A common haplotype at the CD36 locus is associated with high free fatty acid levels and increased cardiovascular risk in Caucasians

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CD36 is a class B scavenger receptor recognizing a variety of ligands including long-chain fatty acids and modified LDL. We investigated whether genetic variability at this locus is a determinant of free fatty acid (FFA) plasma levels and risk of coronary artery disease (CAD) in Caucasians. Typing of 21 polymorphic markers, evenly spanning the CD36 gene, revealed two linkage disequilibrium (LD) blocks that could be tagged by five polymorphisms (233137 > G, 231118 > A, 25444G > A, 27645del > ins and 30294G > C). In 585 non-diabetic individuals of Caucasian origin, the 30294G > C polymorphism was significantly associated with FFA levels (P = 0.02)—an effect that was especially visible among men (P = 0.009). A similar association was observed in this gender at 233137 (P = 0.008) and 231118 (P = 0.028). When the five tag polymorphisms were considered together, men carrying the AGGIG haplotype had 31% higher FFA (P = 0.0002) and 20% higher triglycerides (P = 0.025) than non-carriers. The same haplotype was associated with increased risk of CAD in 197 type 2 diabetic individuals from the US (OR = 2.3, 95% CI 1.2–4.2). A similar tendency was observed in a group of 321 type 2 diabetic individuals from Italy (OR = 1.4, 0.9–2.3), resulting in an overall relative risk of 1.6 (1.1–2.3, P = 0.015) in the two populations considered together. By targeted resequencing, we identified a common variant in the CD36 promoter that is in strong LD with the AGGIG haplotype and could be partly responsible for these findings. In conclusion, this comprehensive study of CD36 variability indicates that the common polymorphisms at this locus modulate lipid metabolism and cardiovascular risk in Caucasians.

INTRODUCTION

CD36, also known as platelet glycoprotein IV or IIIb, is an 88 kDa membrane protein expressed on the surface of a wide variety of cell types including adipocytes, skeletal muscle cells, platelets, endothelial cells and monocytes/macrophages (1). Initially identified for its binding to collagen and thrombospondin (TSP-1) in platelets, CD36 is a class B scavenger receptor recognizing a variety of ligands including long-chain fatty acids, modified LDL, anionic phospholipids, Plasmodium falciparum infected erythrocytes and apoptotic cells (1–4).
Several recent findings suggest a role for CD36 as an important regulator of the metabolic pathways involved in insulin-resistance. CD36 facilitates the membrane transport of long-chain fatty acids (FA) into muscle and adipose tissues (5). Increased FA availability can induce insulin resistance if the capacity of adipose tissue to store triglycerides and/or that of muscle to oxidize FA is exceeded (6). As a result, alterations in CD36 level may be involved in the development of diet-induced insulin-resistance as suggested by findings in rodents (7). Homozygous disruption of the CD36 locus in mice leads to hepatic insulin-resistance with high plasma free fatty acids (FFA) and triglycerides (8,9). Transgenic rescue of CD36 in the spontaneously hypertensive rat (SHR) strain, in which the gene is severely mutated, alleviates the metabolic syndrome typical of this animal model (10,11). Furthermore, CD36 is regulated by the peroxisome proliferator activated receptor gamma (PPARγ) and is a gene target of thiazolidinediones (TZDs), which are agonists of this nuclear receptor (12). Upregulation of adipocyte or muscle CD36 by TZDs appears to mediate some of the insulin sensitizing effects of these drugs (13).

Atherosclerosis is a frequent and deadly complication of insulin resistance and diabetes (14). Vascular dysfunction and atherosclerosis parallel the progression of the disease and may be accelerated by the dyslipidemia as well as the hyperglycemia that are prevalent in poorly controlled diabetes. In the arterial wall, CD36 contributes to the scavenging of oxidized LDL—one of the major triggers of atherosclerotic lesions (15,16). Thus, genetic variability in the expression or activity of this molecule may have an independent impact on the risk of coronary artery disease (CAD), in addition to that due to its effects on FFA levels and insulin-sensitivity.

CD36 deficiency, mostly due to a Pro90Ser mutation, has been reported in Japanese and African subjects with a frequency of 2–4% (17,18). This condition has been associated to the metabolic syndrome in Japanese, with CD36 deficient individuals having impaired glucose disposal in response to insulin and increased levels of FFA, triglycerides, fasting blood glucose and blood pressure (19). Other studies have confirmed the association with higher plasma FFA, but not with other insulin-resistance traits (20,21). No data is available for Caucasians, owing to the rarity of the Pro90Ser mutation in this racial group (<0.3%).

Whether more common CD36 variants are also associated with specific metabolic phenotypes has not been fully investigated. Here, we show in two different subpopulations that a common CD36 haplotype is associated among Caucasians with increased plasma FFA and triglycerides and is a determinant of increased cardiovascular risk in type 2 diabetes.

RESULTS
To determine the haplotype structure of the CD36 locus in Caucasians, 19 common SNPs (minor allele frequency ≥0.05) and two insertion/deletion polymorphisms were typed in 72 unrelated individuals from the general population. Eight of these markers were representative of the major linkage disequilibrium (LD) bins identified in the region between intron 3 and exon 14 by a resequencing project (http://pga.gs.washington.edu/data/cd36/). The other 11 were selected from the dbSNP database to cover the two alternative promoters (22), the 5′ non-coding exons, and the 3′-UTR in exon 15. The 21 polymorphisms spanned a total of 63 kb, resulting in a 3.0 kb average spacing (Table 1). Significant LD was observed across the entire locus (Fig. 1A). However, two blocks of preferential LD appeared to be present, one extending from position −33137 to 15554, the other covering the remaining 15 kb (Fig. 1A). This pattern was confirmed by haplotype analysis and the block-partitioning algorithm implemented in the HapBlock software (23). If the two blocks were considered separately, common (≥5%) haplotypes accounted for 80% of the haplotypes in the first block and 80% of those in the second block (Fig. 1B). In contrast, if the entire locus was considered as a single block, common haplotypes accounted for only 69% of the chromosomes. On the basis of these findings, five haplotype tagging polymorphisms were selected, two from the first block (−33137A>G and −31118G>A) and three from the second block (25444G>A, 27645del and 30294G>C) (Fig. 1B).

The association between fasting FFA levels and the five haplotype tagging polymorphisms was investigated in 585 non-diabetic Caucasian individuals from Italy (231 men and 354 women), who had been characterized with respect to percent ideal body weight (% IBW), waist circumference,
Figure 1. Haplotype blocks at the CD36 locus. (A) Pairwise LD between polymorphisms. $D'$ and $r^2$ are reported below and above the diagonal, respectively. Markers are positioned to scale. Two overlapping blocks of preferential LD are visible, one from position –33137 to 15341, the other from position 15341 to 30294. (B) Common haplotypes defined by the polymorphisms. Within each block, common haplotypes are indicated with different shades of color (orange for the first block, blue for the second). Haplotypes that are rare (<0.05) in only one block are indicated in white. Haplotypes that are rare in both blocks are not reported. Haplotype-tagging polymorphisms and the corresponding alleles are indicated in bold.
Table 2. Clinical characteristic of non-diabetic subjects according to CD36 genotypes

<table>
<thead>
<tr>
<th></th>
<th>−33137A &gt; G</th>
<th></th>
<th>−31118G &gt; A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A/A</td>
<td>A/G</td>
<td>G/G</td>
</tr>
<tr>
<td>N</td>
<td>153</td>
<td>286</td>
<td>108</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35 ± 12</td>
<td>36 ± 11</td>
<td>38 ± 13</td>
</tr>
<tr>
<td>%IBW (%)</td>
<td>116 ± 20</td>
<td>117 ± 21</td>
<td>117 ± 20</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>83.1 ± 13</td>
<td>82.5 ± 13</td>
<td>82.9 ± 12</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>115 ± 13</td>
<td>113 ± 12</td>
<td>116 ± 13</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76 ± 10</td>
<td>76 ± 8</td>
<td>77 ± 8</td>
</tr>
<tr>
<td>FFBG (mg/dl)</td>
<td>88 ± 8</td>
<td>89 ± 9</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>189 ± 40</td>
<td>194 ± 38</td>
<td>195 ± 43</td>
</tr>
<tr>
<td>Fasting insulin (mU/ml)</td>
<td>6.7 (4.6, 9.1)</td>
<td>6.9 (5.2, 13)</td>
<td>6.7 (4.6, 9.1)</td>
</tr>
<tr>
<td>FFA (mU/ml)</td>
<td>6.4 (4.7, 9.3)</td>
<td>6.7 (4.7, 9.0)</td>
<td>7.0 (4.6, 9.3)</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>114 ± 13</td>
<td>116 ± 13</td>
<td>113 ± 13</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>76 (53, 102)</td>
<td>72 (54, 108)</td>
<td>76 (54, 105)</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>52 ± 13</td>
<td>53 ± 13</td>
<td>54 ± 14</td>
</tr>
</tbody>
</table>

Data are mean ± SD, with the exceptions of fasting insulin, FFA, and triglycerides for which data are medians (25th, 75th percentiles). Genotypes were available for 547 subjects at −33137, 542 at −31118, 514 at 25444, 562 at 27645 and 518 at 30294.

*P = 0.069 and **P = 0.02 across the three genotypes groups.

blood pressure, fasting glucose and insulin and lipid profile. As previously reported in the literature (24,25), men had lower FFA levels than women [median = 515 (interquartile range 374, 652) versus 540 (412, 704) μmol/l, P < 0.029]. Genotype distributions were in Hardy–Weinberg equilibrium at all five loci. The clinical characteristics of study subjects according to genotypes are shown in Table 2. A significant difference in FFA levels across genotypes was observed at position 30294 (P = 0.02) and a similar tendency was observed at position −33137 (P = 0.069). When data were stratified by gender, the association between these two SNPs and FFA levels was much more evident in men (P = 0.008 at −33137 and P = 0.009 at 30294) than in women (NS, at both locations) (Table 3). An association with FFA levels was also detected in men for SNP −31118 (P = 0.028). At all three locations, men carrying the major allele had higher FFA levels than men homozygous for the minor allele, with a pattern consistent with an additive model being evident at −33137 and −31118 (P for linear trend = 0.002 and 0.006, respectively). A similar pattern of association was observed at position −33137 for serum triglycerides (P = 0.06, P for linear trend = 0.027). No other metabolic traits or anthropometric measures were significantly associated with the five polymorphisms in either men or women (data not shown). When the five polymorphisms were considered together, the AGGIG haplotype was associated in men with high levels of FFA (P = 0.001), whereas the GAGIC haplotype was associated with low levels (P = 0.023) (Fig. 2A). The AGGIG haplotype was more strongly associated with high FFA than any other haplotype carrying alleles associated with high FFA at individual loci (Fig. 2A). The effect of this haplotype was similar in homozygosis or heterozygosis (P = 0.045), with AGGIG carriers having 31% higher FFA levels than non-carriers (P = 0.0002) (Fig. 2B). A similar pattern was observed for triglycerides (20% increase in AGGIG carriers, P = 0.025), but not for fasting insulin or other metabolic traits. No association between CD36 haplotypes and FFA or triglycerides levels was observed in women.

To determine whether the CD36 AGGIG haplotype was also associated with CAD, we typed two populations of Caucasian individuals with type 2 diabetes, one from Boston (n = 197), the other from Italy (n = 321). Each population included a group of cases with clinically significant CAD, and a group of controls with negative cardiovascular history and a normal exercise treadmill test (ETT) or angiography (Table 4). The overall prevalence of AGGIG carriers was similar in the two populations and not significantly different from that in non-diabetic individuals (0.538 in type 2 diabetic subjects from Boston, 0.551 in type 2 diabetic subjects from Italy and 0.578 in non-diabetic subjects). Among the type 2 diabetic individuals from Boston, AGGIG carriers were significantly more frequent in CAD-positive cases than CAD-negative cases (P = 0.045).
An association between AGGIG haplotype and CAD was after adjusting for age, gender and smoking) (Table 5). In contrast with the findings associated with the AGGIG haplotype was 1.6 (95% CI 1.2–4.2, P = 0.015) (Table 5). In contrast with the findings of FFA, the increase in CAD risk associated with the AGGIG haplotyp was similar in men and women (P = 0.32 for interaction with gender). Results were similar when body weight and the prevalence of hypertension, which were different in cases and controls, were included in the logistic regression model (data not shown). The relation to serum lipid traits could not be evaluated because of the high prevalence of antilipemic treatment in these individuals. To identify common polymorphisms in functional regions that may be in LD with the AGGIG haplotype, we resequenced all exons together with 1.5 kb of each promoter in 18 Caucasian individuals from the general population. One common sequence variant (−22674T > C) was detected that was not present in SNP databases. It was placed in the upstream promoter, 14 bases 5′ of the transcription start site, in the core of a binding element for the transcriptional repressor GFI1B. This SNP was in complete LD with the −33137A>G htSNP. No common variants affecting the coding sequence were identified. We also analyzed the relationship between the five haplotype tagging polymorphisms and an intron 3 microsatellite (in3TGN) that was previously found to be associated with the expression of an alternative spliced, inactive transcript and increased susceptibility to cerebral malaria in Thai (26). Two major alleles were found at this locus in our Caucasian population, corresponding to 12 and 13 TG repeats, with frequencies of 0.427 and 0.443, respectively. The 13 repeat allele (the one associated with the expression of the inactive transcript in Thai) corresponded almost exactly to the A allele at position −33137, whereas the 12 repeat allele was associated with the G allele.

### DISCUSSION

Previous studies, mostly from Japan, have reported an association between rare CD36 variants and high blood FFA and triglycerides (19–21). Impaired insulin-mediated glucose disposal has been also documented, although results have not been uniform (19–21). The aim of our study was to investigate whether more common polymorphisms at this locus could also affect lipid and glucose metabolisms and influence cardiovascular risk. In a population of non-diabetic individuals of Caucasian ancestry, we found that one of the few common haplotypes occurring at this locus (AGGIG) is associated with increased fasting levels of FFA and triglycerides (19–21). Impaired insulin-mediated glucose disposal has been also documented, although results have not been uniform (19–21). The aim of our study was to investigate whether more common polymorphisms at this locus could also affect lipid and glucose metabolisms and influence cardiovascular risk. In a population of non-diabetic individuals of Caucasian ancestry, we found that one of the few common haplotypes occurring at this locus (AGGIG) is associated with increased fasting levels of FFA and triglycerides. These findings are unlikely to be due to chance. First, the P-value for the association with FFA is highly significant. Second, the same haplotype associated with FFA was...
significant associations with a related phenotype (CAD) in an independent study, and a similar tendency was observed in yet another population. Nonetheless, the results of association studies must always be interpreted with caution (27,28), especially when multiple comparisons are performed, and replication in other settings is needed before a link between CD36 variability, FFA metabolism and cardiovascular disease is firmly established.

The metabolic phenotype associated with the AGGIG haplotype—higher FFA and triglycerides in the presence of normal insulin and glucose levels—duplicates, although on a smaller scale, the alterations displayed by the CD36 knockout mice (7–9). Thus, we postulate that the AGGIG haplotype is associated with some degree of CD36 deficiency in the skeletal muscle and other tissues where the expression of CD36 is normally high. The resulting decrease in FFA clearance would raise the levels of plasma FFA, redirecting them to the liver where their uptake is independent of CD36 leading to increased production of triglycerides.

Although the association between CD36 variants and FFA could be detected in the overall population, this genetic effect appears to be much stronger in men than women. The reasons for such sexual dimorphism are unclear, but may be related to the profound differences in FFA metabolism between genders, reflecting in part differences in the hormonal environment and body fat distribution. Similar to the results of other studies (24,25), women from our population had significantly higher fasting FFA concentrations than men. Furthermore, the correlation between FFA levels and body weight or waist circumference was much weaker in this gender than in men ($r = 0.14$ versus $r = 0.28$ for %IBW and $r = 0.12$ versus $r = 0.23$ for waist circumference). Thus, FFA metabolism appears to be under different regulatory mechanisms in men and women. If CD36 plays a more central role in men, its deficiency may have a bigger impact in this gender.

Increased FFA levels are toxic for endothelial cells and have been linked to increased cardiovascular risk in epidemiological studies (29–31). Thus, it is conceivable that the AGGIG haplotype association with CAD is mediated by its association with increased FFA levels. However, the fact that the haplotype association with CAD was not restricted to men, as was the case for FFA, suggests that additional factors might be involved. CD36 also functions as a scavenger receptor for oxidized LDL and advanced glycation end products (AGEs), which are major triggers of inflammation and atherosclerosis in diabetic subjects (15,16,32,33). Thus, a reduced clearance of these metabolites could also contribute to the AGGIG haplotype effect on CAD risk.

The number and identities of the causal variants responsible for these findings are unknown at this time. There are at least two candidate variants in the first LD block. One ($-22674T > C$), which was identified in this study, is located in the CD36 upstream promoter. Allele T, which is in complete LD with the FWA-associated allele A at $-33137$, determines the presence of a binding site for the transcriptional repressor GFI1B (34), whereas allele C determines its absence. The other candidate variant is a TG microsatellite in intron 3. One of the alleles (13 repeats) has been reported to determine the expression of an alternative spliced, inactive transcript lacking exons 4 and 5 (26). This allele, which is associated with decreased P. falciparum clearance and increased malaria severity in Thai (26), is in complete LD with the FWA-associated 33137A allele. In the second block, two of the polymorphisms defining the AGGIG haplotype are placed in the 3’-UTR where they could determine decreased mRNA stability.

Finally, some limitations of our study should be considered. One concerns the characterization of the haplotype structure at the CD36 locus. Data from the literature suggest that a 3.0 kb SNP spacing should be narrow enough to identify all common ($\geq$5%) haplotypes if extensive LD is present as it is the case for CD36 (35). Furthermore, for a large part of the gene we used polymorphisms representative of major LD bins identified by a resequencing project (36). However, we cannot exclude the possibility that other common haplotypes might be identified by further narrowing the SNP spacing. Thus, our study, though comprehensive, might have missed some genetic effects. Another limitation is that we only considered basic metabolic traits such as fasting levels of metabolic substrates and insulin. Two caveats also apply to the results of the association study with CAD. One is that controls may have included individuals with significant CAD as a result of the occurrence of silent ischemia in diabetes and the less than perfect negative predictive

**Figure 2.** Association between common CD36 haplotypes and FFA levels in men. (A) Haploscore output. Positive and negative scores denote an association with high and low FFA levels, respectively. Haplotype specific $P$-values are reported along with the global $P$-value. (B) FFA levels in carriers of different haplotype combinations. Medians are indicated by squares, interquartile ranges by lines. ‘X’ denotes any haplotype other than AGGIG and GAGIC. Data refer to 167 men for whom genotypes were available for all tag polymorphisms and the assigned diplotypes had $\geq 75\%$ probability; $P = 0.0002$ for AGGIG carriers versus non-carriers.
Table 4. Clinical characteristics of CAD-positive cases and CAD-negative controls with type 2 diabetes from Boston and Italy

<table>
<thead>
<tr>
<th></th>
<th>Boston CAD −</th>
<th>Boston CAD +</th>
<th>Italy CAD −</th>
<th>Italy CAD +</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>79</td>
<td>118</td>
<td>201</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Males (%)</td>
<td>52.0</td>
<td>67.5</td>
<td>41.8</td>
<td>67.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age (years)</td>
<td>68 ± 7</td>
<td>66 ± 7</td>
<td>61 ± 8</td>
<td>64 ± 8</td>
<td>0.52</td>
</tr>
<tr>
<td>Age at diabetes Dx (years)</td>
<td>54 ± 9</td>
<td>53 ± 10</td>
<td>49 ± 10</td>
<td>49 ± 11</td>
<td>0.04</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>30.0 ± 5</td>
<td>31.5 ± 7</td>
<td>30.9 ± 5</td>
<td>29.6 ± 5</td>
<td>0.38</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>7.4 ± 1.1</td>
<td>7.5 ± 1.4</td>
<td>8.4 ± 1.8</td>
<td>8.6 ± 1.8</td>
<td>0.09</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet only (%)</td>
<td>10.1</td>
<td>6.8</td>
<td>12.9</td>
<td>10.0</td>
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<tr>
<td>Oral agents (%)</td>
<td>48.1</td>
<td>47.4</td>
<td>49.2</td>
<td>37.5</td>
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<tr>
<td>Insulin (%)</td>
<td>41.8</td>
<td>45.8</td>
<td>37.8</td>
<td>52.5</td>
<td>0.04</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>68.4</td>
<td>79.7</td>
<td>75.1</td>
<td>84.0</td>
<td>0.04</td>
</tr>
<tr>
<td>Ever smoked (%)</td>
<td>45.6</td>
<td>67.0</td>
<td>27.9</td>
<td>41.7</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Data are % or mean ± SD.

Table 5. Risk of coronary artery disease associated with the CD36 AGGIG haplotype

<table>
<thead>
<tr>
<th></th>
<th>Boston CAD −</th>
<th>Boston CAD +</th>
<th>Italy CAD −</th>
<th>Italy CAD +</th>
<th>Boston + Italy</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>44.3</td>
<td>60.2</td>
<td>52.2</td>
<td>60.0</td>
<td>50.0</td>
</tr>
<tr>
<td>AGGIG carriers (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Odds ratio</td>
<td>2.3a</td>
<td>1.4a</td>
<td>1.6b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>1.2–4.2</td>
<td>0.9–2.3</td>
<td>1.1–2.3</td>
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<td></td>
</tr>
<tr>
<td>P</td>
<td>0.01</td>
<td>0.14</td>
<td>0.015</td>
<td></td>
<td></td>
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</tbody>
</table>

aAdjusted for age, gender and smoking.
bAdjusted for age, gender, smoking and population (Boston versus Italy).

value of the ETT (37). Because of this possible misclassification, the risk of CAD associated with the AGGIG haplotype may have been underestimated. The other caveat is that the association with CAD was detected in diabetes—a condition characterized by increased FFA levels, increased production of oxidized LDL and AGE-modified proteins, and accelerated atherogenesis. Whether a similar effect is also present among normoglycemic individuals remains to be determined.

MATERIALS AND METHODS

Subjects

Non-diabetic individuals. The association between CD36 variants and metabolic traits was evaluated in 585 Caucasian residents of the Gargano area (East Coast of Italy). Subjects were recruited among the employees of the hospital ‘Casa Sollievo della Sofferenza’ (San Giovanni Rotondo, Italy), who had fasting plasma glucose <7 mmol/l at screening and were not taking any medications. The study protocol and informed consent procedures were approved by the local research ethic committee. All study subjects were examined between 8:00 and 9:00 am after an overnight fast. Height and weight were used to calculate body mass index (BMI) and % IBW (calculated by multiplying BMI by 4.39 for males and 4.76 for females). Waist circumference (the widest value between the lower rib margin and the iliac crest) was measured with a plastic measuring tape by the same investigator in all subjects while standing. Systolic and diastolic (disappearance of Korotkoff sound, phase V) blood pressures were measured in the sitting position with an appropriately sized cuff after a 5 min rest. Plasma glucose (mmol/l), serum insulin (pmol/l) and lipid profile (total serum cholesterol, HDL cholesterol and serum triglycerides) were measured using commercially available enzymatic kits as previously described (38). Plasma free fatty acids were determined by a microenzymatic assay as described by Shimuzu et al. (39) and implemented in the NEFA C test kit (Wako Chemicals, Richmond, VA, USA). This measurement is on the basis of the esterification of FFA to acyl-CoA by means of acyl-CoA synthase followed by acyl-CoA oxidation with acyl-CoA oxidase and measurement of the resulting hydrogen peroxide with a colorimetric method. The intra- and inter-assay coefficients of variation were 2.2 and 2.8%, respectively.

Case–controls studies. Two populations of individuals with type 2 diabetes (defined according to the WHO criteria) were studied, one from Boston (n = 197), the other from San Giovanni Rotondo, Italy (n = 321). The study protocol and informed consent procedures were approved by the local research ethic committees. Each population included a group of CAD-positive cases and a group of CAD-negative controls. In the Boston study, the CAD-positive cases—defined as subjects who had a stenosis >50% in at least one major coronary artery or their main branches—were recruited among type 2 diabetic patients who underwent cardiac catheterization at the Beth Israel Deaconess Medical Center (BIDMC) between February 1, 2000 and January 31, 2002. CAD-negative controls were Joslin patients (the Joslin Clinic serves as the BIDMC Diabetes Clinic) who were aged 55 or older, had diabetes for 5 years or more, and had a negative cardiovascular history and a normal ETT according to a standard Bruce protocol.
and ETT or with coronary stenosis (at angiography) major coronary artery or their main branches, or who had angiographic evidence of stenosis. Diabetic patients who attended the local institution from (40). The San Giovanni Rotondo sample consisted of type 2 diabetic patients who attended the local institution from January 2002 to July 2003. Cases were patients who had angiographic evidence of stenosis >50% in at least one major coronary artery or their main branches, or who had acute myocardial infarction. Controls included diabetic patients without symptoms and with normal resting ECG and ETT or with coronary stenosis (at angiography) ≤50%. Clinical features of cases and controls from the two studies are shown in Table 4.

SNP genotyping

Fifty-two polymorphisms spanning the entire CD36 locus were selected from the dbSNP database. Of these, 21 turned out to have a minor allele frequency ≥0.05 in Caucasians and were amenable to reliable genotyping using the methods available in our laboratory. Eight of these polymorphisms were representative of the major LD bins identified in the region between intron 3 and exon 14 by a resequencing project (http://pga.gs.washington.edu/data/cd36/). In the initial LD study, 72 subjects from the general population subsets were typed at these 21 polymorphic loci by means of PCR followed by dot blotting and allelic specific hybridization or gel electrophoresis (for the insertion/deletion at 27645). The five haplotype-tagging polymorphisms were then typed in the study groups by single base extension/fluorescence polarization (AcycloPrime-FP SNP Detection System) using a Wallac VICTOR² Multilabel Plate Reader (Perkin–Elmer) or agarose gel electrophoresis (27645Ins/Del). Genotyping quality was tested by including six blinded duplicate samples in each 96-well assay. The average agreement rate of duplicate samples was >97%. Sequences of the primers and probes used for typing are available from the authors.

Data analysis

Genotype distributions were tested at each polymorphic locus for departure from Hardy–Weinberg equilibrium. Pairwise LD coefficients (D′) were estimated and plotted using the GOLD software package (41). Maximum likelihood estimates of haplotype frequencies were derived using the EM algorithm as implemented in the function haplo.em of the HaploStats suite (42). Haplotype block partitioning and the htSNP selection were conducted by means of the dynamic programming algorithms implemented in the HapBlock software (23), with α = 80% [minimal proportion of chromosomes accounted by common (≥0.05) haplotypes].

Continuous variables were compared among genotype groups by ANOVA using the PROC GLM procedure of the SAS software package (SAS Institute, Cary, NC, USA). All analyses included gender and age as covariates. Fasting insulin, triglycerides and FFA were analyzed after logarithmic transformation. The presence of a linear trend across genotypes (consistent with an additive model) was investigated at each position by assigning a value of 0 to p/p, 1 to p/q and 2 to q/q and using these values as a continuous variable in regression analyses. As FFA levels were significantly different in men and women, data were also analyzed after stratifying the data by gender. The association between FFA levels and common (≥0.05) CD36 haplotypes was analyzed in men using the score statistics proposed by Schaid et al. (42) and implemented in the function HAPLO.SCORE of the HaploStats software. This method allows adjustment for non-genetic covariates (age, gender) and provides a global test of association as well as haplotype-specific tests. After testing for association with haplotypes, diplotype were assigned to each individual on the basis of the posterior probabilities of genetic covariates (age, gender) and provides a global test of the different phases. Diplotype were similarly assigned to CAD cases and controls, and the risk of CAD associated with the carrier status for the AGGIG haplotype was then estimated by logistic regression analysis using age, gender, smoking, body weight and presence of hypertension as covariates. Potential differences in the association between genders or between studies were investigated by adding an interaction term (genotype×gender or genotype×study) to the model. A P-value of 0.05 (unadjusted) was considered as significant.

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