

Original Article

Gene variations of nitric oxide synthase regulate the effects of a saturated fat rich meal on endothelial function[☆]

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SUMMARY

Background & aims: Endothelial nitric oxide synthase (eNOS) gene variations have been linked to a higher risk for cardiovascular diseases (CVD) by unknown mechanisms. Our aim was to determine if two single nucleotide polymorphisms (SNPs) located in NOS3 (E298D and i19342) interfere with microvascular endothelial function (MEF) and/or oxidative stress during the postprandial state.

Methods: MEF was assessed with laser Doppler flowmetry at baseline and 2, 4, 6 and 8 h after ingestion of a single fatty meal (60% fat, 15% proteins and 25% carbohydrates) by 40 healthy young males. Oxidative stress was measured by nitrites/nitrates and oxidized LDL (LDL-ox) concentrations in fasting and postprandial states.

Results: Postprandial MEF was impaired in the carriers of minor alleles of the SNPs (global mean 60.99% Vs 87.25%, $p = 0.016$ for i19342; 63.62% Vs 95.71%, $p = 0.011$ for E298D). Carriers of E298D showed a higher LDL-ox at fasting and postprandial measures, and lower nitrites/nitrates at fasting.

Conclusions: Minor allele carriers for E298D and i19342 have an impaired postprandial MEF and increased oxidative stress. Our results both provide insight into the higher risk of CVD attributed to E298D and identify variants that affect MEF in a healthy population.

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1. Introduction

Several lines of evidence link endothelial dysfunction with the development of various clinical diseases and cardiovascular risk factors such as coronary heart disease, hypertension, diabetes or heart failure.^{1,2} While some factors chronically impair vascular endothelium function, such as smoking, LDL-cholesterol concentration, hypertension, diabetes mellitus, age, and others,^{3,4} there is an additive transient impairment of endothelial function during the postprandial state,⁵ especially after meals rich in saturated fatty acids. Our group recently has shown that consuming a low-fat diet, or a high-fat diet enriched with olive oil can partially reduce this postprandial endothelial dysfunction.⁶ In contrast, it has been suggested that there is a circadian rhythm to endothelial function,

with impairment in the first hours after morning wake up, even in the fasting state.⁷

Endothelial function is also influenced by oxidative status of the vascular endothelium. This feature is determined by several factors, both intra- and extra-endothelial, and may exhibit a balance between pro-oxidant and antioxidant species to ensure proper endothelial function. Many oxidative biomarkers (as H₂O₂, F₂-isoprostanes, malondialdehyde, 4-hydroxy-2-nonenal, antioxidants, glutathione and nitrosative stress such as nitrate/nitrite) have been used to assess oxidative status, some with more and some with less accuracy.⁸ Among these are nitrate/nitrite. Although they may not exactly reflect nitric oxide bioavailability in vascular endothelium, nitrate/nitrite levels have been used extensively to infer nitric oxide pathway status.⁹

The main intrinsic regulator of nitric oxide formation is endothelial nitric oxide synthase (NOS3, also named eNOS or ECNOS). NOS3 maps to chromosome 7 (7q35–36), and the presence of the variant allele of some single nucleotide polymorphisms (SNPs) located in this gene or nearby have been linked to decreased NOS3 function, or to increased prevalence of several cardiovascular

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diseases.^{10,11,12} A common genetic *NOS3* variation (894 G→T) within exon 7, results in the replacement of glutamic acid by aspartic acid at amino acid 298.¹³ The homozygotes for the rare allele (DD) of this SNP, called E298D (also called Glu298Asp, G894T or rs1799983) have been linked to coronary heart disease,^{10,14} and arterial vasospasm at various locations, as coronary or central nervous system.^{15,16,17} Furthermore, homozygotes for the rare allele have been associated with left ventricular hypertrophy¹⁸ and earlier age of onset of myocardial infarction compared with the rest of the population.¹⁹

A search for other SNPs that could also influence postprandial endothelial function was preceded by an analysis of linkage disequilibrium among those SNPs genotyped as part of the HapMap Consortium (www.hapmap.org). Among these SNPs, we selected i19342 (rs743507), within *NOS3* intron 21, as a likely representative of another distinct linkage disequilibrium (LD) block. The HapMap data suggest an r^2 measure of LD in the CEU (White) population between SNPs E298D and i19342 of 0.54, indicating moderate but not strong LD.

Bearing in mind that humans spend a majority of time in the postprandial state, and that the postprandial state provokes an independent transient endothelial dysfunction, we analyzed the additive hypothetical effects of *NOS3* variants on microvascular endothelial function and on oxidative markers (nitrites/nitrates, oxidized LDL) during the postprandial state after a fatty meal. This approach allowed us to explore the function of *NOS3* in the postprandial state and provide clues to the intrinsic mechanisms that underlie higher cardiovascular risk associated with *NOS3* variation.

2. Material and methods

2.1. Study population

Sample size was set by the following premises based in previous studies performed in our unit.^{6,20} Expected grand mean: 80; expected standard deviation: 20; minimum difference expected between groups: 20; alpha risk: 0.05; power: 80%. Based on these premises the minimum sample size needed was 30. Forty healthy non-smoking White males from the University of Cordoba gave informed consent and entered the study after a medical examination. Potential subjects were excluded if they had a chronic illness, were extremely physically active or had a family history of premature cardiovascular disease. The studies in which these participants were enrolled were approved by the Ethics Committee for Clinical Investigations of the Reina Sofia University Hospital.

2.2. Study design

After overnight 12 h fast, subjects were given a fatty meal. The amount of fat given was 1 g of fat and 7 mg of cholesterol per kg of body weight. The meal contained 60% of its energy in the form of fat (35% SAT, 19% MUFA, 6.3% PUFA), 15% as protein and 25% as carbohydrates, and was ingested within 20 min. After the meal, subjects had no energy intake for 11 h but were allowed to drink water. Blood samples to assess plasma markers of oxidation were collected before the meal, and 4 h later.

2.3. Biochemical determinations

2.3.1. DNA amplification and genotyping

Genotyping of the SNPs were determined by the TaqMan assay, using real time PCR, and allelic discrimination was performed by fluorescence by means of a 7900 Sequence Detection System (SDS) (Applied Biosystems). This methodology has been described elsewhere.²¹ The primer sequences used were GGCTGGACCCAGAAA and CACCCAGTCAATCCCTTGGT for E298D and for i19342 the

TaqMan assay from Applied Biosystems, catalog number hCV1026883. In both cases, percent successful genotype calls were observed to be > 95%.

2.3.2. Oxidative markers

Plasma levels of oxidized LDL were measured by the Mercodia Oxidized LDL ELISA (Mercodia AB, Uppsala, Sweden) and nitrates/nitrites (NOX) by colorimetric method (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical, Ann Arbor, Michigan). Determinations were performed in duplicate.

2.4. Study of the endothelial function

Laser Doppler linear Periflux 5000 (Perimed S.A., Stockholm, Sweden) was used to measure MEF by means of an ischemic reactive hyperemia test. Laser Doppler flowmetry is an easy, non invasive method for determining microvascular endothelial function.^{22,23,24,25} This ease of use allows performing several measurements during a postprandial study, and has been used by our unit in several studies.^{6,20,26} Briefly, capillary flow of the second finger of the dominant arm of the patient was assessed for 1 min before (t_0) and after (t_d) 4 min of ischemia of the arm by means of a sphygmomanometer, and the ischemic reactive hyperemia was obtained, which is equal to $(AUC_{td}-AUC_{t_0})/AUC_{t_0} \times 100$. This calculation was performed using the basal determinations of 2, 4, 6, and 8 h after consumption of the fat meal.

2.5. Statistical analysis

2.5.1. Genotype analysis

SNPs were tested for LD. Hardy–Weinberg equilibrium was tested with a Chi-squared based test. For all genotype and linkage disequilibrium analyses, we used Helix-Tree software program (Golden Helix, MT, USA) version 4.3.2.

2.5.2. Statistical analysis

The data were analyzed using Student's *t* test and analysis of variance for repeated measures. We tested the effects of time, phenotype and the interaction between time and phenotype for each variable. When there were statistically significant differences in phenotype or in the interaction in the ANOVA test, a multiple comparisons test adjusted by Bonferroni's rule was performed for each time point. When assessing oxidative stress markers, we used triacylglycerol area under curve of the time period as a covariate. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois). All data presented in text and tables are expressed as means \pm SD unless stated otherwise. In order to establish proper phenotypes, population was stratified into carriers of the variant allele compared to homozygotes for the common allele, on the assumption of a dominant allele effect.

3. Results

Genotype distribution, Hardy–Weinberg equilibrium and linkage disequilibrium (LD) test results are listed in Table 1. SNPs E298D and i19342 are in LD ($D'0.85$; $R^2 0.74$; $p < 0.0001$). We did not find differences reaching significance in the age or anthropometric measures (weight, height, body mass index) depending on the *NOS3* SNPs (Supplemental Table 1)

3.1. *NOS3* E298D

Genotype frequencies for E298D were 10 GG, 24 GT and 6 TT individuals, which is in agreement with the Hardy–Weinberg rule

Table 1
Genotype frequencies, minor allele frequencies, Hardy–Weinberg Equilibrium and Linkage Disequilibrium of the Gene Variations.

	Genotype Frequency			MAF	HWE	LD
	10GG	24GT	6TT			
NOS3_E298D	10GG	24GT	6TT	0.45	0.18	D' 0.5
NOS3_I19342	16TT	18TC	6CC	0.38	0.80	R ² 0.74

MAF = Minor allele frequency. HWE = Hardy–Weinberg equilibrium test. LD = Linkage disequilibrium.

($X^2 = 0.064$; $p = 0.18$). Allele frequencies were 0.55 and 0.45, which agreed with those published in the National Library Medicine SNP database²⁷ (Table 1).

Postprandial MEF was impaired in the carriers of the minor allele, with a reduction of 33.53% of the grand mean during the 8 h postprandial study (GT/TT = 63.62 vs GG = 95.71, p for genotype = 0.011) (Fig. 1 A). Time was an independent determinant of MEF in the postprandial state (p for time = 0.003). There was an interaction between gene and time (p for interaction = 0.043). In the multiple comparisons test (Bonferroni adjustment) we found a lower MEF in carriers (GT/TT) compared with common allele homozygotes (GG) at time points 4 ($p = 0.003$), 6 ($p = 0.037$) and 8

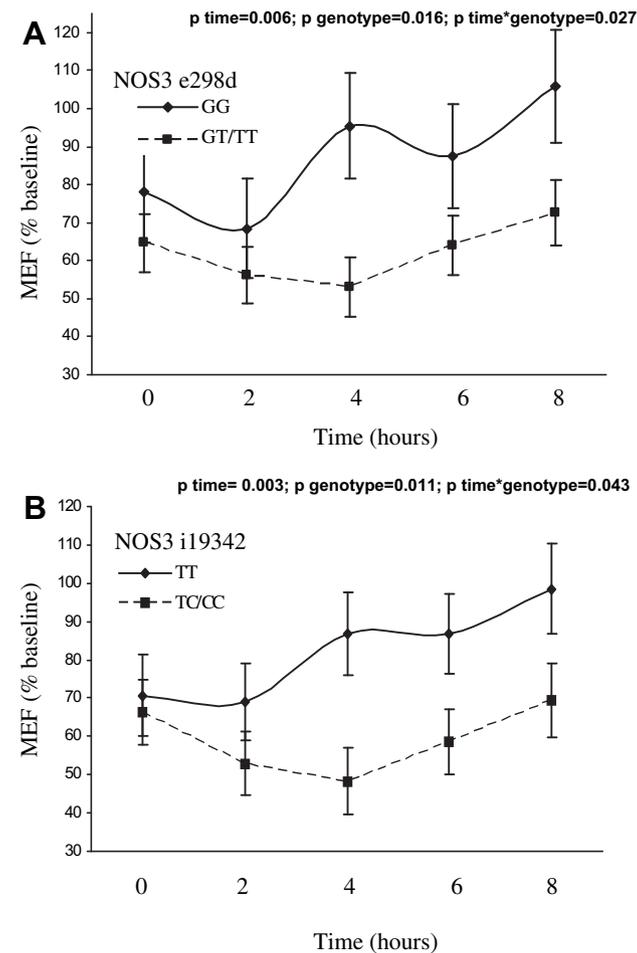


Fig. 1. Microvascular Endothelial Function (MEF) measured by laser Doppler flowmetry in fasting and postprandial states. MEF was assessed as ischemic reactive hyperemia (% baseline), computed as the % of change between post and pre-ischemia areas under the curve. Carriers of E298D (Panel A) and i19342 (Panel B) SNPs of the NOS gene have an impaired postprandial microvascular endothelial function (for both panels, the lower line identifies the carriers of the mutation, versus the upper line that identifies the homozygotes for the most common allele). All values are derived from repeated measures ANOVA. $N = 40$.

($p = 0.007$) hours after the meal (Fig. 1 A). Nitrites/nitrates were lower in the 4 h postprandial determination, compared with the fasting state ($p = 0.001$) (Table 2). We found higher nitrites/nitrates at fasting in GG vs GT/TT (GG 17.3 ± 7.84 vs GT/TT 12.69 ± 4.16 , $p = 0.030$) and non-significant higher nitrites/nitrates in the postprandial measure in GG vs GT/TT (GG 13.3 ± 4.28 vs GT/TT 11.44 ± 4.10 , $p = 0.24$) (Table 2). There was a lower LDL-ox in the homozygotes for the major allele, both at fasting (GG 0.98 ± 0.09 vs GT/TT 1.23 ± 0.05 mmol/L, $p = 0.018$) and postprandial (GG = 0.92 ± 0.09 vs GT/TT = 1.19 ± 0.05 , $p = 0.017$) determinations (Table 2).

3.2. NOS3 i19342

Genotype frequencies were 16 TT, 18 TC, 6 CC, and this variant is also in Hardy–Weinberg equilibrium ($X^2 = 0.064$; $p = 0.80$). The allele frequencies were 0.63 and 0.37, which agrees with previously reported frequencies²⁸ (Table 1).

Postprandial MEF was impaired in the carriers of the minor allele (TC/CC), p for genotype = 0.016, with a reduction of 30.10% of the grand mean in the 0 to 8 h period, after the meal (60.99 Vs 87.25) (Fig. 1 B). Time alone was also a determinant of MEF in the postprandial state (p for time = 0.006), irrespective of genotype. There was an interaction between gene and time (p for interaction = 0.027), and multiple comparisons test (Bonferroni adjustment) showed that the carriers of the SNP (TC/CC) showed a lower MEF at 4 ($p = 0.003$), 6 ($p = 0.016$) and 8 ($p = 0.017$) hours after the meal as compared to the homozygotes for the common allele (TT). Although we observed a similar trend at 2 h, these differences were not significant ($p = 0.091$), (Fig. 1B).

Nitrites/nitrates were lower in the 4 h postprandial determination, compared with the fasting state ($p = 0.001$) (Table 2). Non-significant higher NOX means were observed for TT participants in fasting (TT 16.18 ± 7.11 vs TC/CC 12.39 ± 3.90 , $p = 0.053$) and postprandial (TT 13.26 ± 4.16 vs TC/CC 11.00 ± 4.06 , $p = 0.117$) determinations. We did not observe any differences in LDL-ox between the fasting and the postprandial state, nor between TT and TC/CC.

4. Discussion

Our study shows that the carriers of the minor alleles of either E298D (GT/TT) or i19342 (TC/CC) SNPs in the NOS3 gene have an impaired postprandial microvascular endothelial function compared to persons homozygous for the most common alleles (GG and TT, respectively). The use of laser Doppler flowmetry allowed us to perform several measurements during the postprandial state, and allowed us to pinpoint this impairment as being provoked mainly from the 4th hour in the postprandial state to the 8th hour.

The NOS3 E298D variant has been linked to cardiovascular disease and diabetes mellitus in several studies.^{10,11,12} In addition to accelerated atherosclerosis,¹³ some data regarding the phenotypic effects of this polymorphism are accounted for by an impaired vasodilatation, such as its association to coronary spasm,^{15,16} angiographic vasospasm after aneurysmal subarachnoid hemorrhage,¹⁷ early onset of myocardial infarction¹⁹ or additive effects to smoking,²⁹ itself a known endothelial dysfunction factor. An *in vitro* reduction of basal nitric oxide release in homozygotes for the rare allele of E298D in human endothelium³⁰ or animal models³¹ has been reported. A molecular basis supporting this finding remains to be described. Amino acid 298, affected by the E298D variant, is not near the catalytic zone of eNOS.³² Nevertheless, it has been demonstrated that the variant form of the eNOS protein is subject to intracellular cleavage, which essentially inactivates it.³³

Table 2

Concentration in plasma of different oxidative markers, depending on E298D and i19342 genotypes.

	Baseline		Postprandial		ANOVA p value		
	GG	GT/TT	GG	GT/TT	Time	Genotype	Interaction
<i>E298D</i>							
NOX ($\mu\text{mol/L}$) ^a	17.3 \pm 2.4	12.7 \pm 0.8	13.3 \pm 1.4	11.4 \pm 0.8	0.001	0.083	0.047
LDL (mmol/L)	2.00 \pm 0.12	2.26 \pm 0.09	1.77 \pm 0.15	1.9 \pm 0.08	0.001	0.043	> 0.20
LDL-ox(U/L)	0.98 \pm 0.09	1.23 \pm 0.05	0.92 \pm 0.09	1.19 \pm 0.05	0.013	0.007	> 0.20
<i>i19342</i>							
NOX ($\mu\text{mol/L}$)	16.2 \pm 1.6	12.4 \pm 1.2	13.3 \pm 1.1	11.0 \pm 0.9	0.001	0.079	> 0.20
LDL (mmol/L)	2.21 \pm 0.12	2.19 \pm 0.10	1.76 \pm 0.12	1.83 \pm 0.10	0.005	> 0.20	> 0.20
LDL-ox(U/L)	1.14 \pm 0.07	1.19 \pm 0.05	1.12 \pm 0.08	1.14 \pm 0.06	0.101	> 0.20	> 0.20

^a All values are expressed as mean \pm standard error. NOX: nitrates/nitrites.

Our work shows that carriers of the E298D SNP have an impaired endothelium-dependent vasodilation in skin in the postprandial state, as measured by laser Doppler. This type of flowmetry previously has been linked to cardiovascular risk assessment. Individuals at increased risk for coronary heart disease are characterized by an impaired microvascular function in skin.³⁴ It is necessary to remark that, in our study, there was no significant difference in the MEF between groups in the fasting state, and that the differences were only evident in the postprandial measurements. Thus, we hypothesize that the dysfunction associated with the SNPs is only expressed when there is an increase in the oxidant status, such as occurs in the postprandial state. Furthermore, this is probably due to the incapacity of the eNOS protein to respond adequately to a greater demand for nitric oxide formation. Even a partially impaired function of eNOS may be adequate to create sufficient nitric oxide to ensure MEF in the resting situation. However, when oxidation increases, an impaired eNOS may not be efficient enough to provide the needed nitric oxide to buffer the excess of pro-oxidant species, which then gives rise to endothelial dysfunction.

Looking for underlying mechanisms that may explain our findings, we assessed nitrites/nitrates and LDL-ox during the intervention period. An increase in the oxidative stress of these persons was revealed, as they showed higher LDL-ox both in fasting and postprandial determinations, and lower nitrites/nitrates at fasting (and a similar trend in postprandial measurements). In our sample, carriers of the minor allele of E298D have a lower concentration of NOX in the fasting state. Although NOX does not exactly measure nitric oxide bioavailability in the vascular endothelium, lower plasma measurements may be a reflection of what happens in the vascular wall, and may be partly responsible for the higher postprandial microvascular endothelial dysfunction. On the other hand, the elevated LDL-ox of these persons can explain their increased susceptibility for accelerated atherosclerosis. Upon this landscape of an altered eNOS function, any exacerbating circumstances, such as tobacco smoking, or increased demands for coronary flow, for example stress or exercise, in the set of a compromised coronary vessel, such as one in an environment of increased atherosclerosis, could determine a cardiovascular event due to the inability of the vessels to vasodilate. In line with our hypothesis, Antoniadou and colleagues found increased LDL-ox levels in E298D carriers when they were exposed to “biological stress” such as during the acute phase of myocardial infarction in the fasting state.³⁵ Our hypothesis, therefore, states that carriers of the minor allele for E298D are at higher risk for cardiovascular disease by a conjunction of accelerated atherosclerosis and decreased vasodilatory capacity, especially in the postprandial state. In agreement with this theory, recent work by Erbs et al. showed decreased *in vitro* relaxation of arterial internal mammary artery rings from patients with coronary heart disease in carriers of the E298D minor allele.³⁶

Although the other SNP selected for genotyping, i19342, has no reported phenotypic associations nor ascribed function, genotyping of HapMap populations indicated a useful minor allele frequency and moderate LD with SNP E298D. Surprisingly, i19342 showed a striking effect on postprandial endothelial function. The location of this variant within *NOS3* intron 21 provides for some interesting biological consequences. SNP i19342 may affect *NOS3* mRNA processing, interaction with small RNA species, or transcription rates of *NOS3*. Thus, i19342 becomes a promising determinant of endothelial function, and more studies are needed to determine its real importance.

A segment of the kinetics of the endothelial function shown in the present study can be due to diurnal modification of endothelial function, as suggested by Gaenger et al.⁷ That report showed that endothelial function is impaired during the first hour after morning wake up, and improves gradually until evening, even in the fasting state. This must be kept in mind when interpreting the kinetics of endothelial function in a postprandial study. At first glance, it could be hypothesized that the meal test actually improved endothelial function, while a deeper examination which considers the results of Gaenger et al., causes us to miss the improvement of endothelial function over the 8 h of the study. Furthermore, the homozygotes for the common allele showed an increased MEF between the fasting and the 8 h post-meal time points, which follows the theory espoused by Gaenger and colleagues. In addition, minor allele carriers showed MEF levels that were essentially unchanged from the beginning and 8 h after the meal. This can be interpreted as a lack of improvement during the day, and thereby an impaired MEF even at 8 h after the meal.

The moderately strong LD between E298D and i19342 can also explain some of the apparent contradictory findings regarding E298D in observational studies. It is possible that the frequencies of i19342 and E298D in different populations could be distinct with different degrees of LD between these polymorphisms. In such a case, it is possible that in populations with a higher double mutation frequency the effects attributed to E298D could actually be due to the two variants together, and that, in studies undertaken in populations with a lower frequency of this double mutation, the lack of effect of E298D would actually reflect a lower “double mutant” negative effect. This notion, however, is highly speculative, and is something that only the combined study of i19342 and E298D can elucidate.

With the present study, we provide a rationale for the increased cardiovascular risk observed in carriers of the less common 298D allele in that we have assessed directly an impairment of MEF in the postprandial state, an increase in LDL-ox, and a decrease in NOX availability. At the same time, we have identified another independent modulator of MEF (i19342). This work identifies a population with increased cardiovascular risk that corresponds to a compromised vasodilatory capacity in the postprandial state.

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Statement of authorship

JD-L carried out the studies and data analyses, performed the statistical analysis and drafted the manuscript. AG-R, FF, CM, YJ-G carried out the samples analyses. JD-L, PP-M, FP-J, JL-M participated in the design of the study. LDP and CQL performed the genetic analysis, FP-J, JL-M, JMO conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement

None of the authors declare any potential disclosures or conflict of interest regarding this manuscript.

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None.

Appendix. Supplementary material

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.clnu.2010.08.006.

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