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CLINICAL RESEARCH
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Variation in the *ADIPOQ* gene promoter is associated with carotid intima media thickness independent of plasma adiponectin levels in healthy subjects

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Aims The *ADIPOQ* gene encodes the protein adiponectin, and decreased circulating adiponectin levels have been observed in cardiovascular disease. We investigated the role of the *ADIPOQ* gene single-nucleotide polymorphisms (SNPs) A-11426G, G-11391A, C-11377G, and T45G with plasma adiponectin levels and common carotid artery intima media thickness (IMT) in a cohort of healthy subjects participating in the RISC (Relationship between Insulin Sensitivity and Cardiovascular disease) study.

Methods and results Anthropometric and metabolic assessment and B-mode ultrasound of the carotid IMT were measured in 1306 subjects [589 men; 717 women, mean ± SD age 43.8 ± 8.3 years, BMI 25.5 ± 4.0 kg/m^2] recruited from 19 centres in 14 European countries. Carriers of the −11426G allele and homozygous carriers of the −11391G allele had significantly lower plasma adiponectin levels. These relationships remained significant after adjusting for age, sex, recruitment centre, and BMI. Carriers of SNP −11377G allele had significantly greater IMT values compared with C allele homozygotes [geometric mean (interquartile range) 601 (543–665) vs. 590 (537–647) μm, $P = 0.021$]. This relationship became stronger after correcting for key covariates, including plasma adiponectin levels ($P = 0.011$).

Conclusion Variation within the *ADIPOQ* gene promoter is directly associated with carotid IMT in healthy subjects and is independent of circulating adiponectin levels.

Keywords Genetics • Adiponectin • *ADIPOQ* gene • Carotid intima media thickness

Introduction

A susceptibility locus for type 2 diabetes and the metabolic syndrome has been mapped to chromosome 3q27.[1] The *ADIPOQ* gene is located at position 3q27 and encodes the protein adiponectin. Decreased circulating adiponectin levels have been observed in cardiovascular disease (CVD), obesity, and type 2 diabetes compared with healthy controls.[2,3] Adiponectin has been shown to suppress the transformation of macrophages into foam cells[4] and is detected in catheter-injured vessels but not in intact vessels.[5] Low adiponectin levels are associated with endothelial dysfunction[6] and increased carotid intima media thickness (IMT),[7] whereas higher plasma levels have been associated with a lower risk of myocardial infarction.[3]

A number of single-nucleotide polymorphism (SNPs) in the *ADIPOQ* gene have been described.[8,9] The SNP 45G allele has

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been associated with coronary artery disease (CAD)[10] and abdominal obesity[11] in French Caucasians and with type 2 diabetes in Japanese populations.[12] A common haplotype of SNPs G-11391A and C-11377G in the proximal promoter region of the *ADIPOQ* gene has been associated with circulating adiponectin levels and type 2 diabetes in French Caucasians.[8] Recent studies have shown SNP A-11426G to be associated with fasting plasma glucose levels and type 2 diabetes[9,13] and SNP C-11377G with coronary stenosis and vascular events.[14] Therefore, it is evident that SNPs in the *ADIPOQ* gene may be associated with CVD and type 2 diabetes. However, most studies have investigated the relationships between *ADIPOQ* and adiponectin levels in disease states and there is limited work investigating the role of these SNPs and CVD risk in healthy individuals.

The aim of this study was to explore the relationships among *ADIPOQ* gene common variants (SNPs A-11426G, G-11391A, C-11377G, and T45G) and carotid IMT and plasma adiponectin levels in healthy subjects participating in the RISC (Relationship between Insulin Sensitivity and Cardiovascular disease) study.

Subjects and methods

Subjects

The RISC study is a prospective, observational, multi-centre cohort study designed (1) to establish whether insulin resistance predicts deterioration of CVD risk markers, diabetes, obesity, atherosclerosis, and CVD; (2) to determine the genetic and environmental contributions to insulin resistance and CVD; (3) to develop a method to identify insulin-resistant individuals in clinical practice.[15] The study recruitment methods and inclusion/exclusion criteria have been previously described.[15,16] Briefly, healthy Caucasian men and women, aged between 30 and 60 years, were recruited from 19 centres in 14 European countries. The baseline data collection began in June 2002 and was completed in July 2005. Each recruitment centre obtained local Ethics Committee approval, and participants gave separate written consent for the study and the collection of DNA. In total, 1566 participants were initially screened and excluded ($n = 190$) if: systolic and diastolic blood pressure (BP) was $\geq$140/90 mmHg; fasting plasma glucose $\geq$7.0 mmol/L; 2 h plasma glucose $\geq$11.1 mmol/L; total cholesterol $\geq$7.8 mmol/L; triglycerides $\geq$4.6 mmol/L or had ECG abnormalities and carotid artery plaques. A further 50 participants dropped out or did not show for appointments. The final study number was 1326, of which 1311 consented to providing blood samples for DNA analysis. The analysis presented in this paper is based on 1306 subjects (589 men, 717 women) who had passed the eligibility criteria and had complete data on *ADIPOQ* genotypes.

Biological and lifestyle measurements

Height was measured on a stadiometer, and body weight and fat-free mass were measured on a TANITA bioimpedance (Tanita International Division, UK). Waist circumference was measured by tape according to a standardized written protocol. Sitting BP was measured (OMRON 705 cp, OMRON Healthcare Europe, The Netherlands) three times over 10 min and the median value was used in statistical analysis. Smoking status was self-reported by study questionnaire. Fasting blood samples were taken before and 30, 60, 90, and 120 min into the oral glucose tolerance test (OGTT), together with samples for central analysis of routine blood chemistry and for DNA extraction. Blood collected during the studies was separated into plasma and serum, aliquoted, and stored at −20°C for glucose and −80°C for lipids. Serum aliquots were also stored at −80°C for insulin determination. Samples were transported on dry ice at pre-arranged intervals to central laboratories. Plasma insulin was measured by a specific chemiluminescence assay.[15] On a separate day within 1 month of the OGTT, a euglycaemic hyperinsulinaemic clamp was performed in all subjects. Exogenous insulin was administered as a primed-continuous infusion at a rate of 240 pmol $\text{min}^{-1}\,\text{m}^{-2}$ simultaneously with a variable 20% dextrose infusion adjusted every 5–10 min to maintain plasma glucose level within 0.8 mmol/L (±15%) of the target glucose level (4.5–5.5 mmol/L). The clamp procedure was standardized across centres with the use of a demonstration video and *ad hoc* operating instructions; the raw data from each clamp study were immediately transferred to the coordinating centre where they underwent quality control scrutiny according to pre-set criteria. Insulin sensitivity was expressed as the ratio of the *M*-value[17] averaged over the final 40 min of the 2 h clamp and normalized by the fat-free mass to the mean plasma insulin concentration measured during the same interval (*M/I*, in units of μmol $\text{min}^{-1}\,\text{kg}_{\text{ffm}}^{-1}\,\text{mM}^{-1}$). LDL cholesterol concentration was calculated by the Friedewald formula.[18] Genomic DNA was extracted using a Nucleon BACC2 kit (Tepnel Life Sciences Plc, Manchester, UK).

Plasma adiponectin measurements

Plasma adiponectin was determined as described previously by a novel in-house time-resolved immunofluorometric assay (TR-IFMA) on the basis of two antibodies and recombinant human adiponectin (R & D Systems, Abingdon, UK).[19] The adiponectin molecule is known to form a wide range of polymers, of which the predominant polymers include trimers, hexamers, and highly congregated multimers.[20] Previous experiments have demonstrated that both antibodies used are able to detect several adiponectin polymers in serum, including the major three molecular forms. All standards and unknown samples were analysed in duplicate, with the exception of non-specific binding, which was analysed in quadruplicate. The intra-assay coefficient of variation (CV) was <5% and the inter-assay CV was <10%.

Carotid intima media thickness measurements

The carotid ultrasound method used a standardized scanning protocol common to all recruiting centres, and central reading of ultrasound studies.[15] In each centre, certified trained technicians performed high-resolution B-mode ultrasound of extracranial carotid arteries bilaterally, according to previously described scanning protocol.[21] The whole imaging procedure was recorded on S-VHS tapes and read in a centralized reading centre (Pisa) by a single reader (M.K.) blinded to clinical data, using a high-resolution video recorder (Panasonic AG-MD830) coupled with a computer-driven image analysis system developed by the Institute of Clinical Physiology, CNR, Pisa, Italy. For the purpose of this study, IMTs of the near and far walls of the right and left CCA were measured in digitized end-diastolic frames, ∼10 mm proximal to bifurcation. The measurements were performed in five measurement points for each wall, and the mean near- and far-wall IMTs were calculated by averaging the measurement points. Mean CCA IMT used in the statistical analyses was calculated as the overall mean of all available CCA walls (up to four). Both near- and far-wall IMTs were measured in this study as it was previously demonstrated that only far-wall measurement had significantly lower association with cardiovascular outcomes than the combined near- and far-wall measurements.[22] The intra- and inter-observer variabilities of CCA IMT measurements were tested in 100 subjects and were 4.1 and 6.2%, respectively.

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ADIPOQ single-nucleotide polymorphism genotyping

Single-nucleotide polymorphism T45G

A matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom MassARRAY, San Diego, CA, USA) method was used to genotype SNP T45G. The primers were designed using the Sequenom MassARRAY Assay design program version 2.0.4 (Sequenom). The PCR primer sequences were as follows: forward primer 5′AGTGCACATGTGGATTCCAG 3′, reverse primer 5′ CCTTGAGTCGTGGTTTCCTG-3′. A 5 µL PCR reaction was set up using 2 ng DNA, 1.25× HotStar *Taq* PCR buffer, 3.5 mM $MgCl_2$ per reaction, 200 µM each dNTP, 100 nM each of forward and reverse primers, and 0.15 U HotStar *Taq* polymerase (Qiagen, UK). PCR amplification was performed in 384-well PCR plates, and cycling conditions were as follows: 95°C for 15 min and 35 cycles of 95°C for 20 s, 58°C for 30 s, 72°C for 1 min, followed by a final extension step of 72°C for 3 min. Following this, a primer extension reaction was performed using the mass extend primer 5-CTATTAGCTCTGCCCGG-3, and samples were then prepared according to the manufacturer's methods (Sequenom) for genotyping using MALDI-TOF.

Single-nucleotide polymorphisms A-11426G, G-11391A, C-11377G

The *ADIPOQ* promoter SNPs were genotyped using a TaqMan® allelic discrimination assay (Applied Biosystems, Warrington, UK). Primers and probes were designed using the Assay by Design Service (Applied Biosciences, Warrington, UK). For each SNP, a 25 µL PCR reaction was set up with 10 ng of genomic DNA, 1× TaqMan universal PCR MasterMix with AmpErase® UNG, and 1× primer and probes assay mix. Samples were amplified on a GeneAmp 9700 PCR machine (Applied Biosciences). PCR cycling conditions were as follows: 50°C for 2 min followed by 95°C for 10 min, then 40 cycles of 92°C for 0.15 s, 60°C for 1 min. Following PCR, an allelic discrimination assay was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems).

Quality control

Of the genotyped samples, 5% were duplicates and there was at least one negative control per 96-well DNA plate. The accuracy of the genotyping was determined by the genotype concordance between duplicate samples. We obtained a 100% concordance between the genotyped duplicate samples for each of the SNPs. The genotyping success rate for each of the SNPs was >98%.

Statistical analysis

All analyses were performed using SPSS version 13 (SPSS Inc., Chicago, IL, USA) and Minitab version 14 (Minitab Inc., USA). The Haploview program, http://www.broad.mit.edu/mpg/haploview/, version 3.2,[23] was used to assess linkage disequilibrium (LD) between the genotyped SNPs. Data are reported as mean ± SD. Skewed variables (plasma adiponectin, carotid IMT, fasting serum insulin, *M/I* value, fasting triglycerides) were log-transformed for analysis and are presented as geometric means and the interquartile range (25th–75th quartile). The Pearson correlation coefficient was determined for the associations of biological variables with log-transformed plasma adiponectin levels. Single-SNP effects with continuous variables were analysed using linear regression using three models; these were the additive (common allele homozygotes coded as 1, heterozygotes as 2, and recessive allele homozygotes as 3), dominant (common allele homozygotes coded as 1 and heterozygotes and recessive allele homozygotes as 2), and recessive (common allele homozygotes and heterozygotes coded as 1 and recessive allele homozygotes as 2) models. General linear model (GLM) analysis was performed to test for associations between SNP genotypes and continuous variables (log carotid IMT and log adiponectin) after adjusting for confounding variables (sex, BMI, recruitment centre, and age). Normality was assessed by plotting the residuals. For significant associations, further adjustments were made in the GLM analysis for other known CVD risk factors (HDL cholesterol, LDL cholesterol, total cholesterol, triglycerides, BP, fasting insulin, *M/I*, fasting and 2 h glucose, waist circumference, smoking, and adiponectin levels). Two-tailed *P*-values <0.05 were considered significant; however, allowance for multiple testing was done by the interpretation of the significant results for testing the four *ADIPOQ* SNPs (a global significance level of 0.05 means a single test significance level of 0.0127) in the GLM analysis. *ADIPOQ* gene promoter haplotype frequencies were estimated and the likely pair of haplotypes (diplotypes) were reconstructed using the PHASE 2.1 software.[24] We performed 10 000 iterations of each run to produce reliable probability estimates. Rare diplotypes with population frequencies <0.01 were omitted.

Results

Characteristics and biological variables of the study cohort

The study cohort consists of 1306 subjects (589 men and 717 women) aged 43.8 ± 8.3 years (mean ± SD), with a mean BMI of 25.5 ± 4.0 kg/m^2. The clinical and metabolic characteristics of the study population are shown separately for men and women in *Table 1*. *Table 2* shows the correlation between biological variables, with plasma adiponectin levels in men and women. All listed variables were significantly correlated (*P*-values <0.05) with plasma

Table 1 Clinical and metabolic characteristics of males and females in the RISC study cohort

	Male	Female
n	589	717
Age (years)	43 ± 8	44 ± 8
BMI (kg/m^2)	26.4 ± 3.5	24.9 ± 4.3
Waist circumference (cm)	93 ± 10	81 ± 12
Fasting insulin[a] (pmol/L)	32.1 (22.0–46.0)	29.3 (20.0–40.0)
M/I[a] (µmol min^{-1} kg$_{ffm}^{-1}$ nM^{-1})	109 (83–154)	140 (110–193)
Fasting glucose (mmol/L)	5.3 ± 0.8	4.9 ± 0.6
2 h glucose (mmol/L)	5.7 ± 1.6	5.8 ± 1.5
Triglycerides[a] (mmol/L)	1.1 (0.8–1.5)	0.9 (0.6–1.1)
Cholesterol (mmol/L)	4.9 ± 0.9	4.8 ± 0.9
HDL cholesterol (mmol/L)	1.2 ± 0.3	1.6 ± 0.4
LDL cholesterol (mmol/L)	3.1 ± 0.8	2.8 ± 0.8
Systolic BP (mmHg)	122 ± 10	114 ± 13
Diastolic BP (mmHg)	76 ± 7	73 ± 8
Carotid IMT[a] (µm)	612 (553–670)	581 (525–640)
Plasma adiponectin[a] (mg/L)	6.1 (4.8–7.9)	9.2 (7.3–12.2)

Data are presented as means ± SD.
M/I, insulin sensitivity assessed by euglycaemic clamp; IMT, intima media thickness.
[a]Geometric means (25th–75th quartile).

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Table 2 Correlation coefficients between plasma adiponectin with biological variables in RISC study subjects

	Plasma adiponectin[a] (mg/L)			
	Male	*P*-value	Female	*P*-value
n	589		717	
Age (years)	0.08	0.039	0.09	0.014
BMI (kg/m^2)	−0.17	<0.0001	−0.28	<0.0001
Waist circumference (cm)	−0.15	<0.0001	−0.34	<0.0001
Fasting insulin[a] (pmol/L)	−0.27	<0.0001	−0.38	<0.0001
M/I[a] (μmol min^{-1} kg$_{ffm}$$^{-1}$ nM^{-1})	0.31	<0.0001	0.31	<0.0001
Fasting glucose (mmol/L)	−0.10	0.021	−0.16	<0.0001
2 h glucose (mmol/L)	−0.21	<0.0001	−0.19	<0.0001
Triglycerides[a] (mmol/L)	−0.27	<0.0001	−0.29	<0.0001
HDL cholesterol (mmol/L)	0.38	<0.0001	0.41	<0.0001
LDL cholesterol (mmol/L)	−0.07	0.075	−0.11	0.003
Systolic BP (mmHg)	−0.02	0.527	−0.12	0.002
Diastolic BP (mmHg)	−0.08	0.037	−0.11	0.003
Carotid IMT[a] (μm)	−0.05	0.257	−0.05	0.258

P-values <0.05 are considered significant.
M/I, insulin sensitivity assessed by euglycaemic clamp; IMT, intima media thickness.
[a]Log-transformed values used in the analysis.

adiponectin except for LDL cholesterol and systolic BP in men. However, there was no significant correlation of carotid IMT with plasma adiponectin in both men ($r = -0.05$) and women ($r = -0.05$).

ADIPOQ genotype and haplotype frequencies

All genotyped SNPs were in Hardy–Weinberg equilibrium and common with minor allele frequencies >0.05. There were no significant differences in the genotype or allele frequencies for the SNPs between subjects that made up the North and South European recruitment centres (*P*-values >0.05); therefore, recruitment centres were combined for subsequent analysis. *Table 3* shows detailed information for each SNP and the allele and genotype frequencies. There was strong LD ($D' > 0.8$) between the three promoter SNPs and weak associations between these promoter SNPs and SNP T45G in exon 2 of the *ADIPOQ* gene. The estimated haplotype frequencies consisting of the three promoter SNPs A-11426G, G-11391A, C-11377G were 59.5% for AGC, 23.9% for AGG, 9.0% for GGC, and 7.4% for AAC. Haplotype frequencies were similar to those previously reported in French and Swedish Caucasians.[8,13]

Relationships between *ADIPOQ* single-nucleotide polymorphisms and plasma adiponectin levels and carotid intima media thickness

By linear regression analysis, SNPs A-11426G and G-11391A were significantly associated with plasma adiponectin levels, and SNP C-11377G was significantly associated with carotid IMT in the dominant model (*Table 4*). Homozygous carriers of the −11391G allele had significantly lower plasma adiponectin levels compared with A allele carriers [geometric mean (interquartile range), 7.4 (5.4–10.0) vs. 9.1 (6.9–12.2) mg/L, $P < 0.0001$]. Plasma adiponectin levels remained significantly lower after adjusting for age, sex, BMI, and recruitment centre ($P < 0.0001$). Similarly, for SNP A-11426G, carriers of the G allele had significantly lower plasma adiponectin levels compared with A allele homozygotes before ($P = 0.015$) and after adjustment ($P = 0.005$) for the same covariates. The diplotype containing the haplotypes −11426G, −11391G, −11377C was significantly associated with adiponectin levels. Diplotypes AGC/GGC and GGC/GGC were significantly associated with lower plasma adiponectin levels (*Table 5*) compared with all other diplotypes before ($P = 0.003$) and after adjustment for sex, age, BMI, and recruitment centre ($P = 0.010$).

Only SNP C-11377G was associated with carotid IMT. Carriers of the G allele had significantly greater carotid IMT values compared with C allele homozygotes [geometric mean (interquartile range), 601 (543–665) vs. 590 (537–647) μm, $P = 0.021$] (*Table 4*). This difference remained after adjusting for age, sex, recruitment centre, BMI, as well as plasma adiponectin levels and other determinants of CVD risk (*Table 4*: GLM model 2; $P = 0.011$). The results remain significant at a global 5% significance level when allowance is made for testing the four SNPS. The diplotype containing the haplotypes −11426A, −11426G, and −11377G was significantly associated with carotid IMT values (*Table 5*). Diplotypes AGC/AGG and AGG/AGG were associated with higher carotid IMT values compared with all other diplotypes [604 (543–665) vs. 592 (540–650) μm, $P = 0.013$]. This difference remained significant after correcting for key determinants (*Table 5*: GLM model 2; $P = 0.003$). There was no association between SNP T45G and adiponectin levels and carotid IMT; however, the G

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Table 3 *ADIPOQ* single-nucleotide polymorphisms, genotypes, and allele frequencies (%) in the RISC study population

SNP (dbSNP ID number[a])	*n*	Genotype frequencies (*n*)			Allele frequencies (*n*)	
					Allele 1	Allele 2
A-11426G (rs16861194)	1292	*AA* 82.6 (1067)	*AG* 16.1 (208)	*GG* 1.3 (17)	*A* 90.6 (2342)	*G* 9.4 (242)
G-11391A (rs17300539)	1300	*GG* 85.3 (1109)	*GA* 14.1 (183)	*AA* 0.6 (8)	*G* 92.3 (2401)	*A* 7.7 (199)
C-11377G (rs266729)	1300	*CC* 56.2 (730)	*CG* 38.8 (505)	*GG* 5.0 (65)	*C* 75.6 (1965)	*G* 24.4 (635)
T45G (rs2241766)	1306	*TT* 78.1 (1020)	*TG* 20.5 (268)	*GG* 1.4 (18)	*T* 88.4 (2308)	*G* 11.6 (304)

[a]For more details about dbSNP, see http://www.ncbi.nlm.nih.gov/SNP/.

Table 4 Relationship between *ADIPOQ* gene single-nucleotide polymorphism genotypes and adiponectin and carotid intima media thickness

SNPs/genotypes	Plasma adiponectin[a] (mg/L)	Carotid IMT[a] (μm)
A-11426G		
AA	7.7 (5.6–10.5)	596 (540–655)
AG + GG	7.2 (5.4–9.6)	598 (548–652)
P-value	0.015	0.676
GLM model 1 *P*-value[b]	0.005	0.428
G-11391A		
GG	7.4 (5.4–10.0)	596 (540–655)
GA + AA	9.1 (6.9–12.2)	590 (534–653)
P-value	<0.0001	0.473
GLM model 1 *P*-value[b]	<0.0001	0.917
C-11377G		
CC	7.7 (5.7–10.2)	590 (537–647)
CG + GG	7.6 (5.4–10.5)	601 (543–665)
P-value	0.669	0.021
GLM model 1 *P*-value[b]	0.273	0.044
GLM model 2 *P*-value[c]	—	0.011
T45G		
TT	7.7 (5.6–10.3)	594 (540–653)
TG + GG	7.5 (5.4–10.5)	596 (539–653)
P-value	0.588	0.838
GLM model 1 *P*-value[b]	0.638	0.999

P-values <0.05 are considered significant.
IMT, intima media thickness.
[a]Geometric mean (25th–75th quartile) presented.
[b]*P*-value after adjustment for age, sex, BMI, and recruitment centre.
[c]*P*-value after adjustment for other CVD risk factors (HDL cholesterol, LDL cholesterol, and total cholesterol, serum triglycerides, systolic and diastolic BP, fasting insulin, insulin sensitivity assessed by the euglycaemic clamp, fasting and 2 h plasma glucose, waist circumference, smoking status) as well as age, sex, BMI, recruitment centre, and adiponectin levels.

allele was associated with increased waist circumference (TT vs. TG + GG, 87 ± 0.2 vs. 90 ± 1.5, $P = 0.02$) after adjustment for age, sex, recruitment centre, and BMI.

Discussion

The intriguing observation of our study is that variation in the *ADIPOQ* gene promoter is directly associated with common carotid artery IMT in healthy subjects. Specifically, individuals with the G allele of the C-11377G SNP had significantly higher carotid IMT values compared with C allele homozygotes. This relationship was stronger after correction for key covariates, including plasma adiponectin levels. Our findings are supported by Hoefle *et al.*,[14] who recently reported an association between SNP −11377 and coronary angiography-determined CAD in a prospective study of 402 men. The SNP −11377 G allele was significantly associated with increased coronary stenosis and future vascular events independent of serum adiponectin levels and traditional CVD risk factors.

How might variation in the *ADIPOQ* gene directly influence carotid IMT? Clearly, our study does not allow us to address this directly, but it is interesting to note that a recent study reported that the *ADIPOQ* gene is expressed in vascular tissue.[25] Moreover, ACE-inhibitor therapy given to type 2 diabetic patients resulted in a two-fold increase in the vascular expression of the *ADIPOQ* gene and was associated with an improvement in endothelial function.[25] This led the authors to speculate that adiponectin generated locally within the vasculature might directly influence endothelial function. This is supported by the observation that globular adiponectin applied to cultured vascular endothelial cells increased both the expression and the activity of eNOS.[26] The circulating serum/plasma levels of adiponectin may not reflect the actual amount of adiponectin present at the tissue level, for example, the concentration in the sub-endothelial space where the anti-atherogenic targets for adiponectin are located.[27,28] Therefore, further *in vitro* studies are needed to explore whether variation in the *ADIPOQ* gene in vascular tissue affects local adiponectin expression, endothelial function, and ultimately CVD risk. Although SNP −11377 was in strong LD with the other promoter SNPs, only the haplotype containing the −11377 G allele was associated with increased carotid IMT. It may be that SNP −11377 is in LD with another

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Table 5 **Relationship between *ADIPOQ* promoter single-nucleotide polymorphism haplotypes and adiponectin and carotid intima media thickness**

ACDC promoter SNP diplotypes (A-11426G, G-11391A, C-11377G)	*n*	Plasma adiponectin[a](mg/L)	Carotid IMT[a] (μm)
AGC/GGC and GGC/GGC	145	6.9 (5.2–8.7)	589 (548–650)
All other diplotypes	1153	7.7 (5.6–10.5)	596 (540–655)
P-value		0.003	0.422
GLM model 1 *P*-value[b]		0.010	0.185
AGC/AGG and AGG/AGG	457	7.4 (5.3–10.0)	604 (543–665)
All other diplotypes	841	7.7 (5.7–10.2)	592 (540–650)
P-value		0.089	0.013
GLM model 1 *P*-value[b]		0.102	0.027
GLM model 2 *P*-value[c]		—	0.003

P-values <0.05 are considered significant.
IMT, intima media thickness.
[a]Geometric means (25th–75th quartile) presented.
[b]*P*-value after adjustment for age, sex, BMI, and recruitment centre.
P-value after adjustment for other CVD risk factors (HDL cholesterol, LDL cholesterol, and total cholesterol, serum triglycerides, systolic and diastolic BP, fasting insulin, insulin sensitivity assessed by euglycaemic clamp, fasting and 2 h plasma glucose, waist circumference, smoking status) as well as age, sex, BMI, recruitment centre, and adiponectin levels.

nearby functional variant, which directly affects the expression of adiponectin in vascular tissue.

Of the other promoter SNPs, the A-11426G and G-11391A and the haplotype containing the −11426 G allele and −11391 G allele were significantly associated with lower plasma adiponectin levels, before and after covariate correction. The SNP −11391A allele has been consistently reported associated with higher adiponectin levels in European populations[8,11] and increased *in vitro* transcriptional activity.[29] Putative binding sites for transcriptional factors have not been found in the promoter region of the *ADIPOQ* gene where SNPs −11391 and −11377 lie; however, between these two SNPs and adjacent to the position of the −11377 SNP, a nucleotide sequence which is similar to an enhancer element sequence in the epidermal growth factor receptor gene has been reported.[8]

Although there was a strong association between the A-11426G and G-11391A SNPs and circulating adiponectin levels, there was, however, no association between these SNPs and carotid IMT. Furthermore, plasma adiponectin levels were not an independent predictor of carotid IMT in our study after adjustment for the usual covariates and CVD determinants (data not shown). The majority of studies investigating *ADIPOQ* gene SNPs and adiponectin levels have been reported in disease states, especially in subjects with type 2 diabetes and CVD. Our study is a large healthy cohort and therefore we are able to dissect out relationships independent of the potentially confounding secondary effects of the disease. However, it is important to note that our study is cross-sectional; on the other hand, the RISC project is a longitudinal study of CVD risk and so we will, in time, be able to investigate the relationship between circulating adiponectin levels and carotid IMT progression.

A previous study reported an association of SNP T45G with CAD in patients with type 2 diabetes, independent of other CVD risk factors.[10] We were unable to show a relationship between the G allele and carotid IMT in healthy subjects, but we did replicate the reported association between the SNP 45G allele and increased waist circumference.[11]

The RISC study is a multi-centre European study and allows us to investigate a large and well-characterized healthy population. However, because of the high number of recruiting centres, different operators and ultrasound scanners have been used for the carotid studies. To restrict this limitation, all ultrasound technicians were trained and certified in the ultrasound-reading centre, the carotid imaging procedure was performed according to a standardized protocol,[21] and a single reader processed and analysed the carotid images. All analyses were centre-adjusted. We acknowledge that the difference in carotid IMT between the SNP −11377 genotype groups was small in clinical terms; however, our results are supported by the study of Hoefle *et al.*,[14] who reported an association of SNP −11377 with coronary stenosis and vascular events in CAD subjects.

In conclusion, variation in the *ADIPOQ* gene promoter was associated with carotid IMT in a large cohort of healthy subjects, and this was independent of plasma adiponectin levels and other determinants of CVD risk. Although our observation will require further replication, it does support the concept that variation in the *ADIPOQ* gene directly influences carotid IMT and CVD risk.

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Conflict of interest: none declared.

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Appendix: EGIR–RISC study group

RISC recruiting centres

Amsterdam, The Netherlands: R.J. Heine, J. Dekker, G. Nijpels, W. Boorsma; Athens, Greece: A. Mitrakou, S. Tournis, K. Kyriakopoulou, P. Thomakos; Belgrade, Serbia and Montenegro: N. Lalic, K. Lalic, A. Jotic, L. Lukic, M. Civcic; Dublin, Ireland: J. Nolan, T.P. Yeow, M. Murphy, C. DeLong, G. Neary, M.P. Colgan, M. Hatunic; Frankfurt, Germany: T. Konrad, H. Böhles, S. Fuellert, F. Baer, H. Zuchhold; Geneva, Switzerland: A. Golay, E. Harsch Bobbioni, V. Barthassat, V. Makoundou, T.N.O. Lehmann, T. Merminod; Glasgow, UK: J.R. Petrie (now Dundee), C. Perry, F. Neary, C. MacDougall, K. Shields, L. Malcolm; Kuopio, Finland: M. Laakso, U. Salmenniemi, A. Aura, R. Raisanen, U. Ruotsalainen, T. Sistonen, M. Laitinen, H. Saloranta; London, UK: S.W. Coppack, N. McIntosh, P. Khadobaksh; Lyon, France: M. Laville, F. Bonnet, A. Brac de la Perriere, C. Louche-Pelissier, C. Maitrepierre, J. Peyrat, A. Serusclat; Madrid, Spain: R. Gabriel, E.M. Sánchez, R. Carraro, A. Friera, B. Novella; Malmö, Sweden (i): P. Nilsson, M. Persson, G. Östling, (ii): O. Melander, P. Burri; Milan, Italy: P.M. Piatti, L.D. Monti, E. Setola, E. Galluccio, F. Minicucci, A. Colleluori; Newcastle upon Tyne, UK: M. Walker, I.M. Ibrahim, M. Jayapaul, D. Carman, K. Short, Y. McGrady, D. Richardson; Odense, Denmark: H. Beck-Nielsen, P. Staehr, K. Hojlund, V. Vestergaard, C. Olsen, L. Hansen; Perugia, Italy: G.B. Bolli, F. Porcellati, C. Fanelli, P. Lucidi, F. Calcinaro, A. Saturni; Pisa, Italy: E. Ferrannini, A. Natali, E. Muscelli, S. Pinnola, M. Kozakova, B.D. Astiarraga; Rome, Italy: G. Mingrone, C. Guidone, A. Favuzzi. P. Di Rocco; Vienna, Austria: C. Anderwald, M. Bischof, M. Promintzer, M. Krebs, M. Mandl, A. Hofer, A. Luger, W. Waldhäusl, M. Roden.

Project management board

B. Balkau (Villejuif, France); S.W. Coppack (London, UK); J.M. Dekker (Amsterdam, The Netherlands); E. Ferrannini (Pisa, Italy); A. Mari (Padova, Italy); A. Natali (Pisa, Italy); M. Walker (Newcastle upon Tyne, UK).

Core laboratories and reading centres

Lipids—Dublin, Ireland: P. Gaffney, J. Nolan, G. Boran; hormones—Odense, Denmark: C. Olsen, L. Hansen, H. Beck-Nielsen; albumin:-creatinine—Amsterdam, The Netherlands: A. Kok, J. Dekker; genetics—Newcastle upon Tyne, UK: S. Patel, M. Walker; stable isotope laboratory—Pisa, Italy: A. Gastaldelli, D. Ciociaro; ultrasound reading centre—Pisa, Italy: M. Kozakova; ECG reading—Villejuif, France: M.T. Guillanneuf; data management—Villejuif, France: B. Balkau, L. Mhamdi; mathematical modelling and website management—Padova, Italy: A. Mari, G. Pacini, C. Cavaggion; coordinating office—Pisa, Italy: S.A. Hills, L. Landucci, L. Mota.

Further information on the RISC project and participating centres can be found on www.egir.org.

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