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Variants in the *APOC3* promoter insulin responsive element modulate insulin secretion and lipids in middle-aged men

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Abstract

Variation in the insulin responsive element (IRE) of the *APOC3* promoter has been shown to be associated with insulin and glucose concentrations after an oral glucose tolerance test (OGTT) in young healthy men. We evaluated two variants in the IRE (−455T>C and −482C>T) in the Ely study, a prospective cohort study of middle-aged men ($n=223$) and women ($n=279$), to determine if the effect of these variants on glucose homeostasis could be explained by altered nonesterified fatty acid (NEFA) levels and if these effects are modulated by age and gender. Both variants had significant effects on the 30-min insulin incremental response in men alone (−482C>T, $P=0.007$; −455T>C, $P=0.0155$), with rare allele homozygotes having a 33.3% and 23.3% lower insulin increment as compared to common allele homozygotes, respectively. Thirty-minute NEFA concentrations were also significantly associated with genotype in men and levels were approximately 10% higher in carriers homozygous for the rare alleles as compared to subjects homozygous for the common alleles (−482C>T, $P=0.04$; −455T>C, $P=0.006$). In addition, there was a strong interaction between both variants and cigarette smoking affecting fasting triglyceride levels in both men (interaction: −455T>C, $P=0.02$; −482C>T, $P=0.008$) and women (interaction: −455T>C, $P=0.007$; −482C>T, $P=0.013$). Taken together, the data shows that men who carry the rare alleles of the IRE variants have disturbed glucose homeostasis and an unfavourable lipid phenotype. The finding of an elevated 30-min NEFA may be an important mechanistic link between triglyceride-rich lipoprotein (TRL) metabolism and glucose homeostasis.

Keywords: Glucose homeostasis; Nonesterified fatty acid; Triglyceride metabolism; Atherosclerosis; Type 2 diabetes

1. Introduction

We have previously shown that a variant in the *APOC3* promoter, a gene primarily known to be involved in triglyceride-rich lipoprotein (TRL) metabolism, is also associated with insulin and glucose levels after an oral glucose tolerance test (OGTT) in young healthy men [1]. These findings demonstrate some of the interactions between lipid and glucose metabolism and support the involvement of lipid metabolism in the development of type 2 diabetes. Lipoprotein genes have generally not been investigated as candidate genes for type 2 diabetes, though one report has implicated the *APOA1–C3–A4* locus in determining risk, but only in overweight individuals [2].

ApoCIII is a 79-aa glycoprotein, synthesized by the liver and small intestine and is a major protein component of TRL and HDL. Epidemiological studies have shown that plasma levels of apoCIII, and the distribution of apoCIII between plasma lipoprotein fractions, appear to be important in the progression of coronary artery disease (CAD) [3,4]. Transgenic mouse studies have suggested that the predominant mechanism of apoCIII-induced hypertriglyceridaemia was decreased lipolysis at the cell surface [5]. Thus, apoCIII probably impacts on CAD by inhibiting lipoprotein lipase-mediated lipolysis and prolonging of the exposure time of the arterial wall to atherogenic particles such as very-low-density lipoproteins (VLDL) and remnant particles.

Abbreviations: IRE, insulin responsive element; OGTT, oral glucose tolerance test; NEFA, nonesterified fatty acids; TRL, triglyceride-rich lipoproteins; *APOC3*, apolipoprotein CIII gene

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The gene for *APOC3* has been mapped to chromosome 11q23.3 [6] and is flanked by the genes for *APOA1*, *APO4* and *APOA5* in a 50-kb gene cluster [7]. Two variants (−455T>C and −482C>T) occur within the insulin responsive element (IRE) in the *APOC3* promoter. ApoCIII expression is normally down-regulated 40–50% by insulin, and the presence of a rare allele of either variant (−455C or −482T) proved sufficient to remove the ability of insulin to inhibit apoCIII expression in vitro [8]. However, we have shown that the −482C>T [1], but not −455T>C (unpublished data), significantly influences insulin and glucose levels after an OGTT in young healthy men from the EARSII study. Subjects who carried the −482T allele had significantly elevated AUC insulin and glucose concentrations as compared to −482CC subjects.

The aim of this study was to establish if the effect of *APOC3* promoter variants on insulin and glucose measures was exaggerated or altered in middle-aged men and women, a population in which impaired glucose tolerance and dyslipidemia should be more prevalent. Thus, while results from EARSII reflected the effects in healthy young men, the Ely study enabled us to examine these effects in both men and women in middle age. Furthermore, we had hypothesized that apoCIII may be affecting glucose metabolism by causing elevated nonesterified fatty acid (NEFA) levels, and NEFA measures were available in this cohort.

2. Material and methods

2.1. Sample population

The Ely Study is a prospective population-based study of the aetiology and pathogenesis of type 2 diabetes mellitus and related disorders that has been described previously [9,10]. Briefly, subjects who were not known to have diabetes were randomly recruited from a sampling frame consisting of all those aged 40–65 years in the single GP practice in Ely, Cambridgeshire, in April 1990 [10]. Subjects ($n=1122$; 74% response rate) agreed to participate and attended the local surgery after a 10 h fast for a clinical examination, which included a dietary and medical questionnaire, anthropometric measurements and a standard 75 g OGTT (blood drawn at 0, 30 and 120 min). Fifty-one subjects had prevalent but undiagnosed diabetes. The remaining 1071 nondiabetic subjects were followed up 4.5 years later [9] and 937 individuals underwent the same evaluation as in 1990–1992. The data presented in this paper is on a random subgroup of 502 individuals who had complete data at both phases of the Ely Study and on whom a DNA sample was available.

2.2. Biochemical measures

All blood samples were immediately placed on ice and centrifuged on site. Serum samples were aliquoted, packed in ice and transferred to the laboratory where they were stored at −70 °C within 4 h. Plasma glucose was measured using the hexokinase method [11] and triglyceride measured using the RA 1000 (Bayer Diagnostics, Basingstoke, UK) with a standard enzymatic method. Plasma insulin was determined by two-site immunometric assays with either ^{125}I or alkaline phosphatase labels [12,13]. Plasma NEFA were determined enzymatically based on acyl-CoA synthetase activity (Boehringer Mannheim, Lewes, Sussex, UK) [14].

2.3. Polymorphism detection

Both variants analysed were restriction fragment length polymorphisms (RFLP) and the −482C>T assay was performed as described previously [1]. The same PCR product (as the −482C>T) was digested with 1 unit of Fnu4HI (NEB) at 37 °C overnight to determine the −455T>C variant. The fragments were separated by using 5–10% polyacrylamide Microtitre Array Diagonal Gel Electrophoresis (MADGE) [15].

2.4. Statistical analysis

The data presented are from both phases of the study with an average of 4.5 years of follow-up. *T*-tests were used to compare characteristics and biochemical measures between men and women at baseline. The 30-min insulin incremental response (a measure of insulin secretion) was calculated by dividing the difference between 30 min and fasting insulin concentrations by the 30-min glucose concentration [9]. We hypothesized that a gene effect would determine the 'usual' level of insulin sensitivity, which could be estimated by repeated measurement. We did not hypothesize that the *APOC3* polymorphisms would affect the rate of change of insulin sensitivity over time, as such an analysis would probably require the use of a larger population, a longer follow-up and a more precise measure of insulin sensitivity. In this study, the primary outcome variables were insulin/glucose and NEFA levels following an OGTT and also the 30-min insulin increment, which combines the insulin and glucose measures to generate an estimate of insulin secretion and should be more informative than either insulin and glucose alone. A mixed model using outcome variables from both phases of the study was used to describe the differences between genotypes with time-dependent (e.g. body mass index) and fixed covariates (sex). The MIXED procedure in SAS was used for this analysis, which allows repeated measures for each subject and avoids the loss of subjects due to missing data points (SAS Institute Inc., Release 6.12, Cary, NC, USA). Traits that were not normally distributed, e.g. insulin and triglyceride, were normalized using log transformation. We also examined possible interaction effects between genotype and smoking in the MIXED analysis for each variable of interest.

3. Results

3.1. General characteristics

The general baseline characteristics and biochemical measures of the sample stratified by sex are shown in Table 1. Significant differences between men and women are shown.

The two variants in the IRE (−482C>T and −455T>C) were typed in all subjects and were found to be in Hardy–Weinberg equilibrium. No other *APOC3* variants were typed in this cohort. The −482T allele frequency was 0.25 (95% CI, 0.23–0.29) and the −455C allele frequency was 0.37 (95% CI, 0.34–0.39) in the total sample. The two variants were in strong allelic association ($\Delta = 0.73$, $P < 0.001$). There was no significant difference in either −482T or −455C allele frequencies by glucose tolerance status (normal glucose tolerance, impaired glucose tolerance or type 2 diabetes at follow-up) (data not shown).

3.2. Effects of APOC3 variants on insulin and glucose measures

Multivariate regression models were constructed and used to test for significant effects of each variant on both fasting insulin (a surrogate for insulin sensitivity) and for the 30-min insulin incremental response (a surrogate for insulin secretion) using both measures from each subject. No significant effects on fasting insulin were found with either variant in men or women. However, significant effects were observed on the 30-min insulin incremental response (30-min fasting insulin/30-min glucose) with both variants in the men alone (Table 2). With both variants, the lowering effect was confined to the rare allele homozygotes, but the magnitude (−482T/T vs. C/C, 33.3% lower; −455C/C vs. T/T, 23.3% lower) of the effect was greater and the level of statistical evidence better with the −482C>T variant as compared to the −455T>C ($P = 0.007$ and $P = 0.016$, respectively), though there was not sufficient statistical evidence to suggest that the −482C>T was responsible for this effect alone. There was no association found in women for either variant.

Further insight into the relationship between −482C>T and insulin and glucose over the course of the OGTT and the gender difference observed in these parameters can be seen in the separate plots of insulin and glucose in Fig. 1. Plots for the −455T>C were similar to those shown, but differences between genotype were less marked than for the −482C>T and are not shown. It can be seen that the reduced 30-min insulin secretion in male −482T/T carriers is followed by an increase in insulin levels over the remaining 90 min of the test, as compared to −482C carriers who show a gradual decrease in insulin levels (Fig. 1A). This is combined with an elevated glucose level from 30 to 120 min in −482T/T male subjects as compared to −482C carriers (AUC, $P = 0.012$). This explains why −482T/T male subjects have a reduced 30-min insulin increment (which incorporates 30-min glucose level), despite the fact that 30-min insulin was not significantly different by genotype alone. Women who carry the −482T/T genotype show some reduction in insulin levels at 30 min, but in contrast to the men, this is followed by a gradual decrease in insulin levels in parallel to the −482C carriers, an effect which is significant for AUC insulin ($P = 0.028$).

Table 1
Baseline means (S.E.) or 95% confidence interval, characteristics and biochemical measures of the sample group

	Men	Women
n	223	279
Age (years)	54.33 (0.52)	52.86 (0.45)[a]
Body mass index (kg/m^2)	25.81 (0.18)	25.53 (0.28)
Waist-to-hip ratio	0.905 (0.004)	0.761 (0.003)[b]
Fasting NEFA (mmol/l)	0.405 (0.014)	0.524 (0.016)[b]
30-min NEFA (mmol/l)	0.404 (0.015)	0.429 (0.016)
120-min NEFA (mmol/l)	0.090 (0.005)	0.078 (0.003)[a]
NEFA area (mmol h/l)	0.561 (0.018)	0.620 (0.020)[a]
Fasting insulin (pmol/l)[c]	38.5 (35.7, 41.6)	40.1 (37.4, 43.0)
30-min insulin (pmol/l)[c]	241.1 (218.6, 266.0)	263.5 (244.4, 284.1)
120-min insulin (pmol/l)[c]	207.6 (186.4, 231.3)	230.2 (212.8, 249.2)
Insulin increment[c,d]	23.6 (21.3, 26.1)	26.7 (24.3, 29.4)
log(FU insulin area)[c]	448.1 (414.0, 485.0)	473.6 (444.3, 504.9)
Fasting glucose (mmol/l)[c]	5.84 (5.77, 5.91)	5.57 (5.50, 5.63)[b]
30-min glucose (mmol/l)[c]	8.49 (8.28, 8.71)	7.80 (7.61, 7.98)[b]
120-min glucose (mmol/l)[c]	6.01 (5.78, 6.25)	6.08 (5.86, 6.30)
Glucose area (mmol h/l)[c]	14.61 (14.28, 14.94)	13.86 (13.55, 14.18)[e]
Fasting triglyceride (mmol/l)[c]	1.31 (1.22, 1.40)	1.10 (1.04, 1.16)[b]
Diabetes (%)	0	0
IGT (%)	21.5	25.8

Data are arithmetic mean (S.E.).
[a] $P \le 0.05$.
[b] $P \le 0.001$ (men vs. women).
[c] Data are geometric mean (95% CI).
[d] Insulin increment is the 30-min insulin incremental response (pmol/l insulin/mmol/l glucose).
[e] $P \le 0.01$.

Table 2
Adjusted means (S.D.) for the 30-min insulin incremental response by APOC3 −482C>T ($n = 502$) and APOC3 −455T>C ($n = 499$) genotype using data from both phases of the Ely Study

	Men				Women			
	N	Mean	95% CI		*N*	Mean	95% CI	
			Lower	Upper			Lower	Upper
−482TT	136	26.91	24.75	29.27	149	31.01	28.54	33.68
−482CT	72	28.27	25.21	31.70	103	29.47	26.68	32.56
−482CC	15	18.05	14.00	23.27	27	26.74	21.96	32.55
P value		0.007				0.359		
−455CC	91	27.53	24.84	30.52	104	30.27	27.38	33.47
−455TC	97	27.97	25.32	30.89	132	29.99	27.40	32.75
−455TT	33	21.12	17.80	25.05	38	29.06	24.60	34.33
P value		0.0155				0.92		

Data computed using mixed model analysis with 30-min insulin incremental response (at both phases) as outcome variable, adjusted for age and BMI.

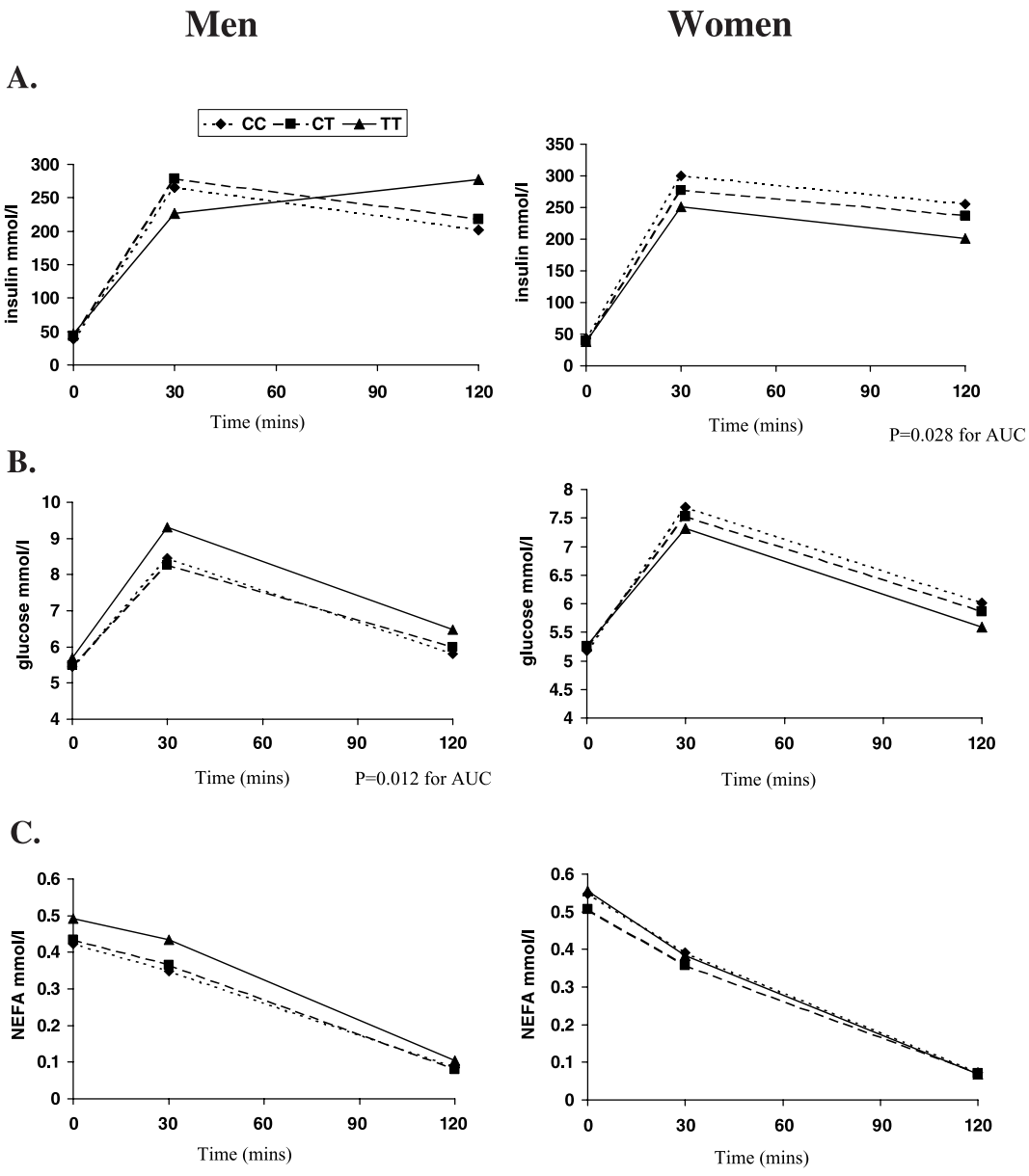


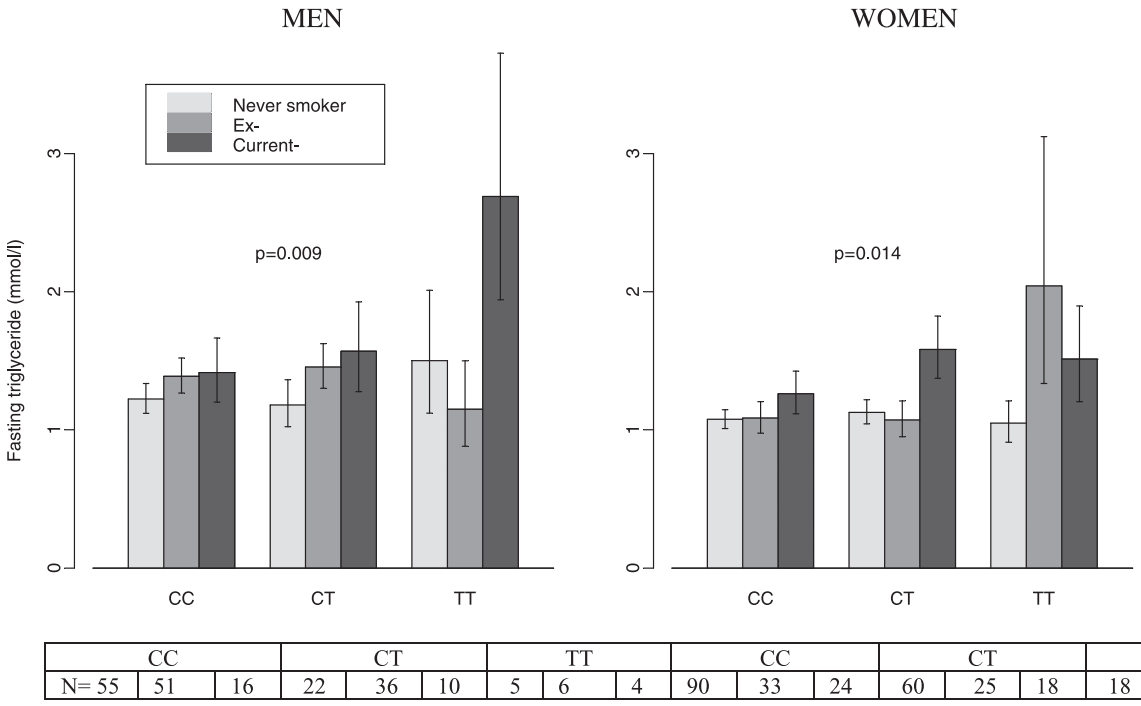
Fig. 1. Insulin (A), glucose (B) and NEFA (C) levels over the course of the OGTT in men and women, according to *APOC3* −482 genotype. (●) CC; (■) CT; (▲) TT. *P* values shown are for TT vs. CT+CC groups for AUC insulin/glucose/NEFA.

However, no difference in glucose levels were observed by genotype, explaining why a significant effect on 30-min insulin incremental response (30-min fasting insulin/30-min glucose) was not found in women (Fig. 1B).

3.3. Effects of APOC3 variants on lipid parameters

Significant effects were observed with both the −482C>T and the −455T>C variants on serum fasting triglyceride, but these effects were dependent on smoking status. Adjusted means by −482C>T genotype and smoking status are shown separately for men and women in Fig. 2. The effects on triglyceride are similar between men and women in that subjects who carry the −482T allele tended to have higher triglyceride levels if they were smokers, but not if they had never been smokers, with the ex-smokers showing an intermediate effect (genotype/smoking interaction: men, $P=0.009$; women, $P=0.014$). However, the numbers in the −482T/T groups are quite modest, and therefore the pattern of effect in these groups is somewhat variable. The effects on triglyceride of the −455T>C were very similar to the −482C>T, and the smoking interaction terms were significant in both men ($P=0.02$) and women ($P=0.007$) [data not shown].

Associations between the *APOC3* variants and NEFA measures were examined. In the men, but not the women,



CC			CT			TT			CC			CT			TT		
N= 55	51	16	22	36	10	5	6	4	90	33	24	60	25	18	18	2	7

Fig. 2. Adjusted mean and 95% CI for triglyceride levels by *APOC3* −482C>T according to smoking status in men and women. *P* values correspond to the significance of the interaction between genotype and smoking on triglyceride levels. Numbers of subjects are shown in the table, corresponding to the columns in the bar chart.

significant effects of similar magnitude (independent of smoking) on 30-min NEFA concentrations for both −482C>T and −455T>C were seen (Table 3). NEFA levels were higher in homozygous carriers of rare alleles compared to subjects homozygous for the common alleles (−482C>T, 26% higher, $P=0.04$; −455T>C, 26% higher, $P=0.006$). In addition, there was a significant association with AUC NEFA for the −455 C>T (C/C, 0.59; T/C, 0.53; T/T, 0.50: $P=0.02$), but results for the AUC for −482C>T variant were not statistically significant (−482T/T, 0.6; T/C, 0.53; C/C, 0.51: $P=0.14$). Plots of NEFA for the −482C>T in men and women are shown in Fig. 1C. Considering the NEFA measures over the time course of the OGTT, NEFA levels were higher in −482T/T male but not female subjects (as compared to C/T+C/C subjects). The −455T>C plot for men was similar to the −482C>T and is not shown.

Table 3
Adjusted means (S.D.) for 30-min NEFA by APOC3 −482C>T ($n=502$) and APOC3 −455T>C ($n=499$) genotypes using data from both phases of the Ely Study

	Men				Women			
	N	Mean	95% CI		*N*	Mean	95% CI	
			Lower	Upper			Lower	Upper
−482CC	136	0.347	0.324	0.370	149	0.389	0.363	0.415
−482CT	72	0.364	0.333	0.396	103	0.358	0.327	0.390
−482TT	15	0.439	0.370	0.509	27	0.396	0.333	0.458
P value		0.043				0.296		
−455TT	91	0.334	0.306	0.362	104	0.398	0.366	0.429
−455CT	97	0.361	0.334	0.388	132	0.362	0.334	0.390
−455CC	33	0.423	0.376	0.469	38	0.386	0.333	0.439
P value		0.006				0.2397		

Data computed using mixed model analysis with 30-min NEFA concentration (at both phases) as outcome variable, adjusted for age and BMI.

4. Discussion

Both high fasting insulin (a surrogate for insulin resistance) and a decreased 30-min insulin incremental response (a surrogate for insulin secretion) are cross-sectionally associated with glucose intolerance [16] and independently predict the development of type 2 diabetes [17–19]. In a previous study of young healthy men (mean age 22), we found an association with the −482T allele and elevated AUC insulin and glucose levels after an OGTT [1]. In the present study of middle-aged men and women (mean age 53 at baseline), we observed a significant association between the −482T allele and reduced 30-min insulin secretion in men. This follows the expected pattern of an age-related decline in glucose tolerance, with hyperinsulinemia, evident in younger subjects who are adequately compensating for an impaired glucose homeostatic response, compared with reduced insulin sensitivity and secretion in older subjects who are compensating less efficiently and progressing toward type 2 diabetes. It should be noted though that the 30-min insulin increment is only a surrogate measure for

insulin secretion, and may reflect defects in both insulin secretion and insulin sensitivity. A euglycemic clamp or an intravenous glucose tolerance test (IVGTT) will be required to further delineate the exact nature of the defect in these subjects, though these are difficult to perform in large numbers of subjects. We have previously evaluated the association of the −482C>T variant in a British middle-aged sample (as part of a multi-ethnic study in Wandsworth, London, UK), and saw a significant increase in fasting insulin in carriers of the −482T allele in men and women, but 30-min measures were not available in this study [20]. Thus, in these three studies, EARSII, Wandsworth, and now the Ely study, we see a significant relationship between the −482T allele and measures of insulin resistance/secretion in men, though the particular measure varies between studies. This may reflect the limitation and overlap between the various measures of insulin sensitivity/secretion derived from an OGTT. It is also likely that factors such as diet and BMI, which are known to affect insulin sensitivity, may differ between studies and effect our ability to detect the influence of these genotypes. In addition, a Caucasian group from London may be more ethnically heterogeneous than from Ely, a more rural setting.

Other groups have also observed associations between *APOC3* variants and insulin sensitivity. Perez-Jimenez et al. [21] saw a decrease in insulin sensitivity in men who carried the S2 allele of the SstI variant (this variant is in strong LD with the IRE variants) after a high saturated fat diet. This effect was not seen in the women. Similarly, in the Framingham offspring study, the S2 allele was associated with elevated fasting insulin concentrations ($P<0.04$) in men, whereas no significant associations were observed in women [22]. Neither study investigated the IRE variants.

The effects on insulin increment and NEFA levels are confined to those subjects homozygous for the rare variants, as were the effects on remnant lipoproteins that we reported previously [23]. Interestingly, a recent case–control study investigating angiographically defined atherosclerosis also showed that homozygosity for the −455C variant was associated with increased apoCIII levels and an elevated risk for CAD (OR = 2.5) [24]. The SstI variant did not confer a significant risk for CAD and the −482C>T was not investigated in this study (but is in strong LD with the −455T>C). Thus, it would seem that homozygosity of the IRE variants is required before a significant effect on insulin increment/lipids or CAD is observed. As these groups have relatively low numbers of subjects (though they have two measures per subject), it is important that these results should be interpreted with caution until replicated in additional studies. It is also impossible to tell from the present study which variant in the IRE is responsible for the observed effects, given the similarity between the two sets of results and the lack of statistical justification for differentiating between them.

Even though CAD is the most common cause of death in patients with diabetes, the mechanism by which dyslipidemia and type 2 diabetes are linked is unclear. This study provides some insight into this process. Our results highlight the synergism between lipid and carbohydrate metabolism since we show an association between a measure of insulin secretion and an *APOC3* genotype that is considered to modulate insulin-mediated regulation of apoCIII and thus LPL activity.

We can only speculate about a possible mechanism for the effect of *APOC3* promoter variants on insulin secretion. As apoCIII has been shown to inhibit LPL-mediated lipolysis in both in vitro [25] and animal studies [5,26], the sites of action are likely to involve the tissues where LPL is responsible for the hydrolysis of triglyceride; particularly adipose tissue and muscle. However, LPL has also recently been detected in the pancreatic beta cell [27], where fatty acids have been shown to be important modulators of glucose-stimulated insulin secretion (reviewed in Ref. [28]). As we have found an association with insulin secretion and not fasting insulin (a measure of insulin sensitivity), the influence of apoCIII on the beta-cell LPL activity is likely to be of particular importance.

Whatever the mechanism, the observation of significantly elevated NEFA levels co-occurring with a reduced insulin secretion in men supports our prior hypothesis that elevated NEFA levels could be involved in perturbing insulin sensitivity and may be the route through which the APOC3 IRE variants influences insulin/glucose haemostasis (however, they may also be a consequence). The role of elevated plasma free fatty acids in the development of insulin resistance and β-cell dysfunction is now well known (reviewed in Ref. [29]).

It is possible that these effects observed at the IRE polymorphisms could be a result of LD with variants in the newly discovered apoAV gene nearby. However, regression analysis of haplotypes from the apoA1/C3/A4/A5 locus suggested that the APOC3 −482C>T and the APOA5 W19 variants were largely responsible for the effects on triglyceride observed at this locus and had effects on triglyceride levels independent from each other [30]. It remains to be determined whether or not this will also be the case for the effects on insulin/glucose.

Interestingly, the effects of these IRE variants on insulin/glucose and lipids differ considerably between men and women, though this is concordant with the two studies that showed association between the S2 allele of the SstI site and insulin sensitivity [21,22]. However, the smoking-dependent effect on triglyceride was common to both men and women. We have previously shown an almost identical smoking interaction in a large sample ($n=2745$) of healthy middle-aged men [31]. It is uncertain why the effects of these variants on glucose homeostasis should occur only in men. However, a previous analysis of data from individuals in the Ely Study that investigated the determinants of NEFA suppression showed considerable differences between men and women. In men, the strongest association of the area under the NEFA suppression curve was with the 30-min

insulin incremental response, whereas in women, age and BMI were most closely associated to the area under the NEFA curve [32]. Therefore, it is plausible to conclude that associations between insulin secretion and NEFA suppression are stronger in men than in women in keeping with our results. Sex-specific differences were also observed in this cohort for hormone-sensitive lipase variants (− 60C>G), where an effect on insulin sensitivity was found only in women and not in men [33].

Thus, we have shown that variation in the *APOC3* gene influences the 30-min insulin increment, a surrogate measure for insulin secretion and plasma lipid levels in middle-aged men. Given the predictive value of defective insulin secretion in determining progression to type 2 diabetes, these variants may be contributing to the pathogenesis of this disease. However, as the frequency of these variants did not differ between NGT and IGT/diabetic subjects, it is unlikely to be a major susceptibility locus, though the power to detect susceptibility loci in this study is extremely limited. These results should be independently validated in another group with similar characteristics and measures, to confirm that these effects are robust. Investigation of a type 2 diabetes case-control or family-based association study will be necessary to more fully evaluate the role of these variants in this common disease. The recent report showing a OR of 2.5 for CAD with an IRE variant [24] also highlights the importance of these variants in CAD, and the necessity of examining the IRE variants in addition to the more commonly evaluated SstI variant.

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