Common CD36 SNPs reduce protein expression and may contribute to a protective atherogenic profile

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Membrane CD36 functions in the uptake of fatty acids (FAs), oxidized lipoproteins and in signal transduction after binding these ligands. In rodents, CD36 is implicated in abnormal lipid metabolism, inflammation and atherosclerosis. In humans, CD36 variants have been identified to influence free FA and high-density lipoprotein (HDL) levels and to associate with the risk of the metabolic syndrome, coronary artery disease and stroke. In this study, 15 common lipid-associated CD36 single nucleotide polymorphisms (SNPs) were evaluated for the impact on monocyte CD36 expression (protein and transcript) in 104 African Americans. In a subset of subjects, the SNPs were tested for association with monocyte surface CD36 (n = 65) and platelet total CD36 (n = 57). The relationship between CD36 expression and serum HDL and very low-density lipoproteins (VLDLs) levels was also examined. After a permutation-based correction for multiple tests, four SNPs (rs1761667, rs3211909, rs3211913, rs3211938) influenced monocyte CD36 protein and two (rs3211909, rs3211938) platelet CD36. The effect of the HDL-associated SNPs on CD36 expression inversely related to the impact on serum HDL and potential causality was supported by Mendelian randomization analysis. Consistent with this, monocyte CD36 protein negatively correlated with total HDL and HDL subfractions. In contrast, positive correlations were documented between monocyte CD36 and VLDL lipid, particle number and apolipoprotein B. In conclusion, CD36 variants that reduce protein expression appear to promote a protective metabolic profile. The SNPs in this study may have predictive potential on CD36 expression and disease susceptibility in African Americans. Further studies are warranted to validate and determine whether these findings are population specific.

INTRODUCTION

CD36 is a multi-ligand scavenger receptor expressed on a variety of cell types including adipocytes, myocytes, monocytes, platelets, hepatocytes and vascular epithelial cells (1,2). CD36 functions in the uptake of fatty acids (FAs) and oxidized lipoproteins. Signal transduction triggered by binding these and other ligands contributes to multiple cellular effects of the protein in pathways related to lipid utilization, insulin resistance, inflammation, atherosclerosis and thrombosis (1–7). CD36 may also contribute to oral fat perception and intestinal chylomicron formation (8–10).

In humans, CD36 deficiency results in defective myocardial FA uptake measured by non-invasive scintigraphy (11), and a link with hypertrophic cardiomyopathy has been proposed (12). Macrophages isolated from CD36-deficient humans exhibited reduced uptake of oxidized low-density lipoproteins (LDLs) (13). These findings supported the relevance of rodent data to humans and generated interest in the role of CD36 expression and function in the etiology of human metabolic disease. Studies of subjects with Type I CD36 deficiency suggested that CD36 absence is associated with high serum lipids and with a poor metabolic profile (14,15). In contrast,

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several recent studies that measured CD36 expression on monocytes or concentration of a soluble form of CD36 in the circulation have associated increased CD36 level with insulin resistance and with elevated serum levels of inflammation markers (16–18).

Genetic association studies provide valuable insight into the physiological role of specific genes in humans by identifying links to particular phenotypes or diseases. However, the findings require duplication and ideally functional validation. Association studies have related CD36 polymorphisms to abnormal serum FA (19) and LDLs (20,21). CD36 single nucleotide polymorphisms (SNPs) have been linked to diabetes-associated coronary artery disease (CAD) risk in Caucasians (19) and Korean populations (22) and to metabolic syndrome (MetS) risk in American Puerto Ricans (23).

Mutations in the gene have been associated with insulin resistance, low adiponectin level and the pathogenesis of type 2 diabetes (24). In a genome-wide association (GWA) study from the Heart and Aging Research in Genomic Epidemiology consortium, 15 CD36 SNPs associated (P-values of 10^-5) with the risk of stroke (25). CD36 SNPs also associated with left ventricular mass (26) and obesity (27).

The CD36 gene region has been linked to high-density lipoprotein (HDL) levels in rodents (28) and humans (29–31). A study in mice used comparative analysis of human and mouse quantitative trait loci, which identified CD36 among candidate genes influencing HDL variability (28). In humans, the CD36 genomic region has been linked to HDL in several studies of Mexican Americans (29–31). We recently identified associations between multiple CD36 SNPs and susceptibility to the MetS and with serum HDL cholesterol in a large African American cohort from the Hypertension Genetic Epidemiology Network (HyperGEN) (32).

Specifically, the minor allele of nine common CD36 SNPs associated with increased serum HDL and the minor allele of seven SNPs associated with reduced HDL. These observations suggested that the role of CD36 in human HDL metabolism may be underappreciated. To gain insight into the functional relevance of lipid-associated CD36 variants to CD36 expression and HDL, we evaluated the impact of 15 common SNPs previously reported to associate with serum free FA (FFA), HDL and/or MetS (19,32) on CD36 protein and transcript levels in a cohort of African Americans. In addition, we examined the relationship between monocyte CD36 expression levels and serum HDL and very LDL (VLDL), which are core components of dyslipidemias implicated in the MetS, type 2 diabetes and cardiovascular disease (33,34). HDL serum cholesterol efflux capacity as a function of monocyte protein expression was also determined in vitro.

RESULTS

HDL-associated SNPs on CD36 expression

CD36 tag SNPs previously associated with serum lipids (Table 1) were evaluated for their influence on fasting CD36 expression in a cohort of unrelated African Americans (n = 104). Subjects’ demographics and clinical characteristics are shown in Table 2. The minor allele frequencies (MAFs) of the tag SNPs genotyped in this study (Table 1) were similar to those in the HyperGEN African American sample (32) and in populations of African descent in HapMap. SNP genotype frequencies were in accordance with Hardy–Weinberg expectations (P > 0.05).

Fifteen SNPs were tested on CD36 expression (Supplementary Material, Table S1) and 10 were found to have effects. The SNP, rs3211938, which encodes a truncated CD36 protein, was previously associated with increased HDL, and a subject homozygous for the minor allele (G-allele) exhibited CD36 deficiency on monocytes and platelets (32). We extended these findings in the current sample. The G-allele of rs3211938 (Table 3) significantly reduced monocyte total CD36 protein (P = 2.36 × 10^-5) but had no effect on monocye CD36 transcript. Thus, transcription is not impacted while the encoded truncated protein is unstable. Surface CD36 expression was reduced on CD14+ monocytes examined in a subset of subjects (n = 65, P = 0.046) but significance was diminished after adjustment for multiple tests.
Table 2. Characteristics of subjects

<table>
<thead>
<tr>
<th>n (M/F)</th>
<th>30/74</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41.6 ± 9.5</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.4 ± 7.9</td>
</tr>
<tr>
<td>Insulin (uIU/ml)</td>
<td>12.2 ± 7.8</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>95.7 ± 15.6</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>55.7 ± 14.28</td>
</tr>
<tr>
<td>HDL-2 (mg/dl)</td>
<td>15.4 ± 9.4</td>
</tr>
<tr>
<td>HDL-3 (mg/dl)</td>
<td>40.16 ± 6.9</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>90.1 ± 47.6</td>
</tr>
<tr>
<td>Total CHOL</td>
<td>186.1 ± 33.2</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>16.1 ± 12.1</td>
</tr>
<tr>
<td>VLDL-TG (mg/dl)</td>
<td>53.4 ± 39.4</td>
</tr>
<tr>
<td>APOA1 (mg/dl)</td>
<td>159.8 ± 34.7</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. BMI, body mass index; HDL, high-density lipoprotein; TG, triglycerides; CHOL, cholesterol; VLDL, very low-density lipoprotein; APOA1, apolipoprotein A1.

Rs3211938 has only been identified in subjects of African ancestry as a result of positive selective pressure (35,36) and the predictive impact on expression would not apply to other populations. Several non-coding SNP alleles related to increased HDL also associated with reduced CD36 expression (Fig. 1 and Table 3). Prior to multiple test adjustment, minor alleles of rs10499859 and rs32112913 significantly reduced monocyte CD36 transcript, total protein and surface expression. SNP rs3211909 reduced monocyte transcript and protein. SNPs rs3211909 and rs3211913 also reduced platelet CD36 (Supplementary Material, Table S2). The associations between three SNPs (rs3211938, rs3211913 and rs3211909) and total protein exceeded the permutation-based significance threshold of P = 0.0013. Considering these SNPs are not completely unlinked, the impact on expression may not be independent (Supplementary Material, Fig. S1).

SNP rs1761667 was previously associated with increased circulating free FAs in Caucasians (19). This SNP is common in African Americans (MAF ~ 0.39) and Caucasians (MAF ~ 0.48), although the minor alleles are reversed. The A-allele is the minor allele in African Americans (versus G in Caucasians). We found that the A-allele associates with a 2.7 mg/dl increase in HDL (P = 0.006) in non-diabetic HyperGEN African American participants (n = 1595). The A-allele (Table 3 and Fig. 1) also reduced monocyte CD36 transcript, total and surface protein. The effect on total protein was significant after multiple testing (P < 0.0013).

SNPs that reduced monocyte CD36 protein and met the multiple test-corrected threshold of P < 0.0013 showed similar directional effects on platelet expression. As shown in Supplementary Material, Figure S2, rs1761667, rs3211909, rs3211913 and rs3211938 were nominally associated with reduced platelet CD36 protein. An additional SNP (rs3211870) that did not influence monocyte CD36 significantly reduced its expression on platelets. This is consistent with occasional divergence of CD36 protein levels on monocytes and platelets (37–39) and may reflect differential regulation (40). SNP alleles (SNPs rs9784998, rs1737398, rs3211868 and rs3211870) that reduced HDL (Table 1) tended to increase CD36 expression (Table 3); however, only the rs9784998 T-allele reached significance (P = 0.026) but not after multiple tests adjustment. This allele also increased platelet CD36 (Supplementary Material, Fig. S2).

Total CD36 protein as a function of genotype for SNPs that met the permutation-based significance threshold showed a significant reduction in CD36 in subjects homozygous for the minor allele versus non-carriers for each SNP (P < 0.006) (Fig. 2). Overall, our data suggest that the association between the above CD36 SNPs and HDL may be related to their impact on CD36 expression and support a significant impact of common variants on individual variability in CD36 levels.

Causality of the relationship between CD36 expression and HDL

The associations identified for the same CD36 SNPs with HDL and CD36 expression allow us to test for a causal relationship using Mendelian randomization (41,42). Three criteria must be satisfied for causality: (1) the SNPs must not associate with confounding factors with respect to expression or HDL, (2) the SNPs must reliably associate with expression and (3) there must be no direct effect of SNPs on HDL independent of that due to CD36 expression (43). Potential confounders between HDL and CD36 expression include serum glucose and insulin levels and BMI, and no SNPs in our sample affected these traits. The association between the SNPs and CD36 expression in the present study supports criterion 2. To test criterion 3 each SNP was used to predict HDL after adjusting for CD36 level. These tests showed no residual HDL association remained after adjustment for CD36 expression, indicating that the SNPs are valid instrumental variables and supporting a causal association between CD36 level and HDL.

CD36 protein level inversely associates with serum HDL and HDL subfractions

To further investigate the observation that the effect of the SNPs on CD36 expression is inversely related to serum HDL, we directly examined the relationship between monocyte CD36 protein and total HDL (P = 0.009). Serum HDL fractions are heterogeneous with respect to particle size, maturation and lipid content, which affect anti-atherogenic potency (44). The major HDL subgroups in humans are HDL2 and the smaller HDL3. Monocyte CD36 inversely correlated with all HDL fractions (Table 4).

HDL particle efficiency in cholesterol efflux

To determine whether the CD36 level was associated with altered HDL efficiency in cholesterol efflux, serum from subjects genotyped for the significant SNPs was tested for ability to efflux cholesterol from cholesterol-preloaded hepatoma cells (45). Methodological details are in the Supplementary Material. As shown in Figure S2, Supplementary Material, monocyte CD36 level did not correlate with serum cholesterol efflux (P = 0.55). Overall, the data indicated that CD36...
Table 3. Significant CD36 SNP expression associations

<table>
<thead>
<tr>
<th>SNP</th>
<th>Effect allele</th>
<th>Transcript</th>
<th>SE</th>
<th>P-value</th>
<th>Total protein</th>
<th>SE</th>
<th>P-value</th>
<th>Surface expression</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1761667</td>
<td>A</td>
<td>−0.48</td>
<td>0.19</td>
<td>0.016</td>
<td>−0.68</td>
<td>0.18</td>
<td>3.5E−04</td>
<td>−0.27</td>
<td>0.09</td>
<td>0.003</td>
</tr>
<tr>
<td>rs10499859</td>
<td>G</td>
<td>−0.53</td>
<td>0.18</td>
<td>0.005</td>
<td>−0.55</td>
<td>0.18</td>
<td>0.003</td>
<td>−0.24</td>
<td>0.09</td>
<td>0.011</td>
</tr>
<tr>
<td>rs9784998</td>
<td>T</td>
<td>0.39</td>
<td>0.21</td>
<td>0.064</td>
<td>0.47</td>
<td>0.21</td>
<td>0.026</td>
<td>0.13</td>
<td>0.11</td>
<td>0.212</td>
</tr>
<tr>
<td>rs3211870</td>
<td>C</td>
<td>−0.19</td>
<td>0.305</td>
<td>0.034</td>
<td>−0.52</td>
<td>0.18</td>
<td>2.7E−04</td>
<td>−0.27</td>
<td>0.09</td>
<td>0.005</td>
</tr>
<tr>
<td>rs3211909</td>
<td>C</td>
<td>−0.45</td>
<td>0.21</td>
<td>0.008</td>
<td>−0.85</td>
<td>0.15</td>
<td>3.1E−07</td>
<td>−0.21</td>
<td>0.09</td>
<td>0.020</td>
</tr>
<tr>
<td>rs3211913</td>
<td>G</td>
<td>−0.47</td>
<td>0.17</td>
<td>0.421</td>
<td>−1.07</td>
<td>0.24</td>
<td>2.36E−05</td>
<td>−0.26</td>
<td>0.13</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Resulting β-estimates (standard error, SE) expressed per minor allele. SNPs listed by chromosomal position. SNPs with \( P < 0.05 \) are in bold. Age, gender and BMI were considered as covariates but were not significant predictors of monocyte expression, monocyte surface expression or monocyte transcript expression \( (P > 0.05) \). Each of the outcomes was log-transformed to normalize distribution. Sample sizes for CD36 transcript, \( n = 70 \), CD36 total monocyte protein, \( n = 74 \), CD36 monocyte surface CD36, \( n = 65 \).

\( ^* \)Associations that met the threshold for multiple testing, critical \( P < 0.0013 \).

 inversely impacts HDL level and does not influence particles’ ability to efflux cholesterol.

**CD36 expression and serum VLDL, VLDL particle number and Apo B**

CD36 has been documented to facilitate cellular uptake of both FA (2) and cholesterol (46,47), and CD36 deficiency alters both HDL and VLDL concentrations in mice (48). We examined in our local recruits the relationship between monocyte CD36 level and VLDL parameters [cholesterol, triglyceride (TG) content, Apo B and particle number] determined by NMR spectroscopy (Table 4). Positive correlations were identified that may serve as predictors of CD36 level and reduced susceptibility to dyslipidemia.

**DISCUSSION**

CD36 variants have been associated with altered blood lipid levels (19,21,24,32,49) and with MetS risk (23,32), type 2 diabetes (50,51), CAD (19,22) and myocardial infarction (52,53). A recent GWA (CHARGE) study associated several CD36 SNPs with the risk of stroke (25). Low HDLs and high VLDLs are independent risk factors for the above diseases (54,55). This study documents the following novel findings:

(i) an inverse relationship between the CD36 protein and serum HDL as well as HDL subfractions with a causal relationship supported by Mendelian randomization; (ii) a positive relationship between CD36 expression and serum VLDL lipid concentration, particle number and Apo B level; (iii) several common CD36 SNPs reduce CD36 level and (iv) after adjustment for multiple testing, four SNPs are identified that may serve as predictors of CD36 level and reduced susceptibility to dyslipidemia.

**CD36 SNPs and HDL interindividual variability**

HDL levels have a strong genetic component accounting for up to 70% of individual HDL variability (56). To date, the cumulative effect of eight replicated genes that influence HDL metabolism \( [\text{ATP-binding cassette transporter A1 (ABCA1)}, \text{apolipoprotein A-I (ApoA1)}, \text{apolipoprotein E (ApoE)}], \text{cholesterol ester transfer protein (CEPT)}, \text{hepatic lipase (LIPC)}, \text{lecithin-cholesterol acyltransferase (LCAT)}, \text{lipoprotein lipase (LPL)} \) and scavenger receptor class B type 1 (SR-B1)] accounts for less than 20% of HDL variability (57,58). Recent GWA studies for HDL confirmed the above associations and a number of other previously identified genes [angiotensin-related protein 4 (ANGPT4), flavin adenine dinucleotide synthetase (FADS), N-acetylglucosaminyltransferase 2 (GALNT2), hepatocyte nuclear factor 4 alpha (HNF4a), liver X receptor-alpha (LXRA), paraoxonase 1 (PON1), mevalonate kinase (MVK) and methylmalonic aciduria (MMAB)] in European populations (59). CD36 is involved in metabolic pathways linked to several of the identified genes \( (i.e. \text{LPL}, \text{LXRA}, \text{ABCA1}) \) (60). The CD36 locus has been implicated in HDL variability in African and Mexican American populations (29,31,32). We estimate that the SNPs we identified may account for \( \sim 3.4\% \) of HDL variability in the HyperGEN African American sample (32). The current study suggests that this variability reflects in part the impact of the SNPs on CD36 expression. Other than the coding SNP, rs3211938, most SNPs in this study localize to introns, as have a large proportion of identified complex trait-associated SNPs (61). Intronic SNPs can influence protein levels by interfering with the binding of regulatory factors or by creating alternative splicing sites (62). Using the functional analysis tool FASTSNP (63), three SNPs (rs10499859, rs3211909, rs3211913) were identified to lie within intronic enhancer sites and one was in the CD36 promoter (rs1049654). We cannot rule out that the identified SNPs are not causal because of linkage with as yet unidentified variants.

The inverse association between CD36 expression and total HDL as well as HDL subfractions supports the interpretation that the CD36 level influences HDL metabolism in general and not steps involved in interparticle conversion. CD36 is less efficient than its family member SR-B1 in mediating uptake in less efficient than its family member SR-B1 in mediating uptake in.
cholesterol is an unlikely contributor (Supplementary Material, Fig. S2). Recent findings in mice document significantly enhanced cellular cholesterol efflux in macrophages and hepatocytes from Cd36-deficient mice, which promotes the generation of more HDLs (66). This is consistent with the effects observed in this study where reduced CD36 increased all HDL fractions.

**CD36 expression and serum VLDL**

The positive correlations between CD36 levels and serum VLDL parameters (Apo B, VLDL-TG, VLDL-C and VLDL particle number) suggest that CD36 influences VLDL metabolism. In humans, hepatic CD36 expression was increased in subjects with non-alcoholic fatty liver disease (67,68). It was recently shown in mice that increased hepatic Cd36 level achieved by diet or adenoviral expression associated with enhanced FA uptake and promoted TG accumulation and secretion (69). Also in mice, increased hepatic Cd36 appears critical for liver X receptor-induced steatosis (70). Thus, the most likely interpretation of our findings is that the CD36 level positively influences hepatic VLDL production.

The relationship between CD36 level and serum lipids may also reflect the lipid regulation of the protein rather than vice
versa. For HDL, the impact of CD36 SNPs was from a large candidate gene association (32). The current findings, i.e. inverse relationship between SNP effects on CD36 expression and HDL, and suggestive causality of this relationship (based on Mendelian randomization) support the interpretation that the CD36 level impacts HDL. In the case of VLDL, potential causality could not be addressed, as there are no documented VLDL–CD36 SNP associations. The present findings, together with earlier reports of a role for CD36 in hepatic steatosis (67–69), suggest that the CD36 level influences VLDL. However, the VLDL–CD36 association may be bidirectional.

In summary, the candidate gene association analysis in a large cohort of African Americans identified several HDL-associated CD36 SNPs (32). The CD36 SNP–HDL association has been confirmed in a GWA study with a second larger cohort of African Americans (S. Kathiresan, personal communication) (unpublished data). These data support the influence of several HDL-associated SNPs on CD36 expression and the causality of the link between expression and HDL. The insight obtained implicating human CD36 in HDL and VLDL metabolism supports a role for CD36 in the etiology of dyslipidemias and metabolic diseases. Therefore, the expression-associated SNPs in this study may have predictive potential with regard to CD36 expression and disease

Table 4. Pearson correlations for fasting serum lipid measures and monocyte CD36 total protein expression

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Pearson’s r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL (mg/dl)</td>
<td>79</td>
<td>-0.29</td>
<td>0.009</td>
</tr>
<tr>
<td>HDL-2 (mg/dl)</td>
<td>79</td>
<td>-0.33</td>
<td>0.003</td>
</tr>
<tr>
<td>HDL-3 (mg/dl)</td>
<td>79</td>
<td>-0.27</td>
<td>0.016</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>79</td>
<td>0.24</td>
<td>0.03</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>79</td>
<td>0.28</td>
<td>0.011</td>
</tr>
<tr>
<td>VLDL-TG (mg/dl)</td>
<td>79</td>
<td>0.26</td>
<td>0.019</td>
</tr>
<tr>
<td>Apo B (mg/dl)</td>
<td>79</td>
<td>0.29</td>
<td>0.009</td>
</tr>
<tr>
<td>Particle number (nmol/l)</td>
<td>60</td>
<td>0.28</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Values in the table are Pearson’s correlation coefficients between fasting lipid parameters and total monocyte CD36 expression with associated P-values.
susceptibility in African Americans; however, further studies are needed to determine whether these findings are population specific.

MATERIALS AND METHODS

Subjects, blood collection and serum measurements

Unrelated African American subjects (n = 104) were recruited. Subjects with diabetes were excluded and no subjects reported statin use. The study was approved by the Washington University School of Medicine Institutional Review Board.

Genomic DNA was extracted from whole venous blood (Puregene) after an overnight fast (8–12 h). Serum and plasma, obtained within 30 min, were stored at −80°C. Insulin was measured by a two-site sandwich chemiluminescent immunometric assay (IMMULITE/IMMULITE 1000 Insulin, Diagnostic Products Corporations, USA), plasma glucose concentrations were measured using an automated glucose analyzer (YSI 2300 STAT Plus, YSI Life Sciences, Yellow Springs, OH, USA). HDL was determined enzymatically following dual dextran sulfate–MgCl₂ precipitation to quantify HDL-3 and calculate HDL-2 (difference between total HDL and HDL-3) and VLDL-C, VLDL-TG by lipoprotein β-quantification (1.006 g/ml) (71). Total cholesterol (Diagnostics Chemicals, Oxford, CT, USA) and triglyceride concentrations (Roche Diagnostics Corporation, Mannheim, Germany, and Indianapolis, IN, USA) were measured by enzymatic colorimetry and apolipoprotein A1 by turbidimetry (Hitachi 917 analyzer, 700 nm, reagents by Wako Chemicals, USA). Plasma lipoprotein particle sizes were measured by proton NMR spectroscopy (LipoScience, Raleigh, NC, USA).

SNP selection and genotyping

SNPs were selected for genotyping based on previously reported association with HDL (rs10499859, rs9784998, rs1049654, rs3173798, rs3211868, rs3211870, rs3211909, rs3211913, rs3211938) and/or MetS (rs3211850, rs7755) (32) or association with FFA levels (rs1984112, rs1761667, rs2151916, rs1527483) (19). Genotyping was performed by Sequenom MassARRAY (72) which identifies alleles by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (68). Genotype success rate was 95 ± 0.14% for all SNPs.

CD36 expression

Monocyte and platelet proteins were isolated (32), separated by 10% SDS–PAGE and probed for CD36 protein using primary anti-human monoclonal CD36 antibody 1:1000 (FA6-152, Abcam) and anti-mouse IgG fluorescently labeled IRDye (LI-COR Odyssey Infrared Imaging System). Human β-actin antibody (ȘC47778, Santa Cruz) bound by goat anti-mouse IgG (Li-COR Biosciences) was the loading control.

Monocyte surface CD36 was measured (in triplicate) by fluorescence-activated cell sorting (FACS) (32) and gating with fluorescein isothiocyanate-conjugated CD14 monoclonal antibody (BD Pharmigen) using FACSCalibur™ cytometer (BD Biosciences; San Jose, CA, USA) running CellQuest™ (BD Biosciences, version 3.3).

CD36 transcript was determined by quantitative RT–PCR using primers for exon 1b (40). Transcripts were quantified using SYBR green chemistry on the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Primer efficiency was verified using threshold cycle versus log cDNA dilution according to the equation: Efficiency = 10^(-slope). Triplicates were run and relative abundance of the CD36 transcript was determined by competitive Ct (delta–delta Ct). The human large ribosomal protein (RPLPO) was the endogenous control.

Statistical analysis

Associations between SNPs and CD36 expression profiles were analyzed using multivariate linear regression analysis under an additive genetic model using PLINK v.1.05 (http://pngu.mgh.harvard.edu/purcell/plink/) and/or in SAS version 9.1. Age, gender and BMI, as covariates, were not significant (P > 0.05) predictors of monocyte protein (or platelet), transcript or surface protein. Outcomes were log-transformed to normalize distribution. To correct for multiple tests (15 SNPs and three phenotypes), permutations were conducted to randomly re-assign individuals’ genotypes and phenotypes. To preserve correlations between both SNPs (due to LD) and phenotypes (measures of CD36 expression), they were permuted as blocks. We conducted 1000 null replicates and took the smallest P-value for any SNP–phenotype combination from each replicate to calculate our empirical null distribution, resulting in a P-value threshold of 0.0013 necessary to exceed an experiment-wide alpha level of 5%. ANOVA and Kruskal–Wallis tests were used to compare the mean gene expression across genotypes (Fig. 2).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

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Conflict of Interest statement. None declared.

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