The effects of flavanone-rich citrus juice on cognitive function and cerebral blood flow: an acute, randomised, placebo controlled crossover trial in healthy young adults

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1   **The effects of flavanone-rich citrus juice on cognitive function and cerebral blood flow:**

2   **an acute, randomised, placebo controlled crossover trial in healthy young adults**

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16   Short title: Flavanone rich juice, cognition & CBF

17

22 **Abstract**

23 One plausible mechanism underlying flavonoid-associated cognitive effects is increased

24 cerebral blood flow (CBF). However, behavioural and CBF effects following flavanone-rich

25 juice consumption have not been explored. The aim was to investigate whether consumption

26 of flavanone-rich juice is associated with acute cognitive benefits and increased regional CBF

27 in healthy young adults. An acute, single-blind, randomised crossover design was applied

28 with two 500ml drink conditions; high flavanone (HF; 70.5mg) and an energy, vitamin C

29 matched zero flavanone control. Twenty four healthy young adults aged 18-30 underwent

30 cognitive testing at baseline and two hours post drink consumption. A further sixteen healthy

31 young adults were recruited for fMRI assessment whereby CBF was measured with arterial

32 spin labelling during conscious resting state at baseline, and two and five hours post drink

33 consumption. The HF drink was associated with significantly increased regional perfusion in

34 the inferior and middle right frontal gyrus at two hours relative to baseline and the control

35 drink. In addition, the HF drink was associated with significantly improved performance on

36 the Digit Symbol Substitution Test at two hours relative to baseline and the control drink, but

37 no effects were observed on any other behavioural cognitive tests. These results demonstrate

38 that consumption of flavanone-rich citrus juice in quantities commonly consumed can acutely

39 enhance blood flow to the brain in healthy young adults. However, further work is required to

40 establish a direct causal link between increased cerebral blood flow and enhanced

41 behavioural outcomes following citrus juice ingestion.

1. Introduction

Studies investigating the neuro-protective effects of foods and beverages containing flavonoids suggest that they may lead to benefits for memory and learning by improving neuronal functioning and promoting neuronal protection and regeneration[1]. In rodents, dietary flavanone supplementation (e.g. hesperidin) over several weeks is associated with significant improvements in spatial working memory. Moreover, these cognitive improvements correlate with increased expression of signalling proteins involved in learning and memory, and increased brain derived neurotrophic factor (BDNF) in the hippocampus[2,3]. These are important findings since increased expression of BDNF is associated with benefits for cognitive function in humans such as slower onset of Alzheimer's disease[4]. This supports the presence of mechanistic pathways by which citrus fruit based flavanones may have positive effects on the brain.

Epidemiological data showing an association between flavanone consumption and crystallized intelligence[5] is supported by positive effects from several human intervention studies indicating cognitive benefits in adults following chronic consumption of flavanone-rich fruits and vegetables, for reviews see[6,7]. For example, improved memory function in older adults with mild cognitive impairment (MCI) has been observed following daily consumption of concord grape juice (CGJ) for twelve weeks[8] and sixteen weeks[9]. Of particular relevance here is a recent finding that eight weeks daily consumption of flavanone-rich orange juice was associated with improvements in executive function and episodic memory in healthy older adults aged 60-81 years[10]. This indicates that consumption of fruit juices which contain flavanones as the predominant flavonoid may lead to benefits for the human brain, even in healthy adults.

Neuro-imaging studies in young human adults have demonstrated that consumption of flavanol-rich cocoa can acutely enhance peripheral and cerebral blood flow (CBF)[11,12]. Furthermore, promising associations have been observed between increased neuronal activity and behavioural benefits following chronic flavanol-rich cocoa supplementation. Enhanced activation in the dentate gyrus (measured with a fMRI blood oxygenation level-dependent (BOLD) signal) and simultaneous improvements in spatial working memory were reported in healthy older adults following consumption of flavanol-rich cocoa for three months relative to a low flavanol control[13].

73　However, other chronic flavanol interventions have failed to report concomitant cognitive

74　benefits in the presence of enhanced neuronal activation. For example, increased steady state

75　evoked potentials (assessed using Steady State Probe Topography) in posterior parietal and

76　central-frontal regions were observed in middle-aged adults following thirty days daily

77　consumption of 250mg or 500mg cocoa flavanol drinks relative to placebo, however, there

78　were no effects for behavioural measures of spatial working memory[14]. Similarly, enhanced

79　activation was observed in various brain regions during performance of an attention

80　switching task following five days consumption of 172mg cocoa flavanols. However,

81　changes in the BOLD signal were not associated with performance on the attention switching

82　task[12].

83　To summarise, the evidence suggests that flavonoid consumption can enhance vasodilation in

84　the periphery and lead to increased blood flow in specific regions of the brain in the acute

85　postprandial period. Daily flavonoid consumption over several weeks is associated with

86　cognitive benefits, but as yet, there is only weak evidence supporting a coupling between

87　increased CBF with improved performance on neuropsychological tests. The current research

88　builds upon these findings by investigating whether the aforementioned positive cognitive

89　effects of daily flavanone consumption over several weeks[10] are supported by acute

90　cognitive benefits in the immediate postprandial phase. It is reasonable to hypothesise that

91　acute cognitive benefits are underpinned by changes in CBF. Therefore, in addition to

92　assessing behavioural outcomes, the present research examined the effects of flavanone-rich

93　juice on CBF using fMRI arterial spin labelling (ASL). We chose a commercially available

94　citrus-based juice given that flavanones are naturally found in high concentrations in citrus

95　fruits such as orange and grapefruit. This also reflects the quality and quantity of juice

96　consumed by the general population. In sum, the aim of the present research was to

97　investigate the effects of flavanone-rich juice on acute cognitive function and CBF in healthy

98　young adults by adopting a placebo matched, crossover, randomized, single-blind, design.

99　**2. Experimental Methods**

100　Different participants were recruited for the behavioural cognitive arm (n=28) and the ASL

101　imaging arm (n=16) of the study (see Table 1), however, inclusion and exclusion criteria

102　were identical for both arms. Participants were not permitted to take part in both arms. At the

103　time of designing the study, there was an absence of published data concerning the effects of

104　flavanone consumption in humans on cognitive function, cardiovascular outcomes, or

105 cerebral blood flow. Therefore, we considered it important to create an experimental design

106 in which cognitive and cerebral blood flow effects could be examined in isolation. For,

107 example, it is important to establish if effects on CBF are observed independently of

108 behavioural effects. Furthermore, in light of the absence of experimental support for a

109 specific behavioural task sensitive to flavanone consumption in humans, it was considered

110 that a range of cognitive functions should be assessed. Incorporating a comprehensive

111 cognitive battery into the fMRI sequencing schedule posed significant practical difficulties.

112 Therefore, a decision was taken to recruit separate cohorts for the behavioural and imaging

113 arms. Healthy young adults aged 18-30 years were recruited from the University of Reading

114 and surrounding area via community advertising with posters, leaflets and emails. Twenty

115 four participants (four males) completed the behavioural cognitive arm (four participants

116 dropped out due to work commitments or illness) and all sixteen participants completed the

117 ASL arm (eight males). Inclusion criteria were BMI 19-25kg/m^2 and fluent English speaker

118 whilst exclusion criteria were signs of mild cognitive impairment (Mini Mental State

119 Examination Score <26), smoking, alcohol consumption >15 units/week, orange juice

120 consumption >250ml/day, fruit/vegetable consumption >4 portions/day, caffeine intake >3

121 drinks/day, actively pursuing weight loss through a dietary intervention, clinical diagnosis of

122 mental illness, neurological disease, chronic fatigue, kidney disease, liver disease, thyroid

123 dysfunction, diabetes mellitus, myocardial infarction or hypertension, and consumption of

124 medication for lipids, hypertension, hypotension or anticoagulation. Recruitment commenced

125 March 2011 and terminated August 2011. Our sample size was based on previous research

126 reporting significant cognitive effects of berry flavonoids in older adults with sample sizes

127 ranging from nine to twenty one[8,9,15] and improvements in CBF following cocoa flavanols in

128 sixteen young adults[12].

129 [Table 1 here]

130 2.1 Design

131 An acute single-blind, randomised cross-over design was applied with two drink conditions;

132 high flavonoid (HF) and control (CT). Cognitive behavioural testing and ASL measurements

133 were performed prior to and post consumption of the drink at each visit (see procedure). The

134 500ml HF drink was a commercially available 100% juice (Tropicana Ruby Breakfast Juice,

135 PepsiCo Inc.) which naturally contained 70.5mg flavonoids (42.15mg hesperidin, 17.25mg

136 naringin, 6.75mg narirutin, 4.3mg caffeic acid; analysed by the University of Reading),

137   225kcal, 48.5g sugars, 4g protein, 0g fat, 3.5g fibre, and 150mg vitamin C. The Tropicana

138   Ruby Breakfast Juice contained juices from oranges and grapefruits. The 500ml CT drink

139   was a commercially-available concentrated cordial product (Lemon Barley Squash,

140   Sainsbury's, UK) which was prepared with 240mls of concentrate and 260mls of mineral

141   water (Buxton Spring still mineral water) containing zero flavonoids, 230kcal, 48g sugars,

142   0.7g protein, 0g fat, 0.3g fiber, and 130mg vitamin C. Our dose of 70.5mg flavonoids could

143   be considered low relative to previous research[6], however, it is important to examine

144   whether cognitive benefits are associated with consuming concentrations of flavanones which

145   are present in the habitual diet. Therefore, the 500ml juice serving provided an acceptable

146   balance between a suitable flavonoid concentration and an achievable volume of

147   consumption within the context of the habitual diet. The drinks were stored at 4°C and

148   prepared and served by the experimenter. Each 500ml portion was served in two 250ml

149   opaque flasks and consumed through an opaque straw, thus participants could not see the

150   drink and remained blinded. The randomisation order was determined by an independent

151   statistician. For the behavioural cognitive arm, twelve participants consumed the HF drink at

152   visit 1 and twelve consumed the CT drink at visit 1, whilst for the ASL arm eight participants

153   consumed the HF drink at visit 1 and eight consumed the CT drink at visit 1.

154   2.2 Procedure

155   In summary, participants attended three separate visits; one screening visit and two test day

156   visit. The behavioural arm test days included two cognitive test time points (baseline and two

157   hour post) and the ASL arm visit days included three time points (baseline, two hour post and

158   five hour post). The screening visit and each test day visit were separated by a one week

159   washout. Initially telephone screening interviews were performed and volunteers who met the

160   inclusion criteria were invited to attend the University of Reading Hugh Sinclair Nutrition

161   Unit for a screening visit. At screening, data on height, weight, health status, medication and

162   blood pressure was collected and participants completed the Mini Mental State Examination

163   (MMSE), a diet and lifestyle questionnaire and a fruit and vegetable questionnaire, data from

164   which was used to corroborate the inclusion/exclusion criteria. For each test day visit,

165   participants arrived at 08:00 having fasted from alcohol for 48 hours and all other food and

166   drink (except water) for twelve hours. At screening, participants were provided with low-

167   nitrate bottled water for consumption during the fast. Prior to each test day visit, participants

168   were also instructed to avoid polyphenol-rich foods for 24 hours (including berries, fruits,

169   fruit juices, jams and preserves, red wine, black, green and fruit teas, coffee, cocoa, soy

170 products, caffeinated energy drinks and vegetables except potatoes) and were provided with

171 standardised typed instructions identifying which foods to avoid. The evening prior to each

172 test day, participants consumed (at home) a low fat standardized chicken and rice meal

173 provided by the research team (350kcal, 6.9g fat of which 3g saturates, 52.1g carbohydrate of

174 which 9.7g sugars, 19g protein, 1.4g fiber, 0.9g salt) to avoid second-meal cognitive

175 effects[16]. On each test day participants were required to orally confirm that they had adhered

176 to the aforementioned dietary restrictions. Following a fifteen minute rest, blood pressure

177 measurements were taken (on behavioural visit days only) on the left upper arm by a

178 validated blood pressure monitor (Omron MX2 automatic digital upper arms BP monitor,

179 Milton Keynes, UK) and recorded as the average of three consecutive measurements. At

180 08:30 hrs, participants consumed a standardised breakfast within fifteen minutes (88g

181 croissant, 25g cream cheese and 120ml bottled mineral water containing 51g fat, 14g protein,

182 64g carbohydrates, 777kcal). For the behavioural test days, baseline cognitive testing

183 commenced at 08:45 hrs, followed by consumption of the drink (either HF or CT) at 09:45

184 hrs. Participants were informed that the drink was a fruit-based beverage available in most

185 UK supermarkets and which must be consumed within fifteen minutes. Blood pressure was

186 measured at 11:40 hrs (behavioural arm only) and lunch, identical to breakfast in both content

187 and amount, was provided fifteen minutes prior to the two-hour post-drink cognitive battery

188 which commenced at 12:00 hrs. An assessment at this time point was based on previous data

189 demonstrating cognitive effects 2 hours following an acute flavonoid dose[12]. For the ASL

190 visit days, the timings were identical to the behavioural cognitive visit days, such that ASL

191 measurements were performed at 08:45 hrs (baseline), 12:00 hrs (two hours) and 15:00 hrs

192 (five hours). The behavioural cognitive visits took place in individual cubicles at the

193 University of Reading Hugh Sinclair Nutrition Unit and the ASL visits took place at the

194 Centre for Integrative Neuroscience and Neurodynamics (CINN). Participants remained

195 within the Nutrition Unit or the CINN for the entire test visit during which only water

196 consumption was permitted (notwithstanding the test day foods and drinks). Participants

197 received a £120 honorarium upon completion. This study was conducted according to the

198 guidelines laid down in the Declaration of Helsinki and all procedures involving human

199 subjects were approved by the School of Psychology and Clinical Languages Ethics

200 Committee. Written and verbal informed consent was obtained and formally recorded.

201 2.3 Cognitive Battery

202 The 45-minute cognitive battery consisted of the following tests administered in the

203 respective order: Freiburg Vision Test (v3.6.3), Word Recall (immediate), Logical Memory

204 (immediate recall), Sequence Learning Task, Digit Symbol Substitution (DSST), Stroop Test,

205 Letter Memory Test, Go-NoGo Task, Spatial Delayed Recall, Word Recall (delayed), and

206 Logical Memory (delayed). Where multiple versions of a test were required (see below),

207 parallel versions were presented in a counterbalanced order across conditions and visits. The

208 Freiburg Vision Test assesses visual acuity[17] for which there are two dependent variables:

209 Landholt C and Vernier Threshold. To acquire the Landhold C measurement participants

210 were required to identify the orientation of a horseshoe symbol using the numbers 1-9 on the

211 keyboard keypad (excluding 5). The presentation size of the horseshoe and thus the ease of

212 identifying the orientation randomly varied across trials. Landholt C was subsequently

213 calculated according to the number of correct responses relative to the presentation size. To

214 acquire the Vernier Threshold, participants viewed a stimulus which consisted of two 1cm

215 lines with one directly above the other. Participants pressed the left scroll key if the line

216 above was to the left of the line below, and the right scroll key if the line above was to the

217 right of the line below. The degree to which the lines were aligned varied randomly across

218 trials. The Vernier Threshold was subsequently calculated according to the number of correct

219 responses relative to the horizontal distance between the two lines[17]. Verbal Recall involved

220 computerised, individual presentation of thirty words. A response was required (using the

221 keys 'M' for yes, 'Z' for no) according to one of five questions which required visual,

222 phonetic or semantic processing of the target word (e.g. "is the word in capitals", "does the

223 word rhyme with..." or "is the word a type of..."). Upon cessation of the presentation, oral

224 recall of the target words was required (the dependent variable). Within each version of the

225 test, each word was accompanied by the same question for all participants whilst the order of

226 presentation varied randomly. Equal versions were created and matched for frequency,

227 familiarity, imageability, meaningfulness, word length and syllables. Delayed Word Recall

228 involved one attempt to orally recall the words presented thirty minutes prior during

229 Immediate Word Recall. The Logical Memory Test (Wechsler Memory Scale – Revised)

230 requires oral recall of a short paragraph. The paragraphs were presented via cassette tape. The

231 dependent variables for immediate and delayed recall were the number of correctly recalled

232 units. The Sequence Learning Task[18] required participants to immediately press the keys 'V,

233 B, N or M' according to the appearance of a stimulus (a 2mm white dot for 200ms) in one of

234 four 3.5cm x 2cm boxes on the screen. Unbeknownst to participants, the order of stimulus

235 presentation followed a set sequence (one block), thus this test assesses the ability to learn a

236 sequence. The duration of each repetitive sequence varied from 2-4 trials. Each test

237 presentation contained six blocks, with each block consisting of 100 trials. The dependent

238 variable was number of correct responses. The DSST[19] is a pen and paper test which

239 contains a key of nine digit-symbol pairs and an accompanying list of digits. Under each

240 listed digit a space is provided to enter the corresponding symbol. Participants entered as

241 many symbols as possible over 90 seconds. The dependent variable was the number of

242 correct responses. The computerised Stroop Test[20] required participants to identify the

243 colour in which a word was presented. There were 120 randomly presented stimuli, each for

244 1650ms, consisting of 60 congruent and 60 incongruent trials (a congruent trial being when

245 the meaning of the word matched the colour in which it was presented). Participants

246 responded with the keys 1-4 which represented the colours green, blue, red and yellow

247 respectively. The dependent variable was reaction time (for correct responses only). The

248 Letter Memory Task[21] involved serial 2000ms presentation of individual letters. The number

249 of letters per trial varied randomly between 5, 7, 9 and 11 for a total of twelve trials and 48

250 letters. For each trial, at the termination of the presentation phase participants were required

251 to orally recall the final four letters from the presentation. The dependent variable was the

252 total number of correct responses defined as recalling the correct sequence in its entirety. The

253 Go-NoGo is a computerised task assessing inhibition and sustained attention. The present

254 version was adapted from the Go-NoGo paradigm[22]. Participants were required to respond

255 to sixty stimuli using one of three specified keyboard keys; 'p' 'q' or 'space bar'. The stimuli

256 consisted of X, Y or a number 'lure'. Initially, there was a 25 stimuli 'Pre-Potent Go' phase.

257 During the Pre-Potent Go phase, X and Y were presented alternately, with the participant

258 required to press 'q' when X appeared and 'p' when Y appeared. The X and Y were known

259 as the 'Go' trials. The Go-NoGo phase followed the Pre-Potent Go phase. During the Go-

260 NoGo phase, the 'Go' trials were interspersed with 'NoGo' trials; these appeared as numbers

261 lures. Pressing the space bar was the required response upon viewing a number lure. During

262 the Go-NoGo phase X and Y were presented randomly, interspersed with number lures, such

263 that the predictable alternating sequence was disrupted. Responses were required only if a Y

264 appeared after an X or vice-versa, and therefore the participant must inhibit the established

265 pre-potent response in all other trials. Reaction Time for correct responses was the dependent

266 variable. The Spatial Delayed Recall Test required participants to recall the location of a

267 white dot on the screen. Each trial commenced with a fixation cross followed by presentation

268 of a white dot for 50ms in a random location. The white dot was replaced by a randomly

269 generated number between 90-99 at which point participants were asked to orally subtract

10

270 three from this number continuously for eight seconds. Once eight seconds had elapsed the

271 number disappeared and the participant was required to indicate (by touching the screen) the

272 location at which the white dot had previously appeared. There were sixteen trials in total and

273 the dependent variable was the distance from the target (mm).

274 2.4 fMRI protocol

275 Scanning was performed at the CINN, University of Reading, UK using a 3.0 Tesla Siemens

276 MAGNETOM Trio MRI scanner with a 12-channel Head Matrix coil. The ASL images were

277 acquired using the PICOREQ2T sequence with the following parameters: number of

278 slices=18, slice thickness=5.0mm, inter-slice gap=1.25mm, TR=2500ms, TE=11ms,

279 TI1=700, Saturation Stop Time=1600, TI2=1800, perfusion mode=PICORE Q2T (pulsed). A

280 high resolution whole-brain three dimensional anatomical image was also acquired using an

281 MPRAGE gradient-sequence with 176 x 1mm thick slices (1*1*1 voxels size, TE: 2.52ms,

282 TR: 2020ms, TI: 1100ms, FOV: 250x250, slice thickness: mm2, Flip Angle: 9deg). FMRI

283 data processing was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part

284 of FSL (FMRIB's Software Library; www.fmrib.ox.ac.uk/fsl). ASL volumes from each

285 scanning session were all registered to the corresponding individual's high resolution

286 structural image using rigid body transformations. In a second step, the images were

287 registered to the Montreal Neurological Institute (MNI) template brain using a 12 degrees of

288 freedom affine transformation algorithm. To allow voxelwise comparisons, each CBF map

289 was individually processed using perfusion signal modelling, which models the differences

290 between control images and tagged (spin labelled) images within a time series. A CBF map

291 was produced for each participant, drink (HF and CT) and time point (baseline, two hours

292 and five hours).

293 2.5 Statistical Analysis

294 All analysis and data processing was performed by independent researchers who did not

295 participant in any of the test day procedures and remained blinded to condition. Cognitive test

296 and blood pressure-dependent variables were assessed with a 2x2 repeated measures

297 ANOVA (Drink x Time). Significant main effects and interactions were explored with post

298 hoc t-tests applying Bonferroni corrections for familywise error. Analysis of the cognitive

299 and blood pressure data was performed with SPSS Statistics 21. FMRI data processing was

300 carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98 part of FSL (FMRIB's

301   Software Library, www.fmrib.ox.ac.uk/fsl). ASL volumes from each scanning session were

302   all registered to the corresponding individual's high resolution structural image using rigid

303   body transformations. In a second step the images were normalised to the Montreal

304   Neurological Institute (MNI) template brain using a 12 degrees of freedom affine

305   transformation algorithm. To allow voxelwise comparisons, we firstly processed each CBF

306   map individually using the perfusion signal modelling, which models the differences between

307   control and tag. We processed a CBF map for each participant, time point (pre and post) and

308   drink (HF & CT). These perfusion flow maps were then given as inputs for the 2nd level

309   analysis (t contrasts) which processed the difference between pre and post for each drink.

310   Specifically these t test contrasts compared the CBF maps at 2 and 5 hours post drink with

311   the pre drink baseline, and had the form of a simple subtraction defined as such: CBF 2 hrs -

312   CBF baseline, and CBF 5 hrs - CBF baseline. The output of this second step was contrast

313   images which corresponded to the actual increase in the perfusion flow post drink

314   consumption. Each of those contrast images was then entered into a 3rd level paired-sample t

315   test which compared the drink interventions. The resulting Z (Gaussianised T/F) statistic

316   image was then cluster thresholded with initial clusters determined using a voxelwise

317   uncorrected height threshold of Z>2.3 followed by a cluster significance threshold of p <0.05

318   (corrected for multiple comparisons). Prior to analysis normality checks were performed on

319   all data and outliers were removed.

320   **3. Results**

321   [Figure 1 here]

322   3.1 ASL CBF

323   Figure 1 shows significantly greater regional perfusion in the inferior frontal gyrus and

324   middle frontal gyrus of the right hemisphere two hours following consumption of the HF

325   drink compared to the CT drink (988 voxels, co-ordinates: (X=37.9, Y=31.8, Z=17.8),

326   statistics threshold: Z=3.69, p<0.001. There were no significant differences in regional

327   perfusion between the HF and CT drinks five hours post consumption, and no significant

328   differences in global perfusion were observed between the two conditions at either time point.

329   3.2 Cognitive Tests

330   [Figure 2 here]

331 A significant Drink*Time interaction was observed for the DSST ($F^{1,23}$=10.76, p<0.01). As

332 shown in Figure 2, post hoc t-tests revealed that consumption of the HF drink resulted in a

333 significant improvement in DSST performance at two hours relative to baseline (t=3.84,

334 p<0.01), whereas no significant improvement in performance was observed following the CT

335 drink (t=0.05, p=0.96). Baseline DSST performance did not differ between the CT and HF

336 drinks (t=0.02, p=0.98). No significant interactions or main effects were observed for all

337 other cognitive tests (see Table 2).

338 [Table 2 here]

339 3.3 Blood pressure

340 The Drink*Time interactions were not significant for either diastolic ($F^{1,23}$=1.19, p=0.29) or

341 systolic blood pressure ($F^{1,23}$=0.5, p=0.49). However, main effects of Time revealed that both

342 systolic ($F^{1,23}$=4.56, p<0.05) and diastolic ($F^{1,23}$=13.38, p<0.01) blood pressure significantly

343 reduced at two hours relative to baseline (see Table 2). To further explore the main effect of

344 Time, post hoc t-tests revealed that consumption of the HF drink significantly reduced

345 diastolic blood pressure at two hours compared to baseline (t=3.43, p<0.01), whereas this

346 reduction did not reach significance following the CT drink (t=2.05, p>0.05).

347 **4. Discussion**

348 Acute improvement in a measure of executive function (DSST) and increased CBF in the

349 right frontal gyrus during conscious resting state were observed two hours following

350 consumption of 500ml of flavanone-rich citrus juice relative to a zero flavonoid, vitamin C

351 matched, equicaloric control drink. These data indicate that 70.5mg flavonoids (specifically

352 42.15mg hesperidin, 17.25mg naringin, 6.75mg narirutin, 4.3mg caffeic acid) can increase

353 CBF in healthy young adults. However, these data do not provide evidence for a direct

354 association between increased CBF and behavioural benefits. Firstly, cognitive testing and

355 CBF were not assessed simultaneously, and moreover, no effects were observed for the

356 majority of cognitive outcomes.

357 This is the first data to show regional specific increases in human CBF following a flavanone

358 dose. The frontal gyrus has been identified within a network of brain areas which are active

359 during conscious resting state[23] which may explain the observed regional specific increased

360 perfusion. The inferior frontal gyrus has typically been implicated in tasks which require

361 inhibition, planning, decision making and other aspects of executive function[24], such as the

362   DSST, for which improvements were observed in this study following the flavanone-rich

363   juice. However, the mechanisms underpinning the right hemispheric lateralisation are

364   unclear.

365   These data provide evidence that flavonoid sub-classes other than cocoa-flavanols can also

366   have acute effects on CBF within the immediate postprandial period. Increased global CBF

367   across grey matter was observed 2 hours after consumption of a 560mg flavanol drink

368   relative to a control drink[12], however, regional blood flow was not assessed, most likely due

369   to the small sample size of healthy young adults (n=4). The same authors also reported that a

370   smaller flavanol dose (172mg) was associated with increased regional specific BOLD signal

371   intensity (including medial and lateral prefrontal cortex, parietal cortex, anterior cingulate

372   cortex and the cerebellum) 1.5 hours post consumption in 16 health young adults, although

373   the cocoa drink was consumed for 5 consecutive days prior to the fMRI scan. Direct

374   comparisons between the regions of interest reported by Francis et al.[12] and the present

375   study are restricted by differences in scanning methods (BOLD or ASL), the flavonoid sub-

376   class and dose (172mg cocoa flavanols or 70.5mg fruit flavanones), duration of consumption

377   (5 days or a single acute dose) and behavioural instructions during imaging; the present study

378   examined conscious resting state whereas Francis et al.[12] examined neural activity during an

379   executive function task. In addition, a limitation of the present study was the absence of

380   double blinding during data collection which could have introduced experimenter biases.

381   Critically though, data analysis was performed blinded by an independent researcher. Further

382   investigation of the acute effects of flavonoid consumption on regional CBF are required in

383   order to identify whether specific regions appear to particularly reactive to flavonoid

384   ingestion in the postprandial period. For example, increased perfusion in the anterior

385   cingulate cortex and central opercular cortex was recently observed two hours post

386   consumption of 494mg cocoa flavanols[25], however, behavioural tasks were not assessed.

387   Studies of neural activation following chronic daily consumption of fruit based flavonoids[9]

388   and flavanol-rich cocoa flavonoids[13,14] indicate that areas of the brain implicated in memory

389   function such as the hippocampus, specifically the dentate gyrus, are especially sensitive.

390   The mechanisms by which flavonoids acutely induce vasodilation and enhance CBF are

391   thought to be via increased nitric oxide synthesis in the endothelium (eNOS). Nitric oxide

392   synthesis is a key regulator of angiogenesis and the dilation of cells, and is also synthesised

393   by neurons in response to neuronal activation (nNOS)[26]. As such, nitric oxide is thought to

394   be crucial for the coupling between increased blood supply and neuronal activity[27].

395 Flavonoid ingestion in humans is known to enhance circulating nitric oxide species[28] in

396 association with beneficial vascular outcomes such as increased flow mediated dilation and

397 augmented microcirculation[11]. Therefore, it is plausible that flavonoid-induced increases in

398 the bioavailability of nitric oxide in the brain may lead to increased blood vessel and neuronal

399 efficiency and, subsequently, improvements in cognitive function. These vascular

400 mechanisms are tentatively supported by the observed reduction in systolic blood pressure

401 following the flavanone-rich juice in the present study, however it should be noted that this

402 was a subtle reduction (3mmHg). Having said that, a large reduction in blood pressure would

403 not be anticipated in this sample of healthy young adults. Research in adults with metabolic

404 syndrome shows that 550mg daily supplementation of the flavanone hesperidin for three

405 weeks can lead to increased flow mediated dilation and endothelial nitric oxide synthesis[29].

406 This is pertinent to the present findings given that hesperidin was the predominant flavanone

407 within the flavanone-rich citrus juice.

408 Research is required to directly examine the relationship between flavonoid consumption,

409 nitric oxide activity, CBF and cognitive function. Interestingly, increased nitric oxide status

410 in the plasma has been observed two hours post consumption of flavonoid-rich apples,

411 however, no effects were observed for cognitive function[30]. Kean et al.[10] reported global

412 cognitive improvements in healthy older adults cognition following daily chronic

413 consumption of flavanone-rich orange juice (305mg/day) over eight weeks, however, nitric

414 oxide status was not examined. This sample of highly educated, healthy young adults, are

415 likely performing close to optimal functioning and therefore, there is greater potential for

416 acutely enhancing cognition in older adults who may be experiencing naturally occurring

417 ageing associated cognitive decline. This may explain why effects were not observed for the

418 majority of cognitive outcomes in the present study, particularly given the relatively small

419 flavanone dose (70.5mg). Previously, positive behavioural effects in healthy young adults

420 have only been observed following high doses of cocoa flavanols e.g. 573mg[31] and

421 550mg/994g[32]. Additionally, it has been argued that flavonoid interventions are more likely

422 to benefit cognition during tasks of high demand[32], therefore it is possible that the current

423 cognitive battery was not suitably challenging, however, there was no evidence of ceiling

424 effects.

425 It can be hypothesised that stronger behavioural effects may occur at a later time point given

426 that plasma flavanone metabolites following orange juice consumption have been observed to

427 peak at six hours[33,34]. Indeed, it is a limitation of the present study that cognitive function

428 was exclusively assessed two hours post consumption (in addition to baseline). Recently,

429 benefits for global cognitive function and subjective alertness were observed 2 and 6 hours

430 post consumption of a flavanone rich (272mg) 100% orange juice in healthy young adults,

431 with the effects being more pronounced (relative to the control drink) at 6 hours[35]. Having

432 said that, presently, increased CBF was observed at two hours but not five hours, possibly

433 indicating that the time course by which the flavonoids in orange and grapefruit juice exert

434 their physiological effects may differ relative to 100% orange juice, although the mechanism

435 for this is unclear. Future acute interventions of flavonoid consumption should examine

436 plasma flavonoid metabolites concomitantly with cognitive outcomes to investigate whether

437 peak metabolite concentrations coincide with the hypothesised behavioural effects. Flavanone

438 metabolites are certainly of interest given that they are known to cross the blood brain

439 barrier[36]. Future studies should carefully consider the time span over which circulating

440 flavonoid metabolites may impact cognitive outcomes. Anthocyanin metabolites have been

441 observed in urine up to 5 days following acute ingestion of blueberries[36]. This has

442 implications for the current findings; the 24 hour dietary restriction may not have been

443 sufficient to account for potential confounding effects of habitual flavonoid intake, although

444 it is unclear whether the associated levels of circulating metabolites can acutely affect

445 cognition.

446 In conclusion, 500ml citrus juice containing 70.5mg flavonoids was associated with increased

447 regional perfusion in the right frontal gyrus in young healthy adults two hours following the

448 flavanone-rich juice in conscious resting state relative to the zero-flavonoid, equicaloric,

449 vitamin C matched control. This data demonstrates that fruit based flavonoids can acutely

450 enhance CBF in healthy adults. Behavioural improvements on a battery of cognitive tests

451 following the flavonoid-rich juice were only observed for one measure of executive function

452 (DSST) in a separate cohort of young adults. Therefore, the present data does not show a

453 clear association between increased CBF and behavioural benefits. Further research should

454 simultaneously examine cognitive performance and respective functional brain activation,

455 regional cerebral blood flow and concentrations of circulating nitric oxide species following

456 consumption of flavonoid-rich juices to further our understanding of underlying mechanisms.

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552   Table 1 – mean participant characteristics for the behavioural cognitive arm and the arterial

553   spin labelling (ASL) arm (standard deviation)

	Behavioural Cognitive Arm (n=24)	**ASL Arm (n=16)**	**p-value comparison between arms**
Age (years)	22 (2.2)	22 (1.9)	0.73
BMI (kg/m^2)	23.2 (3.9)	23.3 (1.7)	0.88
Years in education	16.9 (1.8)	16.6 (1.4)	0.53
MMSE[1] (max 30)	29.3 (1)	29.6 (0.5)	0.19

554   [1]Mini Mental State Examination

555   Table 2 – Means and standard deviations for each cognitive test and blood pressure data at

556   baseline and two hour post consumption for the control and high flavanone drinks

		Control Drink	High Flavanone	Drink*Time interaction (p-value)
DSST[1]	Baseline	77.4 (9.7)	75.9 (8.4)	0.003**
	2 hours	77.5 (9.6)	80.3 (8.9)	
FVT Landholt C[2]	Baseline	0.41 (0.03)	0.4 (0.02)	0.19
	2 hours	0.42 (0.04)	0.4 (0.02)	
FVT Vernier[3]	Baseline	21.2 (23.3)	19.9 (20.1)	0.65
	2 hours	19.6 (16.8)	21.3 (14.7)	
GoNo-Go[4]	Baseline	315 (55)	310 (60)	0.86
	2 hours	308 (62)	305 (57)	
Letter Memory[5]	Baseline	77 (16.7)	74.6 (18.4)	0.89
	2 hours	77.1 (12)	74.1 (16.3)	
Logical Memory Imm[6]	Baseline	17.5 (3.6)	18.3 (3.3)	0.97
	2 hours	15.4 (3)	16.1 (3.6)	
Logical Memory Del.[6]	Baseline	16.1 (3.6)	15.8 (3.9)	0.48
	2 hours	14.1 (3.8)	14.6 (3.3)	
Sequence Learning[7]	Baseline	97.8 (1.5)	98 (1.6)	0.52
	2 hours	96.9 (2.1)	97 (2)	
Spatial Memory[8]	Baseline	27.3 (15.8)	28.2 (18)	0.68
	2 hours	28.2 (15.4)	30 (20.6)	
Stroop[9]	Baseline	654 (74)	647 (71)	0.71
	2 hours	626 (84)	623 (67)	
Word Recall Imm[10]	Baseline	7.3 (3.2)	7.3 (3.5)	0.11
	2 hours	7 (2.7)	5.7 (2.5)	
Word Recall Del.[10]	Baseline	5.2 (2.9)	5.2 (3.2)	0.15
	2 hours	4.5 (2.5)	3.2 (2.3)	
Diastolic BP[11]	Baseline	72 (8.4)	71.7 (7.5)	0.49
	2 hours	69.7 (7.8)	68.4 (7.5)	
Systolic BP[11]	Baseline	115.9 (12.4)	116.5 (12.4)	0.29
	2 hours	115.3 (12.3)	113.8 (12.1)	

557   **p<0.01

558   1Digit Symbol Substitution Test correct responses; 2 Freiburg Vision Test Landholt C a higher score indicates better vision; 3 Freiberg

559   Vision Test Vernier Threshold a higher score indicates better vision; 4 GoNo-Go reaction time (ms); 5 Letter Memory Accuracy; 6 Logical

560   Memory units recalled; 7 Sequence Learning correct responses; 8 Spatial Delayed Recall Test distance from target (mm), 9 Computerised

561   Stroop reaction time (ms); Word Recall number of words recalled; Blood Pressure mmHg.

562   Figure 1 Legend: Significantly greater regional perfusion occurred in the inferior frontal

563   gyrus and medial frontal gyrus of the right hemisphere two hours following the high

564   flavanone drink compared to the control drink. Activations are superimposed on axial slices

565   of the MNI template brain and represent perfusion flow in ml/100g tissue/min with yellow

566   indicating greater perfusion. The images were initially thresholded at $Z>2.3$ to identify

567   activation clusters and then a (corrected) cluster significance threshold of $p<0.05$ was applied.

568   Figure 2 Legend: Following a significant Drink*Time interaction ($F^{1,23}=10.76$, $p<0.01$) post

569   hoc tests revealed that number of correct responses on the Digit Symbol Substitution Test

570   was significantly greater at two hours relative to baseline ($t=3.84$, $p<0.01$) following
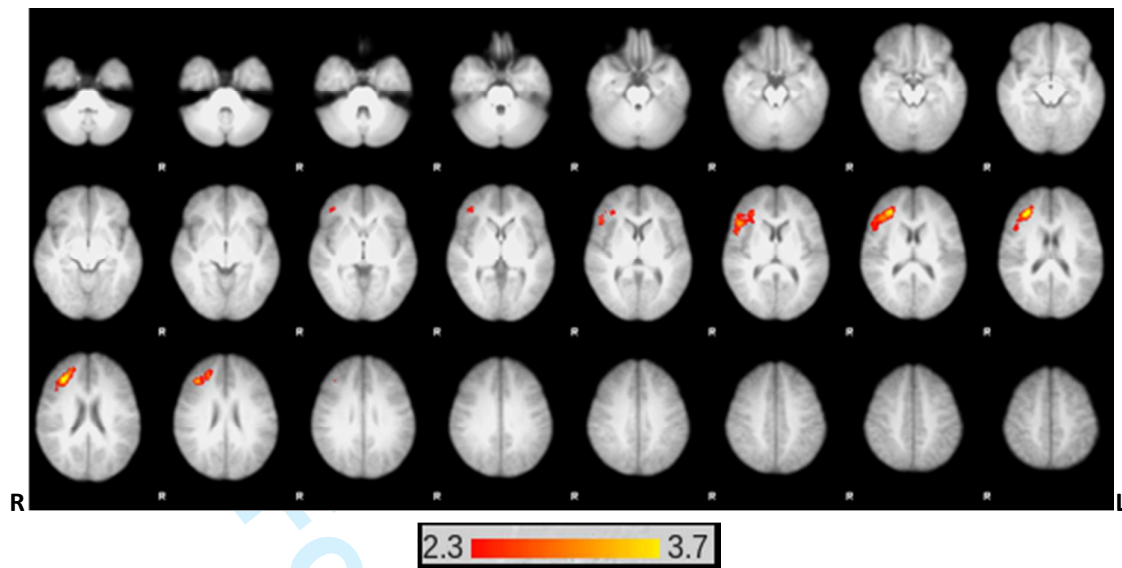
571   consumption of the flavanone rich juice.

Figure 2 – Digit Symbol Substitution Test mean correct responses and standard errors for the control and high flavanone drink at baseline and two hour post consumption