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RESEARCH ARTICLE

The Glu298Asp single nucleotide polymorphism in the endothelial nitric oxide synthase gene differentially affects the vascular response to acute consumption of fruit and vegetable puree based drinks

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Scope: Diets low in fruits and vegetables (FV) are responsible for 2.7 million deaths from cardiovascular diseases (CVD) and certain cancers annually. Many FV and their juices contain flavonoids, some of which increase endothelial nitric oxide synthase (eNOS) activity. A single nucleotide polymorphism in the eNOS gene, where thymine (T) replaces guanine (G) at position 894 predicting substitution of glutamate for aspartate at codon 298 (Glu298Asp), has been associated with increased CVD risk due to effects on nitric oxide synthesis and subsequently vascular reactivity. Individuals can be homozygous for guanine (GG), thymine (TT) or heterozygous (GT).

Methods and results: We investigated the effects of acute ingestion of a FV-puree-based-drink (FVPD) on vasodilation and antioxidant status in subjects retrospectively genotyped for this polymorphism. Healthy volunteers ($n = 24$; 11 GG, 11 GT, 2 TT) aged 30–70 were recruited to a randomized, controlled, crossover, acute study. We showed that acute consumption of 400 mL FVPD differentially affected individuals depending on their genotype. There was a significant genotype interaction for endothelium-dependent vasodilation measured by laser Doppler imaging with iontophoresis ($P < 0.05$) and ex vivo low-density lipoproteins (LDL) oxidation ($P = 0.002$). GG subjects had increased endothelium-dependent vasodilation 180 min ($P = 0.028$) and reduced ex vivo LDL oxidation ($P = 0.013$) after 60 min after FVPD compared with control, no differences were observed in GT subjects.

Conclusion: eNOS Glu298Asp genotype differentially affects vasodilation and ex vivo LDL oxidation after consumption of FV in the form of a puree-based drink.

Keywords:
Fruit and vegetable juice / Glu298Asp polymorphism / Laser Doppler imaging / LDL oxidation / Vasodilation

1 Introduction

Fruit- and vegetable-rich diets have been linked to a decreased risk of cardiovascular diseases (CVD) in epidemiological studies [1]. The association between intake of fruit and vegetables (F&V) and the risk of developing coronary heart disease in 126 399 subjects showed that individuals in the highest

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Abbreviations: CVD, cardiovascular diseases; **eNOS**, endothelial nitric oxide synthase; **F&V**, fruit and vegetables; **FRAP**, ferric reducing antioxidant power; **FVPD**, fruit and vegetable puree based drink; **GAE**, gallic acid equivalents; **GG**, homozygous for guanine at position 894 in the eNOS gene; **Glu298Asp**, single nucleotide polymorphism in the eNOS gene resulting in a G/T (guanine, G, replaced by thymine, T) change at position 894 in exon 7 predicting a substitution of glutamic acid for aspartic acid at position 298; **GT**, heterozygote; **LDI**, laser Doppler imaging with iontophoresis; **NO**, nitric oxide; **ORAC**, oxygen radical absorbance capacity; **TT**, homozygous for thymine at position 894 in the eNOS gene

quintile of F&V consumption had a relative risk of coronary heart disease of 0.80 (95% CI, 0.69–0.93) compared to those in the lowest quintile [2]. The World Health Organisation estimated low F&V consumption caused 31% of ischemic heart disease and 11% of stroke worldwide and 2.7 million deaths annually could be prevented with increased consumption [3]. They recommended a minimum intake of 400 g of F&V per day to prevent chronic diseases such as heart disease, cancer, diabetes, and obesity. The protective effect of F&V could be attributed to fiber [4]; vitamins [5, 6] or non-nutritive phytochemicals they contain, such as polyphenols [7]; or carotenoids [8]. These beneficial components are plant secondary metabolites and are also present in varying quantities in many F&V juices [9–11]. Research indicates F&V juices also have a protective effect against CVD [12]. For example, Concord grape juice (5.5 mL/kg/day) significantly reduced systolic and diastolic blood pressure by 7.2 mmHg ($p = 0.005$) and 6.2 mmHg ($p = 0.001$), respectively [13], and clarified tomato juice consumption decreased platelet aggregation [14].

Many compounds found in F&V have high antioxidant potential [15, 16], which may reduce oxidative stress [17]. Possible mechanisms for their antioxidant activity include reducing formation of reactive oxygen species (ROS) by inhibition of enzymes or by chelation of pro-oxidant transition metal ions; scavenging ROS; and regenerating antioxidant defenses [18]. Oxidation of low-density lipoproteins (LDL) is recognized as an early stage in atherosclerosis development, leading to CVD. Many studies have reported the antioxidant effects of phytochemicals in F&V, including the retardation of the susceptibility of LDL to oxidation in vitro and in vivo [19–22]. However, reviews of clinical trials investigating the effects of F&V on chronic disease risk have indicated that the beneficial effect is not necessarily attributable to antioxidant compounds [16, 23, 24].

Polyphenols are one of the largest groups of phytochemicals and numerous F&V contain polyphenolic compounds known as flavonoid [25,26]. Some flavonoids have been shown to increase the activity of endothelial nitric oxide synthase (eNOS) in animal and cell studies [27–31]. eNOS is responsible for the generation of the vasodilator nitric oxide (NO) in the vascular endothelium [32]. Previous studies have looked at the effects of flavonoid-rich F&V and beverages on measures of vascular reactivity using flow-mediated dilation (FMD) [31, 33] or laser Doppler imaging with iontophoresis (LDI) [34,35] as methods to assess endothelial function in vivo in the brachial artery and the peripheral microcirculation, respectively. However, a meta-analysis of randomized controlled trials investigating the effects of flavonoid-rich foods on CVD risk found just 15 chronic and 14 acute intervention trials measuring FMD [36]. In the acute trials, chocolate or cocoa significantly increased FMD (3.99%, mean of six trials, 70–177 mg epicatechin/day at 90–149 min). The LDI technique has been used to assess the effects of dietary fats on vascular function [37]. However, there have been few studies reported using this method in conjunction with phytochemical-rich foods [34,35]. LDI allows vasoactive substances to be administered noninvasively via chambers adhered to the surface of the forearm facilitating simultaneous delivery of endothelium-dependent (acetylcholine) and endothelium-independent vasodilators (sodium nitroprusside, a direct source of NO) [38, 39]. This lack of studies highlights a need for further research investigating the effects of dietary flavonoids on in vivo measures of vascular function.

Acute studies with black tea or red wine show considerable heterogeneity in vascular response [36]. A potential reason for this variability may be a common polymorphism in the eNOS gene, where a modification in its coding sequence occurs when guanine (G) is substituted for thymine (T) at position 894 in exon 7, resulting in the replacement of glutamic acid for aspartic acid at codon 298 (Glu298Asp) [40]. Consequently, individuals are identified as either homozygous for guanine (GG, wild type), thymine (TT), or heterozygous (GT). The frequency of the Asp298 allele is around 33% in Caucasian populations [41]. Studies in healthy populations show a genotype frequency of GG:GT:TT of 47.8:42.0:10.2% in the United Kingdom [40]; 49.3:41.3:9.3% in Turkey [42]; and 54.6:39.2:6.2% in Brazil [43]. The Glu298Asp polymorphism has been associated with an increased risk of CVD events due to effects on NO biosynthesis and subsequently vascular reactivity [40, 44–47]. However, several studies have found no link between Glu298Asp and CVD in their study populations [48, 49]. Consequently, associations between this polymorphism and CVD risk remain uncertain and warrant further research [50].

The authors investigated the effects of acute consumption of a flavonoid-rich F&V puree based drink (FVPD) in healthy individuals aged between 30 and 70 years on vasodilation as the primary outcome. Additionally, measures of plasma nitrate and nitrite as a surrogate marker of NO, antioxidant status, and bioavailability of the phytochemicals present within the FVPD were also studied [51]. Upon completion of the study, the participants were retrospectively genotyped for the Glu298Asp polymorphism, and the effect of genotype on their vasodilatory response and antioxidant status are the focus of this article.

2 Materials and methods

2.1 Study participants

A total of 24 individuals (20 males and 4 females), aged 30–70, completed the study. The participants were recruited from the university and the local population. Participants were selected if they met the inclusion criteria of: BMI <30 and >20 kg/m^2; blood pressure $< 150/90$ mmHg; hemoglobin >125 g/L; no subjects on weight-reducing diets or taking dietary supplements; not diagnosed with liver disease or diabetes mellitus; no gall bladder problems or abnormalities of fat metabolism; not experienced a mycocardial infarction; no regular vigorous exercise or excessive alcohol

consumption. Blood was taken from all fasting study participants, which was analyzed for glucose, lipid levels, liver function status, and a measure of potential alcohol abuse. Basic anthropometric measurements were also recorded. The study was approved by the University of Reading Research Ethics Committee, and each participant gave informed consent before participating. Registration number for clinical trials: ISRCTN36287115.

2.2 Study design

The study was a randomised, single-blind, controlled, crossover acute dietary intervention study. The participants followed a low flavonoid diet for 5 days prior to the study days. On each study day, the fasted subjects consumed 400 mL FVPD (apple, carrot, and strawberry flavor Vie shots, Unilever Bestfoods, Germany) or a sugar-matched, fruit-flavored control. Blood samples were taken via a flexible cannula inserted into the forearm at baseline and then after drink consumption every 30 min for 4 h and every 60 min for a further 4 h. Urine was collected at baseline and total excretion collected after drink consumption at 120-min intervals for the 8 h of the study day. LDI measurements were recorded at baseline and at five 90-min intervals following drink consumption. The other arm of the study was conducted after a 4-week washout.

2.3 Intervention drinks

Participants consumed 400 mL FVPD (Unilever Bestfoods) or 50 mL fruit-flavored cordial (Robinsons' Lemon Barley Water, Britvic Soft Drinks Ltd., Chelmsford, UK) matched for sugar composition and diluted to 400 mL with low-nitrate mineral water (The Buxton Mineral Water Co. Ltd, Buxton, UK). FVPD was a preparation made from purees and concentrated juices, equivalent to 800 g F&V (apple, 56%; carrot, 29%; and strawberry, 8%). The nutrient content of the drinks is shown in Table 1.

Table 1. Nutrient composition and antioxidant potential of fruit and vegetable puree based drink (FVPD) and control drink

	FVPD (per 100 mL)	Control (per 100 mL)
Energy (kcal)	63	51
Protein (g)	1	0.1
Carbohydrate (g)	13.6	12.6
Of which sugar (g)	12.6	12.6
Fat (g)	0.5	-
Of which saturates (g)	0.1	-
Fiber (g)	1.5	-
Sodium (g)	0.03	-
Ascorbic acid (mg)	36	-
Total carotenoids (mg)	5.1	-
α-Carotene (mg)	1.5	-
β-Carotene (mg)	2.6	-
Nitrate/nitrite (mg)	0.19	-
Total phenolic concentration (mg GAE)	192	11
Epicatechin (mg)	114.2	-
Chlorogenic acid (mg)	7.8	-
Cyanidin (mg)	13.2	-
Pelargonidin-3-glucoside (mg)	9.3	-
Pelargonidin (mg)	2.6	-
ORAC (μM TE)	6702	840

GAE, gallic acid equivalents; ORAC, oxygen radical absorbance capacity; TE, trolox equivalents.

2.4 Total phenolic and polyphenol content of FVPD and control

The total phenolic concentration of the intervention drinks was determined using Folin–Ciocalteu reagent [52]. The polyphenol content of the FVPD was determined by the method of Garcia-Macias et al. [53].

2.5 FVPD antioxidant components

FVPD was extracted with acidified methanol [54]. Total phenolic compounds were determined by the Folin–Ciocalteu method [55], and anthocyanins were determined by the pH differential method [56]. Antioxidant potential was measured with the Oxygen Radical Absorbance Capacity (ORAC) method [57]. The susceptibility of LDL to copper-catalyzed oxidation was determined as the lag phase before oxidation by monitoring the increase in conjugated dienes at 234 nm [58]. FVPD extract containing 0.5 μM GAE (gallic acid equivalents), determined by the Folin–Ciocalteu method, was incubated with isolated LDL containing 100 μg protein.

2.6 Blood sample collection

Blood samples were collected into tubes containing either citrate, EDTA, or heparin and kept on ice until centrifugation (4°C, 3000 rpm). Aliquots of plasma and buffy coat were collected into cryogenic vials and stored (–80°C). Blood samples were centrifuged immediately after collection to minimize oxidative alterations to the sample. Analyses commenced after the intervention study was complete and all samples for each subject were analyzed blindly, within one batch to reduce interbatch variation.

2.7 Isolation of LDL

The method for LDL isolation was modified from Leigh-Firbank et al. [59]. Plasma (2.5 mL) was adjusted to a density of 1.21 g/mL by adding KBr, mixed gently, transferred

to 4.9 mL Beckman optiseal polyallomer centrifuge tube, and overlaid with 1.006 g/mL solution (0.15 M NaCl and 297 μM EDTA, pH 7.4). The tubes were ultracentrifuged (Beckman Coulter Optima L-90K ultracentrifuge, 65.2 near vertical rotor [NVT 65.2], 65 000 rpm, 50 min, 4°C). Further purification was performed by adjusting the density of LDL to 1.15 g/mL by adding 1.33 g/mL solution (2.62 M NaCl, 2.98 M KBr, and 297 μM EDTA, pH 7.4). The adjusted plasma was overlaid with 1.063 g/mL solution in a centrifuge tube and ultracentrifuged (65 000 rpm, 3 h, 4°C) in order to remove albumin and concentrate the LDL as an orange layer at the top of the tube.

2.8 Measurement of conjugated dienes

LDL samples containing 100 μg protein were transferred by pipette to quartz cuvettes (1-cm light path). Phosphate buffer solution (PBS; no Ca/Mg) and $CuSO_4$ were added to the cuvette to achieve concentrations of LDL and $CuSO_4$ of 50 μg protein/mL and 5 μM, respectively. The formation of conjugated dienes (breakdown products of lipid peroxidation) was monitored at 234 nm, 37°C every 2 min for 3 h using a Perkin-Elmer Lambda bio 20 UV/VIS Spectrometer (Waltham, MA, USA) [58].

2.9 Plasma oxidative stability by FRAP

The ferric reducing antioxidant power (FRAP) of plasma was determined using the method of Benzie and Strain [60], adapted for use with 96-well microtiter plates [61]. A Genios spectrophotometer (Tecan Dorset, UK) was used to measure the absorbance at 593 nm. This is an accepted method to assess the antioxidant potential of plasma by its ability to reduce ferric to ferrous ions.

2.10 Laser Doppler imaging with iontophoresis

Measurements were conducted using a moorLDI2 laser Doppler imager (Moor Instruments Ltd., Axminster, UK) as previously described [51]. Acetylcholine chloride (2.5 mL, 1% in 0.5% NaCl solution, Sigma Aldrich, Poole, Dorset, UK) and sodium nitroprusside (2.5 mL, 1% in 0.5% NaCl solution, Sigma Aldrich) were delivered via iontophoresis using ION6 chambers (Moor Instruments) placed on the forearm and connected to a MIC2 iontophoresis controller (Moor Instruments). Laser Doppler imager software (Moor Instruments) controlled the current delivery and repeated scans were taken giving a total charge of 8 mC. The microvascular response was calculated from the area under the flux versus time curve.

2.11 Analysis of Glu298Asp polymorphism

DNA isolated from the buffy coat layer from blood collected in a 10-mL EDTA vacutainer was extracted using a Qiagen DNA Blood Mini Kit (Qiagen Ltd., Crawley, UK). TaqMan PCR technology (7300 Instrument; Applied Biosystems, Warrington, UK) and Assay-on-Demand SNP genotyping assays (Applied Biosystems) were used to determine allelic discrimination of the eNOS gene variants.

2.12 Total nitrate and nitrite

An ELISA kit (Active Motif, Rixensart, Belgium) based on the Greiss reaction [62] was used to measure total nitrate and nitrite in plasma, FVPD, and the control drink. Plasma nitrate and nitrite was used as a surrogate marker of NO, as this is labile and forms reactive species with short half-lives [63].

2.13 Plasma and urinary hippuric acid

Hippuric acid was measured in plasma and urine as a marker of flavonoid absorption and metabolism, as it is a major metabolite of several polyphenol compounds [64, 65]. The hippuric acid concentration was determined by the extraction method of Mohsen et al. [66] and analyzed by HPLC by adapting the methods of Felgines et al. and Kay et al. [67, 68].

2.14 Statistical analyses

All statistical analyses were performed using SPSS 13.0 for Microsoft Windows. Data were checked for normality (Shapiro–Wilk test, as $n < 50$). Data not normally distributed were log transformed and reassessed. A repeated measure ANOVA was used to identify significant treatment by genotype by time interactions. Bonferroni correction was used for post-hoc analysis to reduce the likelihood of chance findings from multiple comparisons. A value of $p \leq 0.050$ was used to define significance and a 95% confidence interval. Only two individuals had the TT genotype, so due to lack of power these individuals were excluded from any statistical analysis. The data presented in tables and graphs are displayed as mean ± SEM, unless otherwise stated.

3 Results

3.1 Baseline characteristics of the participants

There were 11 GG (9 males, 2 females), 11 GT (10 males and 1 female), and 2 TT (1 male and 1 female) individuals. For the purpose of analysis, due to the low numbers of

Table 2. Baseline characteristics (SD) of subjects ($n = 24$) in acute fruit and vegetable puree based drink (FVPD) consumption study split by genotype of the Glu298Asp polymorphism in the eNOS gene

	GG ($n = 11$)	GT ($n = 11$)
Males	9	10
Females	2	1
Age (years)	47 (11)	47 (10)
Systolic blood pressure (mmHg)	127 (12)	119 (12)
Diastolic blood pressure (mmHg)	79 (6)	77 (8)
Weight (kg)	78.5 (13.4)	79.4 (7.9)
Height (m)	1.8 (0.1)	1.8 (0.1)
BMI (kg/m^2)	25.4 (3.4)	25.8 (3)
Total cholesterol (mmol/L)	5.4 (1.1)	5.1 (0.7)
Triacylglycerols (mmol/L)	1.5 (1.1)	1.3 (0.8)
Glucose (mmol/L)	5 (0.3)	5 (0.3)
Total plasma nitrate and nitrite (μmol/L)	38.8 (6.7)	40.6 (8)
Endothelium dependent vasodilatory response (AUC)	1035 (494)	950 (390)
Endothelium dependent vasodilatory response (IAUC)	645 (439)	574 (336)
Endothelium independent vasodilatory response (AUC)	999 (516)	956 (462)
Endothelium independent vasodilatory response (IAUC)	622 (491)	579 (383)

GG, subjects homozygous for guanine (G) at position 894 in the eNOS gene coding for glutamic acid residue at position 298; GT, subjects heterozygous for guanine (G) and thymine (T) at position 894 in the eNOS gene coding for glutamic acid (Glu) and aspartic acid (Asp) residues at position 298, respectively; AUC, area under the flux versus time curve; IAUC, incremental AUC.

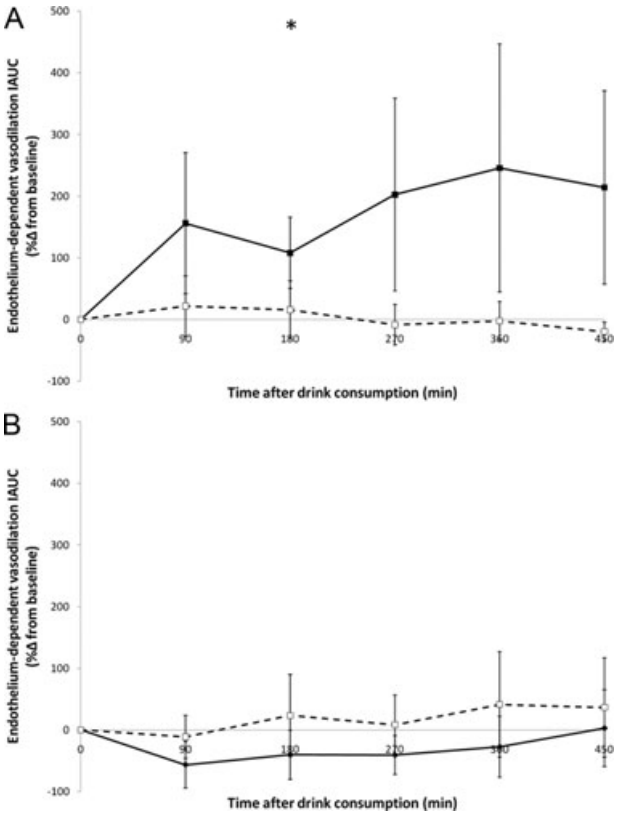

Figure 1. Endothelium-dependent vasodilation response to acetylcholine by GG (A) and GT (B) individuals of the Glu298Asp polymorphism of the eNOS gene after acute ingestion of either control (dotted line) or fruit and vegetable puree based drink (FVPD, solid line) ($n = 11$ in each group). There was a significant time by treatment effect in the GG group ($p < 0.05$). *Significant effect between treatments after post-hoc tests. Results expressed as percentage change from baseline (±SEM).

participants who were homozygous for the T allele, these two participants were excluded. The baseline characteristics of the study population are shown in Table 2. There was no significant difference between the groups.

3.2 Laser Doppler imaging results

The endothelium-dependent vasodilation responses to acute ingestion of FVPD are shown in Fig. 1A and B for the GG and GT genotypes, respectively. There was a significant time by treatment by genotype interaction ($p < 0.05$). The GG group had a higher vasodilatory response at all time points following FVPD consumption. However, the GT group did not respond. After post-hoc tests, it was observed that there was a significantly higher endothelium-dependent vasodilation after 180 min ($p = 0.028$) after the FVPD compared with the control in the GG group. There was no significant effect of acute FVPD consumption on the endothelium-independent response induced by sodium nitroprusside (data not shown).

3.3 Total plasma nitrate and nitrite concentrations

Plasma nitrate and nitrite was measured as a surrogate marker of NO. There was a significant time by treatment effect of FVPD regardless of genotype ($p = 0.001$, Fig. 2). There was an increase in plasma nitrate/nitrite at most time points following FVPD consumption in both genotype groups.

3.4 Plasma antioxidant capacity and LDL lag phase time

Overall, there was a significant time by treatment by genotype interaction in LDL oxidation lag phase time ($p = 0.002$) (Fig. 3). There was a significant difference in LDL oxidation lag phase time between treatments after 60 min ($p = 0.013$, Fig. 3A) in the GG group. A significant difference was also observed in the FRAP value at the same time point (60 min) in the GG group between treatments ($p < 0.05$, Fig. 4). There

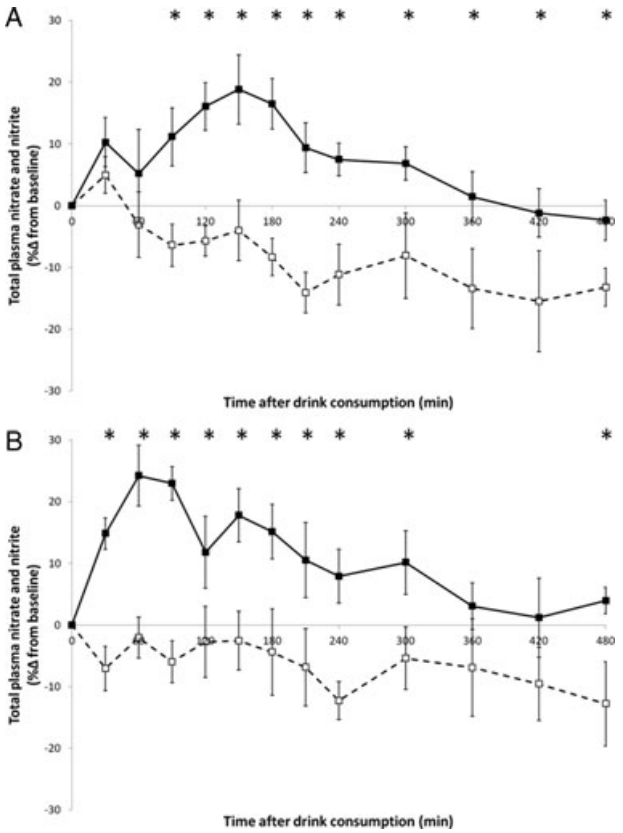

Figure 2. Total plasma nitrate and nitrite concentration (μM) of GG (A) and GT (B) individuals of the Glu298Asp polymorphism of the eNOS gene after acute ingestion of either control or fruit and vegetable puree based drink (FVPD) ($n = 11$ in each group). The solid line represents the effect from FVPD consumption and the dotted line represents the effect from the control. There was a significant time by treatment effect for both genotypes ($p = 0.001$). *Significant effect between treatments after post-hoc tests, $p < 0.05$.

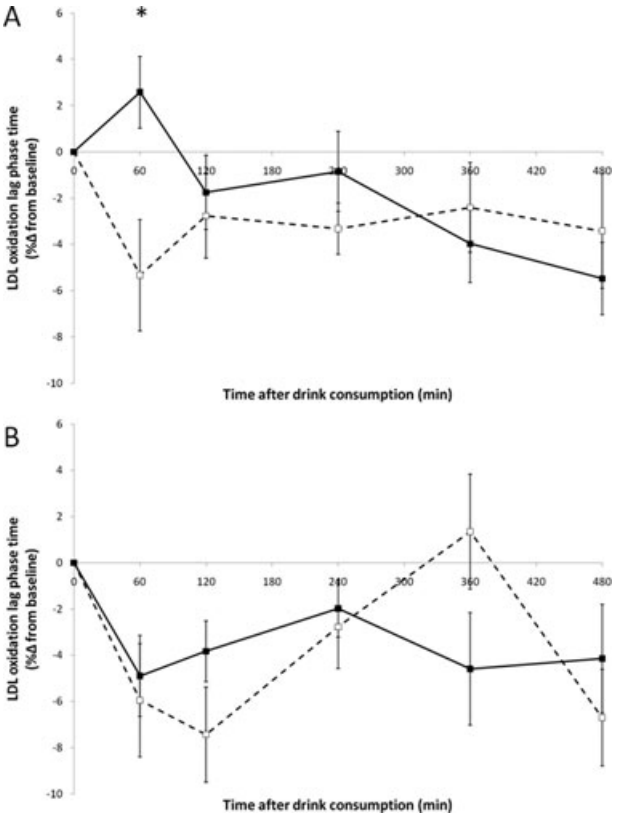

Figure 3. Low-density lipoproteins (LDL) oxidation (lag phase time) of GG (A) and GT (B) individuals of the Glu298Asp polymorphism of the eNOS gene after acute ingestion of either control or fruit and vegetable puree based drink (FVPD) ($n = 11$ in each group). The solid line represents the effect from FVPD consumption and the dotted line represents the effect from the control. There was a significant time by treatment effect for the GG group $p = 0.013$). *Significant effect between treatments after post-hoc tests, $p < 0.05$.

was no significant difference between treatments in LDL oxidation or FRAP value in the GT group.

3.5 Plasma and urinary hippuric acid concentrations

There was a near significant time by treatment by genotype interaction of FVPD consumption on plasma hippuric acid ($p = 0.061$, Fig. 5) and a significant time by treatment interaction for urinary hippuric acid ($p = 0.027$, Fig. 6). After post-hoc tests, it was observed that there was a significantly higher concentration of hippuric acid in plasma following FVPD compared with control consumption after 180, 300, 360, and 480 min for GG individuals ($p = 0.042$, $p = 0.004$, $p = 0.047$, and $p = 0.046$, respectively) and after 360 and 480 min for GT individuals ($p = 0.007$ and $p = 0.035$, respectively), and in urine following FVPD compared with control at 360 min for the GG group ($p = 0.038$) and after 360 and 480 min for the GT group ($p = 0.008$ and $p = 0.019$, respectively).

4 Discussion

The present study is one of the first studies to investigate the effect of an acute ingestion of flavonoid-rich drink on postprandial LDL oxidation and vasodilation and the first to determine the nutrient–gene interaction of the eNOS Glu298Asp polymorphism. The genotype frequency of GG:GT:TT in the current study was 46:46:8%, which is consistent with other studies in the literature [40–43]. The major finding of the study was that the vasodilatory response and the susceptibility of LDL to oxidation were dependent on the eNOS Glu298Asp polymorphism. These findings are consistent with other studies that observed associations with the Glu298Asp polymorphism and NO synthesis [69] and endothelial function [41].

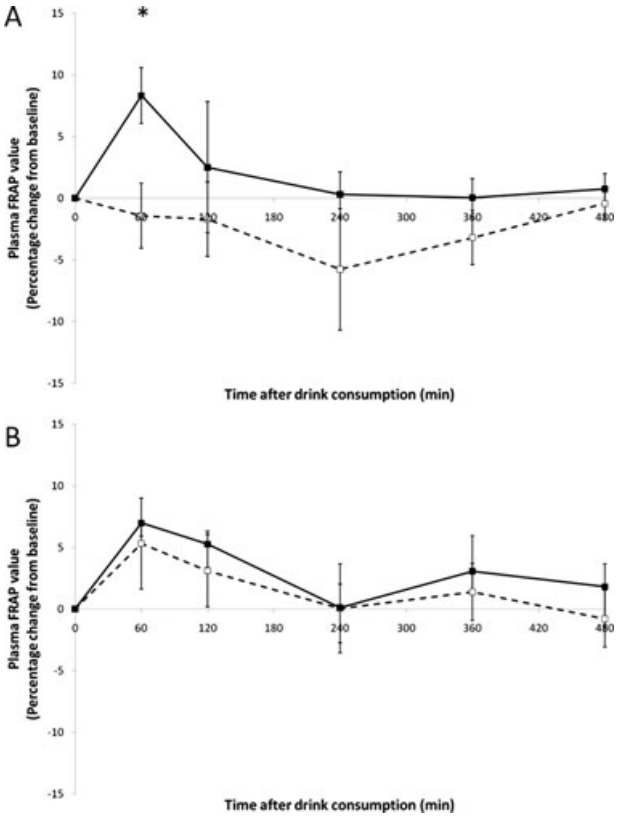

Figure 4. Ferric reducing antioxidant potential (FRAP) of plasma from GG (A) and GT (B) individuals of the Glu298Asp polymorphism of the eNOS gene after acute ingestion of either control or fruit and vegetable puree based drink (FVPD) ($n = 11$ in each group). The solid line represents the effect from FVPD consumption and the dotted line represents the effect from the control. There was a significant time by treatment effect for the GG group ($p = 0.010$). *Significant effect between treatments after post-hoc tests, $p < 0.05$.

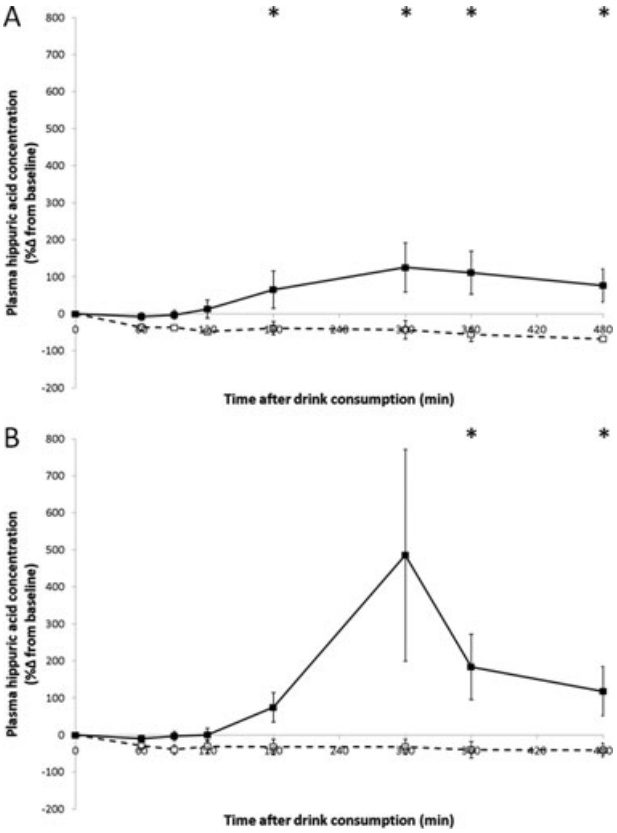

Figure 5. Plasma hippuric acid concentration of GG (A) and GT (B) individuals of the Glu298Asp polymorphism of the eNOS gene after acute ingestion of either control or fruit and vegetable puree based drink (FVPD) ($n = 11$ in each group). The solid line represents the effect from FVPD consumption and the dotted line represents the effect from the control. There was a significant time by treatment effect for both genotypes ($p = 0.001$). *Significant effect between treatments after post-hoc tests, $p < 0.05$.

Acute FVPD consumption resulted in a significant increase in dilation of the microcirculation in the forearm in response to the endothelium-dependent vasodilator acetylcholine after 180 min in GG individuals alone ($p = 0.028$). There was no effect of FVPD consumption on endothelium-dependent vasodilation in the GT genotype or on endothelium-independent vasodilation in response to sodium nitroprusside in either genotype. These results are consistent with previous research showing GG individuals have a significantly higher endothelium-dependent vascular response to acetylcholine compared to GT, but no effect of genotype on the endothelium-independent response to sodium nitroprusside [70].

There was no significant effect of genotype on total plasma nitrate/nitrite at baseline in the current study. This is consistent with previous research [42] that found no effect of Glu298Asp on serum NO concentration. The polymorphism lies within a loop on the external surface of the enzyme and does not make contact with the active site [71]. However, this does not necessarily suggest that any functional effect would be independent of NO synthesis. The measurement of plasma nitrate and nitrite was used in the current study as a surrogate marker for NO. However, this technique would not be able to quantify S-nitrosothiols and other nitrosylated species, which have been shown to increase following flavonoid-rich food ingestion [72]. The FVPD contained (–)-epicatechin (457 mg/400 mL), which has previously been shown to increase flow-mediated vasodilation in healthy individuals [31], which was accredited to increasing eNOS activity by scavenging superoxide. The largest increase in vasodilation in the GG individuals came around 6 h after FVPD consumption, which may correspond to methylated flavonoid metabolites from the small intestine [73]. These metabolites have been shown to have an even greater ability to increase eNOS activity than the parent molecules by inhibiting endothelial NADPH oxidase thereby reducing superoxide production [74]. The significant increase in plasma and urinary hippuric acid in both genotypes confirms that components of the FVPD were

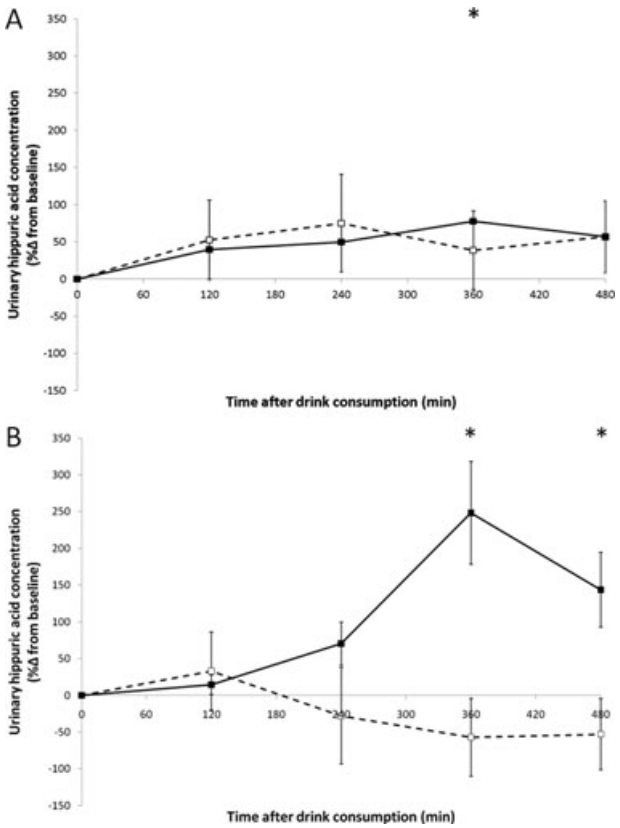

Figure 6. Urinary hippuric acid concentration of GG (A) and GT (B) individuals of the Glu298Asp polymorphism of the eNOS gene after acute ingestion of either control or fruit and vegetable puree based drink (FVPD) ($n = 11$ in each group). The solid line represents the effect from FVPD consumption and the dotted line represents the effect from the control. There was a significant time by treatment effect in the GG group ($P = 0.002$). *Significant effect between treatments after post-hoc tests, $p < 0.05$.

absorbed, as it is a metabolite of several polyphenol compounds, including (–)-epicatechin [64, 65]. The gut microflora converts these compounds to valerolactone in the colon and then to phenolic acids, which are absorbed and transported to the liver via the portal vein. In the liver, phenolic acids are converted to benzoic acid by beta-oxidation, which is then conjugated with glycine to form *N*-benzoylglycine or hippuric acid [64, 65, 75, 76]. The difference in the amount of hippuric acid excreted between the genotypes may suggest that GT individuals metabolized the (–)-epicatechin to hippuric acid in preference to *O*-methylation, which could explain the differential impact of the Glu298Asp genotype on endothelial-dependent vasodilation after FVPD consumption. It was therefore hypothesized that this may be due to the GG individuals methylating the active components in the FVPD to vasoactive metabolites, whereas the GT individuals may be metabolizing them to nonvasoactive hippuric acid. Unfortunately, the authors are unable to measure plasma O-methylated epicatechin to confirm this hypothesis.

The interactions between components within the FVPD in the current study and Glu298Asp genotype were comparable to findings by other researchers. Leeson et al. compared FMD between healthy individuals according to the Glu298Asp polymorphism [41]. In addition, the researchers examined interactions with long-chain *n*-3 fatty acids, as an antiatherogenic factor. They found that FMD was not related to genotype as a whole or within gender groups. However, long-chain *n*-3 fatty acid levels were positively related to FMD in T carriers (reg coeff = 0.023 mm/%, $p = 0.04$, $r = 0.20$), but not in those who were homozygous for G (reg coeff = –0.019 mm/%, $p = 0.1$).

The Glu298Asp polymorphism had an impact on oxidative stability of plasma assessed by ex vivo copper-mediated LDL oxidation after FVPD consumption. FVPD increased the LDL lag phase time compared to control after 60 min in GG individuals only ($p = 0.013$). Additionally, the lag phase time for the GG group was longer after FVPD than control for 4 h after consumption of the drink. Similar differences were observed in the assessment of plasma oxidative stability by FRAP with the GG group exhibiting an increased response following FVPD throughout the 8 h after consumption, whereas GT individuals showed no difference between treatments. No effect of the Glu298Asp polymorphism on plasma ascorbic acid and uric acid was observed (data not shown). The finding that acute FVPD ingestion increased LDL lag phase time is consistent with previous studies that found that lag phase time increased after acute consumption of phytochemical-containing foods on copper-catalyzed LDL oxidation. These include phenolic-rich foods: coffee at 30 and 60 min [77], red wine (containing 433 mg total phenolics) at 1 and 2 h [78], and olive oil (containing 366 mg/kg) at 2 and 6 h post consumption [79]. However, the observation that this response is also linked to genetic variation is extremely novel.

FVPD contained a variety of compounds that could have affected vasodilation or antioxidant status. The most abundant were ascorbic acid (144 mg), carotenoids (20.4 mg), and phenolics (768 mg) of which the major component was epicatechin (457 mg). However, there was no effect of genotype on plasma ascorbic acid and carotenoids which were present in substantially lower concentrations compared to phenolics in the FVPD.

In conclusion, the current study suggests that acute consumption of fruits and vegetables in the form of a puree-based drink had a differential effect determined by the Glu298Asp polymorphism in the eNOS gene of the subjects. A nutrient-gene interaction was observed with the GG group showing a significant increase in endothelium-dependent vasodilation after 180 min, and increased lag phase time for ex vivo copper-catalyzed LDL oxidation in plasma removed 60 min after consumption of the FVPD drink compared to GT individuals. The novelty of these findings suggests that further research is required to investigate the effects of genotype on fruit and vegetable consumption and endothelium-dependent vasodilation and potential mechanisms of action.

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Potential conflict of interest statement: J. A. L. sits on Government Advisory committees that have some committee members from Industry. She also has received research funding from GSK and Jordans. M. H. G. has received research funding from GSK. There are no other conflicts of interest.

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