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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

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Abbreviations: ANOVA: analysis of variance, apo: apolipoprotein, BMI: body mass index, CVD: cardiovascular disease, DIVAS: Dietary Intervention and VAScular function, DVP: digital volume pulse, eNOS: endothelial nitric oxide synthase, FMD: flow-mediated dilatation, HDL-C: high density lipoprotein cholesterol, HOMA-IR: homeostatic model assessment-insulin resistance, IAUC: incremental AUC, LDI: laser Doppler imaging, LDL-C: low density lipoprotein cholesterol, MaxC: maximum concentration, MUFA: monounsaturated fatty acids, NEFA: non-esterified fatty acids, NO: nitric oxide, PUFA: polyunsaturated fatty acids, RI: reflection index, rQUICKI: revised quantitative insulin sensitivity check index, SFA: saturated fatty acids, SI: stiffness index, sICAM-1: soluble intercellular cell adhesion molecule-1, sVCAM-1: soluble vascular cell adhesion molecule-1, TAG: triacylglycerol, TC: total cholesterol

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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.

Conclusion: Our findings suggest the Glu298Asp polymorphism may represent a potential determinant of the inter-individual variability in postprandial responsiveness of %FMD and insulin to acute meal fat composition in postmenopausal women. Further studies are required to confirm these observations.

This study was registered at www.clinicaltrials.gov (NCT02144454).

Keywords: monounsaturated fat, n-6 polyunsaturated fat, postprandial lipemia, saturated fat, insulin sensitivity, flow-mediated dilatation

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

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

intakes per test fat arm), MUFA (refined olive oil and MUFA-rich spreads; 15.5 g SFA, 54.6 g MUFA and 8.5 g n-6 PUFA) or n-6 PUFA (safflower oil and n-6 PUFA-rich spreads; 13.0 g SFA, 10.8 g MUFA and 56.2 g n-6 PUFA). Habitual dietary intake was assessed using a 4-day weighed food diary completed prior to visit 1 and analysed using Dietplan7 software (Forestfield).

Following a 12 h overnight fast, anthropometric measurements were conducted before an indwelling cannula was inserted into the forearm and two fasting blood samples were collected (-30 and 0 min) to determine the mean baseline (fasting) concentration. Fasting vascular function and clinic blood pressure measurements were then performed before the breakfast was consumed within 15 minutes. Blood samples were collected at regular intervals until lunch (every 30 min until 180 min and then every 60 min until 300 min), and then every 30 min up to 420 min ending at 480 min after the breakfast meal. Postprandial measurements of FMD were conducted at 180, 300 and 420 min, and blood pressure, laser Doppler imaging (LDI) with iontophoresis and digital volume pulse (DVP) at 240 and 450 min after the breakfast meal.

The study was conducted in accordance with the Declaration of Helsinki and approved by the University of Reading Research Ethics Committee (project reference number 14/16). All participants provided written informed consent prior to participation, including consent for genotyping for the eNOS Glu298Asp polymorphism.

Vascular reactivity measurements and blood pressure

To assess vascular reactivity, a single trained researcher measured endothelial-dependent vasodilation of both the brachial artery to determine %FMD (primary outcome) and the microcirculation using LDI with iontophoresis as previously described (18). In the peripheral arteries, DVP (Pulse Trace PCA2; Micro Medical Ltd.) determined the stiffness index (DVP-

SI; m/s) and reflection index (DVP-RI; %) as measures of arterial stiffness and vascular tone, respectively (18). Clinic blood pressure (systolic blood pressure and diastolic blood pressure) and heart rate were measured in triplicate using an OMRON M6 automatic digital blood pressure monitor (OMRON). Vascular reactivity and clinic blood pressure measurements were performed after participants had rested for 30 min in a supine position in a quiet, temperature-controlled room (22 ± 1°C).

Biochemical analysis

Blood samples were collected and processed as described previously (17). Briefly, serum lipids (total cholesterol (TC, fasting only), HDL cholesterol (HDL-C, fasting only) and TAG), apolipoprotein (apo)B, glucose, non-esterified fatty acids (NEFA) and high sensitivity C-reactive protein (fasting only) were determined using an ILAB600 autoanalyzer (reagents: Werfen (UK) Ltd.; NEFA reagent: Alpha Laboratories; apoB reagent: Randox Laboratories Ltd). Fasting LDL cholesterol (LDL-C) was estimated using the Friedewald formula (19). ELISA kits were used to analyze circulating serum insulin (Dako Ltd.; Denmark), plasma sICAM-1 and soluble vascular cell adhesion molecule (sVCAM-1), E-selectin and P-selectin (R & D Systems Europe Ltd.; UK & Europe). Plasma nitrite and nitrate concentrations were analyzed using the HPLC based approach, Eicom NOx Analyzer ENO-30 (Eicom; San Diego; USA) as described elsewhere (17, 20).

Using baseline fasting measures, insulin resistance was determined by homeostatic model assessment of insulin resistance (HOMA-IR), and insulin sensitivity was estimated with the use of revised quantitative insulin sensitivity check index (rQUICKI) derived using standard equations (21). The QRISK®2-2016 online calculator was used to evaluate the risk of developing CVD in next 10 y (http://www.qrisk.org/index.php).

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

genotype on fasting and postprandial responses to the meals of varying fat composition. All models contained test fat, genotype and test fat x genotype interaction as fixed factors and subject code was added as a random factor. Since BMI has been shown to have an independent effect on both vascular function and insulin sensitivity, it was included as a fixed effect covariate within our linear mixed model analysis and retained within the model regardless of their degrees of significance. Other previously reported differences in baseline characteristics (7, 9) as well as the functional effect of the Glu298Asp polymorphism are considered to contribute to the higher CVD risk in carriers of the Asp298 allele. Further correction for these factors may mask the metabolic phenotype of these two genotype groups, and their response to the test fats, and therefore were not included as covariates. $P \leq 0.05$ was considered as significant for this exploratory analysis.

In this secondary analysis of the DIVAS-2 study, the test fat only effects will not be discussed since they were part of our primary analysis and presented in our earlier publication (17).

Results

Study participation

A total of 32 postmenopausal women who completed all three study visits of the DIVAS-2 study were included in the secondary analysis according to the Glu298Asp polymorphism. These participants had a mean ± SD age of 58 ± 5 y and BMI of 25.9 ± 4.1 kg/m^2. The genotype distributions of the eNOS single nucleotide polymorphism were in line with the reference data for the Caucasian populations in HapMap, with the frequency of the Glu298 homozygotes 53% (n=17), Asp298 carrier heterozygotes 44% (n=14) and Asp298 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.

Independent of the meal fat composition, there was an effect of genotype on the IAUC for the DVP-SI, with a tendency for a reduction in postprandial SI after sequential meals in the Asp298 carriers than Glu298 homozygotes (P=0.017)(Table 2).

Postprandial CVD risk markers

Fasting serum insulin concentrations were not different between study visits or genotype groups. Significant test fat x genotype interactions were evident for the AUC (P=0.001), IAUC (P=0.005) and MaxC (P=0.005) **(**Table 2**)** for the postprandial insulin response, with differences between the test fats found in the Asp298 carriers only ($P \leq 0.004$). In this genotype group, the AUC, IAUC and MaxC for the postprandial serum insulin response were significantly greater after consumption of MUFA than SFA ($P \leq 0.038$) and n-6 PUFA-rich ($P \leq 0.004$) meals.

For the postprandial glucose and NEFA responses, a significant test fat x genotype interaction was also observed for the AUC after the sequential meals (P=0.038 and 0.032, respectively) (Table 2). However, differences were not evident between the test fats in either the Glu298 homozygote or Asp298 carrier groups. The Glu298Asp polymorphism did not influence the postprandial serum lipid responses (TAG and apoB) following the sequential SFA, MUFA and n-6 PUFA-rich meals (Table 2).

Discussion

To our knowledge, this secondary analysis of the DIVAS-2 study is the first to determine the impact of the eNOS Glu298Asp polymorphism on vascular function and cardiometabolic disease risk markers to sequential meals rich in SFA, n-6 PUFA and MUFA. Our findings have revealed this polymorphism to a potential modulator of the effect of acute meal fat composition, with differential effects of genotype observed on postprandial %FMD and

insulin responses to sequential MUFA-rich meals in postmenopausal women. However, genotype was not found to influence other measures of vascular function or cardiometabolic disease risk biomarkers following the SFA, MUFA and n-6 PUFA rich meals.

A genotype specific relationship was only found between the AUC for the postprandial %FMD response (our primary outcome) and MUFA intake, with a greater AUC compared with both SFA and n-6 PUFA in the Glu298 homozygotes only. This was surprising since a previous dietary fat-genotype study conducted in adults aged 18-65 years had shown a greater impact of meal fat composition in the minor allele carriers, although this study had focused mainly on SFA and long chain n-3 PUFA-rich test meals (7). Only one other study has determined the impact of olive oil containing meals on postprandial vascular function according to the Glu298Asp polymorphism but this study did not include a comparator meal of a differing fat composition (22). Whilst genotype did not influence postprandial microvascular reactivity to the olive oil meals containing varying levels of phenolics, the postprandial vascular response and NOx concentrations were lower (between 4 and 8 h) in the Asp298 carriers than wild-type group. In the current study, only a tendency for lower postprandial plasma NOx concentrations was found in the Asp298 carriers. Interestingly, in both of these studies, vascular reactivity was not different between genotype groups in the fasting state but only during the postprandial state, a finding also observed after a mixed high-fat meal in healthy men (23). Therefore, the compromised vasodilatory response in the Asp298 carriers may only be expressed during times of metabolic stress, such as the postprandial state.

A number of acute studies have shown vascular function to be modulated by the type of fatty acids consumed (13, 24) but findings have been inconsistent with respect to MUFA-rich oils. Two systematic reviews and meta-analysis using data from chronic randomised controlled trials reported favourable effects of the Mediterranean diet (25) and olive oil

varieties higher in polyphenols (26) on the fasting %FMD response. Similar findings have been observed in the postprandial state, with an increase and/or attenuation of the decrease in FMD response to a high-fat meal, evident after meals rich in extra-virgin olive oil (27, 28), olive oil containing higher amounts of phenolic compounds (22), and avocado fruit (29). Polyphenols are considered to mediate their effects on postprandial vascular function by counteracting fat induced oxidative stress and lipid peroxidation, increasing NO bioavailability. However, in the current study, we observed a greater postprandial %FMD response with refined olive oil devoid of phenolic compounds. The lack of effect of meal fat composition in Asp298 carriers may shed some light on the mechanisms underlying the increase in the AUC for the %FMD response with MUFA in the Glu298 homozygotes. The glutamate to aspartate amino acid substitution at position 298 of the eNOS protein has been reported to make the enzyme more susceptible to cleavage (causing dysfunction), decreasing eNOS activity and potentially vasodilation (30). Furthermore, Asp298 carriers may also have less eNOS associated with caveolin-1 in the endothelial cell membrane (31). Findings from an animal study (32) and *in vitro* studies (33) including some of our own unpublished findings (34), have shown olive oil (extra-virgin) and oleic acid to induce eNOS activity and gene expression compared with lard (32) and single fatty acids (palmitic, stearic and linoleic acids (32, 34). Therefore, it is possible that during the postprandial state, the greater FMD response in the Glu298 homozygotes may reflect an effect of MUFA per se on eNOS enzyme activity and expression, a finding which requires confirmation in human studies.

Of the cardiometabolic disease risk markers, insulin may provide a mechanistic link with the observed effects on the %FMD response to the fat-rich meals in the genotype groups via the reported effects of insulin on eNOS stimulation and blood vessel endothelial dependent vasodilation (35). In support, previous studies have reported associations between the Glu298Asp polymorphism with insulin resistance (36-39), with a greater prevalence of

Asp298 carriers in adults classified with the metabolic syndrome. In the current study, Asp298 carriers showed differential insulin responses to the meal fatty acids, with MUFA ingestion leading to higher postprandial concentrations than the SFA and n-6 PUFA-rich meals. The lack of a genotype effect on glucose handling is suggestive that the sequential MUFA meals may have led to a transient reduction in postprandial insulin sensitivity in this genotype group. However, our findings are in contrast with previous studies which have often reported MUFA-rich meals (containing olive oil and palmolein) to improve beta cell function and postprandial insulin sensitivity compared with SFA-rich meals (40, 41), but no studies to date have determined the effects according to the Glu298Asp polymorphism. Interestingly, greater insulin but not glucose concentrations were reported in Asp298 carriers than Glu298 homozygotes after an oral glucose tolerance test in Japanese non-diabetic adults (42). Although the authors could not discount differences in insulin sensitivity during the oral glucose tolerance challenge between genotypes, they did speculate that the dysfunctional eNOS enzyme in Asp298 carriers may have reduced insulin mediated blood flow in tissues leading to a decreased insulin clearance. In our postmenopausal women, fasting insulin and estimates of insulin sensitivity (HOMA-IR) and rQUICKI were similar in our wild-type group and minor allele carriers. However, similar differences in blood flow and insulin clearance have also been identified in obese versus lean individuals. Although a higher BMI in the Asp298 carriers may be thought to contribute to the insulin responses observed, our analysis controlled for BMI suggesting that this may reflect a metabolic phenotype of this polymorphism. However, we cannot discount that other measures of body composition such as percentage body fat or abdominal obesity were impacting on postprandial insulin responses to meal fat ingestion. Further studies are now warranted to determine the mechanisms underlying the impact of the Glu298Asp polymorphism on postprandial glucose handling and insulin control in response to dietary fat intake, and effects on vascular function.

Our study has some limitations. Genotyping was performed retrospectively and so it was not possible to compare the effects of meal fat composition in Asp298 homozygotes as they represent only 10% of Caucasian populations. Since the study sample size was based on the expected change in the postprandial %FMD response between test fats for the main study, we attempted to estimate our power to detect genotype x fat x vascular function interactions in the current study. Based on the expected change in the postprandial %FMD response following a SFA fat load in female Glu298 versus Asp298 genotype groups (7), we calculated a power of 68% to detect differences in response to the test fats between genotype groups. However, our previous study was conducted in females aged 18-65 years and may not be totally representative of our older female group. Therefore, our data analysis should be considered explorative and provides important data to power future studies within this population sub-group. In this study, a significant test fat x genotype interaction was only identified for the AUC but not IAUC for the postprandial %FMD response. Both the IAUC and AUC offer important summary data on the postprandial responses of an outcome measure to the test meals with different fatty acid composition. IAUC estimates the specific response to the test meals, whereas the AUC estimates that, in addition to any background/residual effect. It should also be noted that within the Glu298 homozygotes, there was a tendency for a higher fasting %FMD response prior to the MUFA than SFA and n-6 PUFA-rich meals, which may have contributed to the findings in this study. Furthermore, as the participants were postmenopausal women, our findings may not be representative of those in men or premenopausal women, or other ethnic groups.

In conclusion, our exploratory analysis has revealed that the Glu298Asp polymorphism to be a potential determinant of the inter-individual variability in postprandial %FMD and insulin responses to acute dietary fat intake in postmenopausal 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.

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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