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NOS3 gene polymorphisms are associated with risk markers of cardiovascular disease, and interact with omega-3 polyunsaturated fatty acids

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ABSTRACT

Objective: Omega-3 polyunsaturated fatty acids (*n*-3 PUFA) may protect against the development of cardiovascular disease (CVD). Genotype at key genes such as nitric oxide synthase (NOS3) may determine responsiveness to fatty acids. Gene–nutrient interactions may be important in modulating the development of CVD, particularly in high-risk individuals with the metabolic syndrome (MetS).
Methods: Biomarkers of CVD risk, plasma fatty acid composition, and NOS3 single nucleotide polymorphism (SNP) genotype (rs11771443, rs1800783, rs1800779, rs1799983, rs3918227, and rs743507) were determined in 450 individuals with the MetS from the LIPGENE dietary intervention cohort. The effect of dietary fat modification for 12 weeks on metabolic indices of the MetS was determined to understand potential NOS3 gene–nutrient interactions.
Results: Several markers of inflammation and dyslipidaemia were significantly different between the genotype groups. A significant gene–nutrient interaction was observed between the NOS3 rs1799983 SNP and plasma *n*-3 PUFA status on plasma triacylglycerol (TAG) concentrations. Minor allele carriers (AC + AA) showed an inverse association with significantly higher plasma TAG concentrations in those with low plasma *n*-3 PUFA status and vice versa but the major allele homozygotes (CC) did not. Following *n*-3 PUFA supplementation, plasma TAG concentrations of minor allele carriers of rs1799983 were considerably more responsive to changes in plasma *n*-3 PUFA, than major allele homozygotes.
Conclusions: Carriers of the minor allele at rs1799983 in NOS3 have plasma TAG concentrations which are more responsive to *n*-3 PUFA. This suggests that these individuals might show greater beneficial effects of *n*-3 PUFA consumption to reduce plasma TAG concentrations.

1. Introduction

Cardiovascular disease (CVD) is one of the leading global causes of mortality [1], and places a major burden on healthcare systems. Presence of the metabolic syndrome (MetS) is associated with an increased risk of CVD and type 2 diabetes [2]. This cluster of metabolic abnormalities [3] is of interest from a public health perspective, because early intervention amongst these individuals may prevent the development of serious health complications. To understand progression of the disease, it is necessary to consider both candidate genes for involvement, as well as environmental stressors. Whereas genetic factors by themselves are not yet modifiable, other factors such as diet can interact with genetic variation to determine phenotype; thereby allowing intervention based on nutrigenomic concepts [4]. Omega-3 polyunsaturated fatty acids (*n*-3 PUFA) have been of considerable interest, due to their poten-

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tial ability to reduce CVD risk [5]. Although at present, the evidence for their efficacy remains controversial [6]. It is possible that heterogeneity between studies on the effect of *n*-3 PUFA supplementation may be due to confounding effects, such as genetic background. By identifying key genes influencing sensitivity to *n*-3 PUFA, we may improve understanding of the mechanisms of action of *n*-3 PUFA, and also identify people who most likely would benefit from a nutritional intervention. One of these key genes is nitric oxide synthase (NOS3), the gene responsible for the production of nitric oxide (NO), which is involved in endothelial function and thereby can influence development of atherosclerosis, insulin resistance and type 2 diabetes [7]. NO may regulate endothelial expression of adhesion molecules and inflammatory cytokines, and stimulate glucose uptake in skeletal muscle [8,9]. Single nucleotide polymorphisms (SNPs) in the NOS3 gene have been found to be associated with the MetS, as well as with a number of CVD risk markers including dyslipidaemia and inflammation [10,11]. In our present study, the effects of NOS3 polymorphisms were investigated in cohort of 450 MetS patients from the LIPGENE study, participating in a 12-week dietary intervention to alter dietary fatty acid composition and amount. We hypothesised that variations in the NOS3 gene interact with plasma long chain (LC) *n*-3 PUFA status, to influence CVD risk factors as well as determining responsiveness to alterations in LC *n*-3 PUFA following dietary intervention.

2. Subjects and methods

2.1. Subjects

Participants in the LIPGENE dietary intervention study were recruited from 8 European countries (Ireland, UK, Norway, France, The Netherlands, Spain, Poland and Sweden), in line with the Helsinki Declaration of 1975 as revised in 1983. The study was registered with The US National Library of Medicine Clinical Trials registry (NCT00429195). Subject eligibility was determined using a modified version of the NCEP criteria [12], where subjects were required to fulfil at least three of the following five criteria: waist circumference >102 cm (men) or >88 cm (women); fasting glucose 5.5–7.0 mmol/L; triacylglycerol (TAG) $\geq$1.5 mmol/L; HDL cholesterol < 1.0 mmol/L (men) or <1.3 mmol/L (women); blood pressure $\geq$130/85 mmHg or treatment of previously diagnosed hypertension. Subjects were randomised to one of four isoenergetic dietary treatments, differing both in quality and quantity of dietary fatty acids: High-fat (38% energy) saturated fatty acid (SFA)-rich diet (16% SFA, 12% monounsaturated (MUFA), 6% PUFA) (HSFA); High-fat (38% energy), MUFA-rich diet (8% SFA, 20% MUFA, 6% PUFA) (HMUFA); isoenergetic low-fat (28% energy), high-complex carbohydrate diet (8% SFA, 11% MUFA, 6% PUFA), with 1 g/day high oleic sunflower oil supplement (LFHCC); or 1.24 g/day very long chain *n*-3 PUFA supplement (LFHCC *n*-3). Further details of the dietary intervention procedure have been published previously [13]. Anthropometric measurements and 3-day weighed-food dietary assessments were performed before and after interventions. The present analyses include the 450 subjects with available SNP data.

2.2. Biochemical measurements

Plasma, serum, and buffy coat were prepared from 12 h fasting blood samples taken prior to an intravenous glucose tolerance test (IVGTT) performed in each subject. Serum insulin was measured by solid-phase, two-site fluoroimmunometric assay on a 1235 automatic immunoassay system (AutoDELFIA kits, Wallac Oy, Turku, Finland). Plasma glucose concentrations were measured (IL Test™ Glucose Hexokinase Clinical Chemistry kit) (Instrumentation Laboratories, Warrington, UK). Adiponectin was determined by use of ELISA (DuoSet® ELISA Development System, R&D Systems, MN, USA). Cholesterol and TAG were quantified using the IL Test™ Cholesterol kit and IL Test™ Triglycerides kit (Instrumentation Laboratories, Warrington, UK). The IL Test™ HDL-C kit (Instrumentation Laboratories, Warrington, UK) was used for direct quantification of HDL-C. The WAKO NEFA C enzymatic colour kit (Alpha Laboratories, Hampshire, UK) was used to quantify plasma NEFA concentration. Homeostasis model assessment of insulin resistance (HOMA-IR) derived from fasting glucose and insulin levels [(fasting plasma glucose $\times$ fasting serum insulin)/22.5] as determined [14]. Plasma fatty acid composition was measured on a GC 2010 gas liquid chromatograph (Shimadzu, Japan) [15]. Total plasma *n*-3 PUFA was calculated from the sum of C18:3 (*n*-3), C18:4 (*n*-3), C20:4 (*n*-3), C20:5 (*n*-3), C22:5 (*n*-3) and C22:6 (*n*-3). Long-chain (LC) *n*-3 PUFA was calculated from C20:5 (*n*-3) and C22:6 (*n*-3).

2.3. SNP selection and genotyping

Genotype data for NOS3 from HapMap (http://hapmap.org) were uploaded into HITAGENE, a web-based combined database and sets of genetic analysis tools developed by Hitachi Dublin Laboratory. Haplotype frequencies were estimated by implementation of the expectation maximization (EM) algorithm. Using a 5% cut-off for individual haplotype frequency and >70% for the sum of all haplotype frequencies, genotype data was then transferred from HITAGENE to SNP tagger (http://www.broad.mit.edu/mpg/tagger/server.html) to identify haplotype tagged (Ht) SNPs. A combination of Ht SNPs and SNPs previously mentioned in the literature were genotyped, giving a total of six SNPs: rs11771443, rs1800783, rs1800779, rs1799983, rs3918227, and rs743507. DNA was extracted from buffy coats using the AutoPure LS automated system (Gentra Systems Inc., Minneapolis, MN, USA), and low yielding samples (<10 ng) were subjected to whole genome amplification using the REPLI-g kit (Qiagen Ltd., West Sussex, UK). Genotyping was conducted by Illumina Inc. (San Diego, CA, USA). Genotyping was attempted in all 450 individuals, however due to differences in the success rate of individual SNPs, population numbers differ slightly between SNPs. Adherence to HWE at each SNP locus was determined using the χ^2 test with 1 degree of freedom. All SNPs were found to adhere to HWE. Linkage disequilibrium (LD) between SNPs was assessed using THESIAS [16], and as there was significant LD between rs1800783 and rs1800779, the rs1800783 SNP was removed from further analysis.

2.4. Statistical analysis

Biochemical variables were assessed for normality of distribution, and skewed variables were normalised by log or square root transformation as appropriate. Univariate general linear models (ANCOVA) were used to test for single gene associations and associations between gene–nutrient interactions and biochemical variables within the cohort. The effect of each SNP interacting with *n*-3 PUFA on each biochemical variable was investigated using *n*-3 PUFA as a continuous variable with a SNP-PUFA interaction term, controlling for age, sex, BMI, smoking, alcohol consumption, exercise and LIPGENE centre of origin. To detect *n*-3 PUFA-specific SNP effects, the cohort was dichotomised by the median level of PUFA, and ANOVA was applied, using Tukey's Post Hoc test. Initial analyses were carried out using all three genotype groups; however, as the minor allele homozygotes displayed similar effects to the heterozygotes for all SNPs and had a small group size, these groups were pooled to increase power in detecting effects of the minor allele. A linear regression model containing all previously

Table 1
Clinical characteristics and CVD risk markers of the participants in the LIPGENE Intervention cohort, according to NOS3 genotype.

	rs1800779					rs1799983					rs3918227					rs743507					rs11771443				
	AA		AG + GG			CC		AC + AA			CC		AC + AA			AA		AG + GG			GG		AG + AA		
	Mean	SE	Mean	SE	*P* value	Mean	SE	Mean	SE	*P* value	Mean	SE	Mean	SE	*P* value	Mean	SE	Mean	SE	*P* value	Mean	SE	Mean	SE	*P* value
Sex (male/female)	66/94		114/161		0.950	78/121		98/130		0.471	148/225		36/41		0.157	103/147		80/118		0.839	125/196		55/62		0.091
Age (years)	54.6	0.9	54.1	0.6	0.395	54.7	0.8	53.9	0.7	0.120	54.4	0.6	53.7	1.2	0.378	54.3	0.7	54.2	0.8	0.344	54.2	0.6	54.5	0.9	0.792
Cholesterol (mmol/L)	5.05	0.08	5.33	0.07	0.024	5.10	0.08	5.35	0.07	0.000	5.21	0.06	5.35	0.11	0.124	5.15	0.07	5.34	0.08	0.013	5.18	0.06	5.38	0.10	0.119
LDL (mmol/L)	2.98	0.09	3.29	0.07	0.080	3.05	0.08	3.30	0.08	0.007	3.17	0.06	3.22	0.12	0.599	3.05	0.07	3.35	0.09	0.001	3.10	0.07	3.40	0.11	0.018
HDL (mmol/L)	1.10	0.02	1.13	0.02	0.042	1.12	0.02	1.12	0.02	0.617	1.12	0.02	1.13	0.04	0.316	1.12	0.02	1.12	0.02	0.655	1.12	0.02	1.12	0.03	0.973
TAG (mmol/L)	1.59	0.06	1.52	0.04	0.373	1.51	0.05	1.57	0.04	0.070	1.53	0.04	1.62	0.08	0.125	1.54	0.04	1.54	0.05	0.856	1.52	0.04	1.59	0.06	0.542
NEFA (μmol/L)	593.5	20.9	619.1	16.1	0.430	602.7	17.7	616.3	18.3	0.788	613.3	13.5	592.4	36.7	0.704	602.1	16.5	620.1	20.1	0.465	623.3	14.8	571.7	24.9	0.361
Apo A1 (g/L)	1.39	0.02	1.41	0.02	0.423	1.40	0.02	1.41	0.02	0.471	1.41	0.01	1.39	0.04	0.324	1.40	0.02	1.41	0.02	0.817	1.40	0.02	1.40	0.03	0.998
Apo B (g/L)	0.97	0.02	1.01	0.02	0.192	0.97	0.02	1.02	0.02	0.007	0.99	0.01	1.03	0.03	0.290	0.98	0.02	1.02	0.02	0.065	0.98	0.01	1.04	0.02	0.189
Apo B48 (mg/L)	0.77	0.08	0.81	0.06	0.686	0.75	0.06	0.83	0.07	0.555	0.78	0.05	0.90	0.14	0.727	0.77	0.06	0.83	0.08	0.568	0.78	0.05	0.84	0.10	0.505
Apo CII (mg/L)	40.4	1.3	41.8	1.1	0.560	39.4	1.0	42.9	1.3	0.026	41.2	0.9	41.9	2.4	0.658	40.0	1.1	43.0	1.3	0.087	41.0	1.0	42.1	1.7	0.962
Apo CIII (mg/L)	148.8	3.9	144.0	2.9	0.972	144.2	3.1	147.1	3.5	0.095	144.9	2.6	150.3	6.0	0.174	148.1	3.0	142.6	3.7	0.909	146.3	2.8	144.3	4.3	0.689
Apo E (mg/L)	38.0	1.0	39.5	1.1	0.563	37.9	0.9	39.9	1.2	0.019	38.9	0.9	39.0	1.4	0.218	38.5	0.8	39.6	1.4	0.354	39.3	1.0	38.0	1.1	0.815
Diastolic BP (mmHg)	85.3	0.8	86.7	0.7	0.215	86.2	0.8	86.3	0.8	0.903	86.2	0.6	86.2	1.1	0.357	85.6	0.7	87.1	0.9	0.266	86.2	0.7	86.4	0.9	0.244
Systolic BP (mmHg)	137.8	1.4	139.4	1.0	0.638	139.1	1.2	138.5	1.1	0.333	139.1	0.9	136.8	1.9	0.274	139.0	1.1	138.4	1.2	0.229	139.1	1.0	137.9	1.6	0.054
Adiponectin (μg/mL)	4.23	0.27	3.62	0.12	0.036	3.81	0.20	3.86	0.16	0.605	3.85	0.13	3.79	0.38	0.619	3.93	0.19	3.73	0.15	0.339	3.83	0.14	3.86	0.27	0.657
Leptin (ng/mL)	18.55	1.37	23.30	1.30	0.023	19.92	1.28	23.00	1.43	0.362	22.64	1.10	16.16	1.79	0.025	18.85	1.12	25.01	1.64	0.001	22.12	1.17	20.12	1.74	0.620
Fibrinogen (mg/dl)	340.0	7.0	322.0	5.4	0.088	336.9	6.3	321.3	5.8	0.129	327.9	4.6	331.8	11.6	0.754	339.8	6.0	314.3	5.8	0.013	328.8	4.9	327.8	8.7	0.733
IL-6 (pg/mL)	5.18	0.37	4.84	0.25	0.220	4.88	0.30	5.03	0.29	0.347	4.82	0.22	5.71	0.63	0.340	5.13	0.30	4.76	0.29	0.883	4.85	0.23	5.27	0.44	0.497
Resistin (ng/mL)	10.4	0.5	10.0	0.3	0.745	10.4	0.4	9.9	0.3	0.904	10.0	0.3	10.6	0.6	0.170	10.5	0.4	9.6	0.3	0.204	10.0	0.3	10.5	0.4	0.183
sICAM (ng/mL)	285.9	5.9	286.0	4.8	0.268	282.2	5.8	289.2	4.8	0.174	287.1	4.0	280.6	9.6	0.423	281.2	5.1	292.0	5.3	0.151	287.7	4.4	281.3	7.1	0.366
sVCAM (ng/mL)	595.1	12.6	602.0	10.6	0.563	602.3	12.6	597.2	10.6	0.853	601.0	9.0	592.1	19.7	0.554	598.3	11.3	601.0	11.7	0.806	597.9	9.5	603.8	16.1	0.942
TNFα (pg/mL)	5.00	0.32	5.05	0.27	0.851	5.25	0.35	4.84	0.23	0.194	4.99	0.23	5.23	0.48	0.872	5.45	0.32	4.51	0.22	0.257	5.10	0.26	4.86	0.32	0.931
CRP (mg/L)	5.03	0.36	4.93	0.24	0.885	5.05	0.28	4.90	0.28	0.161	5.08	0.21	4.39	0.58	0.040	5.13	0.28	4.77	0.28	0.043	5.13	0.23	4.53	0.41	0.018
Insulin (μIU/mL)	9.80	0.50	10.04	0.41	0.790	10.16	0.52	9.77	0.38	0.116	10.01	0.36	9.66	0.64	0.434	10.00	0.45	9.88	0.44	0.077	10.20	0.39	9.25	0.47	0.041
HOMA-IR	2.62	0.14	2.68	0.12	0.644	2.71	0.14	2.61	0.12	0.104	2.66	0.10	2.64	0.21	0.624	2.66	0.12	2.66	0.13	0.131	2.70	0.11	2.53	0.15	0.094
Glucose (mmol/L)	5.96	0.08	5.94	0.06	0.344	5.96	0.07	5.94	0.06	0.493	5.94	0.05	6.01	0.12	0.395	5.91	0.05	6.00	0.08	0.521	5.92	0.05	6.04	0.10	0.441
BMI (kg/m^2)	32.2	0.4	32.6	0.3	0.580	31.8	0.4	32.9	0.3	0.350	32.3	0.3	32.9	0.6	0.163	32.0	0.3	33.0	0.3	0.476	32.4	0.3	32.6	0.5	0.479

P values are from ANCOVA analysis testing differences between genotype groups.

used covariates was applied to obtain β coefficients for calculation of predicted values. Correlations were assessed using Pearson's product–moment correlation coefficient with a two-tailed test. Statistical analyses were carried out using SPSS version 14.0 for Windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Differences in markers of dyslipidaemia and inflammation between genotype groups for NOS3 SNPs

Baseline characteristics of the cohort, dependent on genotype for each of the five SNPs studied are presented in Table 1. Significant differences between genotype groups were observed for several MetS markers. Both LDL and total cholesterol were significantly higher in carriers of the minor allele for both rs1799983 and rs743507. For rs1800779, total cholesterol and HDL were significantly higher in the minor allele carriers, with LDL concentrations also appearing higher in this group, albeit not reaching statistical significance. LDL concentrations were significantly higher in minor allele carriers for rs11771443. The rs1799983 SNP appeared to have an effect on apolipoprotein concentrations, with Apo B, Apo CII and Apo E all being elevated in carriers of the minor allele compared to major allele homozygotes. Adiponectin concentrations were significantly lower in minor allele carriers for rs1800779. Leptin concentrations were significantly higher in minor allele carriers for both rs1800779 and rs743507 compared to the respective major allele homozygote groups, but were significantly lower in minor allele carriers for rs3918227. Fibrinogen concentrations were significantly lower in minor allele carriers of rs743507 compared to the major allele homozygotes. CRP was significantly lower in minor allele carriers for rs3918227, rs743507 and rs11771443. Fasting insulin was significantly lower in minor allele carriers for rs11771443. Using false discovery rate (FDR) correction for multiple testing, the association between rs1799983 and cholesterol remained significant (FDR $p = 0.002$), with the associations with LDL and Apo B remaining borderline significant (FDR $p = 0.054$ for both). For rs743507, the associations with LDL and leptin remained significant (FDR $p = 0.01$ for both), with the associations with cholesterol and fibrinogen being borderline significant (FDR $p = 0.075$ for both).

3.2. The rs1799983 SNP interacts with n-3 PUFA to associate with TAG

As *n*-3 PUFA intake is considered to confer possible protection to CVD, total *n*-3 PUFA as a percentage of total plasma lipids was used to examine gene–nutrient interactions between NOS3 SNPs and PUFA. A significant interaction was found between the rs1799983 SNP and plasma *n*-3 PUFA on plasma TAG concentrations. Specifically, plasma TAG concentrations were significantly higher in the minor allele carriers for rs1799983 with low plasma *n*-3 PUFA status and plasma TAG was lower in subjects with high plasma *n*-3 PUFA levels (Fig. 1). In contrast, plasma TAG concentrations are not affected by plasma *n*-3 PUFA status in the major allele homozygotes. Using LC *n*-3 PUFA in the model gave similar results, although the associations were not as strong (data not shown).

3.3. Genetic polymorphism at rs1799983 predicts TAG response to alterations in plasma n-3 PUFA status

A linear regression model including the original covariates was applied to create predicted values of TAG according to genotype at the rs1799983 polymorphism (Fig. 2). The genotype groups exhibit striking differences in the predicted changes in TAG in relation to plasma *n*-3 PUFA concentrations. Thus, based on baseline data, the model predicts that in individuals carrying the minor allele for rs1799983, an increase in plasma *n*-3 PUFA would elicit a considerable reduction in TAG. This reduction would not be seen in the major allele homozygotes.

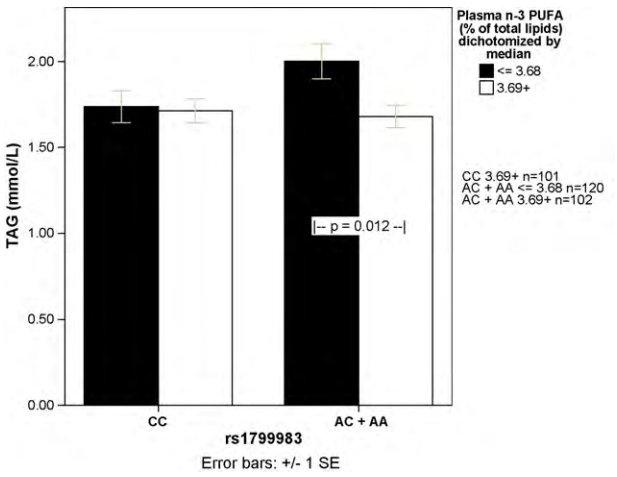

Fig. 1. Interaction between NOS3 rs1799983 and plasma *n*-3 PUFA on TAG concentrations. Values represent genotype group means ± SEM at baseline. CC major homozygotes have stable TAG concentrations irrespective of plasma *n*-3 PUFA above or below the population median, while carriers of the minor allele have significantly higher TAG associated with *n*-3 PUFA below the median.

3.4. The rs1799983 polymorphism affects the relationship between changes in plasma n-3 PUFA and TAG concentrations during a 12-week dietary intervention period

To determine whether the apparent interaction between the rs1799983 polymorphism and plasma *n*-3 PUFA, determined the metabolic response in plasma TAG concentrations to higher diet-induced plasma *n*-3 PUFA levels. The effect of increasing plasma *n*-3 PUFA levels by LC *n*-3 PUFA supplementation for 12-weeks was determined. Consistent with the effect observed at baseline and from the predicted values model, the actual changes in plasma TAG concentration in response to LC *n*-3 PUFA supplementation were strikingly different between genotype groups (Fig. 3). The change in plasma *n*-3 PUFA, presented as percentage change, was divided into four groups to represent a substantial decrease, two

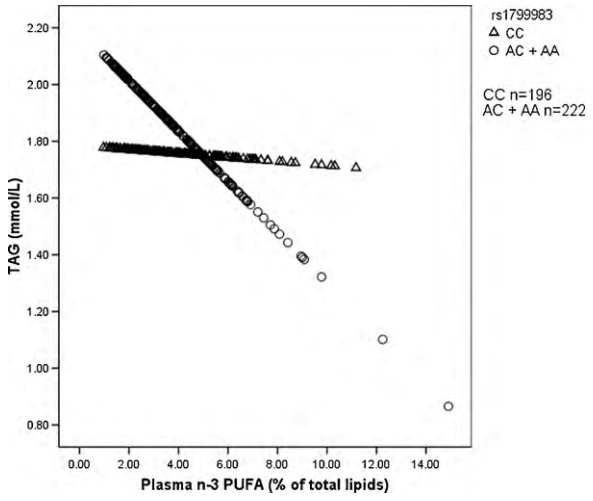

Fig. 2. Predicted plasma TAG concentrations based on amount of plasma *n*-3 PUFA, according to rs1799983. The CC major allele homozygotes are predicted to have a slight reduction in plasma TAG concentrations concurrent with increased plasma *n*-3 PUFA, while the A allele carriers are expected to markedly reduce their TAG concentrations following an increase in plasma *n*-3 PUFA.

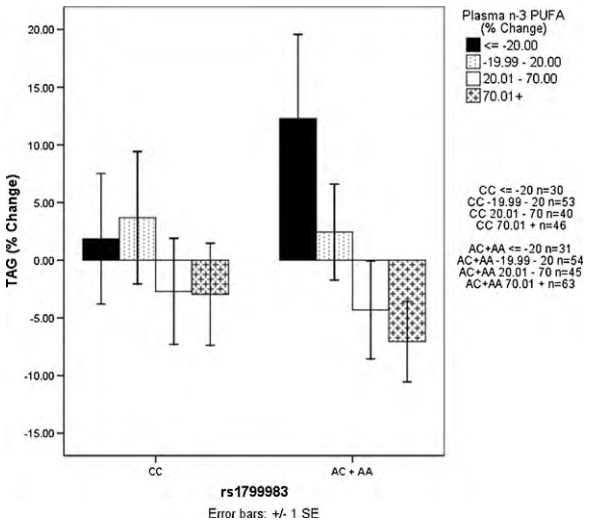

Fig. 3. Changes in plasma TAG concentrations stratified by change in plasma *n*-3 PUFA concentrations in response to LC *n*-3 PUFA intervention according to rs1799983. Values represent genotype group means ± SEM. Within the A allele carriers, a large increase in *n*-3 PUFA (>70%) is associated with a significant reduction in TAG. In these individuals, a substantial decrease (>20%) or a mild change (−19.99 to 20%) is associated with proportional increases in TAG. This clear bi-directional change is not observed within the major allele homozygotes (CC).

intermediate groups, and a substantial increase in plasma *n*-3 PUFA following intervention. Altered plasma *n*-3 PUFA concentrations had pronounced effect on plasma TAG concentrations in the group comprising the heterozygotes and minor allele homozygotes (AC + AA). In these individuals, plasma TAG concentrations were significantly reduced after *n*-3 PUFA supplementation which increased plasma *n*-3 PUFA >70% during the 12-week period, compared with either a decrease or negligible increase in plasma *n*-3 PUFA levels (≤−20%, $p = 0.005$; −19.99 to 20%, $p = 0.023$). The major allele homozygotes appeared to be unresponsive to differences in plasma *n*-3 PUFA status following intervention. Correlation analysis supported this, with a significant negative correlation observed between the change in *n*-3 PUFA and the change in TAG within minor (A) allele carriers ($r = -0.224$, $p = 0.002$), but no correlation within CC homozygotes ($r = -0.09$, $p = 0.244$).

4. Discussion

4.1. SNP associations with circulating lipids and inflammation

The importance of genetic variation at the NOS3 locus in modulating circulating lipids and in particular responsiveness to dietary *n*-3 PUFA on TAG metabolism is highlighted in the present study. The LIPGENE cohort includes individuals with the MetS, who have an increased risk of developing CVD. At baseline, differences in markers of dyslipidaemia were observed between groups with several SNPs. Specifically, total cholesterol and/or LDL cholesterol were increased among minor allele carriers for three SNPs, rs1799983, rs1800779 and rs743507. The rs1799983 SNP, also known as E298D, is the most widely reported SNP of these three, and is located in exon 8 of the gene, resulting in an amino acid substitution. Whereas all MetS patients have an increased risk of CVD, the condition is heterogeneous, and our observations suggest that individuals carrying the minor allele for any of these NOS3 SNPs may have an additional increase in CVD risk. Previous studies investigating NOS3 polymorphisms and cholesterol, have found associations with cholesterol [17] and oxidised low-density lipoprotein (oxLDL) [10], as well as a significant association between a VNTR in NOS3 and HDL, and a trend towards association of rs1799983 with cholesterol in Mexican Americans [18]. The rs1799983 SNP was also found to be associated with Apo B, Apo CII and Apo E in the present study, with all three markers being elevated in carriers of the minor allele as compared to major allele homozygotes. Additionally, BMI was significantly higher in minor allele carriers of rs1799983 as compared with major allele homozygotes. In a comprehensive meta-analysis of the relationship of NOS3 polymorphisms to hypertension, it was found that cholesterol modulated the association between rs1799983 and hypertension [19]. The authors also concluded that haplotype analysis is more informative and preferable to single locus analysis. A separate study examined the relationship between NOS3 SNPs and haplotypes and plasma nitrites, which have been suggested to be a good marker of NO formation [20]. Due to the limitations of conducting dietary intervention in large cohorts, our sample size was not sufficient for reliable haplotype analysis; however given the signals seen from a number of SNPs, the data would suggest that consideration of haplotypes would be desirable where feasible in future studies.

The SNP has previously been reported, particularly in relation to its association with insulin sensitivity. Leptin concentrations were significantly higher in minor allele carriers for both rs1800779 and rs743507, whereas fibrinogen concentrations were significantly lower in minor allele carriers of rs743507 as compared to the major allele homozygotes. Plasma concentration of CRP was significantly lower in minor allele carriers for both rs3918227 and rs11771443. Evidence for an association between NOS3 and CRP has also been found in CVD patients in particular related to inflammatory cytokine levels during acute phases of myocardial infarction. An association between SNPs in NOS3 and leptin has previously been reported.

4.2. Interaction between genotype in NOS3 and fatty acid status influences TAG, a cardiovascular risk marker

An interesting effect of *n*-3 PUFA was observed in our study population, where both fatty acid status and genotype at the rs1799983 polymorphism interacted to determine plasma TAG concentrations. The initial finding of a statistical interaction was verified by examining the effect of increasing plasma *n*-3 PUFA levels by LC *n*-3 PUFA supplementation. It was apparent that genotype was an important determinant of the metabolic response, whether plasma TAG concentrations were sensitive to increased *n*-3 PUFA levels. The minor allele carriers had elevated TAG associated with low *n*-3 PUFA, whereas the major allele homozygotes had stable TAG concentrations irrespective of *n*-3 PUFA. These data were supported by graphically representing the concentrations of TAG predicted for each genotype group, and it was observed that a marked reduction in TAG was predicted in the minor allele carriers in response to increased plasma *n*-3 PUFA. As TAG is thought to be an independent risk factor for CVD [21], a reduction in TAG could protect against disease development.

4.3. Possible mechanisms for a relationship between plasma n-3 PUFA and TAG

Inhibition of NOS in rats has been shown to lead to an increase in circulating TAG [22], potentially mediated by a reduction in fatty acid oxidation. Fish oil supplementation may increase NO in type 2 diabetes [23] as well as healthy individuals [24]. DHA, but not EPA, was found to improve vascular reactivity in overweight hyperlipidemic men [25]. In rats, *n*-3 PUFA supplementation increased basal endothelial NO production, and vaso-dilation in aorta, in addition to increased NOS3 mRNA and protein levels [26]. Both EPA and DHA affect the distribution and translocation of eNOS in caveolae, specialised plasma membrane microdomains, which are important in a variety of functions, including signal transduction, and insulin- and cholesterol-stimulated glucose uptake [27,28]. Recently, the

importance of caveolae in TAG synthesis has been highlighted [29], raising the possibility of an interesting mechanism, where *n*-3 PUFA might affect TAG concentrations via eNOS activity in caveolae. The effect of the rs1799983 SNP in the endothelial environment was studied, and the presence of the minor allele was found to be associated with increased NOS3 protein in response to shear-stress, but lower NOS3 activity and lower protein enrichment in the caveolar membrane, demonstrating a clear functional effect of this polymorphism [30]. It is possible that minor allele carriers of this polymorphism have disrupted functionality of caveolae, which can be ameliorated with sufficient intake of *n*-3 PUFA; however an unfavourable dietary lipid intake may act as a second hit, and lead to dyslipidaemia due to an inability of the caveolae to function correctly. Supplementation of *n*-3 PUFA in major allele homozygotes may not give any additional benefit to caveolar function, and hence may not be beneficial in this specific context. Thus, assuming that the rs1799983 SNP may influence responsiveness to *n*-3 PUFA, this could explain the differential effects of LC *n*-3 PUFA supplementation on TAG that is often observed in human intervention studies.

4.4. The impact of diet-induced alterations in plasma n-3 PUFA on TAG concentrations may be NOS3 dependent

Although the associations between gene–nutrient interactions and biomarkers are interesting, it is of importance how this information might be used in a preventive and therapeutic context. Cross-sectional data alone are not enough to prove that the predicted change in TAG with increasing *n*-3 PUFA would actually occur in reality. Therefore, the data from the 12-week dietary intervention were analysed to examine whether the effects predicted from the baseline analysis could be detected following an intervention. This analysis confirmed the effects seen at baseline. Within the group which had been predicted from baseline data to be highly responsive to changes in *n*-3 PUFA, significant differences were observed. Thus, a reduction in *n*-3 PUFA was associated with an increase in TAG, whereas the greatest increase of *n*-3 PUFA was associated with a distinct decrease in TAG among the carriers of the minor allele for rs1799983.

In conclusion, we have shown that genotype at the rs1799983 polymorphism in the gene encoding NOS3 modulates the TAG-lowering effects of *n*-3 PUFA. Based on this data, and on the observed genotype frequencies, we can infer that a recommendation to increase *n*-3 PUFA could have an augmented beneficial effect on plasma TAG concentrations of more than 50% of the general population, which might in turn have implications with respect to CVD risk.

Conflict of interest

None of the authors have a personal or financial conflict of interest.

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