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Common Adiponectin Gene Variants Show Different Effects on Risk of Cardiovascular Disease and Type 2 Diabetes in European Subjects

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Summary

Alterations in the secretion of adipokines may explain the link between obesity, type 2 diabetes (T2DM) and coronary artery disease (CAD). These conditions have been associated with variation in the adiponectin gene, although evidence for this relationship has been variable, with differences found even in similar samples. This study aims to clarify these inconsistencies by determining the impact of identified adiponectin gene (*ADIPOQ*) variants (-11391G>A,-1377C>G[promoter] and $+45$T>G[exon 2] and $+276$G>T[intron 2]) on the prospective risk of CAD and T2DM in healthy men, and on adverse metabolic markers, in myocardial infarct survivors and controls from different parts of Europe. The hazard ratio for cardiovascular disease varied across the -11391GG/GA/AA($p = 0.03$) and -11371CC/CG/GG($p = 0.05$) genotypes only. In contrast, only the $+45$T>G variant (3.80[1.76-8.24]) was associated with T2DM, while two haplotypes GCTT/GCGG ($p < 0.05$) and $+276$G>T($p = 0.01$) increased risk in interaction with obesity. The variants were associated with a number of biomarkers in Southern but not Northern Europe ($p = 0.01$), despite no significant differences in allele or haplotype frequencies ($p > 0.44$). A risk haplotype could not be identified in either sample. Adiponectin gene variants are hence currently poor markers for the development of T2DM and CAD. Their influence on risk depends significantly on interactions that are not currently understood with either genetic variation elsewhere or the environment of the sample studied.

Keywords: Adiponectin, Metabolic Syndrome, obesity,cardiovascular disease.

Introduction

Obesity is reaching epidemic proportions in developed nations (Flegal *et al*. 2002). This major adverse impact on health is partly due to the induction of a cluster of metabolic derangements, including hyperglycaemia, dyslipidaemia and hypertension, which in a specific combination are termed "metabolic syndrome". The term metabolic syndrome was first used by the World Health Organisation in 1998 to describe a cluster of metabolic risk factors, of which abnormal glucose tolerance was a key factor (Expert Panel on Detection, 2001). A clinical definition of 'metabolic syndrome' was produced by the National Cholesterol Education Program's Adult treatment program III in 2001, and includes abdominal obesity, dyslipidaemia, hypertension, insulin resistance and prothrombotic and inflammatory states (Isomaa *et al*. 2001). The secretion of peptides known as adipokines from adipose tissue is thought to be responsible for the induction of these obesity-induced changes

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(Rajala & Scherer, 2003). The most abundant adipokine is adiponectin, which is secreted only by differentiated adipocytes (Arita *et al*. 1999). Serum adiponectin levels are negatively correlated with many of the features of obesity, including adverse markers of the metabolic syndrome and conventional cardiovascular risk factors. These include higher serum insulin, total cholesterol, Low Density Lipoprotein (LDL), triglycerides, HbA1c and lower High Density Lipoprotein (HDL) and LDL particle size (Gable *et al*. 2006a). Serum adiponectin levels are lower in patients with coronary artery disease(Hotta *et al*. 2000) and are predictive of future risk of type 2 diabetes or myocardial infarction in prospective studies (Kumada *et al*. 2003).

The gene encoding the adiponectin protein (*ADIPOQ)* is located on chromosome 3q27 (Maeda *et al*. 1996). This area of the genome has been identified by whole genome linkage studies as a susceptibility locus for metabolic syndrome, Type 2 Diabetes (T2DM) and cardiovascular disease (Francke *et al*. 2001; Vionnet *et al*. 2000). *ADIPOQ* Variants have been identified in European, North American and Japanese subjects (Hara *et al*. 2002; Vassuer *et al*. 2002). The promoter variants −11391G>A and −11377C>G, exon 2 +45T>G and intron 2 +276G>T are some of the most common and are the most widely studied (see (Gable *et al*. 2006a for review). These variants have been associated with markers of insulin resistance, the metabolic syndrome and type 2 diabetes mellitus. Although the variants in the promoter region appear important in associations with insulin resistance, phenotype studies have failed to find associations with cardiovascular disease (Hegener *et al*. 2006; Jang *et al*. 2006; Juhan-Vague *et al*. 2002; Lacquemant *et al*. 2004; Qi *et al*. 2006a). The variants at +45 and +276 have been associated with cardiovascular disease (Bacci *et al*. 2004; Fillippi *et al*. 2005; Qi *et al*. 2005a). However, results have shown significant variation depending on the sample studied, for example annotating +276T and coronary artery disease in otherwise healthy subjects (Fillippi *et al*. 2005), but +276G and coronary artery disease in subjects with type 2 diabetes(Bacci S, 2004). The fact that these variants are not found in any region of the gene with an observed or predicted function suggests that these associations are due to the variants acting as markers for a yet to be identified functional variant. The impact of different variants in different samples is currently poorly understood. Prospective gene association studies are a more powerful design and using this methodology may clarify these inconsistencies (Humphries *et al*. 2003), as will the ability to estimate the risk of type 2 diabetes and cardiovascular disease in the same cohort. Furthermore, comparing the association between adiponectin gene variants, obesity, and its consequences, in different samples from Northern and Southern Europe will give insight into the impact of these variants in the pathophysiology linking type 2 diabetes and cardiovascular disease.

Methods

The Prospective Second Northwick Park Heart Study (NPHS II)

From April 1989 to April 1994 3,012 healthy Caucasian men, aged 50–64 years, registered with nine primary care practices in the United Kingdom, were recruited for prospective surveillance. The study was approved by the institutional ethics committees and performed in accordance with the Declaration of Helsinki. All subjects gave written informed consent. To be eligible, subjects had to be free of unstable angina, myocardial infarction or evidence of silent infarction, coronary surgery, aspirin or anticoagulant therapy, cerebrovascular disease, malignancy (except skin cancer other than melanoma), or any condition precluding informed consent. Weight, height and blood pressure measurements were recorded, and venous blood samples were collected for plasma and DNA analysis. Participants were recalled annually for 5 years for interview and repeat venous blood collection. Self-report by questionnaire was used to identify cases at baseline. CHD was defined as those who had a myocardial infarction (MI, silent, determined by ECG, or clinical), or those who had coronary intervention procedures as supplied by the Office for National Statistics, hospitals, coroners and general practices. Exclusion criteria precluded subjects requiring insulin or oral hypoglycaemics from entry into NPHS II. New cases of type 2 diabetes were identified by a search of practice note for physician-diagnosed and treated type 2 diabetes, according to current national guidelines.

Hypercoagulability and Impaired Fibrinolytic Function Mechanisms Study (HIFMECH)

The HIFMECH study is described elsewhere (Juhan-Vague *et al*. 2002). Briefly, it comprises a sample of Caucasian male, first myocardial infarction survivors recruited from four European centres (Northern European - *Stockholm, London*; Southern European - *Marseille, San Giovani Rotondo*) (n = 598) along with age-matched (by each centre) healthy controls (n = 653). Post infarction patients were investigated 3 to 6 months after the acute event. Patients and control subjects were examined, in parallel, in the early morning after an overnight fast, and blood samples were obtained. The study was performed in accordance with the guidelines in The Declaration of Helsinki and approved by local ethics committees; informed consent was obtained from all subjects.

Genotyping of Adiponectin Gene Variants

Four variants were chosen on the basis of previously published genotype associations in European Caucasian samples. Two variants were genotyped by Taqman technology (rs17300539,−11391G>A and rs1501299,+276G>T) using Applied Biosytems assay by design (primers and probes available on request). The other two variants (rs266729,−11377C>G and rs2241766, +45T>G) were genotyped by Polymerase Chain Reaction amplification and RFLP analysis using MADGE (Microarray Diagonal Gel Electrophoresis). Primer sequences were: forward CATCAGAATGTGTGGCTTGC and TCTCTCCATGGCTGACAGTG; reverse AGAAGCAGCCTGGAGAACTG and GGTGAGAAGGGTGAGAAAGG, respectively; followed by digest with *Hha*I (−11377) and *Hy*188 III (+45). For all variants, genotype was confirmed by two independent technicians and any discrepancies were resolved by repeat genotyping.

Statistical Analysis

Analysis was performed using 'Intercooled STATA' (version 8.2, STATA Corporation, Texas). Obesity was defined as BMI>30Kg/m^2. Continuous variables were compared by ANOVA, and the variable transformed where required. For NPHS II the results are presented as hazard ratios (HR) obtained from Cox regression models with their corresponding 95% confidence interval (CI), adjusted for age and practice (recruitment site). The main risk factor for the development of type 2 diabetes was obesity with a 3.96 fold [2.87–5.47] increase in risk; therefore hazard ratios for type 2 diabetes were also estimated by obesity. An analysis of variance model with two degrees of freedom was used to test for differences between genotype groups with no assumption of the underlying genetic model, although for survival analysis, when the number of events was low in rare homozygotes, a dominant model was used. To limit the number of comparisons made no test for trend was conducted. The full cohort was included in the survival analysis but where follow-up was incomplete the subject was censored at their last date of follow-up. For HIFMECH the statistical analysis followed the *a-priori* design (Juhan-Vague *et al*. 2002) comparing factors associated with cardiovascular risk in relation to the North and South of Europe. The data from the two Northern and two Southern centres were combined, using the centre as a factor in the analysis of variance, to give centre adjusted p-values for a more statistically robust comparison. For case-control comparisons analyses were performed by conditional logistic regression, to take account of matching by centre. Where appropriate, variables were transformed to normalize their distributions, but were retransformed after analysis and are reported here in their original form. Haplotype frequencies were determined using Thesias (Version 2) and compared by Chi square test.

Results

NPHSII

Baseline characteristics

A total of 159 men developed type 2 diabetes and 269 men developed cardiovascular disease, during 15 years of follow up. *ADIPOQ* genotypes were determined in 92% of subjects and for all variants the genotype distributions were in Hardy Weinburg proportions. The baseline characteristics and genotype frequencies of the sample are described in Table 1. No *ADIPOQ* genotype was associated with the baseline characteristics, except

Table 1 Genotype frequencies and baseline characteristics of men in NPHSII who developed cardiovascular disease (CAD) and type 2 diabetes compared to those who did not

	No CAD N = 2503	With CAD N = 269	P value	No diabetes N = 2767	With diabetes N = 169	P value
Age (years)	56.0 (3.4)	56.6 (3.6)	0.007	56.0 (3.5)	56.3 (3.4)	0.19
SBP†(mmHG)	136.7 (18.6)	141.2(19.3)	0.001	136.3 (18.7)	141.3 19.3)	0.001
BMI†(kg/m²)	26.2 (3.4)	26.7 (3.3)	0.02	26.0 (3.3)	28.6 (3.7)	<0.001
Obesity [%(N)]				12.3 (340)	35.1 (59)	<0.001
Smoking [%(N)]	27.2 (681)	36.4 (98)	<0.001	28.6 (791)	32.0% (54)	0.13
Cholesterol (mmol/L)	5.7 (1.01)	6.06 (1.03)	<0.001	5.72 (1.01)	5.90 (0.98)	0.03
Triglyceride†(mmol/L)	1.77 (0.93)	2.05 (1.06)	<0.001	1.75 (0.92)	2.27 (1.7)	<0.001
Fibrinogen†(g/l)	2.70 (0.51)	2.81 (0.49)	0.001	2.71 (0.52)	2.78 (0.53)	0.11
CRP†(mg/l)	2.92 (3.41)	4.01 (4.79)	<0.001	2.92 (3.44)	4.05 (4.36)	<0.001

Genotype frequencies*	N	Rare allele frequency	[95% CI]
−11391 GG/GA/AA	2350/382/17	0.08	0.07–0.08
−11377 CC/CG/GG	1480/1063/179	0.26	0.25–0.27
+45 TT/TG/GG	2022/548/35	0.12	0.11–0.13
+276 GG/GT/TT	1511/1038/178	0.26	0.24–0.27

†geometric mean (approx sd). CRP measurements made after diabetes was recorded are excluded from the analysis (n = 2). +age and practice adjusted hazard ratio for 1 sd increase in all variables except smoking (current:non), obesity (>30:<30) and age (5 year increase).* All in Hardy Weinburg proportions (p = 0.73,0.52,0.12,0.98 respectively)

that systolic blood pressure was higher in −11377C>G heterozygotes compared to either homozygote (p = 0.02) (Supplementary Table 1).

Cardiovascular disease

The incidence of cardiovascular disease by genotype is shown in Table 2A. The hazard ratio for cardiovascular disease varied across the −11391GG/GA/AA (p = 0.03) and −11371CC/CG/GG genotypes (p = 0.05), but not for the other two variants (+45T>G [p = 0.56]; +276G>T [p = 0.26]). Men homozygous for −11391AA were 3.28 (1.35-8.00; p = 0.03) times more likely to develop cardiovascular disease. Although the hazard ratio [HR] for 11377G homozygous men included 1 (1.16[0.70–1.94]), carriers of the rare allele were significantly more likely to develop cardiovascular disease (1.33[1.05–1.70]; p = 0.02) (Figure 1A).

Type 2 diabetes

In contrast, the only variant associated with risk of type 2 diabetes was +45T>G, with GG homozygotes having a 3.80[1.76-8.24] times increased risk of type 2 diabetes (p = 0.008) (Figure 2A). The incidence of type 2 diabetes by genotype is shown in Table 2B. The risk associated with the rare homozygote at −11377 and +276 was 1.52 [0.87–2.65; p = 0.37] and 0.91 [0.48-1.76: p = 0.92], respectively, while the −11391A carriers (combined as there was only one rare homozygote with type 2 diabetes) had an HR of 1.04[0.68–1.60: p = 0.85]. We have previously reported that, as expected, obesity is a major risk factor for type 2 diabetes in these subjects (Gable DR, 2006b). We examined the effect of obesity on all four variants and type 2 diabetes risk, comparing rare allele carriers to wild-type homozygotes to maintain power. There was evidence of an interaction between the +276G>T variant and obesity (p = 0.01)(Table 3), with an increased risk of type 2 diabetes associated with +276T carriers in the obese men (2.59[1.63–4.11] to 4.82[3.05–7.63]) but not in the lean men (1.00 to 0.76[0.51–1.13]) (Figure 2B). There was no evidence of interaction with the other genotypes (Supplementary Table 2).

ADIPOQ haplotypes

Data from all four variants were combined to determine haplotypes. The haplotype matrix is shown in Figure 3A. Although not all the variants are in high linkage disequilibrium [LD] with each other, these haplotypes are effective at comparing the effect of the individual variants and the haplotypic background (Petrone,

Table 2 The genotype distribution in cases and controls for A) Cardiovascular disease in NPHSII B) type 2 diabetes in NPHSII C) Acute myocardial infarction in HIFMECH

Variant	Genotype	Controls (N)	Cases (N)	Incidence (%)	^P =
A) Cardiovascular Disease					
−11391	GG	2350	232	9.9	0.03
	GA	382	30	7.9	
	AA	17	5	29. 4	
−11377	CC	1480	129	8.7	0.05
	CG	1063	120	11.3	
	GG	179	17	9.5	
+45	TT	2022	204	10.1	0.65
	TG	548	53	9.7	
	GG	35	2	5.7	
+276	GG	1511	155	10.3	0.26
	GT	1038	96	9.3	
	TT	178	12	6.7	
B) Type 2 Diabetes (Type 2 at baseline excluded)					
−11391	GG	2289	133	5.8	0.85
	GA	374	24	6.4	
	AA	17	1	5.9	
−11377	CC	1440	83	5.8	0.37
	CG	1038	60	5.8	
	GG	175	15	8.6	
+45	TT	1968	116	5.9	0.008
	TG	536	25	4.7	
	GG	35	7	20.0	
+276	GG	1470	87	5.9	0.92
	GT	1015	59	5.8	
	TT	175	10	5.7	
C) Acute Myocardial Infarction		**Control N [%]**	**Cases N [%]**	***P =**	
−11391	GG	458 [80.6]	446 [84.3]		
	GA	107 [18.8]	78 [14.7]	0.14	
	AA	3 [0.5]	5 [1.0]		
−11377	CC	329 [58.3]	278 [52.5]		
	CG	197 [34.9]	217 [40.9]	0.04	
	GG	38 [6.7]	35 [6.6]		
+45	TT	384 [68.2]	360 [68.4]		
	TG	168 [29.8]	154 [29.2]	0.87	
	GG	11 [2.0]	12 [2.3]		
+276	GG	289 [52.0]	266 [50.8]		
	GT	225 [40.4]	216 [41.2]	0.96	
	TT	43 [7.7]	22 [8.0]		

^Analysis of variance (2df), no model assumed. All age and centre adjusted.* Odds of being a case unadjusted.

2006), and have previously demonstrated stronger associations with insulin resistance than haplotypes limited to the higher LD blocks (Woo JG, 2006). The average R^2 with the tagging SNPs identified using tagger in Haploview v3.32 [Hapmap CNEP] was 0.51, with $R^2 > 0.7$ in 35% of cases. The two promoter variants and the intron2/exon2 variants showed strong pairwise linkage disequilibrium [LD], but this was weaker between other combinations. There was no evidence of a risk haplotype for cardiovascular disease or type 2

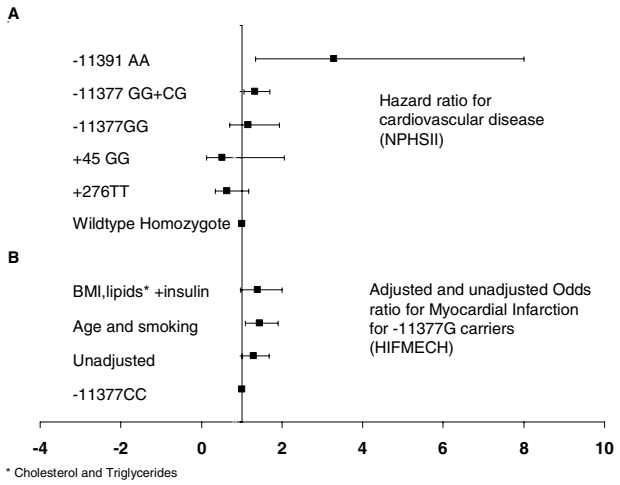

Figure 1 A) Hazard ratios for the development of cardiovascular disease by adiponectin genotypes in NPHSII compared to the relevant wildtype homozygote. B) Odds ratio for being a case (myocardial infarction) for the −11377G carriers compared to wildtype −11377CC homozygotes in the HIFMECH study.

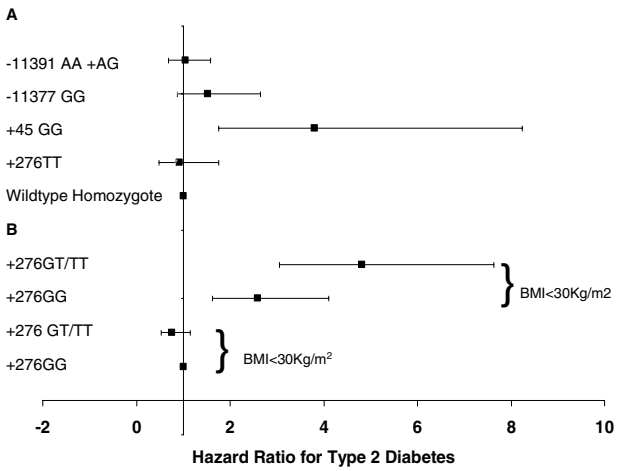

Figure 2 A) Hazard ratios for the development of type 2 diabetes by adiponectin genotypes in NPHSII. B) The interaction between +276 adiponectin gentotype and obesity for the development of type 2 diabetes in NPHSII.

diabetes when considered alone (Table 4A). However, when considered with obesity there was an interaction for risk of type 2 diabetes with the GCTT and GCGG haplotypes, both of which were associated with greater than expected risk in obese men, and a trend towards a global haplotype obesity interaction ($p = 0.08$) (Table 4B).

HIFMECH

Baseline characteristics

ADIPOQ genotypes were determined in 99% of the individual variants and the genotype frequencies and baseline characteristics of the study population are shown in Table 5. All genotypes were in Hardy-Weinburg equilibrium and genotype frequency did not differ between the Northern and Southern samples for any of the variants.

Cardiovascular disease

The genotype distribution in cases and controls is shown in Table 2C. The risk of having cardiovascular disease was only associated with the −11377 promoter variant. The odds of myocardial infarction in the presence of the G allele were 1.30([1.01–1.68]: $p = 0.04$) times greater than the homozygous CC wild-type. This effect persisted when controlled for age and smoking (1.44[1.09–1.90]: $p = 0.01$), but when adjusting further for factors known to be correlated with serum adiponectin (BMI, cholesterol, triglycerides and insulin) the odds ratio just included 1(1.39[0.97–2.00]; $p = 0.07$)(Figure 1B).

Type 2 diabetes

Type 2 diabetes was not examined in the HIFMECH study due to the low number of cases. However, better metabolic characterization of these subjects allowed us to examine a number of adverse metabolic phenotypes not available in NPHSII. When all the *ADIPOQ* variants were examined in the Northern Europe sample for effect on Body Mass Index (BMI), Waist hip ratio (WHR), cholesterol, triglycerides or fasting insulin, in either cases or controls there was only one significant association between −11391G and BMI (Supplementary Table 3A). This is in contrast to the Southern Europe sample, where all the *ADIPOQ* variants were associated with at least one feature of insulin resistance (Supplementary Table 3B). The number of associations observed in the South was significantly higher than the number observed in the North ($p = 0.01$).

ADIPOQ haplotypes

Haplotypes were determined as in NPHSII, and the pattern of LD is shown in Figure 3B. Haplotype frequencies did not differ between the North and South

BMI	Genotype	Total N	diabetic N (%)	HR[1] (95% CI)
<30	GG	1251	61 (4.9)	1.00
	GT/TT	1047	42 (4.0)	0.76 (0.51–1.13)
>30	GG	219	26 (11.9)	2.59 (1.63–4.11)
	GT/TT	143	27 (18.9)	4.82 (3.05–7.63)
Interaction				P = 0.01

Table 3 The interaction between the +276G>T *ADIPOQ* genotype, obesity and prospective risk of type 2 diabetes in the NPHSII cohort

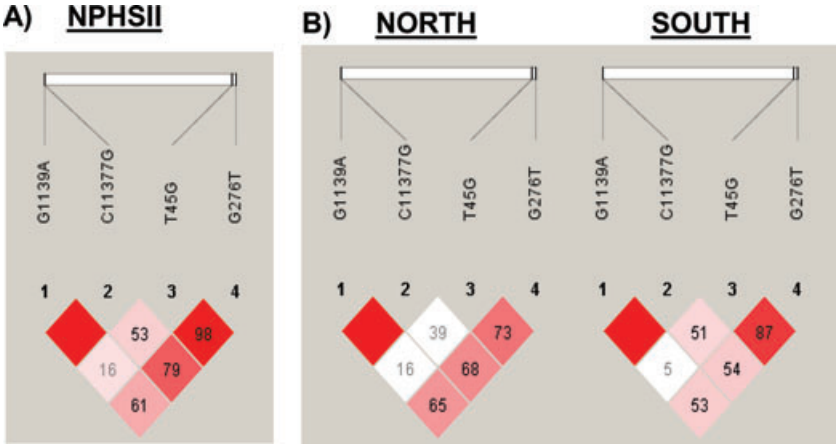

Figure 3 The linkage disequilbrium for the −11391,−11377,+45,+276 variants in the adiponectin gene in A) Healthy men in NPHSII B) The healthy controls in the North and South of Europe in the HIFMECH study.

(p = 0.60) (Supplementary Table 4). The relationship between the variants was the same in the North and the South with no difference in the LD between the two (p > 0.1). There was no difference in haplotype frequency between cases and controls, with no risk haplotype identified for myocardial infarction (Supplementary Table 5). Analysis of all the subjects combined did not identify a specific haplotype with significant effects on serum insulin, triglyceride and cholesterol levels, BMI or waist hip ratio (Supplementary Table 5).

Discussion

Despite an abundance of literature examining the adiponectin gene and evidence of the key role of serum adiponectin concentrations in disease pathogenesis, direct evidence for the association of certain *ADIPOQ* variants with disease outcomes is inconsistent, not replicated or not associated with differences in serum adiponectin (Gable *et al*. 2006a; Heid *et al*. 2006). The examination of *ADIPOQ* variants in NPHSII and HIFMECH has provided a number of interesting findings. Firstly, the novel association of promoter variants with prospective risk of cardiovascular disease. Secondly, the association of a different region of *ADIPOQ* (exon/intron 2) with prospective risk of type 2 diabetes but not cardiovascular disease, and *vice versa*. Thirdly, that the influence of these variants differed widely in different areas of Europe, despite no significant variation in frequency between the two samples studied. Lastly, data from NPHSII suggests one potential factor influencing the genotype-phenotype relationship may be obesity.

The failure to demonstrate consistent associations may be, in part, because *ADIPOQ* does not play a major role in the physiological control of adiponectin levels. Although heritability of serum adiponectin is high (30–93%; (Butte *et al*. 2005; Comuzzie *et al*. 2001; Menzaghi *et al*. 2004; Vassuer *et al*. 2002; Vozarova de Court, 2005), a number of genome-wide scans have failed to demonstrate linkage between serum adiponectin and *ADIPOQ* (Comuzzie *et al*. 2001; Guo *et al*. 2006; Lindsay *et al*. 2003), and where linkage was found, then other areas of the genome were also identified as being important (Menzaghi *et al*. 2004; Pollin *et al*. 2005). This is consistent with variation elsewhere in the genome

Table 4 A) Adiponectin haplotypes and risk of cardiovascular disease and type 2 diabetes in NPHSII B) Adiponectin haplotypes and risk of type 2 diabetes in NPHSII in those with and without obesity (BMI>30Kg/m^2).

A)

−11391 −11377 +45 +276	No CHD freq	CHD freq	HR[1] (95% CI)	No diabetes freq	With diabetes freq	HR[1] (95% CI)
GCTG	0.391	0.388	1.00	0.391	0.361	1.00
GGTG	0.228	0.266	1.20 (0.93–1.56) p = 0.17	0.231	0.255	1.19 (0.87–1.63) p = 0.26
GCTT	0.190	0.167	0.84 (0.63–1.11) p = 0.22	0.189	0.185	1.08 (0.76–1.54) p = 0.66
GCGG	0.083	0.082	1.07 (0.72–1.60) p = 0.73	0.084	0.076	0.94 (0.59–1.50) p = 0.80
ACTT	0.053	0.057	1.58 (0.98–2.57) p = 0.06	0.055	0.045	0.85 (0.45–1.61) p = 0.62
ACGG	0.021	0.012	0.60 (0.29–1.22) p = 0.16	0.019	0.032	1.80 (0.81–3.99) p = 0.15
GGGG	0.016	0.013	0.87 (0.50–1.51) p = 0.61	0.015	0.029	2.34 (0.98–5.60) p = 0.06
GGTT	0.016	0.011	0.94 (0.57–1.57) p = 0.82	0.015	0.016	0.90 (0.22–3.72) p = 0.88
ACTG	0.002	0.002	-	0.001	0.0004	-
GCGT	0.0006	0.0005	-	0.0006	0.0004	-
Global P value			0.35			0.40

B)

−11391/−11377/+45/ +276	LEAN HR[1](95% CI)	OBESE HR[1](95% CI)	Interaction
GCTG	1.00	1.00	-
GGTG	1.10 (0.74–1.64) p = 0.64	1.55 (0.86–2.81) p = 0.15	P = 0.36
GCTT	0.84 (0.53–1.33) p = 0.46	1.86 (0.93–3.70) p = 0.08	P = 0.04
GCGG	0.59 (0.29–1.20) p = 0.14-	1.82 (0.86–3.87) p = 0.12	P = 0.05
ACTT	0.68 (0.29–1.56) p = 0.36-	1.58 (0.47–5.34) p = 0.46-	P = 0.33
ACGG	2.32 (0.99–5.46) p = 0.054	0.58 (0.05–6.65) p = 0.66	P = 0.25-
GGGG	2.74 (0.93–8.07) p = 0.07	0.80 (0.11–5.68) p = 0.82-	P = 0.39
GGTT	-	3.91 (0.58–26.4) p = 0.16	-
ACTG	-	-	-
GCGT	-	-	-

[1]age and practice adjusted, - insufficient numbers with haplotype

being associated with differences in serum adiponectin, including the –308G>A variant of the TNFα alpha gene (*TNF*) (Gonzalez-Sanchez *et al*. 2006) and the Pro12Ala PPAR-γ (Mousavinasab *et al*. 2005) variant. Where *ADIPOQ* variants have been associated with serum adiponectin they explain only a small proportion of the variance, approximately only a third of that due to sex alone (Heid IM, 2006).

The role of different variants in different disease processes, as seen in NPHSII, is consistent with the structure of *ADIPOQ*. Although this is a locus of high recombination, and therefore low linkage disequilibrium (Gibson F, 2004), there appear to be two main haplotype blocks, one including the promoter variants and the other including the +45 and +276 variants (Heid *et al*. 2006; Woo *et al*. 2006). Thus, although the aetiology of insulin resistance and cardiovascular disease overlap, the *NPHSII*, an unselected population at recruitment, could contain some men with a predisposition to metabolic disturbance, where interaction with one region of the gene is important. The sample may also contain some men with higher predisposition to cardiovascular disease or inflammatory activation, where interaction with another region is more important. The associations of different gene regions with different disease outcomes would be detected given adequate power, and explains how the impact of the variants could vary within the sample studied. Therefore, it is likely that the influence of variation in *ADIPOQ* depends on a number of variants working in conjunction with each other within the gene, and gene-gene interactions.

Table 5 The baseline characteristics of the subjects recruited to the HIFMECH study

	NORTH					SOUTH					N v S
	Cases		Controls			Cases		Controls			
	Mean	SD	Mean	SD	p*	Mean	SD	Mean	SD	p*	
Age(yrs)	53.1	5.1	52.7	5.0	0.33	51.0	5.6	50.5	5.6	0.28	<0.01
BMI (Kg/m$^{2)}$	27.2	3.4	25.8	3.1	<0.01	26.9	3.3	26.4	3.2	0.04	0.35
Blood pressure (mmHg)	130/82	17/9	130/84	15/8	0.62 0.03	126/81	16/10	126/84	13/9	0.83 <0.01	<0.01 0.66
Smoking-Current/ Ex (%)	76.8	2.8	62.1	3.1	<0.01	86.3	2.0	62.4	2.7	<0.01	0.08
Diabetes-Type 2 (%)	11.2	2.1	0	-	<0.01	11.2	1.8	0	-	<0.01	0.98
Cholesterol (mmol/L)	5.66	1.22	5.71	0.99	0.65	5.18	1.11	5.39	0.94	0.02	<0.01
Triglyceride (mmol/L)	1.99	0.83	1.52	0.60	<0.01	1.79	0.72	1.39	0.61	<0.01	0.01

Adiponectin genotype	NORTH		SOUTH		N v S	HW^
	Genotype frequencies [N]		Genotype frequencies[N]			
	Cases	Controls	Cases	Controls		
−11391 GG/GA/AA	201/30/2	205/41/1	245/48/3	253/66/2	0.44	0.83
−11377 CC/CG/GG	118/97/17	139/90/15	160/120/18	190/107/23	0.74	0.64
+45 TT/TG/GG	165/60/8	173/67/5	195/94/4	211/101/6	0.10	0.08
+276 GG/GT/TT	117/95/17	124/94/22	149/121/25	165/131/21	0.91	0.91

The blood pressure and lipid parameters include those on anti-hypertensive and lipid lowering agents, respectively. N v S = North v South. Insulin, triglycerides and BMI are log transformed. ^Deviation from Hardy-Weinburg equilibrium.

These differences could also be explained by subtle differences in environment, with certain environments predisposing to cardiovascular disease through interaction with the promoter block, and others increasing risk of type 2 diabetes through interaction with other regions. The North-South European differences also suggest a significant gene-environment interaction. Serum adiponectin can be altered by glycaemic load and index of the diet, as well as fibre content (Qi *et al*. 2005b; Qi *et al*. 2006b), levels of physical activity (Jürimäe *et al*. 2005; Mantzoros *et al*. 2005; Pischon *et al*. 2004; Schulze *et al*. 2004), smoking (Thamer *et al*. 2004), intake of alcohol (Qi *et al*. 2005b) and fish oil (Neschen *et al*. 2006). Differences in treatment of underlying conditions can also affect serum adiponectin; the use of rosiglitazone (Kang *et al*. 2005) can vary both serum adiponectin and the effect of gene variants, while drugs affecting the renin-angiotensin-aldosterone system could also potentially alter adiponectin physiology (Ran *et al*. 2006). All of these could potentially differ between the North and South of Europe; for instance in HIFMECH there are more smokers in the South, while BMI is higher in southern controls than in Northern controls (Table 5). As described here, gene-obesity interactions have been observed with a number of *ADIPOQ* variants (Boutia-Naji *et al*. 2006; Fillippi *et al*. 2004; Hu *et al*. 2004; Jang *et al*. 2006; Ukkola *et al*. 2003; Vassuer *et al*. 2005). A reduction in *ADIPOQ* expression may be one of the mechanisms for limiting adipocyte size in the face of further energy delivery (Fu *et al*. 2005), and variation in *ADIPOQ* may only have a role in influencing serum adiponectin under these conditions. These North-South differences may explain some of the differences in the influence of *ADIPOQ* variation.

Most studies to date had a limited number of subjects, and were designed to estimate associations with a certain disease outcome. This, taken with the confounding of gene-gene and gene-environment interaction, may also explain the lack of consistency. NPHSII has over 3000 participants and is prospective in design, which may explain why the promoter variants have not been associated with cardiovascular disease previously. The replication of the association with the −11377C>G in HIFMECH suggests that this area of the gene is also important in the pathophysiology of cardiovascular disease. The identification of the +45G variant as being associated with prospective risk of type 2 diabetes is

in keeping with a recent meta-analysis of three other prospective studies (Tso *et al*. 2006), although a protective association for the +276T allele with cardiovascular disease, identified from a five study meta-analysis (Qi *et al*. 2006a), was not replicated. The NPHS II study was powered (80% power at 5% significance level, dominant model) to detect a minimum effect size with a relative risk of between 1.39 and 1.51 for cardiovascular disease and 1.49 and 1.71 for type 2 diabetes, depending on the frequency of the variant studied, so it is possible that more modest genotype risk associations may exist but were not detected. The minimum detectable odds ratio in the HIFMECH study (80% power at 5% significance level, dominant model) of between 1.42 and 1.55 may also explain why there is discordance between the two studies, and in reported studies of the adiponectin gene as a whole. The potential for confounding the relationship between *ADIPOQ* variants and disease odds ratio outcomes means that meta-analyses will be required to determine which variants are important. Advances in understanding the gene structure and its regulation are also required to identify functional changes in the adiponectin gene.

Multiple comparisons have been made in this study and, whilst making such an adjustment reduces the type I error, it leads to increases in the type II error; fewer errors of interpretation occur when no adjustments are made. This analysis followed an *a-priori* hypothesis or design, but it is possible that the statistically significant associations described here were observed by chance as a result of multiple comparisons, and hence they require confirmation. When the associations with phenotype were investigated in the HIFMECH study, by comparing the number of statistically significant associations between North and South, an allowance was made for any association that occured by chance. A further limitation of this study is that serum adiponectin was not measured in any of the subjects examined. There is also a lack of consistent relationships between *ADIPOQ* variants and serum adiponectin levels. This may be in part due to the measurement of total adiponectin when different molecular weight forms may be the active moiety. High-molecular weight (HMW) adiponectin can now be measured by ELISA and has a better predictive value for insulin resistance than total adiponectin alone (Hara *et al*. 2006). The association of variants with HMW adiponectin in large studies is required to address this lack of evidence. Finally, the cohorts in both of the studies consisted of Caucasian males. Therefore, care needs to be taken when applying these results to the general population, and specifically to populations that include non-Caucasian and female subjects.

In summary, these data provide evidence of the importance of promoter variants in the adiponectin gene in risk of cardiovascular disease. They add to the growing evidence for the importance of the +45 variant in insulin resistance/glucose intolerance. They further provide, the evidence that genotype-phenotype relationships are confounded by within-gene variant interaction, gene-gene interaction and gene-environment interaction. This means that currently variants in the adiponectin gene are not good markers for risk of type 2 diabetes or cardiovascular disease. Therefore, to clarify the relationship between *ADIPOQ* variants and disease phenotypes large studies or further meta-analyses are required, preferably including measurements of different molecular weight forms of adiponectin. These are required to identify the functional variants, understand how adiponectin expression is regulated, and to elucidate the physiological role of this adipokine.

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Supplementary Material

Supplementary Table 1 The relationship between the identified adiponectin gene variants and baseline characteristics in NPHSII

Supplementary Table 2 The association of adiponectin genotypes with prospective risk of type 2 diabetes by obesity (BMI>30Kg/m^2) in NPHSII

Supplementary Table3A The relationship between the identified adiponectin gene variants and obesity and insulin resistance markers in the north of Europe sample in the HIFMECH study.

Supplementary Table 3B The relationship between the identified adiponectin gene variants and obesity and insulin resistance markers in the South of Europe sample in the HIFMECH study.

Supplementary Table 4 Adiponectin haplotypes in controls the North and South of Europe in the

HIFMECH study with a comparison of their frequencies in haplotypes with frequency greater than 1%.

Supplementary Table 5 The relationship between adiponectin haplotypes and Waist hip ratio, cholesterol, triglycerides, insulin, BMI and CAD risk in the HIFMECH study. Results are expressed as haplotypic mean [95%CI], that is the value of the phenotype associated with one copy of the haplotype.

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