Mediation of coffee-induced improvements in human vascular function by chlorogenic acids and its metabolites: two randomized, controlled, crossover intervention trials

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Randomized Control Trials

Mediation of coffee-induced improvements in human vascular function by chlorogenic acids and its metabolites: Two randomized, controlled, crossover intervention trials

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SUMMARY

Background & aims: Polyphenol intake has been linked to improvements in human vascular function, although data on hydroxycinnamates, such as chlorogenic acid (CGA) have not yet been studied. We aimed to investigate the impact of coffee intake rich in chlorogenic acid on human vascular function and whether CGAs are involved in potential effects.

Methods: Two acute randomized, controlled, cross-over human intervention trials were conducted. The impact of coffee intake, matched for caffeine but differing in CGA content (89, and 310 mg) on flow-mediated dilatation (FMD) was assessed in 15 healthy male subjects. In a second intervention trial conducted with 24 healthy male subjects, the impact of pure 5-caffeoylquinic acid (5-CQA), the main CGA in coffee (5-CQA; 450 mg and 900 mg) on FMD was also investigated.

Results: We observed a bi-phasic FMD response after low and high polyphenol, (89 mg and 310 mg CGA) intake, with increases at 1 ($1.10 \pm 0.43\%$ and $1.34 \pm 0.62\%$, respectively) and 5 ($0.79\% \pm 0.32$ and $1.52\% \pm 0.40$, respectively) hours post coffee consumption. FMD responses to coffee intake was closely paralleled by the appearance of CGA metabolites in plasma, notably 3-, 4- and 5-feruloylquinic acid and ferulic-4′-O-sulfate at 1 h and isoferulic-3′-O-glucuronide and ferulic-4′-O-sulfate at 5 h. Intervention with purified 5-CQA (450 mg) also led to an improvement in FMD response relative to control ($0.75 \pm 1.31\%$ at 1 h post intervention, $p = 0.06$) and concomitant appearance of plasma metabolites.

Conclusions: Coffee intake acutely improves human vascular function, an effect, in part, mediated by 5-CQA and its physiological metabolites.

Study registration: The National Institutes of Health (NIH) on ClinicalTrials.gov NCT01813981 and NCT01772784.

1. Introduction

Moderate coffee consumption (3—5 servings per day) has been associated with reductions in both stroke and heart disease risk [1,2], something that may be related to its high levels of phenolic acids. A serving of coffee may contain between 27 and 121 mg of phenolic acids (200 mL), most notably the hydroxycinnamate chlorogenic acid (CGA), which is high in dietary terms [3]. Previous clinical trials indicate that the intake of phenolic-rich foods such as berries [4], grape/wine [5], tea and cocoa [6], which all contain structurally related phenolics, improve endothelium-dependent vascular reactivity, suggesting coffee, may also be capable of similar vascular benefits. Despite this, controlled human intervention studies with coffee are few and most have focused on the effects of caffeine, rather than that of the phenolics present, yielding data ambiguous as the overall influence of caffeine and/or coffee on vascular function [8—10]. However, previous studies with CGA in both humans [11] and animals [12] have indicated a potential for coffee to influence vascular endpoints, such as blood pressure, plasma cholesterol levels [13,14] and flow mediated dilatation (FMD), a measure of endothelial dysfunction [15].

The CGA content of coffee is highly variable, depending to a large degree on its processing into the final consumed form, particularly bean roasting, which is detrimental to CGA levels [16]. Thus, presently, it is unclear as to the levels of CGAs sufficient to induce physiological effects on the endothelium. Furthermore, the impact of coffee intake on vascular function over prolonged periods is also unclear. Studies using coffee and pure chlorogenic acid indicate that CGA absorption/metabolism may occur partly in the small intestine and also in the large intestine [17,18], where the microbiota activity cleaves the quinic acid moiety to yield caffeic acid which may then undergo further metabolism and/or absorption in the large intestine [19]. This absorption profile of CGA in humans and the inherent differences in CGA content of differently processed coffees, provides an opportunity to investigate whether CGA is linked to the vascular effects of coffee intake in humans, via interactions of circulating CGA metabolites on the vascular epithelium.

As such, the current investigations were designed to test the hypothesis that CGA is causally related to improvements in endothelial function, measured here as FMD, by conducting two clinical trials: firstly a randomized, controlled, single blinded, crossover intervention trial with two coffees differing in CGA content and controlled against caffeine (*efficacy study*); and a second supporting doubled blinded intervention trial using pure 5-caffeoylquinic acid (5-CQA; the major CGA in coffee) isolated from coffee (*proof of concept study (POC)*). Vascular measures are linked to measures plasma CGA metabolites in order to build cause-and-effect relationships between CGA intake, individual vascular responses and circulating levels of CGA metabolites.

2. Subjects and methods

2.1. Clinical trial ethics

Both clinical studies were conducted in line with the guidelines in the Declaration of Helsinki and study protocols were approved by the University of Reading Research Ethics Committee, UK (reference: 11/31) and Kantonale Ethikkommission Bern, Switzerland (reference 039/12). The trials were registered with the National Institutes of Health (NIH) records on ClinicalTrials.gov website (NCT01813981; *efficacy study*, NCT01772784; *POC study*).

2.2. Subjects

During year 2011,16 healthy male volunteers were recruited and enrolled onto *efficacy study*, a three arm, randomized, controlled, sinlge-blinded, crossover clinical trial and in year 2012 24 male volunteers were enrolled on *POC study*, a four arm, randomized, double-blinded, crossover clinical trial; all volunteers gave written informed consent prior to their participation (Fig. 1).

For both studies, volunteers were screened before to the start of the trial to ensure they were in good health and were selected according to the inclusion criteria in Table 1. Note, that using the same inclusion criteria for *POC study* as for *efficacy study* resulted in some volunteers in this study having basal FMD values higher than 8.5%. As such, in order to maximize our chances of observing increases in % FMD following CGA intake, we introduced an inclusion criteria of moderate smoking in order to recruit a population with basal % FMD at rest of between 5 and 6%. Those selected for the study were asked not to change their usual dietary or fluid intake and asked to refrain from consumption of polyphenols rich foods including fruits, vegetables, cocoa, chocolate, coffee, tea and wine, all alcoholic beverages in addition to refraining from vigorous exercise such as running, swimming and other high aerobic forms of exercise for 24 h prior to, and during, the study.

2.3. Interventions

For *efficacy study* a low polyphenol soluble coffee (LPC): roasting to internal bean temperature of 225 °C), containing 89 mg CGA per 3.6 g of coffee and a high polyphenol coffee (HPC) derived from combining 50% roasted and 50% green beans (165 °C), containing 310 mg CGA (3.6 g ground coffee) were utilized. Both coffees were prepared by addition of 3.6 g of ground coffee to 50 mL of hot (90 °C), nitrate/nitrite free water. The control intervention was 110 mg caffeine and 0 mg CGA in nitrate/nitrite free hot water, whilst 28 mg of caffeine was added to LPC in order to match with both the control and the HPC (*efficacy study*) (Table 2).

For *POC study* 450 mg purified 5-CQA + 1 g maltodextrin; 900 mg purified 5-CQA + 1 g maltodextrin, doses of CGA shown to decrease cardiovascular disearse risk [21]; 1 g maltodextrin (negative control) and 200 mg purified (−)-epicatechin + 1 g maltodextrin (positive control). Each treatment was reconstituted in 200 mL of warm, nitrate/nitrite free water (~60 °C) and stirred until completely dissolved.

2.4. Study design

Both studies were randomized controlled crossover trials. *Efficacy study* was 3 armed and single blinded (researcher blinded; participant not due to water control), where subjects consumed a LPC, a HPC, or a caffeine control. The two intervention coffees were indistinguishable in appearance and taste. *POC study* was 4 armed and doubled blinded where volunteers consumed two doses of 5-CQA, a negative and positive control; interventions were indistinguishable. In both studies participants were assigned unique sequential random numbers, which was previously allocated to one sequence of the study products according to a computer generated paper list produced by a researcher not otherwise involved in the study. All study personnel involved in the assessment of study outcomes, including nurses, care providers, researchers and the principle investigator, were blinded to intervention and intervention order.

On arrival at the Hugh Sinclair Unit for Human Nutrition, University of Reading, UK (*efficacy study*) or Department of Cardiology and Clinical Research, Inselspital, University Hospital Bern, Switzerland, *POC study*, subjects were rested for 30 min in a quiet, temperature controlled room (~21 °C) before they were cannulated
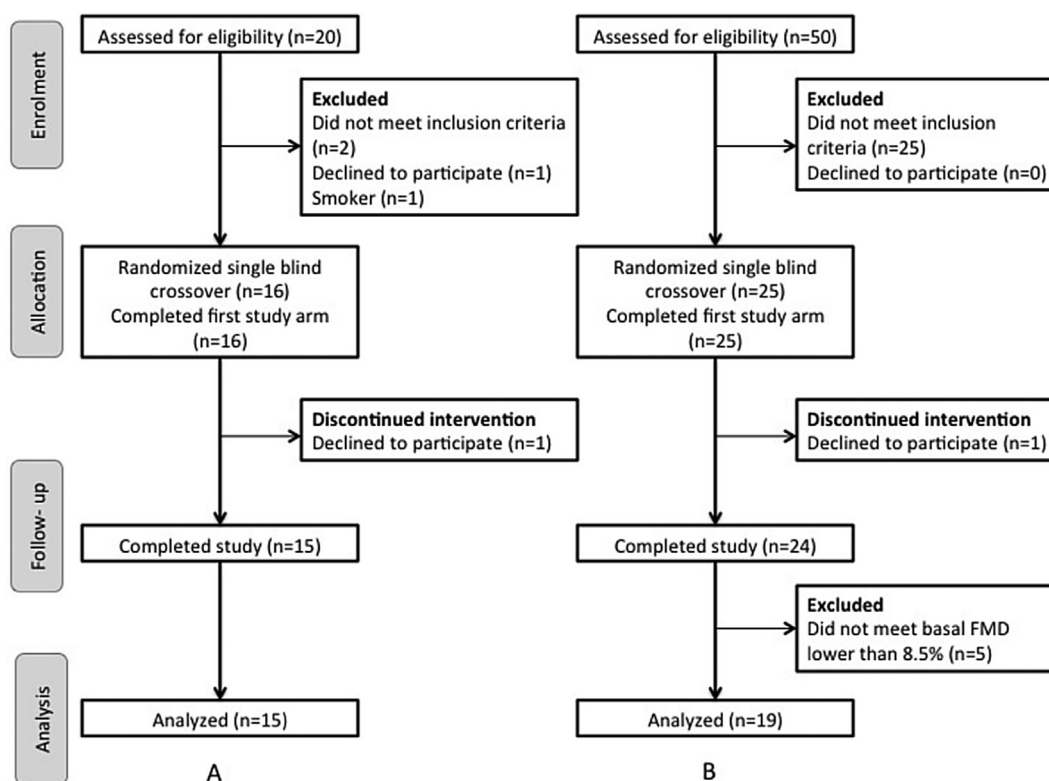
Fig. 1. Flow diagram for study participation in clinical *efficacy study* (A) and Clinical *POC study* (B).

Table 1
Inclusion and exclusion.

	Efficacy study	POC study
Inclusion	18- 40 years	18-70 years
	BMI 18–25 kg/m^2	BMI 18–30 kg/m^2 (and >55 kg)
		[a]
	Normal range liver enzymes, hemoglobin, hematocrit and leukocyte counts	
	No diabetes or hypertension (>150/90 mm Hg)	No diabetes, hypo or hypertension (101/51–160/100 mm Hg)
	Baseline flow mediated dilatation <8.5%	Baseline flow mediated dilatation <8.5%
		Smoking 5–10 cigarettes per day
Exclusion	Being female	Being female
	Taking anti-inflammatory or antihypertensive medication or antibiotics within two months	Taking any regular medication within two months
	Participating in vigorous exercise (>3 × 30 min/week)	Participating in vigorous exercise (>3 × 30 min/week)
	Drinking > 168 g alcohol per week	Drinking > 280 g alcohol per week
	Smoking	

BMI, body mass index; POC, proof of concept.
  [a] Although not part of inclusion criteria, all participants had normal biochemistry results as tested at the first visit.

and a blood sample drawn in the fasted state. Following an additional 10 min of rest, baseline FMD measurements of the brachial artery (primary outcome), blood pressure and laser Doppler imaging with iontophoresis [20] to measure cutaneous perfusion of acetylcholine and sodium nitroprusside; only measured in *efficacy study* (secondary outcomes). Following baseline measurements, volunteers consumed one of the interventions within a 5 min or 2 min period (*efficacy and POC study* respectively). FMD and blood samples were acquired at 1 3, and 5 h for *efficacy study* and 1 and 4 h for *POC study*. Laser Doppler imaging was performed only in *efficacy study* at 1, 3 and 5 h peripheral BP measurements were performed at 1, 3, and 5 h for *efficacy study* and 0.5, 1, 2, 3 and 4 h for *POC study*, post-consumption. Following the conclusion of each arm, volunteers undertook a 1-week washout period before switching to an alternate study arm.

2.5. Flow-mediated dilatation

FMD of the brachial artery was the primary end point measure of the studies and was measured following standard guidelines [22] using an ALT Ultrasound HDI5000 system (ATL Ultrasound, UK), with a semi-automated computerized analysis system (Brachial Analyzer, Medical Imaging Applications-llc, IL, US) for Intervention *efficacy study*. An Esaote MyLab30 Gold, (Esaote SpA, Italy) with a high frequency probe (7–10 MHz) linear array probe with edge detection software with a system for real-time measurement of the brachial artery diameter in B-mode ultrasound images (Cardiovascular Suite, Quipu, Pisa, Italy) was used for Intervention *POC study* as previously described [23]. For both studies briefly, after 30 min supine rest in a quiet, air-conditioned room, the brachial artery was imaged longitudinally at 2–10 cm proximal to the

Table 2
Composition of intervention coffee and control drinks used in Clinical study I.

	Caffeine control drink	Low polyphenol coffee	High polyphenol coffee
3-Caffeoylquinic acid (mg)	0	20	43
4-Caffeoylqunic acid (mg)	0	22	45
3-Feruloylquinic acid (mg)	0	4	10
5-Caffeoylquinic acid (mg)	0	29	124
4-Feruloylquinic acid (mg)	0	4	6
5-Feruloylquinic acid (mg)	0	5	24
3, 4-Dicaffeoylquinic acid (mg)	0	2	23
3, 5-Dicaffeoylqunic acid (mg)	0	1	17
4, 5-Dicaffeoylqunic acid (mg)	0	2	19
Caffeine (mg)	110	110	110
Energy (kcal)[a]	0	2.27	2.21
Protein (g)[a]	0	0.25	0.31
Carbohydrate (g)[a]	0	0.32	0.20
of which sugars (g)[a]	0	0.32	0.20
Fat (g)[a]	0	0.01	0.01
Fiber (g)[a]	0	0.97	1.08
Salt equivalent (g)[a]	0	0.01	0.01
Total chlorogenic acids (mg)	0	89	310

[a] As defined on coffee label.

antecubital fossa. Baseline images were recorded for 60 s, after which a blood pressure cuff placed around the forearm was inflated to 220 mm Hg or 250 mm Hg (for Intervention I and *POC study* respectively). After 5 min of occlusion, the pressure was rapidly released to allow reactive hyperemia, with image collection continuing for 5 or 4 min (for Intervention I and *POC study* respectively) post release. A single, fully trained researcher per center, who was blinded to the intervention details, analyzed all image files for the respective studies and peak diameter was defined as the maximum diameter obtained after the occlusion was released. FMD response was calculated as relative diameter change from baseline as compared to peak diameter during hyperemia and presented as percentage change.

2.6. Laser Doppler imaging

laser Doppler flowmetry/imaging to measure cutaneous perfusion accompanied by iontophoresis of acetylcholine and sodium nitroprusside was carried out as previously described [24] and was utilized in *efficacy study* only (as a secondary outcome). Measurements were taken after 30 min of acclimatization in a supine position in a quiet, temperature controlled room (22–24 °C). The incremental area under the flux versus time curve for 20 scans was used as a measure of micro vascular response to acetylcholine (Ach; endothelium dependent vasodilation) and sodium nitroprusside (SNP; endothelium independent vasodilation).

2.7. Systolic and diastolic blood pressure

SBP and DBP (also secondary outcomes) were measured in both intervention studies after subjects had rested for 30 min in the supine position using Omron MX2 (study 1) and Omron M6 (*POC study*), both automatic digital upper-arm blood pressure monitor. Blood pressure readings were taken every 2 min until 3 successful readings were obtained. The average of these readings were calculated and reported.

2.8. Plasma CGA/metabolite analysis

The blood samples were collected in EDTA treated tubes and spun (1700 × g; 10 min; 4 °C) immediately after collection and plasma was stored in 0.5 mL aliquots with antioxidant, ascorbic acid, at −80 °C. Sample preparation was performed at Nestlé Research Centre, Laussanne, Switzerland according to Marmet et al. [25]. Briefly, in a 2 mL Eppendorf tube, 100 μL of plasma (stored with EDTA/Vitamin C) spiked with 5 μL of internal standards (2 μM) was vortexed, and mixed with 500 μL of ethanol for protein precipitation. After centrifugation at 17,500 g for 5 min at 4 °C the supernatant was transferred into a 1.5 mL Eppendorf tube and the solvent was evaporated under nitrogen. The residue was extracted one more time, the supernatants pooled and the solvent dried again under nitrogen. After evaporation of the solvents, the residue was then mixed with 100 μL of 1% acetic acid 5% acetonitrile aqueous solution and injected (5 μL) for analysis by ultra performance liquid chromatography electrospray ionization mass spectrometry (UPLC-ESI-MS^n). CGA metabolites were separated by reversed-phase UPLC using a C18-column Acquity UPLC BEH C18 1.7 μm, 2.1 × 150 mm, (Waters AG, Baden-Dättwil, Switzerland). The system consists of an Nexera UPLC (Shimadzu Schweiz GmbH, Reinach, Switzerland) connected to a Triple QUAD 5500 mass spectrometer detector equipped with an electron spray ionisation source (AB Sciex Switzerland GmbH, C/o Applied Biosystems Europe BV, Zug, Switzerland). The molecular transitions listed in Supplementary Table 1 were used to qualify and quantify phenolic acids, their metabolites and the three internal standards (specific analytical parameters are indicated for each compound). The calibration curve was injected in singlet, but for all plasma samples, duplicate analysis was performed. Data were collected and processed using Analyst software (AB Sciex Switzerland GmbH, C/o Applied Biosystems Europe BV, Zug, Switzerland). Each participants' samples were analyzed within a single assay batch in random sequence. The samples were analyzed blind.

2.9. Power calculation and statistical analysis

Power calculations were performed for the primary endpoint, change in FMD response. In *efficacy study*, power was based on the intra-individual variability of the operator that performed the FMD analysis (5% CV, SD = 0.3, based on previous studies where the same subjects were measured on 4 different days at the same time). At 0.85 power and 0.05 significance, the number of subjects required to detect a difference of 1.5% FMD in the response of matched pairs in a crossover study was 15. This number is consistent with other studies carried out with similar endpoints and study design [26,27]. Two-way repeated measures ANOVA were fitted to analyze the data using GraphPad Prism version 6

(GraphPad Software Inc., San Diego, CA, US). Post-hoc analysis was carried out using the Bonferroni test. In *POC study*, a sample size of 24 subjects was estimated to have a power >0.85 to detect a mean difference of 1.5% FMD for the positive control considering a paired t-test of a normal mean difference with a two-sided significance level of 0.05, assuming a common standard deviation of 3% and correlation of at least 0.7. For all efficacy (primary and secondary) parameters, absolute changes from pre-dose (t0) were to be analyzed according to a mixed model for cross-over design with repeated measurements: the model was to include treatment, period, sequence, time point and its interaction with treatment as fixed effects, and volunteer and its interaction with period as random effects; change from pre-dose comparison between each of the 2 active 5-CQA doses and the negative control at 1 h post dose were to be derived from the model, and estimates and 95% confidence intervals (CI) for the difference were to be provided as well as nominal p-values. Significance was defined as $p < 0.05$, with p-values represented in the figures as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The incremental area under the laser Doppler imaging flux versus time curve was calculated using the trapezoidal method. Correlation analysis was performed using Pearson's correlation coefficient.

3. Results

3.1. Baseline anthropometry and intervention tolerance

The baseline characteristics of subjects enrolled in both studies were all within normal limits (Table 3). All interventions were well tolerated by all subjects and no adverse events were reported.

3.2. Coffee induced a biphasic increase in FMD

A biphasic increase in endothelium-dependent brachial artery vasodilation, measured as % FMD, was observed in response to consumption of both the HPC and LPC at 1 and 5 h, relative to the caffeine control (Fig. 2, A). A significant effect with respect to time ($p = 0.002$) and highly significant treatment effect ($p < 0.0001$) was observed. Post-hoc analysis indicated that, at 1 h post intervention, there were highly significant increases in % FMD in response to both LPC ($1.10 \pm 0.43\%$; $p < 0.05$) and HPC ($1.34 \pm 0.62\%$; $p < 0.05$), relative to a control change of $0.07 \pm 0.06\%$. At 5 h, HPC significantly increased % FMD by $1.52 \pm 0.40\%$) ($p < 0.0001$), whilst LPC increased it by $0.79 \pm 0.32\%$, ($p < 0.05$), relative to a control change of $-0.46 \pm 0.38\%$ (Fig. 2, A). The difference in % FMD increase induced by the two doses of CGA, delivered as coffee, was not significant, although there was a trend for a differential increase % FMD at 5 h ($p < 0.1$). Neither the LPC or the HPC induced significant changes in endothelium-independent (SNP; $p > 0.05$) or endothelial dependent (acetyl choline; $p > 0.05$) micro-vascular blood flow (Ach) (Table 4). Similarly, there were no significant changes in

Table 3
Baseline clinical characteristics study population.[a]

Characteristic	Efficacy study	POC study
N	15	24
Age (years)	26.3 ± 1.6	23.8 ± 1.4
BMI (kg/m²)	23.5 ± 0.5	23.2 ± 0.4
PSBP (mmHg)	124 ± 2.5	130.5 ± 2.0
PDBP (mmHg)	75 ± 2.3	76.9 ± 1.3
Brachial diameter (mm)	3.0 ± 0.2	3.7 ± 0.1
FMD (%)	5.7 ± 0.3	6.4 ± 0.3

BMI, body mass index; POC, proof of concept; PSBP, peripheral systolic blood pressure; PDBP, peripheral diastolic blood pressure; FMD, flow mediated dilatation.
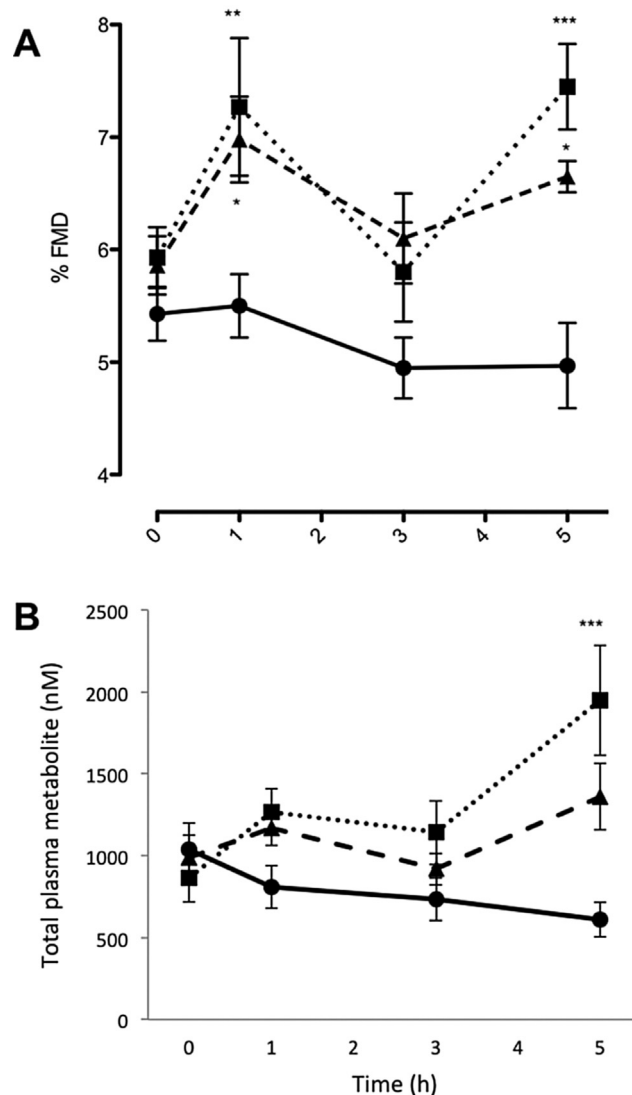[a] Results are expresses as mean ± SEM.

Fig. 2. % FMD (A) and plasma levels of total chlorogenic acid metabolites (sum of 56 identified) (B) following consumption of high polyphenol coffee (·······) and low polyphenol (— ·) (containing 310 mg and 89 mg chlorogenic acid respectively) and a caffeine matched control drink (——) (n = 15). Data were analyzed using two-way repeated measures ANOVA with time and treatment as the two factors. Post hoc analyses were conducted using a Tukey's HSD multiple comparisons test. Values presented are means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, significantly different respect to control drink at the specified time point.

either systolic or diastolic blood pressure in response to any of the interventions (Tables 4 and 5).

3.3. Plasma CGA metabolites

Similar to that observed with the FMD response, a biphasic increase in total plasma CGA metabolites was observed, peaking at 1 h initially and increasing again 5 h post intervention (Fig. 2, B). A significant effect with respect to both time ($p < 0.05$) and treatment ($p < 0.001$) were found for the total of the plasma CGA metabolites. HPC led to a significant increase ($p < 0.001$) in total plasma CGA metabolites 5 h after intake relative to the control. Despite observing similar plasma CGA metabolite profiles in response to LPC intake, these did not achieve significance, although there was also a trend to increase at 5 h, relative to the control.

Table 4
Acute effects of coffee on blood pressure and micro vascular dilation.[a]

	Baseline	1 h	3 h	5 h
IAUC LDI SNP (PU.h^{-1})^3				
HPC (236 mg CGA)	423 ± 153	424 ± 107	346 ± 75	431 ± 127
LPC (108 mg CGA)	541 ± 142	266 ± 75	501 ± 68	523 ± 125
Control (0 g CGA)	431 ± 132	250 ± 56	295 ± 71	262 ± 56
IAUC LDI Ach (PU.h^{-1})				
HPC (236 mg CGA)	272 ± 104	378 ± 64	300 ± 57	321 ± 84
LPC (108 mg CGA)	478 ± 147	237 ± 72	333 ± 63	288 ± 78
Control (0 g CGA)	387 ± 86	194 ± 53	222 ± 77	145 ± 32
PSBP (mmHg)				
HPC (236 mg CGA)	129 ± 4	128 ± 3	130 ± 3	128 ± 2
LPC (108 mg CGA)	126 ± 2	127 ± 2	128 ± 2	132 ± 3
Control (0 g CGA)	126 ± 3	132 ± 2	125 ± 2	129 ± 3
PDBP (mmHg)				
HPC (236 mg CGA)	70 ± 3	75 ± 2	74 ± 2	75 ± 2
LPC (108 mg CGA)	72 ± 2	79 ± 2	73 ± 1	75 ± 2
Control (0 g CGA)	70 ± 2	79 ± 2	74 ± 2	74 ± 2

PSBP, peripheral systolic blood pressure; PDBP, peripheral diastolic blood pressure; IAUC SNP (PU.h^{-1}), incremental area under the curve of flux vs. time for endothelium independent micro vascular dilation (using sodium nitroprusside) as measured by laser Doppler imaging; IAUC Ach (PU.h^{-1}) of flux vs. time for endothelium dependent micro vascular dilation (using acetylcholine) as measured by laser Doppler imaging; HPC, high polyphenol coffee; LPC, low polyphenol coffee.

[a] Values are expressed as means ± SEM (n = 15). No significant differences were found between baseline and post intervention (1, 3 and 5 h) or between control and coffee treatments (p > 0.05).

A total of 56 CGA metabolites were identified and quantified in plasma post intervention for HPC, LPC and control (Supplementary Table 2, 3 and 4 respectively). Both LPC and HPC induced an increase in 13 metabolites; 3-caffeoylquinic acid (3CQA), 4-caffeoylquinic acid (4CQA), caffeic-4′-O-sulfate (CA4S), 3-feruloylquinic acid (3FQA), 4-feruloylquinic acid (4FQA), 5-feruloylquinic acid (5FQA), ferulic acid (FA), isoferulic acid (iFA), methylferulic acid (MeFA), ferulic-4′-O-glucuronide (F4G), isoferulic-3′-O-glucuronide (iF3G), ferulic-4′-O-sulfate (F4S), and isoferulic-3′-O-sulfate (iF4S) (p < 0.001−0.05) relative to the control at 1 h post interventions (Fig. 3, A−M). At 5 h post intervention, only four metabolites; caffeic-3′-O-sulfate (CA3S), MeFA, F4S and iF3G increased relative to the control (p < 0.001−0.05) (Fig. 3, C, I and L).

3.4. Plasma metabolite changes predict changes in FMD

A significant, positive correlation was observed between total plasma CGA metabolites and % FMD across the whole study period (r = 0.21, p = 0.005; Pearson correlation). Furthermore, Pearson correlation assessments for individual metabolites (HPC and LCP combined) can be seen in Supplementary Table 5. Over the 0−1 h time frame, positive correlations (r > 0.2) were observed between % FMD and 3-CQA, 4-CQA, caffeic-3′-O-sulfate (C3S), 5-FQA, iF3G and F4S, which were significant for 3-CQA, 4-CQA, C3S and 5-FQA (p < 0.05), suggesting that these metabolites may be, at least in part, involved in mediating the observed increases in endothelium-

dependent vascular function between 0 and 1 h. When the same analysis was conducted for the 3−5 h period (representing the second peak in % FMD), positive correlations (r > 0.2) were seen with C4S, isoferulic-3′-O-glucuronide (iF3G), F4S, m-coumaric acid-3′-O-sulfate (mCo3S) and 4-methoxycinnamic acid (4MeCinA), all were significant (p < 0.05) (Supplementary Table 5). This suggests that these metabolites may predict the magnitude of FMD increase over this time frame.

3.5. FMD in response to pure CGA administration

As clinical *efficacy study* indicated a relationship between total circulating CGA metabolites and % FMD response in individuals, we conducted a *proof of concept study* to test the efficacy of pure 5-CQA (450 mg and 900 mg) administration, alongside a control and positive control (epicatechin [27]) on human vascular function. One hour after the oral ingestion of 5-CQA, the 450 mg dose, increased FMD in all volunteers to a magnitude that almost achieved significance in comparison to the control (6.02 ± 0.28% to 6.77 ± 0.42%, p = 0.06), whereas intake of the water control or the higher 900 mg dose of 5-CQA had no effect on % FMD (5.90 ± 0.33% to 5.86 ± 0.30% and 6.17 ± 0.31% to 6.46 ± 0.35%, respectively) (Fig. 4, A). Four hours after the ingestion of the treatments, values of FMD were 6.11 ± 0.3%, 6.83 ± 0.34% (p = 0.08) and 6.34 ± 0.34% for water, 450 and 900 mg of CQA. The positive control, (−)-epicatechin, which has been previously reported to induce increased vascular function (assessed by FMD) [27], induced a significant increase in % FMD at 1 h. Total plasma CGA metabolites post intervention of 450 mg of CGA were lower than following intake of the 900 mg dose, but both significantly increased (p = 0.032 and p = 0.006 respectively) (Fig. 4, B).

4. Discussion

A bi-phasic increase in FMD with peaks at 1 and 5 h was observed post consumption of HPC and LPC, which was accompanied by concomitant increases in plasma levels of CGA metabolites. At both 1 and 5 h post intervention, there was a significant increase in brachial artery dilation in comparison to the control drink after consumption of the HPC (p < 0.01 and p < 0.001 respectively) and with the LPC (p < 0.05 and 0.05 respectively). Previous work focusing on the effect of coffee on vascular health has predominantly centered on the physiological actions of caffeine and have not been controlled for phenolic content, leading to mixed results with respect to vasoactive efficacy [8,28]. In agreement with our data, an intake-dependence investigation using decaffeinated coffee indicated that higher levels of intake resulted in increases % FMD suggesting that compounds other than caffeine, likely CGA and related phenolics may contribute to vascular function [29]. In contrast to our data which used 5-CQA (the most abundant CGA in coffee), previous studies with pure 3-CQA showed no efficacy towards FMD [30]. Our study provides further evidence for the potential beneficial effects of low/moderate

Table 5
Acute effects of chlorogenic acid on blood pressure.

	Baseline	0.5 h	1 h	2 h	3 h	4 h
PSBP (mmHg)						
0 mg CGA	119.9 ± 1.2	119.9 ± 1.5	118.6 ± 1.4	117.9 ± 1.2	117.0 ± 0.9	121.3 ± 0.9
450 mg CGA	118.5 ± 1.4	118.4 ± 1.6	116.8 ± 1.6	117.3 ± 1.2	116.7 ± 1.2	118.4 ± 1.2
900 mg CGA	119.6 ± 1.4	117.9 ± 1.5	119.8 ± 1.7	117.1 ± 1.7	117.8 ± 1.7	120.0 ± 1.7
200 mg (−)-epicatechin	118.4 ± 1.9	118.6 ± 1.6	118.2 ± 1.5	120.1 ± 1.9	117.0 ± 1.8	117.0 ± 1.8
PDBP (mmHg)						
0 mg CGA	65.0 ± 1.2	65.2 ± 1.2	65.8 ± 0.9	66.0 ± 0.9	65.8 ± 1.1	67.0 ± 1.1
450 mg CGA	65.6 ± 1.2	65.4 ± 1.2	64.6 ± 1.3	64.3 ± 1.0	65.5 ± 1.0	66.0 ± 1.0
900 mg CGA	65.8 ± 1.2	66.1 ± 1.3	67.6 ± 0.8	65.6 ± 1.0	65.3 ± 0.9	66.1 ± 1.1
200 mg (−)-epicatechin	63.5 ± 1.3	63.5 ± 1.3	64.8 ± 1.2	67.0 ± 1.4	65.5 ± 1.2	66.2 ± 1.0

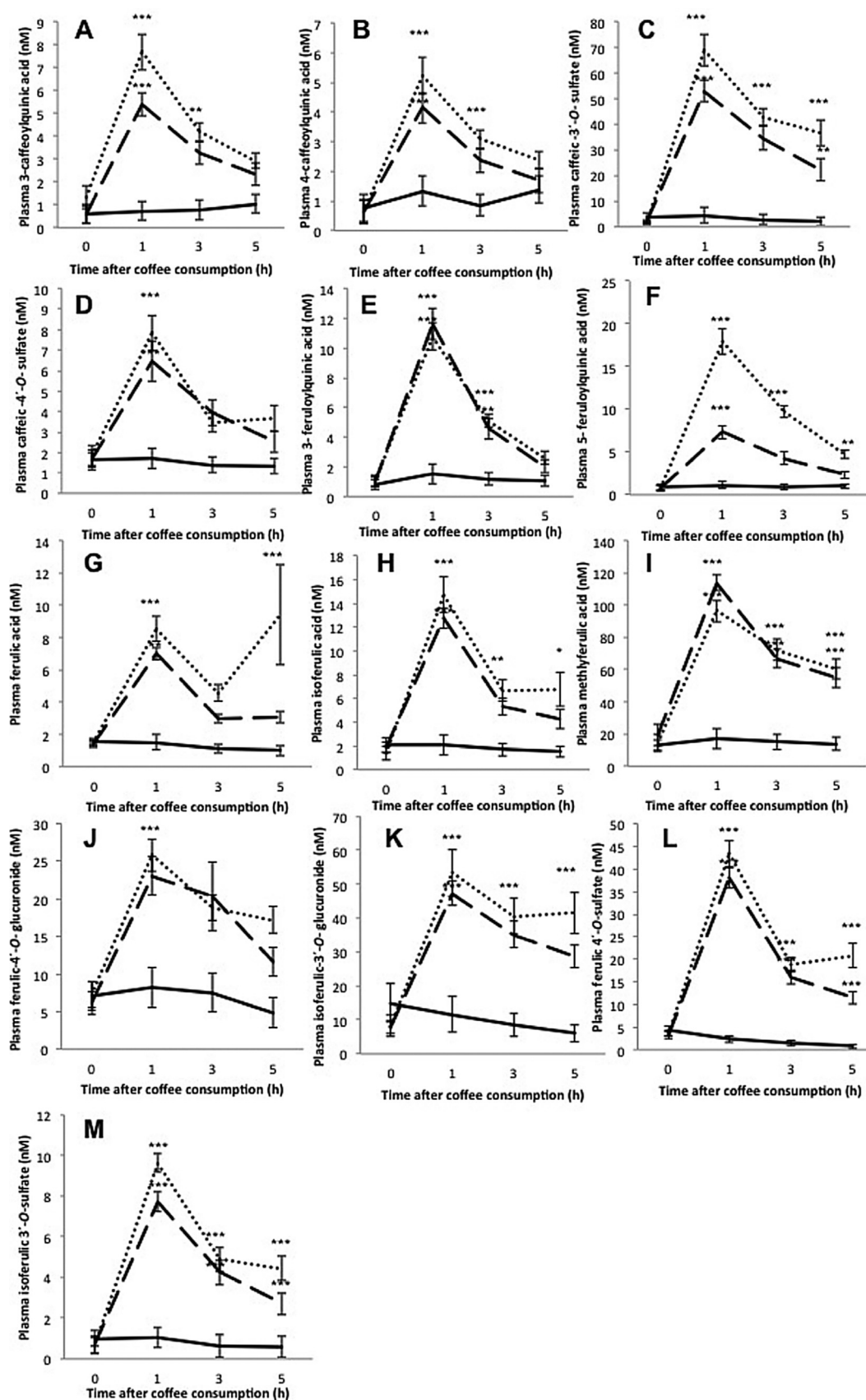PSBP (peripheral systolic blood pressure) and PDBP (peripheral diastolic blood pressure).

Fig. 3. Potential drivers of FMD response at 1 and 5 h as mean (±SEM) plasma concentrations of (A, 3-caffeoylquinic acid), (B, 4-caffeoylquinic acid), (C, caffeic-3′-O-sulfate), (D, caffeic-4′-O-sulfate), (E, 3- feruloylquinic acid), (F, 4- feruloylquinic), (G, ferulic acid), (H, isoferulic acid), (I, methylferulic acid), (J, ferulic-4′-O-glucuronide), (K, isoferulic-3′-O-glurcuronide), (L, ferulic-4′-O-sulfate), (M, isoferulic-3′-O-sulfate) after consumption of high polyphenol 310 mg (·········) or low polyphenol 89 mg (— ·) coffees or control (——) (n = 15). Data were analyzed using a 2- factor repeated measures AVOVA with time and treatment as the two factors [significant effect of time (p < 0.05), treatment (p < 0.001)]. Post hoc analyses were conducted by using Tukey's multiple comparisons test. Plasma concentrations significantly different compared with the control drink at the specified time point: *p < 0.05, **p < 0.01, ***p < 0.001.
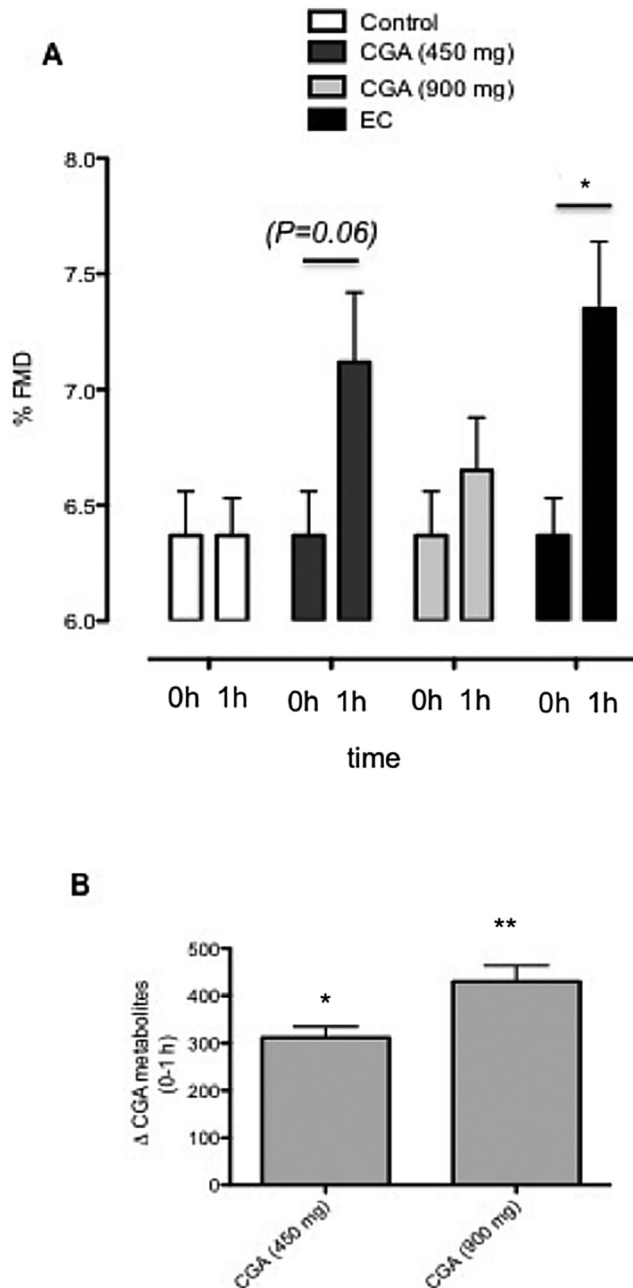
Fig. 4. Change in % FMD (A) and chlorogenic acid metabolites (B) 1 h post ingestion of 0 mg, 450 mg or 900 mg 5-caffeoylquinic acid (B) and 450 mg (−)-epicatechin (A) (n = 24). Data were analyzed using two-way repeated measures ANOVA with time and treatment as the two factors. Post hoc analyses were conducted using a Tukey's HSD multiple comparisons test. Values presented are means ± SEM. *p < 0.05, **p < 0.01.

coffee intake on human vascular function, providing insight, for the first time, into the temporal nature of its vasoactive benefits post intake, how efficacy is affected by the dose of phenolics delivered in the coffee and insights into the potential circulating metabolites that might mediate such effects.

As direct cause-and-effect relationships between intake and efficacy are difficult to establish following the intake of complex food matrices such as coffee, we performed a proof of concept study with purified 5-CQA. Data indicated that pure 5-CQA intake improves vascular function but only at the 450 mg and not the 900 mg intake level, something previously observed following the intake of flavonoid-rich foods/beverages [31], suggesting that there is an

optimal level of circulating polyphenol metabolites for driving physiological effects on the endothelium. In support of this finding, observational studies suggest that moderate coffee intake (4 cups), equating to anywhere between about 105 and 500 mg of CGA (depending on the processing conditions such as roasting), is associated with lower cardiovascular disease risk [1,2]. Combining datasets, illustrates a potentially Gaussian nature to the dose-dependency of CGA on vascular function, with 89 and 310 mg (delivered via coffee and caffeine matched) increasing blood flow by 1.1 and 1.34% FMD, respectively, whilst the 450 mg and 900 mg (pure), resulting in 0.75 and 0.28% FMD, respectively.

In order to investigate the metabolite(s) mediating these in vivo effects, we assessed the pharmacokinetic profile of circulating CGA metabolites up to 5 h post intake. Such data are scarce, with a number of studies conducted in rats [32–35] and others only reporting measurement of a limited numbers of plasma metabolites [36–38]. Our data suggest positive correlations between circulating levels of 3-CQA, 4-CQA and 5-FQA and the sulfate/glucuronide metabolites, C3S, F4S and iF3G (p < 0.05). C3S was the only metabolite that correlated, but had not significantly increased from baseline to 1 h (all others had p < 0.05); suggesting that it is not an important compound with regards to driving the first FMD peak (1 h). Whilst between 3 and 5 h positive correlations were observed for C4S, iF3G, F4S, mCo3S and 4MeCinA (p < 0.05), with F4S and iF3G appearing to mediate vascular efficacy at the second peak (5 h). The nature of these metabolites, along with the detection of un-metabolized CGA in plasma is consistent with previous investigations [37–39], with phenolic acid sulfates, which have previously been shown to reach C_{max} at approximately 1 h after coffee ingestion [40]. Potential mechanisms by which such circulating metabolites mediate their vascular effects have been postulated, and include their potential to inhibit NAPDH oxidase, thus affecting superoxide production and subsequent NO bioavailability at the vascular epithelium [31,41–43]. Notably, CGA administration to spontaneously hypertensive rats improves endothelium dependent vasodilation and decreases blood pressure via effects on NADPH oxidase activity and by increasing NO production [44]. Structural similarities between specific circulating CGA-derived metabolites and apocynin [45] (namely due to ferulic and caffeic acid moieties) provide support for such as mechanism, although direct inhibition of NADPH oxidase was not assessed in the current studies.

We acknowledge limitations within our studies and appreciate the difficulty in establishing cause−effect relationships. We recognize that in intervention *efficacy study* the control drink was not matched for macro and micro nutrients, and therefore the FMD response could well be due to the response to other compounds present in the coffee drinks and not in the control.

Further, both studies are short acute investigations which show the effect of coffee up to five hours after consumption and cannot be used to determine the long term effects of repeated consumption of coffee on vascular function. In addition, the study population was limited to healthy, young male participants and cannot be directly used to predict the vascular response that would be observed in other populations, for example females, elderly or those at risk for cardiovascular disease.

Our data, emanating from the two clinical trials, suggest that the acute increases in endothelium-dependent dilation following either coffee, or pure CGA may reflect the potential of phenolic metabolites to influence the bioavailability of nitric oxide within the vasculature. The vasoactive effects of coffee consumption are evident at moderate levels of consumption, with efficacy following a Gaussian profile with respect to total phenolic intake (at least at 1 h post intake). These data provide an insight into the potential beneficial vascular effects of coffee intake, although longer-term

interventions are required to assess whether these acute effects may be sustained over time, following repeated consumption of different types of coffee.

Conflict of interest

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2016.11.013.

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