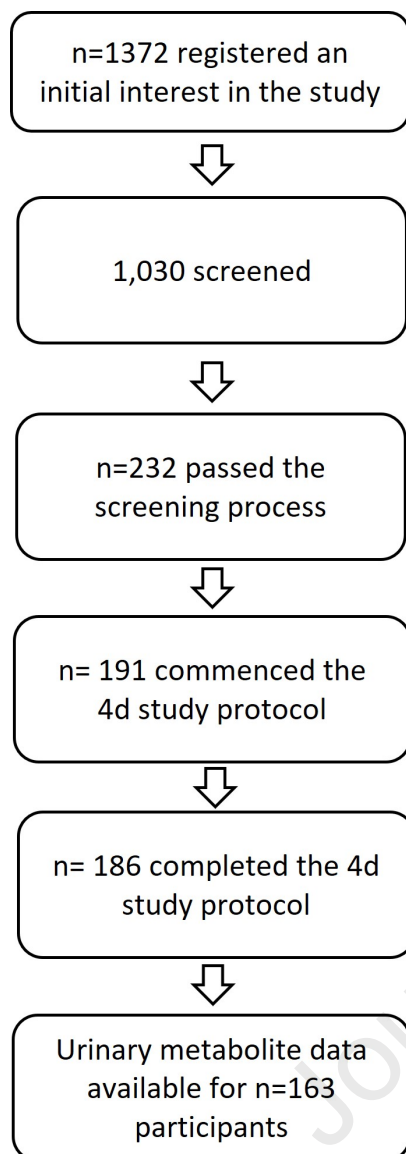
9 **Figure 1:** chocolate, orange juice and blackberry (COB) intervention to examine flavonoid  
10 metabolism

11 **Figure 2:** Participant Flow Diagram.

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**Declaration of interests**

x The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Anne-Marie Minihane reports was provided by University of East Anglia Norwich Medical School. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.