Glu298Asp (rs1799983) polymorphism influences postprandial vascular reactivity and the insulin response to meals of varying fat composition in postmenopausal women: findings from the randomized, controlled DIVAS-2 study

Article

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1 Glu298Asp (rs1799983) polymorphism influences postprandial vascular reactivity and

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5 Kumari M Rathnayake[1,2,3], Michelle Weech[1,3], Julie A Lovegrove[1] & Kim G Jackson[1]

6

7 [1]From the Hugh Sinclair Unit of Human Nutrition, Institute for Food, Nutrition and Health

8 and Institute for Cardiovascular and Metabolic Research, University of Reading, Department

9 of Food & Nutritional Sciences, Harry Nursten Building, Pepper Lane, Whiteknights,

10 Reading RG6 6DZ, UK (KMR, MW, KGJ, JAL) and Department of Applied Nutrition,

11 Faculty of Livestock, Fisheries and Nutrition, Wayamba University of Sri Lanka, Makandura,

12 60170, Sri Lanka (KMR)

13 [3]KMR and MW contributed equally to this manuscript.

14 Address correspondence to JA Lovegrove, University of Reading, School of Chemistry, Food

15 and Pharmacy, Department of Food & Nutritional Sciences, Harry Nursten Building, Pepper

16 Lane, Whiteknights, Reading, RG6 6DZ, United Kingdom. Telephone: +44 (0)118 3786418;

17 Fax: +44 (0)118 3787708; Email: j.a.lovegrove@reading.ac.uk.

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Abstract

Background: Previous acute studies suggest the Glu298Asp polymorphism (rs1799983) may influence vascular reactivity in response to long-chain n-3 polyunsaturated fat (PUFA) intake. However, the effects of this genotype on postprandial vascular function following meals rich in saturated (SFA), n-6 PUFA and monounsaturated (MUFA) fats are unclear.

Objective: This study determined the impact of the Glu298Asp polymorphism on changes in vascular function and cardiometabolic risk biomarkers in response to sequential meals of varying fat composition.

Methods: In a randomized, double-blind, cross-over, acute study, 32 postmenopausal women (mean±SD age 58±5 y; BMI 25.9±4.1 kg/m^2) consumed mixed meals (breakfast: 0 min, 50 g fat; lunch: 330 min, 30 g fat) containing SFA, n-6 PUFA or MUFA on 3 occasions. Blood samples for cardiometabolic disease risk markers and real-time measures of vascular reactivity (including flow-mediated dilatation (FMD, primary outcome)) were collected/performed before and regularly for 480 min after breakfast. Participants were retrospectively genotyped for the Glu298Asp (rs1799983) polymorphism. Data were analysed using linear mixed models.

Results: For the postprandial %FMD response, a test fat x genotype interaction was observed for the area under the curve (AUC; P=0.019) but not incremental AUC, with the AUC being ~24% greater after MUFA than SFA and n-6 PUFA-rich meals in the Glu298 homozygotes (P≤0.026). Test fat x genotype interactions were also evident for postprandial insulin (P≤0.005), with the MUFA-rich meals demonstrating significantly higher AUC (12.8%/14.9%), incremental AUC (14.6%/20.0%) and maximum concentration (20.0%/34.5%) versus the SFA and n-6 PUFA-rich meals (respectively) in Asp298 carriers (P<0.05). Genotype did not influence other study outcome measures in response to the test fats.

3

76 **Conclusion:** Our findings suggest the Glu298Asp polymorphism may represent a potential

77 determinant of the inter-individual variability in postprandial responsiveness of %FMD and

78 insulin to acute meal fat composition in postmenopausal women. Further studies are required

79 to confirm these observations.

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Introduction

Endothelial dysfunction, characterized by a reduction in the bioavailability of nitric oxide (NO), is now considered to be an early modifiable step in the development of cardiovascular disease (CVD). The potent vasodilator NO is synthesized in the vascular endothelium by the enzyme endothelial NO synthase (eNOS) through the oxidation of L-arginine to L-citrulline (1), and plays a key role in the maintenance of vascular homeostasis. A common single nucleotide polymorphism in the eNOS gene, Glu298Asp (rs 1799983), is regarded to have functional effects on NO production and bioavailability, with a 30% higher CVD risk in minor allele Asp298 carriers attributed to impaired vascular function (2, 3) and elevated blood pressure (4-6) compared with Glu298 homozygotes. Previous diet-genotype studies have suggested this polymorphism may interact with dietary fat composition, with higher habitual intakes and meals rich in long chain n-3 polyunsaturated fatty acids (PUFA) associated with beneficial effects on flow-mediated dilatation (FMD) (7, 8) and fasting triacylglycerol (TAG) concentrations (9) in Asp298 carriers. However, very little is known about the effects of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and n-6 PUFA on vascular function and CVD risk outcomes in this genotype group. This is particularly important since current dietary fat recommendations for population CVD risk reduction advise replacement of dietary SFA intake with unsaturated fatty acids.

Elevated levels of TAG-rich lipoproteins during the postprandial phase are an independent CVD risk factor (10-12), and are proposed to induce a temporary state of endothelial dysfunction following meal ingestion (13-15). Interestingly, the effects of long chain n-3 PUFA on the postprandial FMD response appear to be sex and Glu298Asp polymorphism dependent, with a two-fold higher FMD response observed in female compared with male Asp298 homozygotes (7). Although exaggerated postprandial lipemia and impaired vascular function are two important CVD risk factors in postmenopausal

5

111 women (10-12), very little is known about the effects of high fat meals of differing fat

112 composition on macro- and microvascular reactivity in this population sub-group with an

113 increased CVD risk (16). Findings from our Dietary Intervention and Vascular Function-2

114 (DIVAS-2) study have shown sequential test meals higher in unsaturated fatty acids to have

115 favourable effects on postprandial blood pressure and a marker of endothelial activation

116 (soluble intercellular cell adhesion molecule (sICAM)-1), as well as maintenance of higher

117 plasma nitrate levels compared to meals rich in SFA in postmenopausal women. However,

118 effects on real time measures of vascular function were limited (17). In view of the potential

119 role of sex in mediating the impact of the eNOS polymorphism on postprandial vascular

120 function to long chain n-3 PUFA intake, we performed retrospective genotyping in the

121 DIVAS-2 cohort of postmenopausal women to determine the effects of sequential meals rich

122 in SFA, n-6 PUFA and MUFA on postprandial vascular reactivity and CVD risk markers.

123

124 **Subjects and Methods**

125 *Study participants and design*

126 The DIVAS-2 study was an acute, double-blind, randomized, cross-over study conducted at

127 the Hugh Sinclair Unit of Human Nutrition (University of Reading, UK) between June 2014

128 and September 2015. The study recruited 36 non-smoking 'healthy' postmenopausal women

129 aged ≤65 years. The study design, inclusion/exclusion criteria, test meal composition and

130 study measurements have been described in detail elsewhere (17). Briefly, participants were

131 randomized to consume sequential mixed test meals consisting of a warm chocolate drink

132 with toasted white bread and jam given at breakfast (0 min, 3.8 MJ, 50.0 g fat, 19.2 g protein

133 and 98.0 g carbohydrate) and lunch (330 min, 3.0 MJ, 30.0 g fat, 19.2 g protein and 98.0 g

134 carbohydrate) on three separate occasions, 4-6 weeks apart. The test fats included in the

135 meals were either rich in SFA (butter; 52.0 g SFA, 21.0 g MUFA and 3.1 g n-6 PUFA as total

136 intakes per test fat arm), MUFA (refined olive oil and MUFA-rich spreads; 15.5 g SFA, 54.6

137 g MUFA and 8.5 g n-6 PUFA) or n-6 PUFA (safflower oil and n-6 PUFA-rich spreads; 13.0

138 g SFA, 10.8 g MUFA and 56.2 g n-6 PUFA). Habitual dietary intake was assessed using a 4-

139 day weighed food diary completed prior to visit 1 and analysed using Dietplan7 software

140 (Forestfield).

141   Following a 12 h overnight fast, anthropometric measurements were conducted before

142 an indwelling cannula was inserted into the forearm and two fasting blood samples were

143 collected (-30 and 0 min) to determine the mean baseline (fasting) concentration. Fasting

144 vascular function and clinic blood pressure measurements were then performed before the

145 breakfast was consumed within 15 minutes. Blood samples were collected at regular intervals

146 until lunch (every 30 min until 180 min and then every 60 min until 300 min), and then every

147 30 min up to 420 min ending at 480 min after the breakfast meal. Postprandial measurements

148 of FMD were conducted at 180, 300 and 420 min, and blood pressure, laser Doppler imaging

149 (LDI) with iontophoresis and digital volume pulse (DVP) at 240 and 450 min after the

150 breakfast meal.

151   The study was conducted in accordance with the Declaration of Helsinki and

152 approved by the University of Reading Research Ethics Committee (project reference number

153 14/16). All participants provided written informed consent prior to participation, including

154 consent for genotyping for the eNOS Glu298Asp polymorphism.

155

156 *Vascular reactivity measurements and blood pressure*

157 To assess vascular reactivity, a single trained researcher measured endothelial-dependent

158 vasodilation of both the brachial artery to determine %FMD (primary outcome) and the

159 microcirculation using LDI with iontophoresis as previously described (18). In the peripheral

160 arteries, DVP (Pulse Trace PCA2; Micro Medical Ltd.) determined the stiffness index (DVP-

161 SI; m/s) and reflection index (DVP-RI; %) as measures of arterial stiffness and vascular tone,

162 respectively (18). Clinic blood pressure (systolic blood pressure and diastolic blood pressure)

163 and heart rate were measured in triplicate using an OMRON M6 automatic digital blood

164 pressure monitor (OMRON). Vascular reactivity and clinic blood pressure measurements

165 were performed after participants had rested for 30 min in a supine position in a quiet,

166 temperature-controlled room ($22 \pm 1^{\circ}C$).

167

168 *Biochemical analysis*

169 Blood samples were collected and processed as described previously (17). Briefly, serum

170 lipids (total cholesterol (TC, fasting only), HDL cholesterol (HDL-C, fasting only) and

171 TAG), apolipoprotein (apo)B, glucose, non-esterified fatty acids (NEFA) and high sensitivity

172 C-reactive protein (fasting only) were determined using an ILAB600 autoanalyzer (reagents:

173 Werfen (UK) Ltd.; NEFA reagent: Alpha Laboratories; apoB reagent: Randox Laboratories

174 Ltd). Fasting LDL cholesterol (LDL-C) was estimated using the Friedewald formula (19).

175 ELISA kits were used to analyze circulating serum insulin (Dako Ltd.; Denmark), plasma

176 sICAM-1 and soluble vascular cell adhesion molecule (sVCAM-1), E-selectin and P-selectin

177 (R & D Systems Europe Ltd.; UK & Europe). Plasma nitrite and nitrate concentrations were

178 analyzed using the HPLC based approach, Eicom NOx Analyzer ENO-30 (Eicom; San

179 Diego; USA) as described elsewhere (17, 20).

180   Using baseline fasting measures, insulin resistance was determined by homeostatic

181 model assessment of insulin resistance (HOMA-IR), and insulin sensitivity was estimated

182 with the use of revised quantitative insulin sensitivity check index (rQUICKI) derived using

183 standard equations (21). The QRISK®2-2016 online calculator was used to evaluate the risk

184 of developing CVD in next 10 y (http://www.qrisk.org/index.php).

185

DNA extraction and genotyping

DNA was extracted from the buffy coat using the Qiagen DNA Blood Mini Kit (Qiagen Ltd.,

Crawley, UK). The Glu298Asp polymorphism (rs1799983) was determined using the

Applied Biosystems RT-PCR 7300 instrument and Assay-on-Demand single nucleotide

polymorphism genotyping assay (Life Technologies, UK).

Statistical analysis

As no formal sample size calculation was performed, the analysis presented in the manuscript

is explorative, investigating the interactions between the Glu298Asp polymorphism with

acute fat manipulation on the primary (%FMD response) and secondary (other measures of

vascular function and cardiometabolic disease risk markers) outcomes from the DIVAS-2

study, powered to detect a difference in the %FMD response between test fats (17).

All statistical analysis was performed using IBM SPSS statistics version 25 and

results are presented as mean ± SEM, unless specified otherwise. All data were checked for

normality and log transformed where necessary. Sine transformation was used for summary

measures with negative values. Baseline subject characteristics, which represented the mean

fasting data from each study visit, according to genotype group were assessed using

independent t-tests. Summary measures for the postprandial responses following the

sequential meals were expressed as area and incremental area under the time response curve

(AUC and IAUC, respectively) over 420, 450 and 480 min. The IAUC denotes the specific

response to the test meals irrespective of baseline concentrations. For NEFA, AUC and IAUC

were computed from the time of suppression until end of postprandial period (120-480 min).

For postprandial variables with 13 (TAG, glucose, NEFA and insulin) or 10 (apoB)

timepoints, the maximum concentration (MaxC) reached after the test meals was also

calculated. Linear mixed model analyses were implemented to evaluate the impact of

211   genotype on fasting and postprandial responses to the meals of varying fat composition. All

212   models contained test fat, genotype and test fat x genotype interaction as fixed factors and

213   subject code was added as a random factor. Since BMI has been shown to have an

214   independent effect on both vascular function and insulin sensitivity, it was included as a fixed

215   effect covariate within our linear mixed model analysis and retained within the model

216   regardless of their degrees of significance. Other previously reported differences in baseline

217   characteristics (7, 9) as well as the functional effect of the Glu298Asp polymorphism are

218   considered to contribute to the higher CVD risk in carriers of the Asp298 allele. Further

219   correction for these factors may mask the metabolic phenotype of these two genotype groups,

220   and their response to the test fats, and therefore were not included as covariates. $P \leq 0.05$ was

221   considered as significant for this exploratory analysis.

222   In this secondary analysis of the DIVAS-2 study, the test fat only effects will not be

223   discussed since they were part of our primary analysis and presented in our earlier publication

224   (17).

225

226   **Results**

227   *Study participation*

228   A total of 32 postmenopausal women who completed all three study visits of the DIVAS-2

229   study were included in the secondary analysis according to the Glu298Asp polymorphism.

230   These participants had a mean $\pm$ SD age of 58 ± 5 y and BMI of 25.9 ± 4.1 kg/m^2. The

231   genotype distributions of the eNOS single nucleotide polymorphism were in line with the

232   reference data for the Caucasian populations in HapMap, with the frequency of the Glu298

233   homozygotes 53% (n=17), Asp298 carrier heterozygotes 44% (n=14) and Asp298

234   homozygotes 3% (n=1). The Asp298 carrier groups were combined for the data analysis.

Table 1 shows the mean baseline characteristics of the participants for the three study visits according to the Glu298Asp polymorphism. BMI was found to be 12.2% higher in the Asp298 carriers than Glu298 homozygotes ($P=0.040$). Other baseline anthropometric, CVD risk markers and estimates of insulin sensitivity and CVD risk were similar between genotype groups. There were also no differences in habitual dietary energy or macronutrient intakes, although intakes of cholesterol were 36% greater in the Asp298 carriers than Glu298 group ($P=0.049$) (Table 1).

Vascular function and blood pressure

In the fasting state, there was no effect of genotype on the %FMD response, with similar mean fasting %FMD responses observed in the Glu298 homozygotes (5.32 ± 0.77 %, n=16) and Asp298 carriers (4.30 ± 0.59 %, n=15)($P=0.074$). Postprandially, there was a significant test fat x genotype interaction ($P=0.019$) for the %FMD response AUC, with differences between test fats only evident in the Glu298 homozygotes ($P=0.013$) but not Asp298 carriers (**Table 2**). A ~24% greater AUC for the %FMD response was observed in the Glu298 homozygotes after consumption of the MUFA than SFA and n-6 PUFA-rich meals ($P \leq 0.026$). The IAUC, a measure of the change in %FMD response to meal ingestion, was not found to be different between the test fats in either genotype group. Independent of the meal fat composition, Asp298 carriers had a lower mean AUC for the postprandial %FMD response compared with the Glu298 homozygotes (1875 ± 175 versus 2411 ± 158 % x min, respectively, $P=0.021$).

No statistically significant test fat x genotype interactions were found for other vascular measurements (LDI and DVP), clinic blood pressure, biomarkers of NO production (nitrate and nitrite) or plasma markers of endothelial activation (cell adhesion molecules) in the fasting state or following ingestion of the high fat meals of differing fat composition.

260 Independent of the meal fat composition, there was an effect of genotype on the IAUC for the

261 DVP-SI, with a tendency for a reduction in postprandial SI after sequential meals in the

262 Asp298 carriers than Glu298 homozygotes (P=0.017)(Table 2).

263

264 *Postprandial CVD risk markers*

265 Fasting serum insulin concentrations were not different between study visits or genotype

266 groups. Significant test fat x genotype interactions were evident for the AUC (P=0.001),

267 IAUC (P=0.005) and MaxC (P=0.005) (Table 2) for the postprandial insulin response, with

268 differences between the test fats found in the Asp298 carriers only (P≤0.004). In this

269 genotype group, the AUC, IAUC and MaxC for the postprandial serum insulin response were

270 significantly greater after consumption of MUFA than SFA (P≤0.038) and n-6 PUFA-rich

271 (P≤0.004) meals.

272   For the postprandial glucose and NEFA responses, a significant test fat x genotype

273 interaction was also observed for the AUC after the sequential meals (P=0.038 and 0.032,

274 respectively) (Table 2). However, differences were not evident between the test fats in either

275 the Glu298 homozygote or Asp298 carrier groups. The Glu298Asp polymorphism did not

276 influence the postprandial serum lipid responses (TAG and apoB) following the sequential

277 SFA, MUFA and n-6 PUFA-rich meals (Table 2).

278

279 **Discussion**

280 To our knowledge, this secondary analysis of the DIVAS-2 study is the first to determine the

281 impact of the eNOS Glu298Asp polymorphism on vascular function and cardiometabolic

282 disease risk markers to sequential meals rich in SFA, n-6 PUFA and MUFA. Our findings

283 have revealed this polymorphism to a potential modulator of the effect of acute meal fat

284 composition, with differential effects of genotype observed on postprandial %FMD and

285 insulin responses to sequential MUFA-rich meals in postmenopausal women. However,

286 genotype was not found to influence other measures of vascular function or cardiometabolic

287 disease risk biomarkers following the SFA, MUFA and n-6 PUFA rich meals.

288   A genotype specific relationship was only found between the AUC for the

289 postprandial %FMD response (our primary outcome) and MUFA intake, with a greater AUC

290 compared with both SFA and n-6 PUFA in the Glu298 homozygotes only. This was

291 surprising since a previous dietary fat-genotype study conducted in adults aged 18-65 years

292 had shown a greater impact of meal fat composition in the minor allele carriers, although this

293 study had focused mainly on SFA and long chain n-3 PUFA-rich test meals (7). Only one

294 other study has determined the impact of olive oil containing meals on postprandial vascular

295 function according to the Glu298Asp polymorphism but this study did not include a

296 comparator meal of a differing fat composition (22). Whilst genotype did not influence

297 postprandial microvascular reactivity to the olive oil meals containing varying levels of

298 phenolics, the postprandial vascular response and NOx concentrations were lower (between 4

299 and 8 h) in the Asp298 carriers than wild-type group. In the current study, only a tendency for

300 lower postprandial plasma NOx concentrations was found in the Asp298 carriers.

301 Interestingly, in both of these studies, vascular reactivity was not different between genotype

302 groups in the fasting state but only during the postprandial state, a finding also observed after

303 a mixed high-fat meal in healthy men (23). Therefore, the compromised vasodilatory

304 response in the Asp298 carriers may only be expressed during times of metabolic stress, such

305 as the postprandial state.

306   A number of acute studies have shown vascular function to be modulated by the type

307 of fatty acids consumed (13, 24) but findings have been inconsistent with respect to MUFA-

308 rich oils. Two systematic reviews and meta-analysis using data from chronic randomised

309 controlled trials reported favourable effects of the Mediterranean diet (25) and olive oil

310 varieties higher in polyphenols (26) on the fasting %FMD response. Similar findings have

311 been observed in the postprandial state, with an increase and/or attenuation of the decrease in

312 FMD response to a high-fat meal, evident after meals rich in extra-virgin olive oil (27, 28),

313 olive oil containing higher amounts of phenolic compounds (22), and avocado fruit (29).

314 Polyphenols are considered to mediate their effects on postprandial vascular function by

315 counteracting fat induced oxidative stress and lipid peroxidation, increasing NO

316 bioavailability. However, in the current study, we observed a greater postprandial %FMD

317 response with refined olive oil devoid of phenolic compounds. The lack of effect of meal fat

318 composition in Asp298 carriers may shed some light on the mechanisms underlying the

319 increase in the AUC for the %FMD response with MUFA in the Glu298 homozygotes. The

320 glutamate to aspartate amino acid substitution at position 298 of the eNOS protein has been

321 reported to make the enzyme more susceptible to cleavage (causing dysfunction), decreasing

322 eNOS activity and potentially vasodilation (30). Furthermore, Asp298 carriers may also have

323 less eNOS associated with caveolin-1 in the endothelial cell membrane (31). Findings from

324 an animal study (32) and *in vitro* studies (33) including some of our own unpublished

325 findings (34), have shown olive oil (extra-virgin) and oleic acid to induce eNOS activity and

326 gene expression compared with lard (32) and single fatty acids (palmitic, stearic and linoleic

327 acids (32, 34). Therefore, it is possible that during the postprandial state, the greater FMD

328 response in the Glu298 homozygotes may reflect an effect of MUFA per se on eNOS enzyme

329 activity and expression, a finding which requires confirmation in human studies.

330   Of the cardiometabolic disease risk markers, insulin may provide a mechanistic link

331 with the observed effects on the %FMD response to the fat-rich meals in the genotype groups

332 via the reported effects of insulin on eNOS stimulation and blood vessel endothelial

333 dependent vasodilation (35). In support, previous studies have reported associations between

334 the Glu298Asp polymorphism with insulin resistance (36-39), with a greater prevalence of

335   Asp298 carriers in adults classified with the metabolic syndrome. In the current study,

336   Asp298 carriers showed differential insulin responses to the meal fatty acids, with MUFA

337   ingestion leading to higher postprandial concentrations than the SFA and n-6 PUFA-rich

338   meals. The lack of a genotype effect on glucose handling is suggestive that the sequential

339   MUFA meals may have led to a transient reduction in postprandial insulin sensitivity in this

340   genotype group. However, our findings are in contrast with previous studies which have often

341   reported MUFA-rich meals (containing olive oil and palmolein) to improve beta cell function

342   and postprandial insulin sensitivity compared with SFA-rich meals (40, 41), but no studies to

343   date have determined the effects according to the Glu298Asp polymorphism. Interestingly,

344   greater insulin but not glucose concentrations were reported in Asp298 carriers than Glu298

345   homozygotes after an oral glucose tolerance test in Japanese non-diabetic adults (42).

346   Although the authors could not discount differences in insulin sensitivity during the oral

347   glucose tolerance challenge between genotypes, they did speculate that the dysfunctional

348   eNOS enzyme in Asp298 carriers may have reduced insulin mediated blood flow in tissues

349   leading to a decreased insulin clearance. In our postmenopausal women, fasting insulin and

350   estimates of insulin sensitivity (HOMA-IR) and rQUICKI were similar in our wild-type

351   group and minor allele carriers. However, similar differences in blood flow and insulin

352   clearance have also been identified in obese versus lean individuals. Although a higher BMI

353   in the Asp298 carriers may be thought to contribute to the insulin responses observed, our

354   analysis controlled for BMI suggesting that this may reflect a metabolic phenotype of this

355   polymorphism. However, we cannot discount that other measures of body composition such

356   as percentage body fat or abdominal obesity were impacting on postprandial insulin

357   responses to meal fat ingestion. Further studies are now warranted to determine the

358   mechanisms underlying the impact of the Glu298Asp polymorphism on postprandial glucose

359   handling and insulin control in response to dietary fat intake, and effects on vascular function.

360   Our study has some limitations. Genotyping was performed retrospectively and so it

361   was not possible to compare the effects of meal fat composition in Asp298 homozygotes as

362   they represent only 10% of Caucasian populations. Since the study sample size was based on

363   the expected change in the postprandial %FMD response between test fats for the main study,

364   we attempted to estimate our power to detect genotype x fat x vascular function interactions

365   in the current study. Based on the expected change in the postprandial %FMD response

366   following a SFA fat load in female Glu298 versus Asp298 genotype groups (7), we

367   calculated a power of 68% to detect differences in response to the test fats between genotype

368   groups. However, our previous study was conducted in females aged 18-65 years and may

369   not be totally representative of our older female group. Therefore, our data analysis should be

370   considered explorative and provides important data to power future studies within this

371   population sub-group. In this study, a significant test fat x genotype interaction was only

372   identified for the AUC but not IAUC for the postprandial %FMD response. Both the IAUC

373   and AUC offer important summary data on the postprandial responses of an outcome

374   measure to the test meals with different fatty acid composition. IAUC estimates the specific

375   response to the test meals, whereas the AUC estimates that, in addition to any

376   background/residual effect. It should also be noted that within the Glu298 homozygotes, there

377   was a tendency for a higher fasting %FMD response prior to the MUFA than SFA and n-6

378   PUFA-rich meals, which may have contributed to the findings in this study. Furthermore, as

379   the participants were postmenopausal women, our findings may not be representative of those

380   in men or premenopausal women, or other ethnic groups.

381   In conclusion, our exploratory analysis has revealed that the Glu298Asp

382   polymorphism to be a potential determinant of the inter-individual variability in

383   postprandial %FMD and insulin responses to acute dietary fat intake in postmenopausal

384   women. However, further studies are warranted using prospective genotyping to investigate

385   the mechanisms underlying the effects of the Glu298Asp polymorphism and MUFA-rich

386   meals on endothelial function and insulin metabolism. Our findings may be important in

387   identifying population subgroups with greater responsiveness to the beneficial effects of

388   targeted dietary fatty acid manipulation through personalized nutrition.

Statement of Authorship

The authors' responsibilities were as follows: JAL, KGJ, KMR and MW designed the study; KMR conducted the research and carried out DNA extraction, analysed the data, and wrote the manuscript under the guidance of KGJ and JAL; MW conducted the research, analyzed the data and conducted the statistical analysis; KGJ performed the genotyping and conducted the statistical analyses; all authors critically appraised the writing of the manuscript at all stages and approved the final manuscript, and JAL was responsible for the final content. None of the authors had a conflict of interest with regards to the writing or submission of the manuscript.

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Table 1 Baseline characteristics of the Glu298 homozygotes and Asp298 carriers[1]

	Glu298 homozygotes	Asp298 carriers	P value[2]
N	17	15	
Age, y	59 ± 5	57 ± 5	0.383
BMI, kg/m^2	24.5 ± 4.0	27.5 ± 3.7	**0.040**
Waist circumference, cm	87.5 ± 8.6	93.1 ± 8.4	0.080
Body fat, %	34.7 ± 6.9	38.9 ± 5.5	0.078
Blood pressure, mmHg			
Systolic	135 ± 16	137 ± 15	0.762
Diastolic	77 ± 8	79 ± 8	0.427
Heart rate, beats/min	60 ± 6	58 ± 5	0.294
Fasting serum CVD risk markers			
Total cholesterol, mmol/L	5.76 ± 0.53	5.72 ± 0.83	0.769
HDL cholesterol, mmol/L	1.68 ± 0.30	1.56 ± 0.24	0.239
LDL cholesterol, mmol/L	3.52 ± 0.56	3.51 ± 0.75	0.872
Triacylglycerol, mmol/L	1.23 ± 0.28	1.43 ± 0.46	0.199
C-reactive protein, mg/L	0.91 ± 0.81	1.72 ± 1.99	0.146
Glucose, mmol/L	4.98 ± 0.34	5.28 ± 0.58	0.084
Insulin, pmol/L	32.7 ± 18.7	42.7 ± 24.8	0.257
NEFA, μmol/L	588 ± 97	652 ± 186	0.315
Estimates of CVD risk and insulin sensitivity			
QRISK®2[3]	4.8 ± 2.0	4.5 ± 2.3	0.727
HOMA-IR	1.22 ± 0.73	1.74 ± 1.21	0.143
rQUICKI	0.43 ± 0.04	0.40 ± 0.06	0.156

Habitual dietary intakes[4]

Energy, MJ/d	7.0 ± 1.5	7.7 ± 2.1	0.331
Total fat, %TE	36.0 ± 8.8	34.8 ± 5.6	0.698
SFA, %TE	13.8 ± 4.3	12.4 ± 1.8	0.306
MUFA, %TE	12.6 ± 3.2	12.6 ± 2.9	0.942
n-6 PUFA, %TE	5.1 ± 2.0	5.3 ± 1.6	0.612
n-3 PUFA, %TE	0.89 ± 0.30	0.89 ± 0.24	0.959
Trans fat, %TE	0.87 ± 0.33	0.92 ± 0.44	0.715
Dietary cholesterol, mg/d	194 ± 74	264 ± 113	**0.049**
Protein, %TE	15.5 ± 2.7	16.4 ± 2.9	0.380
Carbohydrate, %TE	45.2 ± 8.3	45.5 ± 6.8	0.783
Dietary fibre (AOAC), g/d	20.7 ± 5.3	23.6 ± 6.2	0.166

[1]Data represent the mean of the fasting (baseline) samples collected on the three study visits, except for the dietary intakes that were determined from a single 4-day weighed food diary completed prior to visit 1. Values are mean ± SD.

[2]Independent t-tests were used to compare the baseline characteristics in the genotype groups.

[3]QRISK®2 estimates the 10 y CVD risk (myocardial infarction or stroke).

[4]n=16 for GG homozygotes.

Abbreviations: %TE: percentage of total energy, AOAC: Association of Official Analytical Chemists, BMI: body mass index, CVD: cardiovascular disease, HDL: high density lipoprotein, HOMA-IR: homeostatic model assessment-insulin resistance, LDL: low density lipoprotein, MUFA: monounsaturated fat, NEFA: non-esterified fatty acids, PUFA: polyunsaturated fat, rQUICKI: revised quantitative insulin sensitivity check index, SFA: saturated fat.

Table 2 Vascular function outcomes and circulating CVD risk markers measured in the fasting state and after sequential meals of varying fat composition in Glu298 homozygotes and Asp298 carriers

	Glu298 homozygotes			Asp298 carriers			P values[1]		
	SFA	MUFA	n-6 PUFA	SFA	MUFA	n-6 PUFA	Test fat	Test fat x Genotype	Genotype
Vascular function									
%FMD response									
Fasting, %	4.91±0.60.	6.24±1.01	4.81±0.71	4.59±0.70	3.66±0.5	4.66±0.55	0.908	0.055	0.074
AUC, % x min	2246±149[b]	2783±217[a]	2252±182[b]	1801±172	1813±178	1973±208	0.087	**0.019**	**0.021**
IAUC, % x min	186±171	161±328	231±238	-126±235	276±160	16±119	0.658	0.556	0.913
LDI-Ach[2]									
Fasting, AU	1580±186	1880±268	1890±284	1690±145	1690±131	1710±171	0.427	0.479	0.356
AUC[3], AU x min x 10[3]	72.1±63.8	86.2±114.8	82.1±84.1	75.1±53.9	68.9±45.7	73.1±39.5	0.688	0.130	0.091
IAUC[3], AU x min x 10[3]	9.2±46.1	17.8±80.6	-29.5±64.1	-7.5±37.5	-70.1±54.0	-39.8±69.2	0.664	0.578	0.692
LDI-SNP[2]									
Fasting[3], AU	1600±188	1710±154	1860±309	1710±193	1960±201	1440±135	0.144	0.185	0.874
AUC[3], AU x min x 10[3]	685±52	798±94	776±100	810±73	829±86	661±37	0.270	0.243	0.962
IAUC, AU x min x 10[3]	-34.4±55.9	28.4±64.3	-60.5±79.0	42.1±75.6	-53.7±64.7	15.3±63.5	0.880	0.233	0.895
DVP-RI									

Fasting, %	61.2±1.4	60.8±1.9	61.8±2.2	62.8±2.8	60.5±2.9	63.8±2.9	0.572	0.841	0.839
AUC, % x min x 10^3	25.6±0.7	25.6±0.8	26.9±1.0	26.4±1.3	24.7±0.9	25.5±1.1	0.270	0.243	0.564
IAUC, % x min x 10^3	-2.0±0.5	-1.8±0.7	-1.0±0.6	-1.9±1.2	-2.5±1.0	-3.2±0.8	0.962	0.355	0.190
DVP-SI									
Fasting[3], m/s	6.7±0.2	6.9±0.3	6.9±0.3	7.2±0.5	7.8±0.4	7.3±0.3	0.470	0.591	0.244
AUC[3], m/s x min	3150±95	3220±123	3350±143	3300±177	3080±80	3200±160	0.812	0.169	0.915
IAUC, m/s x min	115±99	128±90	233±134	60±129	-404±121	-71±111	0.041	0.063	**0.017**
SBP									
Fasting[3], mm Hg	134.9±4.1	136.2±3.9	134.3±4.5	133.2±4.4	138.2±3.7	138.5±4.4	0.176	0.232	0.973
AUC[3], mm Hg x min x 10^3	56.6±1.5	55.8±1.4	56.2±1.6	57.4±2.0	58.1±1.7	58.9±1.9	0.579	0.221	0.930
IAUC, mm Hg x min x 10^3	-4.1±0.7	-5.5±0.7	-4.2±0.8	-2.6±0.8	-4.1±0.9	-3.4±0.7	0.053	0.803	0.777
DBP									
Fasting, mm Hg	76.2±2.0	77.2±1.8	76.2±2.1	77.1±2.1	79.4±2.1	79.5±2.1	0.151	0.394	0.744
AUC, mm Hg x min x 10^3	32.8±0.8	32.4±0.8	32.3±0.9	33.3±1.0	33.5±0.9	34.3±1.0	0.557	0.111	0.877
IAUC, mm Hg x min x 10^3	-1.5±0.4	-2.3±0.3	-1.9±0.3	-1.4±0.4	-2.2±0.5	-1.5±0.5	0.058	0.843	0.556
Plasma nitrite									
Fasting, µmol/L	0.12±0.01	0.12±0.01	0.12±0.01	0.14±0.01	0.13±0.01	0.13±0.01	0.229	0.320	0.493
AUC, µmol/L x min	49.8±3.4	50.0±3.5	52.6±3.7	56.1±3.7	56.3±4.5	55.1±3.9	0.773	0.222	0.564
IAUC[4], µmol/L x min	-0.7±0.4	-0.4±0.6	2.2±2.6	-1.8±1.2	1.3±1.3	-0.8±0.8	0.144	0.283	0.097

Plasma nitrate									
Fasting[3], μmol/L	22.3±2.4	20.7±2.5	19.9±3.3	15.5±1.1	16.7±2.5	13.8±1.5	0.117	0.252	0.998
AUC[3], mmol/L x min	6.9±0.7	6.3±0.7	6.3±0.9	5.3±0.3	5.8±0.7	4.9±0.5	0.381	0.431	0.834
IAUC, mmol/L x min	-2.5±0.4	-2.4±0.5	-2.0±0.5	-1.3±0.2	-1.2±0.3	-0.9±0.2	0.151	0.282	0.592
Plasma cell adhesion molecules									
sVCAM									
Fasting, ng/mL	639±27	639±32	627±21	627±26	619±25	624±24	0.889	0.872	0.865
AUC, μg/mL x min	267±11	269±12	258±9	265±9	267±10	260±8	0.139	0.901	0.971
IAUC, μg/mL x min	-1.0±5.4	0.7±4.4	-5.2±3.2	2.2±6.5	7.4±6.7	-1.9±3.6	0.345	0.929	0.492
sICAM									
Fasting, ng/mL	198±7	199±7	198±7	218±12	209±12	215±8	0.717	0.562	0.255
AUC, μg/mL x min	81.4±2.6	83.6±3.0	64.8±4.5	88.4±4.2	89.4±5.1	73.0±7.1	**≤0.001**	0.910	0.286
IAUC, μg/mL x min	-1.8±1.3	-0.1±1.6	-18.5±4.6	-3.1±1.9	1.7±1.7	-17.5±4.9	**≤0.001**	0.857	0.771
E-selectin									
Fasting, ng/mL	28.5±2.2	26.8±2.1	26.9±2.1	29.5±2.8	29.2±2.8	28.7±3.2	0.375	0.723	0.522
AUC, μg/mL x min	11.7±0.8	11.3±0.9	11.5±0.8	10.9±1.5	11.0±1.2	10.1±1.0	0.322	0.312	0.200
IAUC[4], μg/mL x min	-0.30±0.28	0.04±0.21	0.17±0.19	0.01±0.51	0.07±0.24	-0.32±0.16	0.569	0.600	0.165
P-selectin									
Fasting, ng/mL	32.9±2.1	31.9±2.3	31.0±2.5	32.7±1.9	31.3±2.4	32.1±2.1	0.277	0.601	0.578

AUC, μg/mL x min	13.0±0.9	13.2±1.0	13.3±1.1	13.6±0.8	13.8±1.2	13.2±0.9	0.802	0.532	0.762
IAUC, μg/mL x min	-0.78±0.37	-0.18±0.23	0.32±0.24	-0.12±0.29	0.63±0.42	-0.24±0.36	0.109	0.082	0.312

Serum biochemical measures

TAG[3]

Fasting, mmol/L	1.29±0.11	1.16±0.05	1.24±0.11	1.42±0.14	1.50±0.12	1.62±0.19	0.706	0.297	0.153
AUC, mmol/L x min	968±107	912±56	966±114	996±82	1150±111	1170±147	0.465	0.232	0.398
IAUC, mmol/L x min	350±65	353±47	369±80	314±34	423±72	386±70	0.154	0.744	0.744
MaxC, mmol/L	2.91±0.33	2.89±0.21	3.04±0.32	2.83±0.24	3.44±0.35	3.36±0.43	0.114	0.310	0.746

Glucose

Fasting[3], mmol/L	5.07±0.13	4.94±0.09	4.99±0.07	5.33±0.19	5.42±0.17	5.33±0.17	0.904	0.212	0.186
AUC, mmol/L x min	2890±78	2860±108	2970±93	3030±102	3130±145	2990±140	0.732	**0.038**	0.557
IAUC, mmol/L x min	454±82	491±93	576±96	472±68	529±90	429±78	0.656	0.186	0.783
MaxC, mmol/L	8.79±0.32	8.77±0.44	9.18±0.33	9.00±0.57	9.53±0.66	9.07±0.54	0.535	0.230	0.681

NEFA

Fasting[3], μmol/L	545±28	636±41	585±25	649±58	606±64	595±64	0.652	0.134	0.943
$AUC_{120-480}$[3], mmol/L x min	128±10	132±11	131±8	145±11	126±13	125±11	0.289	**0.032**	0.741
$IAUC_{120-480}$, mmol/L x min	-68±11	-97±13	-79±12	-88±17	-92±14	-90±17	0.251	0.598	0.663
MaxC, μmol/L	725±46	724±40	706±44	784±62	695±67	689±63	0.181	0.340	0.710

Apo B

Fasting, mg/L	978±34	953±49	938±40	1020±51	1050±65	1060±65	0.996	0.174	0.174
AUC, mg/mL x min	469±15	456±21	451±18	492±26	509±29	510±31	0.958	0.112	0.136
IAUC, mg/mL x min	-0.8±5.4	-1.1±5.5	0.5±6.4	0.4±5.9	4.1±4.6	-0.1±3.8	0.923	0.805	0.626
MaxC, mg/L	1040±34	999±44	999±42	1090±66	1130±66	1140±69	0.994	0.085	0.104
Insulin									
Fasting[3], pmol/L	43.2±13.8	32.5±3.3	27.9±3.1	41.6±8.0	44.7±7.5	44.7±9.9	0.515	0.474	0.738
AUC[3], nmol/L x min	96±10	85±9	91±7	109±11[a]	123±13[b]	107±11[a]	0.726	**0.001**	0.093
IAUC, nmol/L x min	75±11	69±8	78±7	89±8[a]	102±10[a]	85±8[b]	0.575	**0.005**	0.116
MaxC[3], pmol/L	418±44	389±40	421±45	503±70[a]	604±55[a]	449±49[b]	0.144	**0.005**	0.094

Values are unadjusted means ± SEM. Total n=26-32, with n=13-17 Glu298 homozygotes and n=12-15 Asp298 carriers per outcome.

Time intervals for AUC and IAUC: 420 min for FMD, nitrite, nitrate and plasma cell adhesion molecules; 450 min for DBP, SBP, DVP and LDI; 480 min for serum biochemical markers, except 120-480 min for NEFA.

[1]Fasting and postprandial data were analysed using linear mixed model analyses, with genotype, meal and BMI as fixed factors and subject code included as a random effect. The interaction term was added to assess the test fat x eNOS genotype interaction. Labeled means in a row without a common letter differ, *P*<0.05.

[2]LDI-Ach and LDI-SNP were expressed as AUC for the 20-scan protocol. IAUC was also determined for the 20-scan protocol but differences between test fats for subsequent AUC and IAUC were not significant (data not shown).

[3]Log10 transformed prior to analysis.

[4]Sine transformed prior to analysis.

Abbreviations: Ach: acetylcholine, apoB; apolipoprotein B, AU: arbitrary units, DBP: diastolic blood pressure, DVP: digital volume pulse, FMD: flow-mediated dilatation, IAUC: incremental AUC, LDI: laser Doppler imaging, MUFA: monounsaturated fat, NEFA; non-esterified fatty acids, PUFA: polyunsaturated fat, SBP: systolic blood pressure, SFA: saturated fat, sICAM-1: soluble intercellular cell adhesion molecule-1, SNP: sodium nitroprusside, sVCAM-1: soluble vascular cell adhesion molecule-1, TAG; triacylglycerol