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S U M M A R Y

Background & aims: Genetic background may interact with habitual dietary fat composition, and affect development of the metabolic syndrome (MetS). The phosphoenolpyruvate carboxykinase gene (*PCK1*) plays a significant role regulating glucose metabolism, and fatty acids are key metabolic regulators, which interact with transcription factors and influence glucose metabolism. We explored genetic variability at the *PCK1* gene locus in relation to degree of insulin resistance and plasma fatty acid levels in MetS subjects. Moreover, we analyzed the *PCK1* gene expression in the adipose tissue of a subgroup of MetS subjects according to the *PCK1* genetic variants.
Methods: Insulin sensitivity, insulin secretion, glucose effectiveness, plasma concentrations of C-peptide, fatty acid composition and three *PCK1* tag-single nucleotide polymorphisms (SNPs) were determined in 443 MetS participants in the LIPGENE cohort.
Results: The rs2179706 SNP interacted with plasma concentration of $n-3$ polyunsaturated fatty acids ($n-3$ PUFA), which were significantly associated with plasma concentrations of fasting insulin, peptide C, and HOMA-IR. Among subjects with $n-3$ PUFA levels above the population median, carriers of the C/C genotype exhibited lower plasma concentrations of fasting insulin ($P=0.036$) and HOMA-IR ($P=0.019$) as compared with C/C carriers with $n-3$ PUFA below the median. Moreover, homozygous C/C subjects with $n-3$ PUFA levels above the median showed lower plasma concentrations of peptide

Non-standard abbreviations: AIRg, acute insulin response to glucose; PEPCK-C, cytosolic phosphoenolpyruvate carboxykinase; DI, disposition index; HWE, Hardy–Weinberg equilibrium; HOMA-B, homeostasis model assessment of β-cell function; HOMA-IR, homeostasis model assessment of insulin resistance; IVGTT, intravenous glucose tolerance test; MetS, metabolic syndrome; $n-3$ PUFA, $n-3$ polyunsaturated fatty acids; PCK1, phosphoenolpyruvate carboxykinase; SI, sensitivity index; SNPs, single nucleotide polymorphisms; T2D, type 2 diabetes.

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C as compared to individuals with the T-allele ($P = 0.006$). Subjects carrying the T-allele showed a lower gene *PCK1* expression as compared with carriers of the C/C genotype ($P = 0.015$).
Conclusions: The *PCK1* rs2179706 polymorphism interacts with plasma concentration of $n - 3$ PUFA levels modulating insulin resistance in MetS subjects.

1. Introduction

The pathogenesis of the metabolic syndrome (MetS) is complex and not completely understood but the interaction of overweight, physical inactivity, and genetic factors contribute to its development. The National Cholesterol Education Program-Adult Treatment Panel III has identified the MetS as an indication for intensive lifestyle intervention.[1] Effective interventions include modifications in dietary habits and physical activity, and judicious use of pharmacologic agents for minimizing or delaying the comorbidities associated with the MetS. Epidemiological studies indicate that Western-style dietary patterns promote the MetS, while diets rich in fruits, vegetables, grains, fish and low-fat dairy products have a protective role. In this regards, dietary strategies to increase the intake of dietary antioxidants, and $n - 3$ fatty acids will be helpful for improving health and reducing MetS. A recent intervention study[2] demonstrated that consumption of a low-fat high-carbohydrate supplemented with $n - 3$ PUFA acids reduced the risk of MetS and the prevalence of enlarged waist circumference, hypertension and hypertriacylglycerolemia were reduced. Thus, lifestyle modification has generally been accepted as a cornerstone of treatment for MetS, with the expectation that an appropriate intake of energy and nutrients will improve its control and reduce the risk of complications.[3] However, some evidence suggests that some individuals are genetically predisposed to insulin resistance, and due to the complex nature of gene–environment interactions, dietary adjustment in subject with the MetS may require a personalized approach.[4,5]

Multiple candidate genes may affect primary insulin action, and particularly the interaction with diet is an important modulator of glucose metabolism and insulin resistance.[6,7] The phosphoenolpyruvate carboxykinase gene (*PCK1*) is located on chromosome 20q and encodes the cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C),[8] which is the key enzyme involved in the regulation of gluconeogenesis, glyceroneogenesis and cataplerosis. Expression of PCK1 is under hormonal control, regulated at the transcriptional level by activators, such as glucagon, and inhibitors, such as insulin. It has been suggested that type 2 diabetes mellitus (T2D) could be caused by either excessive PEPCK-C production in the liver or reduced levels of PEPCK-C in adipose tissue.[9] According to this hypothesis several single nucleotide polymorphisms (SNP) at the *PCK1* locus have been associated with increased risk of T2D.[10] Furthermore, Millward et al. have demonstrated that the promoter region of the *PCK1* is essential for the regulation of triglyceride/fatty acid flux between adipose and liver tissues and it is important for the maintenance of glucose and lipid homeostasis and for prevention of insulin resistance.[11] Thus, these data support the central position of *PCK1* in pathways regulating fatty acids as well as glucose metabolism in humans. However, there are no studies exploring the interaction of polymorphisms in the *PCK1* gene with the dietary fatty acid composition as a determinant of insulin resistance.

We investigated whether *PCK1* genetic variants may influence glucose metabolism, including insulin secretion or insulin sensitivity measured after an intravenous glucose tolerance test (IVGTT), in response to different dietary supply of fatty acids among MetS subjects. Moreover, we analyzed the *PCK1* gene expression in the adipose tissue of a subgroup of MetS subjects according to the PCK1 genetic variants.

2. Methods

Detailed design of the present study has been described previously[6,7] and summarized below.

2.1. Subjects

This study was performed in accordance with the Helsinki Declaration of 1975 as revised in 1983, and was approved by the local ethics committees at each centre. All subjects provided written informed consent before any study related procedure. The participants, aged 35–70 years and BMI 20–40 kg/m^2, were recruited for the LIPGENE dietary intervention study from eight European countries (Ireland, UK, Norway, France, The Netherlands, Spain, Poland and Sweden). Subject eligibility was determined using a modified version of the NCEP criteria for the MetS,[1] where subjects were required to fulfil at least three of the following five criteria: waist circumference >102 cm (men) or >88 cm (women); fasting plasma concentration of glucose 5.5–7.0 mmol/L; TAG ≥1.5 mmol/L; HDL cholesterol <1.0 mmol/L (men) or <1.3 mmol/L (women); and blood pressure ≥130/85 mmHg, or treatment of previously diagnosed hypertension. The pre-intervention data for these subjects were published from the LIPGENE dietary intervention cohort elsewhere.[12] Anthropometric measurements were recorded according to a standardized protocol for the LIPGENE study and blood pressure was measured following the guidelines of European Society of Hypertension. The present analyses include the 443 subjects with available SNP data. Of these participants, 68% received anti-hypertensive medication. Details of the inclusion and exclusion criteria of the LIPGENE study were published previously.[13]

2.2. Biochemical measurements

Plasma, serum, and buffy coat were prepared from 12 h fasting blood samples in each subject. Serum insulin and C-peptide were measured by a solid-phase, two-site fluoroimmunometric assay on a 1235 automatic immunoassay system (AutoDELFIA kits, Wallac Oy, Turku, Finland). Plasma glucose concentrations were measured using the IL Test™ Glucose Hexokinase Clinical Chemistry kit (Instrumentation Laboratories, Warrington, UK). Homeostasis model assessment of insulin resistance (HOMA-IR) was derived from fasting glucose and insulin levels [(fasting plasma glucose × fasting serum insulin)/22.5].[14] As HOMA-IR takes into account both insulin and glucose levels, it may be a more complete index than plasma insulin. Homeostasis model assessment of β-cell function (HOMA-B) was calculated as [(20 × fasting serum insulin)/(fasting plasma glucose − 3.5)]. An IVGTT was performed. Insulin sensitivity (sensitivity index, SI) and glucose effectiveness were determined using the MINMOD Millenium Program (version 6.02, Richard N Bergman).[15] The acute insulin response to glucose (AIRg = first phase insulin response) was defined as the incremental area under the curve from time 0–8 min. Disposition index (DI) was calculated as the product of AIRg and insulin sensitivity. Cholesterol and triglycerides were quantified using the IL Test™ Cholesterol kit and IL Test™ Triglycerides kit (Instrumentation Laboratories, Warrington, UK).

Fatty acids were extracted from plasma and transmethylated with boron trifluoride in methanol. Fatty acid methyl esters were

analyzed by gas chromatography on a Shimadzu GC-14A (Shimadzu, Kyoto, Japan) fitted with a Shimadzu C-r6A integrator and a 25 M BP 21 polar aluminium silica column. Detector and injector temperatures were 260 °C and 250 °C, respectively. Fatty acids were identified by the comparison of the relative retention times of plasma fatty acid methyl esters with fatty acid methyl esters standards. Fatty acid mass was measured as a relative percentage of the total quantified fatty acids.[16] 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 ($n-3$); and $n-6$ PUFAs from the sum of 18:2, 18:3$n-6$, 20:3, 20:4$n-6$, and 22:4.

2.3. SNP selection and genotyping

PCK1 genotype data from HapMap v1.1 (www.hapmap.org) was uploaded into HITAGENE, a web-based combined database and genetic analysis software suite. Haplotype frequencies were estimated by implementation of the expectation maximization algorithm. Using a 5% cut-off for individual haplotype frequency and >70% for the sum of all haplotype frequencies, haplotype tagged (HT) SNPs were identified using SNP tagger (www.broad.mit.edu/mpg/tagger/server.html). Thus, the rs2071023, rs2179706, and rs6070157 polymorphisms were genotyped at the *PCK1* gene by Illumina Inc., (San Diego, CA, USA). DNA was extracted from buffy coat samples 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). Adherence to Hardy–Weinberg equilibrium (HWE) at each SNP locus was determined using the χ^2 test with 1 degree of freedom.

2.4. Subcutaneous adipose tissue biopsies

Subcutaneous adipose tissue samples were obtained from the superficial abdominal subcutaneous adipose tissue lateral to the navel of 30 MetS subjects, with instrument Bard® Magnum (ref. MG1522) (Bard Biopsy Systems, Tempe, AZ, USA), needles Bard® Magnum Core (ref. MN1410). Immediately after extraction, samples were stored in Eppendorf tubes with RNA later tissue Collection (Applied Biosystems, Carlsbad, CA, USA) until RNA extraction.

2.5. RNA isolation from adipose tissue

Adipose tissue was homogenized using Ultra-Turrax T25 (IKA Labortechnik, Staufen, Germany). After removal of lipids from the top of the tube, RNA was isolated with a commercial kit RiboPure kit (Ambion, Applied Biosystems, Carlsbad, CA, USA). RNA was collected from the aqueous phase by binding to a fiber glass filter. The quantification of RNA was performed using the spectrophotometer v3.5.2 Nanodrop ND-1000 spectrophotometer (Nanodrop Technology®, Cambridge, UK).

2.6. Gene expression by reverse transcription polymerase chain reaction

The reverse transcription was performed using the commercial kit MessageBOOSTER cDNA Synthesis kit for qPCR (Epicentre, Madison, WI, USA), according to the manufacturer instructions. Briefly, it was amplified 500 pg of total RNA and then converted to cDNA, which was stored at −20 °C. PCR analyses were performed using the OpenArray™ NT Cycler system (Applied Biosystems, Carlsbad, CA, USA) in samples from 30 MetS patients by duplicated, according to the manufacturer instructions. Briefly, each reaction was performed on 1.2 μL of 1:2 (v/v) dilution of the first cDNA strand, 2.5 μL ABI Gene Amp Master Mix (Applied Biosystems), 1 μL Remix (Applied Biosystems), 0.30 μL of water. Reaction mix was loaded on the array by using OpenArray™ NT Autoloader (Applied Biosystems), according to the manufacturer's instruction. The reaction was incubated at 95 °C for 10 min, followed by 40 cycles of 5 min at 95 °C, 2 min at 58 °C, 1 min at 72 °C on the OpenArray™ NT Cycler (Applied Biosystems). Primer pairs were selected from the database TaqMan Gene Expression assays (Applied Biosystems, Carlsbad, CA, USA) https://bioinfo.appliedbiosystems.com/genome-database/gene-expression.html, for the following genes: *PCK1* (Hs00159918_m1) and *RPLP0* (Hs99999902_m1) as housekeeping gene. Expression values were obtained as relative expression of the *PCK1* gene versus the constitutively expressed RPLP0 gene (relative expression = 2 − (Ct, Target gene − Ct, Reference gene)).

2.7. Statistical analysis

Biochemical variables were assessed for normal distribution, and skewed variables were normalised by log 10 or square root transformation as appropriate. Statistical analyses were carried out using SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL). Data are presented as means ± standard error (SE) for continuous variables and as frequencies or percentages for categorical variables. Comparisons of frequencies between qualitative variables were carried out using the Chi-squared test. Potential confounding factors were age, sex, BMI, and LIPGENE centre of origin. ANOVA-based models were used to test for associations between individual SNP and the variables studied, with the SNP as fixed factor, and age, BMI, sex and centre of origin as covariates. The effect of each SNP interacting with groups of plasma fatty acids (omega-3 and omega-6 polyunsaturated, saturated, monounsaturated) on each biochemical variable was investigated using the median of plasma fatty acids to dichotomize the population, and using the resulting groups (above the median versus below the median) as a fixed factor in combination with the SNP genotypes in a univariate ANOVA analysis with the same covariates and Bonferroni corrections as exposed above. Thus, in this model we could assess the associated effects of SNP alone, fatty acids alone and the interaction between the SNP and the fatty acids on the selected variables. Bonferroni's test was used in the post-hoc analysis. The normal distribution of PCK1 gene expression was assessed using the Kolmogorov–Smirnov test. The gene expression data were analyzed by using one-way ANOVA to determine the genotype effect on the *PCK1* gene expression, with the SNP as fixed factor and age, BMI, gender, and centre of origin as covariates.

3. Results

We analyzed the effect of the three rs2071023, rs2179706, and rs6070157 SNPs at the *PCK1* gene locus on glucose metabolism in relation to plasma fatty acid status. Baseline demographic and biochemical characteristics for the rs2179706 SNP are presented in Table 1. Genotype distributions did not deviate from Hardy–Weinberg expectations. No significant baseline differences were observed in relation to age, blood pressure, fasting lipids, glucose, insulin, peptide C and HOMA-IR concentration by genotype (Table 1). Although we did not observe any gene–nutrient interactions for the rs2071023 and rs6070157 SNPs these data are presented as supplementary material (Tables 1S and 2S).

3.1. Insulin sensitivity

IVGTT and HOMA indices were used to estimate insulin sensitivity index (SI) and HOMA-IR. The rs2179706 SNP interacted with

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Table 1
Characteristics of participants with the *PCK1* rs2179706 polymorphism at baseline.

	rs2179706			*P*-value
	C/C	C/T	T/T	
n	151	192	100	
Age, years	54.54 (0.7)	54.65 (0.6)	53.46 (0.8)	0.531
BMI, kg/m^2	32.17 (0.3)	32.89 (0.3)	32.33 (0.3)	0.258
Diastolic BP (mmHg)	86.25 (0.7)	85.42 (0.7)	87.61 (1)	0.205
Systolic BP (mmHg)	137.58 (1.3)	139.37 (1)	140.32 (1.6)	0.366
Total cholesterol, mmol/L	5.33 (0.07)	5.31 (0.07)	5.44 (0.09)	0.519
LDL-C, mmol/L	3.30 (0.08)	3.20 (0.07)	3.38 (0.10)	0.359
HDL-C, mmol/L	1.09 (0.02)	1.11 (0.02)	1.15 (0.02)	0.157
TG, mmol/L	1.68 (0.06)	1.88 (0.07)	1.75 (0.07)	0.103
ApoB, g/L	1.01 (0.01)	1.02 (0.01)	1.03 (0.02)	0.921
Glucose (mmol/L)	5.93 (0.06)	5.93 (0.04)	5.96 (0.08)	0.953
Insulin (mU/L)	9.97 (0.5)	10.08 (0.4)	10.61 (0.5)	0.671
Peptide C (pmol/L)	2.53 (0.07)	2.61 (0.06)	2.78 (0.08)	0.109
HOMA-IR	2.67 (0.1)	2.67 (0.1)	2.84 (0.1)	0.674

All values are means ± SE. BP, blood pressure; TG, triglycerides; HOMA-IR, homeostasis model assessment of insulin resistance.

plasma $n-3$ PUFA levels, which were significantly associated with plasma concentrations of fasting insulin, peptide C, and HOMA-IR. Among subjects with $n-3$ PUFA levels above the population median, carriers of the C/C genotype exhibited lower plasma concentrations of fasting insulin ($P = 0.036$) and HOMA-IR ($P = 0.019$), as compared with C/C carriers with plasma $n-3$ PUFA below the median (Fig. 1A and B). Moreover, homozygotes for C/C with $n-3$ PUFA levels above the median, showed lower plasma concentrations of peptide C as compared with individuals with the T-allele ($P = 0.006$) (Fig. 1C). SI did not differ between participants with different genotypes. There were no significant interactions between other groups of plasma fatty acids (SFA, MUFA and $n-6$ PUFA) and *PCK1* SNPs on glucose metabolism. We also explored the genetic component independent of the nutrient effect, and no differences were observed. In a next step a linear regression model including the original covariates was applied to create predicted values of HOMA-IR according to genotype at the rs2179706 SNP (Fig. 2). The genotype groups exhibited striking differences in the predicted changes in HOMA-IR in relation to plasma $n-3$ PUFA concentrations. From baseline data, the model predicts that in C/C individuals, an increase in plasma $n-3$ PUFA would elicit a considerable decrease in HOMA-IR (Fig. 2). An opposite effect was observed in subjects carrying the T-allele.

3.2. Insulin secretion

We also explored the effect of the rs2179706 on insulin secretion according to plasma levels of different fatty acids. For that purpose acute insulin response to glucose (AIRg), disposition index (DI) and HOMA-B were measured. In contrast to the insulin sensitivity findings, we did not observe any gene–nutrient interactions for these parameters.

3.3. Adipose tissue gene expression

We analyzed the *PCK1* gene expression in the adipose tissue of a subgroup of MetS subjects with the rs2179706 polymorphism. Subjects carrying the T-allele showed a lower *PCK1* gene expression as compared with carriers of the C/C genotype ($P = 0.015$) (Fig. 3).

4. Discussion

We identified that the *PCK1* rs2179706 SNP influenced insulin resistance by interacting with plasma $n-3$ PUFA levels in MetS

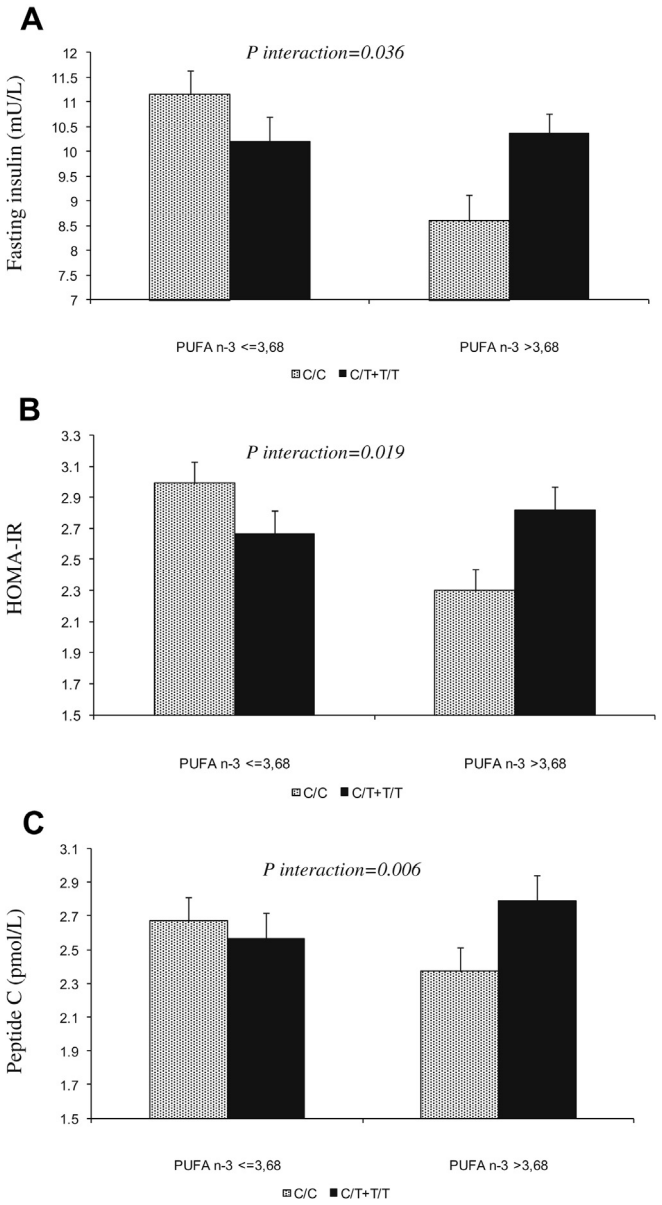

Fig. 1. Interaction between the rs2179706 polymorphism and plasma concentrations of omega-3 polyunsaturated fatty acids ($n-3$ PUFA), above or below the median within the same genotype group of fasting plasma concentration of of insulin (**A**), homeostasis model assessment of insulin resistance (HOMA-IR) (**B**) and, peptide C (**C**). Values are means ± SE. *P* values were adjusted for age, sex, BMI, and LIPGENE centre of origin.

patients. Thus, among subjects with $n-3$ PUFA levels above the population median, carriers of the C/C genotype exhibited lower plasma concentrations of fasting insulin and HOMA-IR as compared with C/C carriers with $n-3$ PUFA below the median. Moreover, homozygotes for C/C with $n-3$ PUFA levels above the median showed lower plasma concentrations of peptide C as compared with individuals with the T-allele. This new knowledge about the relationship between genetic and environmental components may facilitate the choice of more effective diet for MetS prevention based on the personalized nutrition.

Over the last few years further attention has been paid to the quality of dietary fat. The plasma fatty acid composition has been determined as a biomarker of habitual dietary fat intake, reflecting the combination of dietary fat consumption and endogenous de novo fatty acid biosynthesis and metabolism. Previous evidence supports the notion that $n-3$ PUFAs are effective in preventing

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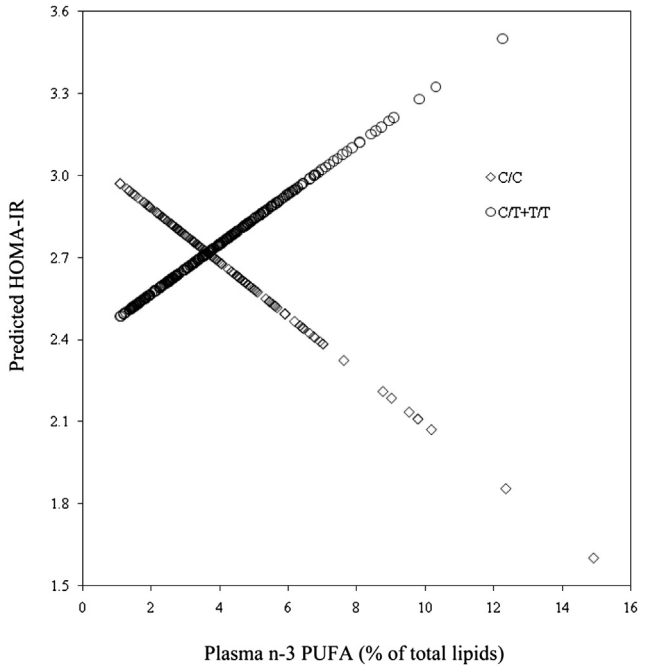

Fig. 2. Predicted values for homeostasis model assessment of insulin resistance (HOMA-IR) for the *PCK1* rs2179706 polymorphism. A difference was observed between the genotypes, with the T-allele genotype group (circles) appearing to be "high responders" to the plasma concentration of omega-3 polyunsaturated fatty acids ($n-3$ PUFA) and the C allele homozygote group (squares) appearing to be "low responders".

cardiovascular events, cardiac death and coronary events and also to reduce the risk of MetS.[17–20] However, limited and inconsistent data are available concerning the relation between $n-3$ PUFA and glucose metabolism. Results from the Women's Health and the Nurses' Health studies, showed an increased risk of T2D, especially with high intake of $n-3$ PUFA.[21,22] Recently Wu et al. conducted a meta-analysis concluding that $n-3$ PUFAs do not support either major harms or benefits on development of diabetes.[23] However results from other meta-analysis indicate differences between geographical regions in relationship with dietary intake of $n-3$ PUFA and the risk of T2D, with an increased risk among studies conducted in the U.S., no association in European populations, and an inverse association in Asian/Australian populations.[24] Thus, general recommendations may not be equally positive. Our present study shows for the first time an interaction between the *PCK1* gene locus, plasma fatty acid and glucose metabolism in subjects with the MetS.

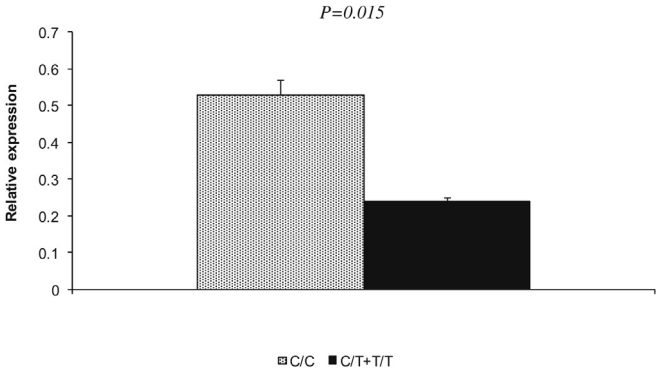

Fig. 3. *PCK1* gene expression changes in adipose tissue of subjects with MetS and the rs2179706 polymorphism. Data are expressed as means ± SE of relative expression ($n=30$, 10 homozygous C/C and 20 with the T-allele). Significant differences between groups were determined by one-way ANOVA.

As with many genetic association studies investigating polygenic diseases, the association between *PCK1* polymorphisms and T2D has showed mixed results[10,25–29] However, there is consistent evidence from animal models and human studies implicating that PEPCK-C is involved in the development of insulin resistance.[30,31] Previous studies have demonstrated that *PCK1* responds to a wide range of nutrients to maintain glucose and lipid homeostasis.[32] In our study we observed a gene–fatty acid interaction among subjects with the rs2179706 SNP and plasma levels of $n-3$ PUFA to influence insulin resistance, suggesting the potential sensitivity of this SNP to dietary factors. Genetic variation promoting enhanced *PCK1* transcription might lead to increased PEPCK-C enzymatic activity, which would increase insulin resistance through its effect on gluconeogenesis. For example, a mutation that impairs the ability of insulin to suppress hepatic *PCK1* transcription would have this effect. By contrast, mutations that reducing hepatic *PCK1* expression may cause hypoglycemia.[33] Gomez-Valades et al. have shown that liver-specific silencing of *PCK1* can improve glycaemic control, and insulin sensitivity in a diabetic mouse model, supports the role of PEPCK-C in T2D pathology and provides a potential target for treatment of the disease.[30] On the other hand it has been suggested that T2D could be caused by reduced levels of PEPCK-C in adipose tissue. In our study we have shown that subjects carrying the T-allele had a lower *PCK1* gene expression in the adipose tissue as compared with carriers of the C/C genotype. This is relevant from a functional point of view because this SNP may influence *PCK1* gene expression and corroborate previous evidence suggesting that lower expression in adipose tissue is related to insulin resistance and T2D. Based on these results we may speculate that $n-3$ PUFA fatty acids interact with the rs2179706 SNP, which may confer increased risk of insulin resistance by decreasing *PCK1* gene expression in the adipose tissue. This is remarkable because insulin resistance is believed to be an important factor linking metabolic abnormalities in patients with the MetS.

Thus, our present study suggests an enhanced beneficial effect of increasing the amount of $n-3$ PUFA in the diet of C/C subjects in comparison with those carrying the T-allele. Based on these data, and on the observed genotype-dependent associations, it is possible that a recommendation to increase dietary intake of $n-3$ PUFA could have a beneficial effect on insulin resistance only among MetS patients with the C/C genotype, which might in turn have implications for cardiovascular risk. Despite the evidence, we should be cautious in the interpretation of our results. The LIPGENE cohort is very well characterized population, and the multicentre origin of the patients allows extrapolation of the results to the European population. The main limitation of our present study is that it is cross-sectional, without the possibility to prove causality. A second limitation is related to the SI. We did not find parallel results between HOMA-IR and SI, although IVGTT is a very well validated method to estimate insulin sensitivity.

In conclusion, our results support the notion that $n-3$ PUFA may play a contributing role in triggering insulin resistance by interacting with a genetic variant at *PCK1* gene locus. Advances in the nutrigenomics are expected to open new ways in genome-customized diets for MetS prevention.

Statement of authorship

Prof. Roche and Prof. Lopez-Miranda had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conception and design of the study: P.P.-M., A.G.-R., H.M.R., J.L.-M. Provision of study materials or subjects: E.E.B., A.D.-K., C.D., U.R., W.H.M.S., J.A.L., C.A.D., H.M.R. and J.L.-M. Collection and assembly of data: J.D.-L., I.M.F.G., J.G., B.K., B.K.-W., A.C., E.E.B., O.H., W.H., C.D., U.R., W.H.M.S., J.A.L., C.A.D.

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Conflict of interest

None of the authors has any conflict of interest that could affect the performance of the work or the interpretation of the data.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2012.10.003.

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