Variation in \textit{USF1} shows haplotype effects, gene:gene and gene:environment associations with glucose and lipid parameters in the European Atherosclerosis Research Study II

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Upstream stimulatory factor 1 (USF 1), is a transcription factor controlling expression of several genes involved in lipid and glucose homeostasis and co-localizes with familial combined hyperlipidemia (FCHL) and type 2 diabetes on chromosome 1q22–23. We sequenced \textit{USF1} in 24 UK FCHL probands, but found no rare or common cSNPs. Three common intronic single nucleotide polymorphisms (SNP), 306A>G, 475C>T and 1748C>T, were identified and their association was examined with fasting and postprandial lipids and after an oral glucose tolerance test (OGTT) in the European Atherosclerosis Research Study II offspring study. There were no significant differences in allelic frequencies of the SNPs between cases and controls. Individually none of the SNPs showed significant associations with any parameter. In haplotype analysis, compared with other haplotypes, 475C/1748T showed significantly higher and 475T/1748T showed lower peak glucose (\(P = 0.004\) and \(0.07\), respectively) during the OGTT. There was significant case–control heterogeneity in the interaction of genotype with body mass index, on fasting low density lipoprotein with 306A>G and 1748C>T, and on borderline significance with fasting glucose with 475C>T (\(P = 0.002\), 0.0007 and 0.015, respectively). Furthermore, 475C>T showed interaction with both \textit{HSL} 260C>G (case–control heterogeneity \(P = 0.0002\)) on AUC TG and \textit{APOC3} –482C>T on plasma apoE levels (\(P = 0.0012\)). Thus, in these healthy young men, variation in \textit{USF1} was the influencing feature of both glucose and lipid homeostasis showing case–control heterogeneity.

**INTRODUCTION**

Glucose and lipid homeostasis is maintained by the coordinated action of the liver, adipose tissue, muscle and pancreas to sustain plasma glucose levels during fasting and to store energy postprandially. The cellular mechanisms for sensing and responding to glucose and insulin are pivotal in this process. Glucokinase is the crucial component of cellular glucose sensing in the pancreas and liver (1). Insulin acts acutely to stimulate glucose uptake and also makes adaptive metabolic changes by modulating gene expression.

Upstream stimulatory factor 1 (USF 1) is a member of the basic helix–loop–helix leucine zipper (bHLH-zip) family of transcription factors. USF1 binds as a homodimer, or as a heterodimer with the related transcription factor USF2, to palindromic sequences termed E boxes with a consensus sequence of CACGTG (2,3). USFs are ubiquitously expressed and controls the expression of genes involved in glucose and

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lipid metabolism. In the liver, USF1 regulates the expression of fatty acid synthase (FAS), a key enzyme in lipogenesis, in response to glucose (2). In the liver, the expression of the genes for apolipoprotein (apo) A-II (APOA2) (3), apoCIII (APOC3) (4), hepatic lipase (LIPC) (5) and ABCA1 (ABCA1) (6) are also regulated by USF1. USF also controls expression of the glucokinase gene, a critical element in hepatic glucose sensing (7). In adipose tissue, USF1 influences de novo lipogenesis by mediating the insulin-responsive expression of FAS (8) and acetyl-CoA carboxylase (9), as well as the glucose-regulated expression of hormone-sensitive lipase (HSL) (10), the key enzyme in fatty acid mobilization from triglycerides (TG) in adipose tissue. In the pancreas, USF1 also influences the expression of the insulin (11), glucagon receptor (12) and islet-specific glucose-6-phosphatase catalytic-subunit-related (13) genes. These data indicate an important role for USF1 in the co-ordinate regulation of lipid metabolism in response to glucose and insulin signalling.

USF1 on chromosome 1q22–23 (15), co-localizes with familial combined hyperlipidemia (FCHL) (16,17). FCHL is the most common genetic dyslipidemia (18,19) and is characterized by high levels of cholesterol and/or TG (20). We postulated that naturally occurring variation in USF1 might influence lipid homeostasis by influencing several genes, and therefore represented a good candidate gene for a mixed phenotype disorder such as FCHL (21). We sequenced the entire coding region of USF1 in 24 UK men and women with a diagnosis of FCHL (22) but identified only non-coding, intronic single nucleotide polymorphisms (SNPs), suggesting that no single coding variant in USF1 was associated with FCHL. However, in view of the influence of USF1 on lipid and glucose metabolism, we examined the effect of the three most common of these USF1 intronic SNPs on lipid and glycemic measures in the European Atherosclerosis Research Study II (EARS II), a multicentre offsprings study of over 800 healthy young men with baseline and postprandial lipids, fasting insulin and glucose measures after a standard oral glucose tolerance test (OGTT).

RESULTS

We sequenced the USF1 gene from position −603 to +2803 (+1 denoting the A of the ATG initiation codon) in 24 previously studied individuals with FCHL (22), using 13 pairs of overlapping oligonucleotides (Supplementary Material, Table S1). No rare or common coding variants were present, but several intronic polymorphic sites were identified. Three of the most common SNPs, namely 306A>G (rs2073653) in intron 2, 475C>T (rs2073655) in intron 3 and a novel intron 6 SNP, 1748C>T, were further examined in the EARSII study, where fasting lipid and glucose levels and response to both an oral fat tolerance test (OFTT) and an OGTT were available. Baseline characteristics of the EARSII cases and controls have been presented elsewhere (23). Since numbers from each recruitment centre were small, data were analysed by combining recruitment centres into four regions: Baltic (Estonia and Finland); United Kingdom; Middle Europe (Belgium, Denmark, Germany and Switzerland) and South Europe (Greece, Italy, Portugal and Spain). Among the three USF1 variants, there was a departure from Hardy-Weinberg equilibrium for the 1748C>T SNP in controls from the Baltic region, with an excess of observed homozygotes (P < 0.01). Allele frequencies for the three polymorphisms in subjects from the four regions of Europe studied are shown in Table 1, and were similar for cases and controls. There were some regional differences in the 1748T allele frequency (P = 0.007), although no clear North-to-South gradient was apparent. (The minor allele frequencies for the 14 recruitment centres are presented in Supplementary Material, Table S2.) Significant negative pair-wise linkage disequilibrium (LD) coefficients (D') for the USF1 SNPs were seen; D' between 306A>G and 475C>T and 1748C>T were −0.87 (r2 = 0.04) and −0.84 (r2 = 0.05); respectively, and that between 475C>T and 1748C>T was −0.82 (r2 = 0.04), (all D' : P < 0.0001).

Univariate association of the SNPs with quantitative traits

Univariate analyses of the three SNPs with anthropomorphic and lipid measures and parameters of the OFTT and OGTT were carried out. For all three SNPs, analyses by ANOVA showed no statistically significant effects on any parameters (data not shown).

Haplotype analysis and association with parameters of the OGTT

We next examined the combined effect of the SNPs by haplotype analysis. Those haplotypes with frequencies less than 0.01 were excluded from further analysis (Table 2). The associations of the haplotypes with anthropomorphic measures, lipid parameters and parameters of the OFTT and OGTT were examined. While the haplotypes were not associated with fasting levels or response to the OFTT (data not shown), there was a significant association with parameters relating to the OGTT (Table 3). From this data it is clear that compared with the haplotype defined by the common alleles 306A/475C/1748C (ACC), haplotype 306A/475C/1748T (ACT) had a raising effect on area under the curve (AUC) and peak of glucose (P = 0.02 and 0.004, respectively), while the rare 306A/475T/1748T (ATT) haplotype showed a trend toward a lowering effect on peak glucose levels (P = 0.067). This suggests that 306A>G is not influencing the haplotype associations, and it is the combination of 475C>T and 1706C>T which determines the glucose response to the OGTT. On the basis of these initial haplotype results we re-analysed the response to the OGTT using a two-SNP haplotype. Figure 1 shows the association of the 475C>T/1748C>T haplotype with response to the glucose load. Carriers of the 475C/1748T (CT) haplotype had the poorest response to the OGTT, and 475T/1748T (TT) carriers have the best response. Interestingly, among the eight subjects unambiguous for this haplotype (TT), seven were in the control cohort. There was no significant heterogeneity of the results between cases and controls.
USF1 SNPs show interaction with BMI on LDL cholesterol and fasting glucose

The interactions between body mass index (BMI) and the three USF1 SNPs on baseline levels were tested. There were significant interactions between BMI (entered as a continuous variable in the model) and SNPs (tested as dominant) and these were confined only to the cases. BMI and 306A>G on low-density lipoprotein (LDL) cholesterol (in the cases $P = 0.0002$, controls $P = 0.55$, heterogeneity between cases and controls $P = 0.002$); BMI and 1748C>T on LDL cholesterol levels (in the cases $P = 0.0003$, controls $P = 0.40$, heterogeneity $P = 0.0007$) and BMI and 475C>T on fasting glucose levels (in the cases $P = 0.0002$, controls $P = 0.37$, heterogeneity $P = 0.015$) (Fig. 2A–C). For each of the three interactions, correlations between BMI and LDL or glucose were modulated by genotypes in cases, but not in controls. No haplotype effects were seen across the tertiles of BMI, probably owing to the small numbers in each haplotype group.

USF1 475C>T shows interaction with the HSL −60C>G and APOC3 −482C>T

Since USF1 is involved in the control of expression of LIPC (5), HSL (10) and APOC3 (4), all previously studied in EARSII (24–26), we examined the possibility of interaction between the USF1 SNPs and promoter variants, LIPC −514C>T, HSL −60C>G and APOC3 −482C>T, with baseline parameters and response to fat and glucose loads. For this purpose, we used the DICE algorithm which is a data mining tool aimed at exploring all main effects and interactions between polymorphisms belonging to different genes (27). Using the data mining algorithm, we detected an interaction between HSL −60C>G and USF1 475C>T on AUC TG after the fat load, with case–control heterogeneity ($P = 0.0012$) which remained significant after adjustment for fasting TG levels ($P < 0.0002$) (Fig. 3). In cases, AUC TGs were highest in USF1 475T+ carriers who carried the HSL −60G allele, but in the controls USF1 475T+/HSL −60G+ men had the lowest AUC TG levels.

While USF1 475C>T showed a borderline significant effect on plasma apoE levels alone ($P$ for trend $= 0.018$), APOC3 −482C>T showed no significant effects on apoE levels ($P$ for trend $= 0.18$). However, there was a highly significant interaction between the APOC3 −482C>T and the USF1 475C>T ($P = 0.001$) with no case–control heterogeneity. This was particularly evident in APOC3 −482CC men: those with the genotype APOC3 −482CC/USF1 475TT had the lowest apoE levels compared with APOC3 −482CC/ USF1 475CC with an overall $P < 0.0001$ (Fig. 4). In APOC3 −482TT men the interaction with USF1 475C>T genotype showed the opposite trend ($P = 0.029$). There was no interaction between any of the USF1 SNPs and LIPC −524T>C (data not shown).

DISCUSSION

Our interest in USF1 was prompted by the fact that the gene co-localizes with the locus for FHCL on chromosome 1q22–23 (15, 16). Furthermore, since FCHL is a disorder with multiple phenotypes, a transcription factor, such as USF1, involved in the control of gene expression of several genes in lipid metabolism, was a good candidate gene for FCHL. While preparing this manuscript, the study by Pajukanta et al. (28), identified both linkage and association of USF1 with FCHL in Finnish families. Our sequencing of the USF1 coding sequence and flanking regions in FCHL patients confirmed their report of no evidence of rare or common coding SNPs (28). With only common SNPs available for further study, since USF1 controls the expression of genes involved in glucose homeostasis in addition to lipid metabolism, we investigated the association of these USF1 variants in EARSII, a study with fasting lipid measures and response to both an OFTT and OGTT. Individually, none of the USF1 SNPs showed association with any of these parameters.

Haplotype analysis and response to the OGTT

The major haplotype associations were with parameters relating to the response to the OGTT namely peak glucose ($P = 0.002$) and at the 30 min time point for estimating the AUC glucose ($P = 0.006$). These haplotype effects were limited to the combined effects of 475C>T and 1748C>T, with a raising effect of the CT haplotype which was highly significant. Men carrying the rare 475T/1748T (TT) haplotype had 42% lower peak glucose and 21% AUC glucose at the 30 min time point of the OGTT, than men carrying the

Table 1. Minor allele frequencies of USF1 SNPs in cases and controls by region

<table>
<thead>
<tr>
<th>Region</th>
<th>Status</th>
<th>306A&gt;G</th>
<th>475C&gt;T</th>
<th>1748C&gt;T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baltic</td>
<td>Cases</td>
<td>0.18</td>
<td>0.30</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>0.14</td>
<td>0.24</td>
<td>0.29</td>
</tr>
<tr>
<td>UK</td>
<td>Cases</td>
<td>0.12</td>
<td>0.22</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>0.10</td>
<td>0.18</td>
<td>0.41</td>
</tr>
<tr>
<td>Middle</td>
<td>Cases</td>
<td>0.15</td>
<td>0.28</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>0.12</td>
<td>0.28</td>
<td>0.31</td>
</tr>
<tr>
<td>South</td>
<td>Cases</td>
<td>0.17</td>
<td>0.22</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>0.18</td>
<td>0.21</td>
<td>0.33</td>
</tr>
<tr>
<td>All</td>
<td>Cases</td>
<td>0.15</td>
<td>0.26</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>0.13</td>
<td>0.23</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Difference in allele frequency between regions (adjusted for case–control status): 306A>G $P = 0.06$; 475C>T, $P = 0.03$; 1748C>T $P = 0.007$.

Table 2. Estimated haplotypes of the three USF1 SNPs combined (1390 chromosomes)

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>306A&gt;G</th>
<th>475C&gt;T</th>
<th>1748C&gt;T</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>Controls</td>
<td>Cases</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>C</td>
<td>C</td>
<td></td>
<td>0.320</td>
</tr>
<tr>
<td>A</td>
<td>C</td>
<td>T</td>
<td></td>
<td>0.295</td>
</tr>
<tr>
<td>A</td>
<td>T</td>
<td>C</td>
<td></td>
<td>0.229</td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>C</td>
<td></td>
<td>0.134</td>
</tr>
<tr>
<td>A</td>
<td>T</td>
<td>T</td>
<td></td>
<td>0.012</td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>T</td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>G</td>
<td>T</td>
<td>C</td>
<td></td>
<td>0.003</td>
</tr>
</tbody>
</table>

*Excluded from further analyses (too rare).
common 475C/1748C (CC) haplotype. There were no haplotype effects on fasting or postprandial lipid levels.

### Comparison between USF1 genotypic effects in Finnish FCHL families (28) and EARSII

There are similarities and dissimilarities between our results and that of Pajukanta et al. (28), in their study identifying linkage and association of USF1 with FCHL. Most strikingly, as with our sequence analysis of UK FCHL patients, no rare or common cSNPs were identified in USF1 in Finnish FCHL families, suggesting that the impact of variation in USF1 on FCHL is not the result of a single or rare causative coding variant(s). Pajukanta et al. (28) reported a haplotype association, primarily owing to two intronic SNPs, which they termed usf1s1 (intron 11, rs3737787) and usf1s2 (intron 7, rs2073658). Similarly, in EARSII, haplotype analysis showed the strongest effects. The 475C>T (rs2073655) in intron 3 is reported to be in complete LD, in the family data, with usf1s2 (28), and the effects. The 475C>T and 1748C>T appears to be a novel SNP, which we tested for LD with USF1 in Finnish FCHL families (primarily with TG levels and other lipid parameters) and healthy EARSII men (with response to a glucose load).

While in the Finnish study the haplotype effect showed both association and linkage to the FCHL phenotype, there was strong association with TG (particularly in those FCHL men with TG above the 90th percentile), as well as with cholesterol, apoB and small dense LDL, reflecting a FCHL lipid profile (31). In the healthy young EARSII men, a trend was seen with TG levels (data not shown), but these associations were very weak and did not approach statistical significance.

The difference in phenotypic associations seen in the Finnish FCHL families (primarily with TG levels and other lipid parameters) and healthy EARSII men (with response to OGTT) highlights the differences in the study subjects. The association of USF1 SNPs with TG in the Finnish men was primarily in those with raised TG above the 90th percentiles. EARSII men have low TG levels with a mean around 0.91 mmol/l. In the Finnish study, no association with glucose or related parameters was examined.

### USF1 gene : BMI interactions

USF1 SNPs showed interaction with BMI in determining LDL levels (306A>G and 1748C>T) and fasting glucose
Figure 1. Plasma glucose levels over the period of the OGTT by USF1 475C>T/1748C>T haplotypes (f = frequency).

(475C>T). The BMI:genotype interaction in all three instances was seen only in the ‘cases’ (those men with a family history of premature CHD) and not in the controls, with significant heterogeneity of effects (P = 0.0002, 0.0003 and <0.015, respectively). Increased levels of obesity, reflected by high BMI are known to amplify genetic effects, for example the association of the lipoprotein lipase N291S variant with TG is seen only in men in the highest tertiles of BMI (32). Furthermore, FCHL phenotypes have been shown to be modified by BMI (33). It is interesting that even in these young healthy men, with a low mean BMI which does not differ significantly between cases and controls (23.34 ± 0.14 and 23.21 ± 0.13 kg/m², respectively) (23), USF1 genotypes modulate the correlation between BMI and LDL or fasting glucose, but only in the cases.

Gene:gene interaction

LIPC, HSL and APOC3 are three genes whose expression is under the control of USF1 (4,5,10). Since EARSII had previously genotyped promoter variants in these three genes (24–26), we investigated the potential gene:gene interaction between these genes and USF1. The LIPC −514C>T is known to disrupt an USF1-binding site (5), however, there was no interaction between USF1 and LIPC −514C>T on any of the intermediate traits. There was, however, once again significant case–control heterogeneity in the interaction between HSL −60C>G and USF1 475C>T on AUC TG after the fat meal. The HSL −60C>G change is functional, with −60G displaying 40% lower promoter activity (34). There is a potential USF1-binding site, starting 42 bp upstream of the −60C>G site (identified by a transcription factor identifying programme TRANSPLORER™, Biobase Gmbh). The close proximity of the −60C>G and the E box motif suggests that there might be a molecular interaction between these two sites, and we are currently investigating their potential interaction. HSL is the primary TG hydrolysing enzyme in adipose tissue and is central to free fatty acid homeostasis; the −60C>G has been shown to be associated with non-esterified fatty acid levels (35). HSL is also expressed in pancreatic β-cells and variation in the gene has been associated with insulin sensitivity (36). In EARSII, we previously showed that the HSL −60C>G was associated with fasting insulin using the homeostatic model assessment of insulin resistance (25). HSL expression is controlled post-translationally, with activation by catecholamines and inactivation by insulin. The functional effect of the promoter variant identified, in addition, a role for transcriptional control of HSL. We speculate that after the fat challenge, when insulin levels rise, HSL levels would be reduced. In the current study, the interaction of HSL −60C>G with USF1 475C>T on postprandial TG suggests a molecular relationship between the two genes and a fine tuning of HSL transcriptionally. This interaction once again showed strong case–control heterogeneity (P = 0.001 and after adjustment for fasting TG, P < 0.0002).

ApoCIII is a major component of TG rich lipoproteins (TRLP), which plays an important role in the regulation of TG metabolism. It is suggested that apoCIII does this by acting as an inhibitor for lipoprotein lipase (37) and displacing apoE, a major ligand for LRP and LDLR, from the TRLP (38), thus reducing TG catabolism both by reduced hydrolysis and receptor-mediated clearance. The APOC3 −482C>T disrupts an insulin responsive element and has been shown to be functional (39). We and others have shown that the APOC3 −482C>T is associated with differences in TG levels, exacerbated by smoking (40,41). In EARSII, −482C>T determined response to the OGTT with −482T carriers having elevated glucose and insulin levels after the glucose load. Here, we identified an interaction of APOC3 −482C>T and USF1 475C>T on fasting apoE levels. On its own, genetic variation in USF1 affected apoE levels, (P = 0.02), while APOC3 −482C>T did not. Together these genes showed interaction (P < 0.001) which suggests a molecular interaction of the two genes influencing lipoprotein particle composition. In this instance there was no case–control heterogeneity. Using a bandshift assay, Pastier et al. identified two USF1 sites in the APOC3 promoter, which did not have classical E-box motifs. These are located at positions −58 to −31 and −19 to −4, on either side of the TATA box, in the promoter of APOC3 (4). Using, TRANSPLORER™, we identified an additional potential USF1-binding site with a classical E-box motif, at position −440 to −427, 40 bp downstream of the −482, which suggests that its proximity to the −482 polymorphic site might enable molecular interaction. ApoE is carried primarily on very low density lipoprotein (VLDL).
and its remnants, thus high apoE reflects slow clearance of these remnants particles or overproduction of TG rich lipoproteins, one of the features of FCHL (21).

In conclusion, in EARSII the impact of \textit{USF1} haplotypes was primarily reflected in response to an OGTT, which suggests that in these healthy young men effects were seen only under stress, as with the glucose challenge, and not on fasting glucose levels. Furthermore, additional effects were different in those men with a family history of premature CHD and the age-matched controls. Thus \textit{USF1} may be an

Figure 2. Interaction between BMI and \textit{USF1} SNPs showing heterogeneity between cases and controls. (A) Interaction between 306A$\rightarrow$G and BMI on LDL cholesterol levels. Cases: AA = 253, AG = 90, GG = 8; controls: AA = 291, AG = 84, GG = 7. (B) Interaction between 1748C$\rightarrow$T and BMI on LDL cholesterol levels. Cases: CC = 171, CT = 141, TT = 28; controls: CC = 172, CT = 147, TT = 50. (C) Interaction between 475C$\rightarrow$T and BMI on fasting glucose levels. Cases: CC = 191, CT = 127, TT = 26; controls: CC = 221, CT = 128, TT = 21.
important determinant of insulin resistance. The gene:gene interactions highlight the wide influence of USF1 in controlling the expression of genes influencing both lipid and glucose metabolism. These findings need confirmation, but suggest that in view of the fact that USF1 also co-localizes with type 2 diabetes (42), it might prove to be a gene with wider consequences than just FCHL determination.

**MATERIALS AND METHODS**

**Study population**

The patients with a clinical diagnosis of FCHL have been described elsewhere (22). The EARSII study was undertaken to compare postprandial responses (OGTT and OFTT) of male cases \( n = 407 \), aged between 18 and 28 years, whose father had a proven myocardial infarction before the age of 55 years and male controls \( n = 415 \) who were age-matched and recruited irrespective of family history. The subjects were recruited from 14 European university student populations from 11 European countries which were divided into four regions on the basis of ischemic heart disease rates for each country, as well as geographic and linguistic proximity: Baltic (Estonia and Finland); United Kingdom; Middle Europe (Belgium, Denmark, Germany and Switzerland) and South Europe (Greece, Italy, Portugal and Spain). Owing to sample loss no control samples were available from Portugal. The study design has been described previously (23).

**Postprandial tests**

A standard 75 g oral glucose load was administered after a 12 h overnight fast. Venous blood samples were drawn at 0, 30, 60, 90 and 120 min for determination of insulin and glucose concentrations. One week later the participants had an OFTT. The standard fat meal consisted of 1493 kcal of energy; 21.6 g dairy milk protein, 56.2 g carbohydrates and 65.5 g dairy milk fat (of which 41.6 g was saturated). The cholesterol content was 416.6 mg. The meal was administered after a 12 h overnight fast. Blood samples were withdrawn at 0, 2, 3, 4 and 6 h for determination of TG concentrations. Subjects were instructed to keep physical activity to a minimum and to refrain from smoking during the 6 h of the test.

**Laboratory measurements**

Laboratory measures were performed centrally. Plasma TG was measured in Glasgow (UK) according to the Lipid Research Clinics Manual of Laboratory Operations and standardized to CDC, Atlanta, USA. Glucose was measured in Glasgow by the hexokinase/glucose 6-phosphate dehydrogenase end-point method. Insulin was measured by radioimmunoassay.

**Identification of common SNPs in USF1**

Using 13 overlapping sequencing primer pairs (Supplementary Material, Table S1), the entire USF1 genomic sequence, spanning the coding region (the ATG initiation codon numbered as +1) from −603 to +2203 was sequenced in DNA samples from 24 UK men and women diagnosed with FCHL (22), to identify any rare mutations and common SNPs. Protocols
were followed for the use of the DYEnamic ET dye Terminator Cycle Sequencing kit and the MegaBACE DNA analysis system (Amersham Biosciences).

Genotyping conditions

All amplification reactions contained 8 pmol of each primer, 0.25 U of Taq polymerase and 3 mM concentration of MgCl₂ in a final volume of 20 µl. Details of the amplification primers, and SNP identification are presented in Supplementary Material, Table S3. Initially, each reaction was heated to 95°C for 5 min, then cycled for 30 repeats. All restriction enzyme digests were carried out in a final volume of 15, 10 µl of PCR product, 1.5 µl of appropriate 10× buffer and 5 U of enzyme. Fragments were separated on 7.5% polyacrylamide using microplate array diagonal gel electrophoresis (MADGE) (43) and visualized directly after staining with ethidium bromide.

Statistical analysis

Analyses were performed using the SAS statistical software (SAS Institute Inc, Cary, NC). Hardy–Weinberg equilibrium was tested by a χ² test with 1 degrees of freedom separately in cases and controls from the four European regions described earlier. Allele frequencies were deduced from the genotype frequencies and the differences between regions and case–control status were tested by a χ² test. Pairwise linkage disequilibrium coefficients between polymorphisms were estimated using log-linear analysis and their extents were expressed as the ratio of the unstandardized coefficients to their maximal value (D') (44,45).

For all the analyses, the following phenotypes were studied: BMI, waist/hip ratio, blood pressure, cholesterol, LDL-cholesterol, high-density lipoprotein (HDL) cholesterol, apoB, apoA1, apoE, LpCIII/B at baseline and at 4 h after the OFTT, fasting glucose, fasting insulin, fasting TG, AUC TG, peak TG, AUC of glucose, peak glucose, AUC insulin and peak insulin. Anthropomorphic measures and fasting lipid levels, according to genotype, were studied by an analysis of variance (SAS-PROC GLM) adjusted for age, recruitment centre and case–control status. The postprandial response was compared across genotypes using analysis of variance for fasting levels, for the AUC above the fasting concentration (calculated by the trapezoidal rule), for the peak (calculated as the highest value minus the fasting value). For distributions positively skewed, a power transformation (log or square-root) was applied. In particular, the peak of glucose and AUC of TG were square-rooted and apoE was log-transformed. Total numbers for TG at OFTT, fasting glucose, fasting insulin, fasting TG, AUC of glucose, peak glucose, AUC insulin and peak insulin.

Heterogeneity of the effects between cases and controls was systematically tested for all analyses on phenotypes. No adjustments for multiple comparisons were made, following the arguments of Rothman (46) and Perneger (47,48), which suggest that a Bonferroni adjustment is over conservative. Because of this, and since this was a hypothesis generating study, to take into account the concern of multiple comparisons, a more conservative P-value of 0.01 was taken as the level for statistical.

To analyse simultaneously the three studied polymorphisms, haplotype analysis was performed by use of a maximum likelihood model (49) based on the stochastic-EM algorithm (50) implemented in the THESIAS program (http://www.genecanvas.org). In these analyses, the haplotype combining the most frequent alleles at each locus was used as the reference. Interactions of genotypes with BMI, was tested with each polymorphism in the cases and controls separately, since heterogeneity between cases and controls was detected.
Interactions between the USF1 SNPs and the LIPC – 514 C>T, HSL – 60C>G and the APOC3 – 482C>T were investigated using our recently developed DICE algorithm, which explores in an automated way the effect of all combinations of one, two or three polymorphisms on the phenotypes. To avoid difficulties related to the null-hypothesis testing of one, two or three polymorphisms on the phenotypes. To investigated using our recently developed DICE algorithm, which was forced in the models as a stratification variable potentially interacting with combinations of polymorphisms (27).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

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REFERENCES

29. Gretarsdottir, S., Thorleifsson, G., Reynisdottir, S.T., Manolescu, A., Jonsdottir, S., Jonsdottir, T., Gudmundsdottir, T., Bjarnadottir, S.M.,


46. Rothman, K.J. (1990) No adjustments are needed for multiple comparisons. Epidemiology, 1, 43–46.


### APPENDIX

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