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Objective—Activating transcription factor 6 (ATF6) is a sensor of the endoplasmic reticulum stress response and regulates expression of several key lipogenic genes. We used a 2-stage design to investigate whether ATF6 polymorphisms are associated with lipids in subjects at increased risk for cardiovascular disease (CVD).

Methods and Results—In stage 1, 13 tag-SNPs were tested for association in Dutch samples ascertained for familial combined hyperlipidemia (FCHL) or increased risk for CVD (CVR). In stage 2, we further investigated the SNP with the strongest association from stage 1, a Methionine/Valine substitution at amino-acid 67, in Finnish FCHL families and in subjects with CVR from METSIM, a Finnish population-based cohort. The combined analysis of both stages reached region-wide significance ($P=9\times10^{-4}$), but this association was not seen in the entire METSIM cohort. Our functional analysis demonstrated that Valine at position 67 augments ATF6 protein and its targets Grp78 and Grp94 as well as increases luciferase expression through Grp78 promoter.

Conclusions—A common nonsynonymous variant in ATF6 increases ATF6 protein levels and is associated with cholesterol levels in subjects at increased risk for CVD, but this association was not seen in a population-based cohort. Further replication is needed to confirm the role of this variant in lipids. **(*Arterioscler Thromb Vasc Biol*. 2009;29:1322-1327.)**

Key Words: activating transcription factor 6 ■ cardiovascular risk ■ cholesterol ■ association ■ lipids

High serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and apolipoprotein B (apoB) are major risk factors for cardiovascular disease (CVD). Cholesterol levels are tightly regulated through a feedback pathway from the endoplasmic reticulum (ER) to the nucleus.[1] Sterol regulatory element binding protein 2 (SREBP2), an ER resident transcription-factor, has a central role in cellular cholesterol homeostasis.[2] In response to cholesterol demand the active domain of SREBP2 is released to the cytoplasm and migrates to the nucleus where it binds sterol response element (SRE) sequences in the promoter of target genes, such as the LDL receptor gene.[2]

ATF6 is also an ER membrane-bound transcription-factor and a major regulator of the unfolded protein response (UPR).[3] The UPR is an evolutionary conserved stress-signaling pathway from the ER to the nucleus. Various stressful conditions, eg, nutrient deprivation, oxidative stress, high and low glucose concentrations, viral infections, obesity, and increased synthesis of secretory proteins, lead to accumulation of unfolded proteins and trigger the UPR.[4,5] Besides a role for ATF6 in the UPR, lipogenic properties have been ascribed to ATF6, and crosstalk with the SREBP2 pathway has been demonstrated.[6] There is growing evidence that ATF6-mediated attenuation of SREBP2 fine-tunes the cholesterol biosynthetic pathway in cells under conditions of ER stress.[6] More specifically, Zeng et al demonstrated in vitro that changes in glucose levels influences lipogenesis via ATF6-mediated inhibition of SREBP2.[6] Additional parallel

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pathways through which ATF6 could modulate cholesterol homeostasis may exist, suggested by the presence of ATF6-binding elements in the promoter of the apoB gene.[7]

We investigated whether genetic variation in the ATF6 gene is associated with plasma TC, LDL-C, and apoB levels, and whether it contributes to the complex genetic background of CVD. We used a 2-stage design. In stage 1, we performed genotyping of tag-SNPs in the ATF6 gene region to test for association in Dutch samples ascertained for familial combined hyperlipidemia (FCHL) or increased risk for CVD (CVR). An amino-acid substitution (methionine[67]valine) with the strongest evidence of association was further investigated in stage 2 study samples. We also functionally demonstrated that this variant augments ATF6 protein levels and its downstream targets.

Methods

For complete description of the Methods, please see the online supplemental material available at http://atvb.ahajournals.org.

Study Participants

The study design was approved by the ethics committees of the participating centers, and all subjects gave written informed consent.

Stage 1 study samples consisted of Sample 1 (Dutch CVR) with a total of 393 unrelated subjects at increased risk for CVD, ie, age 40 to 70 years and either hypertension (HT), or body mass index (BMI) >25 kg/m^2 from the Cohort study of Diabetes and Atherosclerosis Maastricht,[8] and Sample 2 (Dutch FCHL) with a total of 195 unrelated probands and spouses from families with FCHL.[9]

Stage 2 study samples consisted of Sample 3 (Finnish FCHL) with 715 individuals from 61 Finnish FCHL families,[9] and Sample 4 (Finnish CVR) with 1371 subjects with CVR selected from 5112 male subjects in the ongoing Finnish population-based cohort, METSIM (METabolic Syndrome In Men),[9] using the same ascertainment criteria as in Sample 1. All of these study samples are described in detail in the supplemental Methods.

Statistical Analyses

Association analyses with continuous traits were performed using linear regression for the genotypic model. The genotypic test is a 2 degrees of freedom test of an additive ($\hat{\beta}_{add}$) and a dominance-deviation ($\hat{\beta}_{dev}$) effect. The $\hat{\beta}_{dev}$ coefficient reflects a deviation from an additive effect. A recessive character is suggested when the sign of $\hat{\beta}_{dev}$ is opposite of $\hat{\beta}_{add}$, and a dominant character is suggested when the signs of both coefficients are in the same direction. A full recessive or dominant model is observed when the magnitude of the effects is equal $|\hat{\beta}dev| = |\beta add|$. To avoid violating the assumptions of the test statistics, we estimated empirical probability values in the combined analysis of Sample 1 and 2 by combining the χ^2 statistics from 20 000 random permutations of each sample.[9] Association analysis in families (Sample 3) was performed with the genotypic model of the family-based association test (FBAT) software.[10] The combined analysis of stage 1 and 2 and the meta-analysis of stage 1, Sample 3 and the METSIM cohort were performed using Fisher method[11] for combining probability value, as described in the supplemental Methods.

Cell Studies

Primary preadipocytes were isolated from subcutaneous adipose tissue from 11 subjects who underwent fat biopsies. HeLa cells were used for transfection experiments with the following plasmids: Cytomegalovirus-galactosidase; Flag-ATF6-(1–373[67]-Methionine); Flag-ATF6-(1–373[67]-Valine); Flag-ATF6-(1–373[67]-Leucine); Grp78 promotor(−284–+221)-Luciferase, and pGL2p.

Reporter assays included luciferase and β-galactosidase assays. Western blot experiments were done using specific antisera against ATF6, FLAG, KDEL (Grp78 and Grp94), βactin, and GAPDH.

Results

ATF6 Protein Levels Correlate With Plasma Lipid Levels

We investigated the potential role of the ATF6 gene in hyperlipidemia by first evaluating the relation between basal ATF6 levels in cultured human primary preadipocytes and plasma TC, LDL-C, and apoB levels. We observed a significant positive correlation between ATF6 levels in vitro and plasma TC, LDL-C, and apoB levels of the corresponding subjects ($r=0.65$, $P=0.032$; $r=0.72$, $P=0.018$; and $r=0.76$, $P=0.006$, respectively; supplemental Figure 1).

Stage 1 Association Analysis

We used a 2-stage design to investigate whether variants within the ATF6 gene are associated with lipid levels in subjects at increased risk to develop CVD. In stage 1, tag-SNPs selected to capture the common genetic variation in ATF6 were investigated in 2 independent Dutch samples comprising 393 individuals with increased CVR (Sample 1), and 195 unrelated FCHL probands and their spouses (Sample 2). In stage 2, the strongest signal was further investigated in 2 Finnish studies: 715 subjects from 61 FCHL families (Sample 3) and in 1371 subjects with CVR (Sample 4) from the METSIM cohort. Finally, a combined analysis of the 2 stages was performed to reach a region-wide significance. Clinical characteristics of the study samples are shown in supplemental Table 1.

In stage 1, we tested a total of 13 SNPs for association with TC, LDL-C, and apoB levels using multivariate linear regression for the genotypic model. (The most significant association was observed for SNP3 (rs1058405) with TC ($P=0.009$, $b_{dev}=-0.2$ to -0.3 and $b_{add}=0.2$ to 0.3), LDL-C ($P=0.008$, $b_{dev}=-0.2$ to -0.3 and $b_{add}=0.2$) and apoB ($P=0.002$, $b_{dev}=-0.3$ and $b_{add}=0.3$) (Table 1 and Figure 1). The distributions of TC, LDL-C, and apoB between the genotype groups suggest a recessive effect for SNP3, which is also demonstrated by the equal magnitude and opposite sign of the additive and dominance-deviation effects. SNP8 and SNP11 were associated with probability values <0.05 (supplemental Table 1 and supplemental Figure 1) but only SNP3 resulted in probability values <0.05 for all traits. We confirmed that the direction of the association is the same in both Sample 1 and 2 for all SNPs with probability value <0.05, as the χ^2 statistics could go in either direction. Furthermore, it should be noted that SNP3 is in high LD with SNP8 ($r^2=0.7$) and in low LD with SNP11 ($r^2=0.2$).

Next, we used an imputation-based regression method to extend our association analysis to nontagged SNPs in the ATF6 region (supplemental Methods). Furthermore, as we test for association with all common SNPs (MAF $>5\%$) in the region using a Bayesian-regression approach, we can statistically assess which SNP (tagged or nontagged) most likely affects the traits. We obtained the strongest evidence of association for SNP3 (ie, the largest estimated Bayes Factor [BF]) for all the traits (Figure 1). The BF we observed for apoB (BF=1.03) can be considered as strong evidence of association, and the ones for LDL-C (BF=0.77) and TC (BF=0.6) as substantial evidence of association. Taken to-

Table 1. Association of Met[67]Val Substitution (SNP3) in Stage I

		Sample1 Dutch CVR				Sample2 Dutch FCHL				Stage I
Trait	Met[67]Val	n=384	$\hat{\beta}^*_{add}$ (SE)	$\hat{\beta}^*_{dev}$ (SE)	R^2†	n=181	$\hat{\beta}^*_{add}$ (SE)	$\hat{\beta}^*_{dev}$ (SE)	R^2†	*P*‡
TC, mmol/L	Val/Val	5.55±0.15				6.72±0.29				
	Met/Val	5.20±0.07	0.19 (0.9)	−0.21 (0.12)	1.20%	5.90±0.17	0.28 (0.11)	−0.33 (0.17)§	3.64%	0.009
	Met/Met	5.22±0.07				5.96±0.13				
LDL-C, mmol/L	Val/Val	3.72±0.14				4.43±0.26				
	Met/Val	3.28±0.06	0.23 (0.9)	−0.30 (0.12)§	2.01%	3.94±0.15	0.21 (0.11)	−0.20 (0.17)‖	2.03%	0.008
	Met/Met	3.34±0.06				3.90±0.12				
ApoB, g/L	Val/Val	1.24±0.04				1.31±0.07				
	Met/Val	1.10±0.02	0.25 (0.9)	−0.33 (0.12)§	2.45%	1.11±0.04	0.31 (0.12)	−0.32 (0.17)	4.16%	0.002
	Met/Met	1.12±0.02				1.11±0.03				

Trait values represent the marginal mean evaluated at the average age and sex±SEM.
*$\hat{\beta}_{add}$, indicates the standardized beta coefficients per each copy of the rare allele (additive term) and $\hat{\beta}_{dev}$ for the dominance-deviation term.
†R^2 indicates the proportion of variance explained by the genotypic model.
‡The *P* values represent the results of the combined analysis of Sample 1 and 2, as described in Methods.
§$P<0.05$ for the significance of deviation from an additive model ($\hat{\beta}_{dev} \neq 0$).
‖$P>0.1$ for the significance of deviation from an additive model.

gether, these data suggest that in Stage 1, SNP3 is the best regional-candidate for affecting plasma lipid levels.

SNP3 is a coding variant that translates into the Methionine[67]Valine amino-acid substitution (supplemental Figure 2B). According to the dbSNP-database SNP3 is possibly a triallelic variant (A-G-T →Met-Val-Leu). However, the leucine variant is most likely a sequencing error as it was originally identified in a sample size of 4 individuals with validation status unknown. Furthermore, we did not observe the leucine variant in 200 subjects of Sample 1 that we specifically screened using an allele-specific primer for it.

Stage 1 and 2 Association Analysis

SNP3 that provided a probability value <0.05 for all traits in the stage 1 analysis was tested in a combined analysis of stage 1 and 2. We analyzed the stage 2 samples using the same genotypic model as in stage 1 and performed a combined analysis of the 2 stages using Fisher method[11] for combining probability values weighted by the sample size. This analysis included a total of 2674 subjects at increased risk to develop CVD. We observed a significant association between SNP3 and TC ($P=9\times10^{-4}$), LDL-C ($P=0.007$), and apoB ($P=0.005$) levels (Table 2). Similar to the results obtained in the stage 1 samples, higher lipid levels were also observed with the Val allele in the stage 2 samples (Table 2). In the FCHL families (Sample 3), both the lipid genotypic mean values and the Z-scores for the homozygote rare group (all $Z_{V/V} > 1.7$) suggest a recessive effect for SNP3, similar to Samples 1 and 2 (Table 2). However, an additive effect could not be rejected in the CVR sample selected from the METSIM cohort (Sample 4), as none of the $\hat{\beta}_{dev}$ terms where significantly different from zero (Table 2). The association between SNP3 and TC levels is region-wide significant, as it surpasses the Bonferroni correction for 13 SNPs tested in

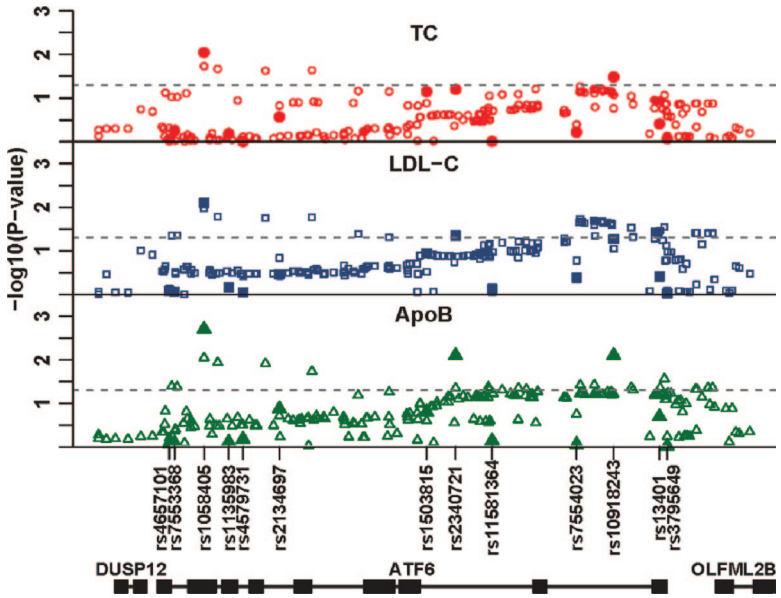

Figure 1. Association results of stage 1. Filled symbols represent the −log10 of the probability values obtained from linear regression test for the genotypic model. The open symbols represent the −log10 of the probability values obtained from Bayesian regression test with SNP imputation. The dashed lines indicates $P=0.05$. The location of the SNPs is shown in relation to the gene structure of ATF6 and its adjacent genes.

Table 2. Association of Met[67]Val Substitution (SNP3) in Stage I and II

		Sample 3 Finnish FCHL				Sample 4 Finnish CVR				Stage I+II
Trait	Met[67]Val	n=715	$Z^*_{V/V}$	$Z^*_{M/V}$	R^2†	n=1371	$\hat{\beta}$‡$_{add}$ (SE)	$\hat{\beta}$‡$_{dev}$ (SE)	R^2†	*P*§
TC, mmol/L	Val/Val	6.47±0.23				5.61±0.09				
	Met/Val	5.96±0.11	1.78	−1.68	2.37%	5.56±0.04	0.09 (0.05)	0.05 (0.06)‖	0.54%	0.0009
	Met/Met	6.15±0.12				5.43±0.04				
LDL-C, mmol/L	Val/Val	4.22±0.20				3.60±0.08				
	Met/Val	3.82±0.10	2.07	−1.71	2.34%	3.57±0.04	0.07 (0.05)	0.04 (0.06)‖	0.32%	0.0072
	Met/Met	3.99±0.10				3.48±0.03				
ApoB, g/L	Val/Val	1.32±0.07				1.10±0.03				
	Met/Val	1.11±0.03	2.32	−1.32	3.03%	1.12±0.01	0.03 (0.05)	0.08 (0.07)‖	0.28%	0.0049
	Met/Met	1.13±0.03				1.09±0.01				

Trait values represent the marginal mean evaluated at the average age and sex±SEM.
*$Z_{V/V}$, indicates the Z value obtained from the FBAT genotypic model test for the Val/Val genotype and $Z_{M/V}$ for the Met/Val genotype.
†R^2 indicates the proportion of variance explained by the genotypic model.
‡$\hat{\beta}_{add}$, indicates the standardized beta coefficients per each copy of the rare allele (additive term) and $\hat{\beta}_{dev}$ for the dominance-deviation term.
§The *P* values represent the results of the combined analysis of stage 1 and 2, as described in Methods.
‖*P*>0.1 for the significance of deviation from an additive model.

stage 1 with 3 traits, as well as 6 additional tests performed in stage 2 (Adjusted probability value 0.0009×45=0.04). However, it should be noted that as we tested 3 correlated traits (average correlation=0.85) the Bonferroni correction for multiple testing, that presumes that tests are independent, is conservative for our analyses.

Meta-Analysis of SNP3 in a Population-Based Cohort

As we obtained a region-wide significant association between SNP3 and plasma TC levels, we also included all available subjects of the METSIM cohort (ie, CVR and non-CVR) in a meta-analysis of all samples weighted by the proportion of the sample size. We did not observe significant associations for TC in meta-analysis of all subjects (n=5812, χ^2=4.42, *P*=0.62). However, as we excluded subjects with T2DM from our study (Stage 1 and 2) we also performed the meta-analysis while excluding subjects with T2DM or family history of T2DM from METSIM. The subjects with family history of T2DM also exhibit impaired glucose homeostasis, as shown in supplemental Table 3. We observed a significant association between SNP3 and TC levels (n=3471, χ^2=18.09, *P*=0.006), suggesting that the effect of SNP3 may be different between individuals with and without T2DM and history of T2DM.

Furthermore, the effect of SNP3 was more pronounced in the stage 1 and 2 analysis that included subjects from METSIM with CVR (Sample 4; Table 2). To further investigate this relationship between SNP3, T2DM, and CVR, we examined the effect of their multiplicative interaction on serum TC levels (supplemental Methods). We observed that plasma TC levels are significantly affected by the interaction of SNP3 and T2DM or family history of T2DM (standardized$\hat{\beta}$gr=−0.12 [SE=0.05], *P*=0.01), whereas an opposite direction of interaction was observed between SNP3 and CVR on serum TC levels (standardized $\hat{\beta}$gr=0.10 [SE=0.05], *P*=0.03). These data suggest opposite effects for the Val variant on TC levels between individuals with T2DM and CVR, with a decreasing effect in subjects with T2DM or family history of T2DM and an increasing effect in subjects with CVR.

Functional Characterization of the Methionine[67]Valine Variant

The Met[67]Val substitution is located in the transcription activation domain (TAD) of ATF6, more specifically in the VN8-like region (amino-acid 61 to 68), which is critical for transcription activity and degradation of ATF6 (supplemental Figure 2).[12] We observed that this amino acid substitution was computationally predicted to be damaging to the protein (PSIC score=2.08) based on phylogenetic and structural information (PolyPhen server tool).[13] To explore the possibility that the Met[67]Val polymorphism alters the protein function, we assessed the effect of amino acid substitution at position 67 on the protein levels and transcriptional activity of ATF6 in vitro. Although the Leucine-variant is probably a sequencing error, we also tested it functionally. Accordingly, we prepared 3 constructs of FLAG-tagged ATF6(1–373) that mimic active ATF6 generated by ER stress. These 3 constructs only varied in amino acid 67. We tested the 3 constructs in HeLa cells by assaying for luciferase expression through the GRP78 promoter. The constructs, FLAG-ATF6(1–373)-[67]-valine and FLAG-ATF6(1–373)-[67]-leucine had significantly higher luciferase activity than the Met allele construct (1.4- and 2.5-fold change, respectively; Figure 2). We also compared the protein levels of endogenous Grp78 and Grp94, which are direct targets of ATF6, in cells transfected with FLAG-ATF6 constructs. In agreement with the results from the luciferase assay, the levels of Grp78 and Grp94 were significantly higher in cells transfected with FLAG-ATF6(1–373)-[67]-valine and FLAG-ATF6(1–373)-[67]-leucine, than those transfected with the Met allele construct (Figure 3A and 3B). Furthermore, FLAG-ATF6 levels were also increased, suggesting a gain-of-function substitution. Taken together, these functional data demonstrate that this variant augments ATF6 protein levels and its downstream transcriptional targets.

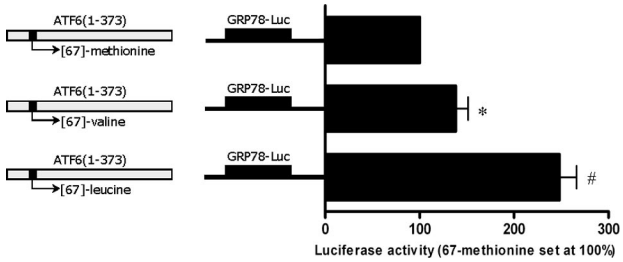

Figure 2. Potency of FLAG-ATF6(1–373)-[67]-methionine, FLAG-ATF6(1–373)-[67]-valine, and FLAG-ATF6(1–373)-[67]-leucine to induce luciferase expression in HeLa cells through the GRP78 promoter. HeLa cells were transfected with 1 of the FLAG-ATF6(1–373) plasmids (1 μg). Cells were cotransfected with either pGL2p, or GRP78-luciferase reporter plasmid (10 μg), and CMV-β-gal (1 μg). Each value represents the mean and SEM of 4 independent transfections from triplicate cultures. *$P<0.05$, #$P<0.001$.

Discussion

Our 2-stage association analysis of tag-SNPs in the ATF6 gene revealed a region-wide significant association between the Met[67]Val amino-acid substitution and increased TC levels in samples ascertained for FCHL and risk factors for CVD. Furthermore, we also functionally demonstrate that the Met[67]Val substitution augments ATF6 levels and its downstream transcriptional targets. These findings are also supported by a strong positive correlation between the ATF6 protein levels in cultured preadipocytes and plasma lipids levels in the corresponding individuals. Obviously, human liver would have been the most preferable tissue for these types of protein experiments. However, liver biopsy is an invasive procedure that can cause potentially life-threatening complications. Therefore, we used adipose biopsies, also relevant for studies of lipid metabolism.

The Met[67]Val substitution in ATF6 (rs1058405) is a common variation. Its MAF ranges from 7% in black to 23% and 29% in Asian and European populations, respectively. It is an excellent functional candidate as it resides within the VN8-like domain of ATF6 (supplemental Figure 2). The VN8 domain was identified in the virion protein 16 (VP16) transcription factor from herpes simplex virus type I and is required for transcriptional activation and rapid proteasomal degradation of VP16.[14,15] More recently, Thuerauf et al identified a VN8-like domain in ATF6, which is 75% identical to the VN8 domain in VP16.[12] Analogous to VP16, the ATF6-VN8 region was found to be critical for transcriptional activity and subsequent degradation of ATF6. Mutating amino acid 62 and 64 in the ATF6-VN8 region caused a 5-fold reduction in transcriptional activity.[12] Our transfection experiments with mutated ATF6 constructs revealed that the valine variant at position 67 increases ATF6 transcriptional activity in cultured cells. Furthermore, ATF6 levels were increased with the Val allele when compared to the Met allele construct. According to the dbSNP-database rs1058405 is possibly a triallelic variant (Met-Val-Leu). The leucine variant could be a sequencing error as it was originally identified in a sample size of 4 individuals with validation status unknown, and we did not detect it in 200 subjects that we specifically screened for the leucine variant. Nevertheless, mutating amino acid 67 to leucine further increased ATF6 levels, as well as its targets, Grp78 and Grp94, illustrating the importance of amino acid 67 for ATF6 transcription activity.

Zeng and colleagues recently elucidated a potential molecular mechanism through which ATF6 can modulate cholesterol homeostasis.[6] In response to glucose starvation, activated ATF6 can interact with SREBP2 and bind as a heterodimer to SREs in the promoters of cholesterogenic genes, eg, the LDL receptor. Bound to SRE, the ATF6-SREBP2 heterodimer recruits HDAC1, and together they exert a major inhibitory effect on SREBP2 transcription activity.[6] It is tempting to propose an additional inhibitory effect on SREBP2 activity attributable to the Met[67]Val gain-of-function-polymorphism, which would further reduce the number of LDL receptors that bind and internalize LDL and VLDL particles, consequently increasing plasma cholesterol levels. Recently, it has also been demonstrated that prolonged high glucose conditions induce ER stress and ATF6 activity.[5] These findings both suggest that glucose levels may present a confounding factor in the genetic analysis of ATF6. We observed an interaction between the valine variant and T2DM on plasma TC levels, suggesting that impaired glucose homeostasis may confound the associations with ATF6. However, the potential confounding role of glucose homeostasis and the exact mechanism through

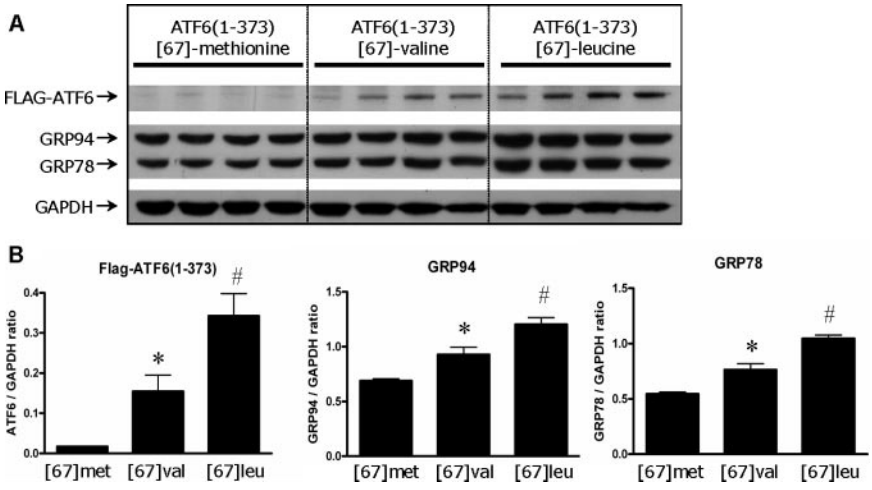

Figure 3. Potency of FLAG-ATF6(1–373)-[67]-methionine, FLAG-ATF6(1–373)-[67]-valine, and FLAG-ATF6(1–373)-[67]-leucine to induce endogenous GRP94 and GRP78 levels. A, HeLa cells, transfected with 1 of the FLAG-ATF6(1–373) plasmids (1 μg), were extracted and analyzed for expression of FLAG-ATF6, Grp78, Grp94, and GAPDH by Western blot using specific antiserum antibodies for FLAG, KDEL (Grp78 and Grp94), and GAPDH, respectively. B, Quantification of the blots by densitometry analysis. Each value represents the mean and SEM of 4 independent transfections). *$P<0.05$, #$P<0.01$.

which the Met[67]Val substitution in ATF6 affects cholesterol metabolism remains to be addressed in future studies.

Recently, the GWAS data of predominantly population-based cohorts were analyzed for lipid traits.[16] SNP3 was not significantly associated with LDL-C in this meta-analysis using an additive model ($P>0.05$). However, as the types of genes identified in a GWAS naturally depend and reflect the characteristics of the study samples, it is likely that other variants and genes will be identified in studies based on CVR. In our analyses of all samples and the METSIM cohort, the association signal originated specifically from subjects at increased cardiovascular risk without T2DM or family history of T2DM. Notably, although our combined probability value of both stages indicates a significant overall association, the association signal and the proportion of variance explained by SNP3 were less significant in Sample 4, selected from the population based METSIM cohort. Furthermore, a recessive mode of inheritance was suggested throughout the samples except for Sample 4, as evident by the sign and the significance of the dominant-deviation term. These differences may reflect the fact that population-based cohorts are not optimal for detecting modest effects, particularly those that are largely recessive. As CVD risk factors are highly prevalent in the population because of modern lifestyle, sampling cases from a population-based cohort hampers the possibilities to collect cases based on the true genetic background. This environmental "noise" may have resulted in a limited power to detect the effect of SNP3 in the METSIM cohort. Therefore, the genetic model and the significance of SNP3 on cholesterol levels need to be addressed in future replication studies using large samples ascertained for CVR.

The effect of SNP3 suggest that this SNP alone is not a major determinant of cholesterol levels, but rather a modifier variant that influences cholesterol levels. However, the combined effect of SNP3 and additional risk factors is likely to be more pronounced, and thus, more easily detectable in study samples that are enriched for CVD risk factors. This may explain the consistent association observed in the CVR and FCHL samples and the lack of association signal in the entire unascertained METSIM cohort or recent GWAS for lipids.[16] As exemplified by our current study and given the complex architecture of common diseases, such as CVD, it may be more important to select replication samples using the specific attributes of the initial study than the size of the replication sample in general.

In conclusion, our data suggest that the Met[67]Val substitution in the ATF6-gene is associated with cholesterol levels in subjects with increased risk to develop CVD. We also demonstrate that the Met[67]Val substitution is a gain-of-function polymorphism that increases ATF6 transcriptional activity.

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Disclosures

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Online supplemental material for MS#ATVB/2008/180240, entitled "The ATF6-Met[67]Val substitution is associated with increased plasma cholesterol levels", by Steven Meex et al.

Methods

Study Participants

The study design was approved by the ethics committees of the participating centres and all subjects gave written informed consent. Clinical characteristics of the study sample are listed in **Table 1**.

Stage 1 study samples

Sample 1, Dutch CVR (cardiovascular risk). A total of 393 unrelated subjects at increased risk for CVD, i.e. with the following risk factors: age 40-70 years and either hypertension (HT), or body mass index (BMI) >25 kg/m^2 from the Cohort study of Diabetes and Atherosclerosis Maastricht (CoDAM) were genotyped. Details of the CoDAM study design are described in detail elsewhere.[1] To eliminate possible interactions with type 2 diabetes (T2DM)[2,3], individuals with T2DM based on an oral glucose tolerance test were excluded from this study sample. Lipid lowering drug treatments were withheld for 2 weeks before fasting blood sample were taken.

Sample 2, Dutch FCHL. A total of 195 unrelated probands and spouses from families with an established diagnosis of FCHL were genotyped. The FCHL families were recruited through the Lipid Clinic of the Utrecht University Hospital (until 1997) and Maastricht University Hospital (1997-2004) as described in detail previously.[4] Lipid phenotypes were measured as described previously[4]. Lipid lowering drug treatments were withheld for 2 weeks before fasting blood sample were taken. Participants were instructed to abstain from alcohol for 72 h and from smoking on the morning of their visit to the lipid clinic.

Stage 2 study samples

Sample 3, Finnish FCHL. A total of 61 Finnish FCHL families with 715 individuals genotyped were recruited in the Helsinki and Turku University Central Hospital, as described in detail previously.[5] Lipid phenotypes were measured as described previously.[5] Lipid lowering drug treatments were withheld for 4 weeks before fasting blood sample were taken.

Finnish population-based cohort, The METSIM (METabolic Syndrome In Men) cohort consists of 5,112 male subjects, age 50-70 years, randomly selected from the population of Kuopio in Eastern Finland (population 95,000). Recruitment, data collection and phenotypic determinations were performed in the University of Kuopio as described previously.[6] Each participant underwent an evaluation of their disease history, drug treatment, cardiovascular risk factors and an extensive panel of lipid, glucose and other metabolic traits. Subjects reimbursed for lipid lowering therapies (n=599) were excluded from the analyses.

Sample 4, Finnish CVR. A total of 1,371 subjects with CVR (cardiovascular risk) were selected from the METSIM cohort using the same ascertainment criteria used for the Dutch CVR (Sample 1), i.e. either BMI >25 kg/m^2 or reimbursed for medication for the treatment of HT. To eliminate possible interactions with T2DM, individuals with family history of T2DM and/or T2DM based on an oral glucose tolerance test were excluded from this study sample.

SNP selection and genotyping

To select SNPs in the ATF6-gene region (193±3kb) for stage 1 genotyping we utilized the HapMap CEU population with northern and western Europe ancestry. We used the Haploview software[7] to select 13 tag SNPs with minor allele frequency (MAF) > 0.1 and r^2 threshold of 0.85 (rs4657101, rs7553368, rs4579731, rs2134697, rs1503815, rs2340721, rs11581364, rs7554023, rs10918215, rs7514053, rs10918243, rs13401and rs3795649). In addition, 3 coding SNPs were also selected (rs1058405, rs2070150 and rs1135983). The stage 1 genotyping of 16 SNPs was performed using the TaqMan 7900HT (Applied Biosystems) in 588 subjects of Samples 1 and 2. We obtained > 97% success rate for all SNPs. The SNPs rs10918215 and rs7514053 were in complete LD (r^2=1) with rs13401 and the SNP rs2070150, was in complete LD with rs1135983. Therefore we excluded these SNPs, resulting in a total of 13 SNPs analyzed. In stage 2 genotyping the rs1058405 (SNP3) was genotyped in 61 Finnish FCHL families (Sample 3) using the pyrosequencing technique on the PSQ HS96A platform and in the METSIM cohort using the TaqMan assay. The genotype call rate was 98% in Sample 3, and 99% in the METSIM cohort. We replicated 10% of the stage 1 genotypes for SNP3 using different DNA dilutions from the same individual and two different genotyping techniques, i.e. TaqMan and pyrosequencing. Only one of the genotypes did not replicate. All SNPs were in Hardy-Weinberg equilibrium in all subjects of Sample 1 and 4, as well as in the Dutch and Finnish FCHL spouses.

Statistical Analyses

We utilized a two-stage study design, in which all tag-SNPs were first analyzed in stage1. The variant that provided the strongest association signal in the stage 1 analyses was further analyzed in a combined analysis of both stages. This design was originally introduced by Skol et al. to reduce the cost of genotyping while maintaining the overall power of the study.[8] Association analysis of the stage 1 samples with continuous traits (TC, LDL-C and apoB) was performed using PLINK v1.01 software.[9] We tested the genotypic model using multivariate linear regression while including age and sex as covariates. The genotypic test is a two degrees of freedom (d.f) test of of an additive ($\hat{\beta}_{add}$) and a dominance-deviation ($\hat{\beta}_{dev}$) effect. The $\hat{\beta}_{dev}$ coefficient is called dominance-deviation as it reflects a deviation from an additive effect. A recessive character is suggested when the sign of $\hat{\beta}_{dev}$ is opposite of $\hat{\beta}_{add}$, and a dominant character is suggested when the signs of both coefficients are in the same direction. A full recessive or dominant model is observed when the magnitude of the effects is equal $|\hat{\beta}dev| = |\hat{\beta}add|$. To avoid violating the assumptions of the test statistics, we estimated empirical p-values in the combined analysis of Sample 1 and 2 by combining the $\chi 2$ statistics from 20,000 random permutations of each sample weighted by the proportion of individuals examined in each sample.[6] Accordingly, the empirical p-value of each SNP was calculated as (R+1)/(20,000+1) where R is the number of times the permuted combined statistic was greater than the observed combined statistic. Association analysis of the Finnish FCHL families (Sample 3) with residual traits adjusted for age and sex was performed using the genotypic model of the family-based association test (FBAT) software.[10] The genotypic model of FBAT compares all genotypes simultaneously to their null expectation in one test with 2 d.f and the test statistic follows a $\chi 2$ distribution. The PedCheck program[11] was used to detect Mendelian errors in the families. All statistical analyses of the METSIM cohort were performed using the SPSS 15.0 software. We assessed the effect of SNP3 on continuous traits using multivariate linear regression for the genotypic model. Multiplicative interaction with CVR and T2DM and/or family history of T2DM was tested by including in the model the genotypes coded as 0, 1, 2 copy of the Val variant (g); the risk factor (CVR or T2DM) coded as 1 or 0 (r); and their interaction term (gr). The combined analysis of stage 1 and 2 and the meta-analysis were performed using Fisher's method for combining P-value with the proportions of the sample sizes as weights.[12]

This statistic follows a chi-square distribution with 2k degrees of freedom, where k is the number of independent tests. The weighted Fisher's $\chi 2$ method [$C_wF = -2w_i\ln(p_i)$] was calculated in R.2.8.0 as follow: w=k*size/sum(size) # where size is the sample size; cwf =-2*sum(w*log(p)); df=2*k ;p.cwf=1 - pchisq(chi, df). We performed a meta-analysis of stage 1, Sample 3 and the entire METSIM cohort as well as while excluding the subjects with T2DM or family history of T2DM from the METSIM cohort. The proportion of variance explained (R^2) was calculated using univariate general linear model in SPSS 15.0. In the FCHL families (Sample 3), the R^2 values were calculated with adjustment for family membership as a factor.[13]

To test for associations with untyped SNPs within the ATF6 region, we used the Bayesian IMputation-Based Association Mapping (BIMBAM) software version 0.99.[14] We utilized the phased chromosomes of 60 HapMap CEU founders to impute genotypes of 249 SNPs with MAF>5% and genotype call rate>95% in the 254kb region on chromosome 1. We only used the subjects of stage 1 with complete genotype data (n=498) and residual trait (Z-scores) adjusted for age and sex were prepared separately in each sample. Ten SNPs failed the first run of imputations (average of 1,000 imputations), indicated by the standard-error (SE) of the Bayes factor (BF) estimate. The BFs were computed using the prior D2 from Servin and Stephens[14], averaging over σa = 0.05; 0.1; 0.2; 0.4 and $\sigma d = \sigma a/4$ where σa denotes the prior for the additive effect and σd denotes the prior for the dominance-deviation effect. We also used the BFs to compute P-values using 10,000 permutations. The posterior-mean-genotypes were used to calculate the average posterior probability of each SNP, calculated as the percentage of individuals with >80% posterior probability for calling a genotype 0, 1 or 2. Only P-values for those 200 SNPs with at least 80% average posterior probability are presented in **Figure 1**

Cell studies and functional experiments

Isolation and culture of pre-adipocytes

Adipose tissue was collected as described previously.[15] Following the fat biopsy and collagenase treatment, the cell suspension was filtered through 500 µm nylon mesh and spun at 220×g for 1 min to separate pre-adipocytes from mature adipocytes. Pre-adipocytes were suspended in 5 ml DMEM medium containing 10% fetal calf serum (FCS) and 1% glutamine / streptomycine / peniciline (GSP) and cultured during 4 passages under standardized conditions. ATF6 protein levels were measured by western blot in lysates of cultured primary pre-adipocytes, derived from 11

subjects. Clinical characteristics of these subjects at the time of the fat biopsy were as follows: 6 women/5 men, age 50±12 years, BMI 26.8±2.2 kg/m^2, TC 5.7±1.5 mmol/L, LDL-C 4.1±1.7 mmol/L, apoB 1.12±0.33 g/L, TG 2.1±1.8 mmol/L.

HeLa cell culture and transfection

HeLa cells were maintained in DMEM containing 10% fetal calf serum. HeLa cells were resuspended at 2.5*10^6 cells per 400µl of cold Dulbecco's phosphate-buffered saline and electroporated in a 0.4-cm gap electroporation cuvette at 250V and 950 microfarads using a GenePulser II Electroporator (Bio-Rad). Cells were then plated at a density of 0.5*10^6 per 24-mm well for luciferase and galactosidase assays, or 1.5*10^6 per 35-mm well for Western blot experiments.

Plasmids

Cytomegalovirus-Galactosidase: Cytomegalovirus-galactosidase, which is driven by the cytomegalovirus promoter and codes for galactosidase, was used to normalize for transfection efficiency.

Flag-ATF6-(1→373[67]-Met/Val/Leu): These Flag-ATF6 constructs are driven by the cytomegalovirus promoter, and encode amino acid 1-373 of the ATF6 protein that confer strong constitutive transcriptional activity, mimicking all aspects of cleaved endogenous ATF6. All three constructs contain a 3xFLAG-epitope and only differ by their amino acid at position 67 (Met, Val or Leu). Construction of Flag-ATF6-(1→373[67]-Leucine) has been described previously[16]. This parent vector was used as a template to create Flag-ATF6-(1-373[67]-Methionine) and Flag-ATF6-(1→373[67]-Valine) by site-directed mutagenesis (QuickChange kit, Stratagene, Inc.) using the following primers:

[67]-Methionine-sense GATTTGGATTTG**A**TGCCTTGGGAGTCAGAC;

[67]-Methionine-antisense GTCTGACTCCCAAGGCA**T**CAAATCCAAATC;

[67]-Valine-sense GATTTGGATTTG**G**TGCCTTGGGAGTCAGAC;

[67]-Valine-antisense GTCTGACTCCCAAGGCA**C**CAAATCCAAATC.

Nucleotides in **bold** were responsible for the desired amino acid substitutions.

Grp78 promotor(-284→+221)-Luciferase: This construct encodes the GRP78 promoter from -284 to +221 driving luciferase. It was created by PCR using HeLa genomic DNA as a template and a sense primer that begins at base -284 and includes a Kpn1 restriction site and an antisense primer at

+221 and includes an Xho1 restriction site. The resulting PCR product was digested with Kpn1 and Xho1, creating the GRP78-promoter (-284 to +221), which was ligated into the vector pGL2 (Promega).

The pGL2 promoter (catalog number E1631; Promega) contains an SV40 promoter upstream of a luciferase reporter gene.

Reporter assays

β-galactosidase: 48 hours after transfection, cells were lysed in 500 µl of ice-cold lysis buffer (25 mM Gly-Gly, pH 7.8, 15 mM MgSO4, 4 mM EDTA, 0.25% Triton X-100, and 1 mM dithiothreitol). Following centrifugation of the cell lysate, 100 µl of supernatant was combined with 500 µl of galactosidase buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1mM MgSO4, 1 mg/ml chlorophenol red-D-galactopyranoside, and 50 mM 2-mercaptoethanol) and after 1h of incubation absorbance was measured at 570 nm.

Luciferase: Following cell lysis and centrifugation as described above, 100µl cell lysate was combined with 100 µl of luciferase buffer (the above described lysis buffer containing 45 mM MgSO4, 0.3 mM D-luciferin, and 3 mM ATP). An Optocompt II luminometer (MGM Instruments, Inc) was used to measure light emission of each sample for 10 seconds. Relative luciferase activities were determined by dividing luciferase values by β-galactosidase values.

Western Blotting: Cell cultures were extracted in lysis buffer composed of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml aprotinin. After clearing by centrifugation, the protein concentration of the lysate was determined. Protein dilutions were prepared with appropriate amount of 2x Laemmli buffer and equal amounts of protein from each sample and then fractionated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were then probed with ATF6-alpha antiserum (Santa Cruz Biotechnology, California), beta-actin antiserum (Biolegend, San Diego), FLAG antiserum (F-3165, Sigma), KDEL antiserum (SPA-827,Stessgen Biotechnologies Inc, San Diego, CA), or GAPDH (RDI-TRK 5G4-65C; Research Diagnostics Inc, Flanders, NJ).

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Supplemental Figure legends

Figure S1

ATF6 protein from cultured pre-adipocytes as measured by western blot using ATF6-specific antiserum antibody. Western blots were quantified by densitometry analysis and ATF6 protein levels were normalized to beta-actin (A.U. indicates arbitrary units). Positive correlation with plasma TC (A) LDL-C (B) and apoB (C) levels is shown. The Pearson correlations with TC, LDL-C and apoB were 0.64, 0.72, and 0.76; respectively with corresponding P-values p=0.032, 0.018, and 0.006.

Figure S2

A. Schematic representation of full-length and cleaved (activated) ATF6.

B. Detailed representation of the VN8-like region (amino acid 61-68) in ATF6. The Met[67]Val amino-acid substitution (grey box), is associated with cholesterol levels.

Table S1. Clinical characteristics of the study samples

Study sample	N (%female)	Age	TC (mmol/L)	LDL-C (mmol/L)	ApoB (g/L)	BMI (kg/m^2)	Glucose (mmol/L)	SBP (mmHg)	DBP (mmHg)	Met[67]Val (MAF)
Stage I study samples										
Sample 1 Dutch CVR	393 (30%)	59±7	5.23±0.91	3.34±0.85	1.12±0.24	28±4.04	5.38±0.50	140±20	84±10	0.30
Sample 2 Dutch FCHL	195 (54%)	51±11	6.06±1.37	4.01±1.22	1.14±0.33	26±3.50	5.11±0.78	133±19	85±12	0.27
Stage II study samples										
Sample 3 Finnish FCHL	715 (51%)	41±17	6.14±1.45	3.89±1.19	1.12±0.38	26±4.78	5.11±1.50	130±17	81±12	0.24
Sample 4 Finnish CVR	1,371 (0%)	59±6	5.49±0.96	3.52±0.85	1.10±0.28	29±3.30	5.72±0.50	142±16	90±9	0.31
Population-based cohort										
METSIM	4,532 (0%)	59±6	5.39±0.96	3.42±0.85	1.06±0.27	27±3.75	5.94±1.17	140±17	88±10	0.31

Trait values represent the mean ± SEM. BMI, indicates body mass index; SBP, systolic blood pressure and DBP, diastolic blood pressure.

Table S2. Association results of ATF6 polymorphisms with TC, LDL-C and apoB levels in stage 1 analyses

SNP	Genotype	N		MAF		TC (mmol/L)		Combined	LDL-C (mmol/L)		Combined	ApoB (g/L)		Combined
		I	II	I	II	Sample I	Sample II	P-value	Sample I	Sample II	P-value	SampleI	Sample II	P-value
SNP 1	C/C	27	12			5.13±0.18	5.98±0.39		3.37±0.16	4.17±0.36		1.14±0.05	1.15±0.10	
rs4657101	A/C	146	57	0.26	0.23	5.31±0.08	6.29±0.18	0.894	3.39±0.08	4.09±0.16	0.834	1.13±0.02	1.18±0.04	0.846
	A/A	205	106			5.21±0.05	5.93±0.13		3.34±0.05	3.94±0.13		1.12±0.02	1.11±0.03	
SNP 2	C/C	32	19			5.05±0.16	6.19±0.31		3.26±0.16	4.04±0.28		1.09±0.04	1.16±0.07	
rs7553368	T/C	165	72	0.31	0.30	5.28±0.08	5.98±0.16	0.559	3.37±0.08	4.01±0.16	0.861	1.13±0.02	1.13±0.04	0.821
	T/T	178	87			5.23±0.08	6.06±0.16		3.34±0.05	3.96±0.13		1.11±0.02	1.13±0.04	
SNP 3	G/G	33	20			5.54±0.16	6.71±0.28		3.73±0.16	4.43±0.26		1.24±0.04	1.31±0.07	
rs1058405	A/G	168	58	0.30	0.27	5.21±0.08	5.91±0.18	**0.009**	3.29±0.05	3.94±0.16	**0.008**	1.10±0.02	1.11±0.04	**0.002**
	A/A	183	103			5.21±0.08	5.96±0.13		3.34±0.05	3.91±0.1		1.12±0.02	1.11±0.03	
SNP 4	T/T	4	1			4.92±0.44	6.55		3.16±0.41	4.77		1.08±0.12	1.29	
rs1135983	C/T	53	23	0.08	0.07	5.21±0.13	6.22±0.28	0.653	3.29±0.10	3.89±0.26	0.697	1.09±0.03	1.12±0.07	0.775
	C/C	324	155			5.26±0.05	6.01±0.1		3.39±0.05	3.99±0.1		1.13±0.01	1.13±0.03	
SNP 5	G/G	8	2			5.26±0.31	5.98±0.96		3.47±0.28	4.09±0.85		1.07±0.08	1.27±0.23	
rs4579731	A/G	86	39	0.14	0.12	5.26±0.10	5.91±0.21	0.997	3.32±0.08	3.78±0.18	0.900	1.12±0.03	1.10±0.05	0.663
	A/A	280	135			5.26±0.05	6.06±0.1		3.37±0.05	4.01±0.1		1.13±0.01	1.14±0.03	
SNP 6	C/C	24	8			5.02±0.18	5.96±0.49		3.19±0.18	3.94±0.44		1.04±0.05	1.11±0.13	
rs2134697	T/C	139	75	0.25	0.25	5.23±0.08	5.8±0.16	0.270	3.32±0.08	3.81±0.16	0.348	1.11±0.02	1.10±0.04	0.133
	T/T	216	100			5.28±0.05	6.27±0.13		3.39±0.05	4.17±0.13		1.14±0.02	1.17±0.03	
SNP 7	T/T	6	3			4.51±0.36	6.22±0.78		2.72±0.34	3.86±0.7		0.96±0.10	1.20±0.23	
rs1503815	C/T	70	40	0.11	0.13	5.26±0.10	5.72±0.21	0.071	3.37±0.10	3.89±0.18	0.114	1.12±0.03	1.09±0.05	0.157
	C/C	302	139			5.26±0.05	6.14±0.1		3.37±0.05	4.01±0.1		1.13±0.01	1.15±0.03	
SNP 8	A/A	44	19			5.00±0.13	5.8±0.31		3.11±0.13	3.89±0.26		1.04±0.04	1.07±0.08	
rs2340721	C/A	171	86	0.34	0.34	5.23±0.08	5.93±0.16	0.063	3.37±0.05	3.91±0.13	0.045	1.12±0.02	1.12±0.04	0.008
	C/C	167	80			5.28±0.08	6.24±0.16		3.42±0.05	4.14±0.13		1.14±0.02	1.18±0.04	
SNP 9	T/T	53	32			5.21±0.13	6.06±0.23		3.39±0.10	3.96±0.21		1.13±0.03	1.14±0.06	
rs11581364	G/T	171	75	0.37	0.39	5.26±0.08	5.98±0.16	0.977	3.34±0.05	3.94±0.13	0.750	1.12±0.02	1.10±0.04	0.727
	G/G	155	70			5.21±0.08	6.14±0.16		3.32±0.08	4.09±0.16		1.11±0.02	1.18±0.04	
SNP 10	G/G	10	7			4.95±0.28	6.19±0.52		3.16±0.26	4.56±0.44		1.11±0.07	1.19±0.12	
rs7554023	A/G	120	47	0.19	0.17	5.34±0.08	6.11±0.21	0.606	3.47±0.08	4.01±0.18	0.417	1.15±0.02	1.16±0.05	0.879
	A/A	246	123			5.21±0.05	5.98±0.13		3.29±0.05	3.94±0.1		1.11±0.01	1.12±0.03	
SNP 11	C/C	42	24			5.41±0.13	6.58±0.26		3.57±0.13	4.33±0.23		1.20±0.04	1.25±0.07	

rs10918243	T/C	183	75	0.35	0.35	5.26±0.05	5.93±0.16	0.033	3.37±0.05	3.89±0.13	0.054	1.12±0.02	1.11±0.04	0.008
	T/T	153	80			5.15±0.08	6.01±0.16		3.26±0.08	4.01±0.13		1.10±0.02	1.13±0.04	
SNP 12	G/G	21	14			5.10±0.18	5.75±0.36		3.19±0.18	3.89±0.34		1.07±0.05	1.06±0.09	
rs13401	A/G	144	65	0.25	0.25	5.18±0.08	6.01±0.18	0.390	3.26±0.08	3.91±0.16	0.390	1.10±0.02	1.12±0.04	0.202
	A/A	210	110			5.28±0.05	6.14±0.13		3.42±0.05	4.09±0.13		1.14±0.02	1.15±0.03	
SNP 13	T/T	57	33			5.18±0.13	6.11±0.23		3.39±0.10	4.07±0.21		1.13±0.03	1.17±0.06	
rs3795649	C/T	186	81	0.40	0.40	5.23±0.08	5.93±0.16	0.856	3.32±0.05	4.01±0.13	0.955	1.12±0.02	1.12±0.04	0.960
	C/C	134	65			5.26±0.08	6.14±0.16		3.37±0.08	3.99±0.16		1.12±0.02	1.15±0.04	

Trait values represent the marginal mean evaluated at the average age and sex ±SEM
The P-values represent the results of the combined analysis of stage 1 and 2, as described in Supplementary methods.

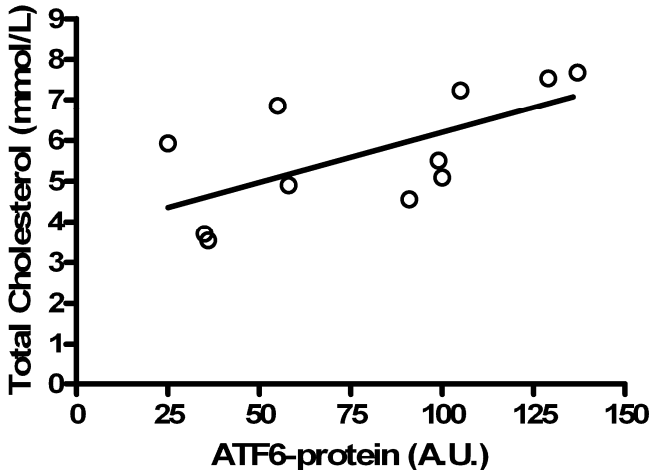
Table S3. Descriptive statistics of glucose traits for the METSIM cohort by family history of T2DM

Trait	FHDM* n=2,099	Non-FHDM n=2,432	P value†
Fasting plasma glucose (mmol/l)	6.08±0.03	5.8±0.02	1.52E-17
Glucose 120min (mmol/l)	6.71±0.06	6.34±0.05	2.45E-06
Fasting serum insulin (mU/l)	10.25±0.34	8.58±0.14	8.17E-07
Insulin 120min(mU/l)	55.9±1.28	52.6±1.15	1.22E-03
Glucose AUC (mmol/l * min)	932±4.94	903±3.82	2.04E-06
Insulin resistance index (HOMA)	3.03±0.12	2.34±0.05	3.82E-10
Insulin sensitivity index (QUICKI)	0.63±0.003	0.65±0.003	6.69E-10

Trait values represent the mean ± SEM. AUC, indicates area under the glucose curve attained during an oral glucose tolerance test; HOMA, Homeostatic model assessment and QUICKI, quantitative insulin sensitivity check index.
*FHDM, indicates subjects with family history of diabetes.
†The P value represents the results obtained by independent t-tests with log transformed values.

Figure S1

A



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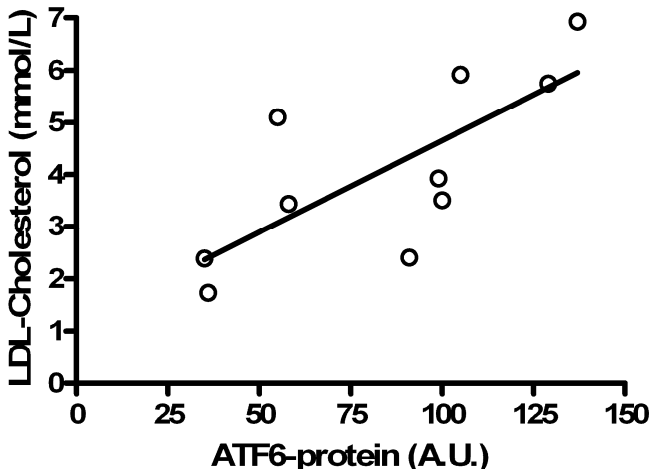

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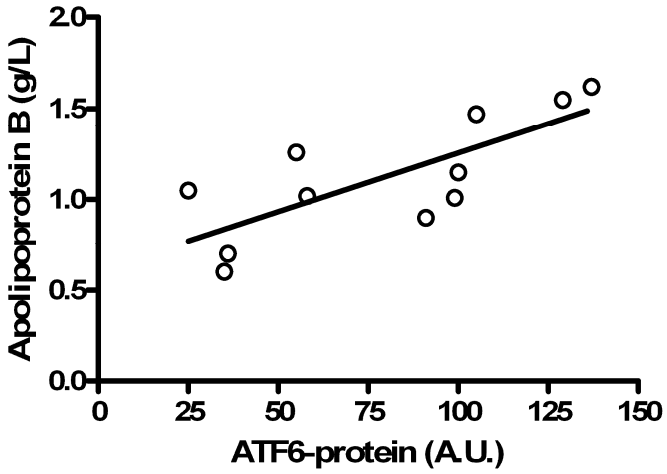

Figure S2