Single-nucleotide Polymorphism of CD36 Locus and Obesity in European Adolescents

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CD36 is a membrane receptor with a wide variety of functions, including the regulation of energy metabolism, fat storage, and adipocyte differentiation. To assess the relationship between CD36 gene single-nucleotide polymorphisms (SNPs) and obesity in adolescents, we evaluated the relationship between CD36 SNPs and the risk of obesity in a case–control study composed of 307 obese (age = 15.0 ± 1.1 years) and 339 normal-weight adolescents (age = 14.6 ± 1.1 years). To validate the results, we assessed the relation between the same SNPs and percentage of body fat (BF%) and BMI in 1,151 European adolescents (age = 14.8 ± 1.4 years). SNPs with a minor allele frequency >0.10 were selected to tag CD36. Genotyping was performed on an Illumina system. Four SNPs (rs3211867, rs3211883, rs3211908, and rs1527483) were associated with increased risk of obesity in the case–control study (odds ratio (OR) (95% confidence interval)): 1.96 (1.26–3.04), P = 0.003; 1.73 (1.16–2.59), P = 0.007; 2.42 (1.47–4.01), P = 0.0005 and 1.95 (1.25–3.05), P = 0.003, respectively. The same four SNPs were associated with higher BMI (P < 0.05) and BF% (P < 0.04) in the validation study. Further analyses identified a haplotype (frequency: 0.05) carrying the minor allele of these SNPs as being associated with obesity (OR: 2.28; P = 0.0008) in the case–control study and with excess adiposity (i.e., higher BF% (P = 0.03) and BMI (P = 0.04)) in the validation study. Our data suggest that genetic variability at the CD36 gene locus could be associated with body weight variability in European adolescents but these findings require replication.

INTRODUCTION

CD36 is an 88-kDa membrane protein expressed at the surface of a wide variety of cell types, including adipocytes, skeletal muscle cells, and monocytes/macrophages (1). It belongs to the B scavenger receptor family and binds with high affinity to lipid-based ligands such as modified low-density lipoprotein, long-chain polyunsaturated fatty acids, and apoptotic cell membranes (1,2).

Gene knockout and overexpression experiments in mice have indicated that CD36 plays a role in energy metabolism, fat storage, and adipocyte differentiation (3,4). Muscle-specific CD36 overexpression enhances fatty acid oxidation, decreases plasma free fatty acid, glucose and insulin levels, and lowers body weight (3). In contrast, CD36 invalidation is associated with high plasma free fatty acid and triglyceride levels, low fasting glucose levels, and less weight gain during a high-fat diet (5). In humans, CD36 is also related to metabolic disorders. First, CD36 deficiency (type 1) is associated with certain features of metabolic syndrome (6,7). Second, CD36 expression in adipocytes is positively correlated with body fat (BF) (8) and is reduced after a period of weight loss (9). Third, several single-nucleotide polymorphisms (SNPs) in the CD36 gene have been associated with metabolic disorders related to excess fat depots (10–14), and a (TG)-repeat in intron 3 has been linked to an elevated BMI in Korean patients with coronary heart disease (15).

The above-mentioned studies assessed the association between CD36 SNPs and metabolic disorders in adults (10–14). However, it is not yet known from population studies whether CD36 SNPs influence BF in adolescents. Genetic association studies in young people are important in that the influence of behavioral and exogenous factors are less marked than in

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adults leaving a proportionally greater influence of genetic determinants on the phenotype. Hence, the aim of this study was to assess the relationship between CD36 genetic variability and obesity and BF accumulation in a population of adolescents. We compared the distribution of seven CD36 SNPs in a case–control study on obesity and then looked for a relationship between these SNPs and the percentage of BF (BF%) in an independent sample of European adolescents.

METHODS AND PROCEDURES

The Pécs case–control study on obesity
The study population consisted of 307 adolescents referred for obesity to the outpatient clinic of the University of Pécs’ Department of Paediatrics (Pécs, Hungary) and 339 healthy, normal-weight adolescents recruited via Pécs schools. Subjects were aged between 13 and 17. Obesity was defined as a BMI over the value given by Cole et al. (16), corresponding to 30 kg/m² at the age of 18. None of the subjects had chronic diseases and none were taking drugs or were dieting. Blood samples for DNA extraction was collected in EDTA K3 tubes. Genomic DNA was extracted from peripheral blood leukocytes, according to a standard procedure.

The HELENA cross-sectional study
Recruitment and phenotyping of the participating adolescents in the HELENA study (HELENA-CSS, the validation study here) (“Healthy Lifestyle in Europe by Nutrition in Adolescence,” www.helenastudy.com) have been described previously (17). Briefly, a total of 3,865 adolescents aged 12–18 years old, were recruited between 2006 and 2007. Data were collected in 10 centers from 9 European countries (18). Subjects were randomly selected according to a proportional cluster sampling methodology taking into account geographical repartition in each city, private/public school ratio, and number of classes by school. One-third of the classes were randomly selected for blood collection, resulting in a total of 1,155 blood samples for the subsequent clinical biochemistry assays and genetic analyses.

In both the Pécs case–control study and the HELENA-CSS, data were collected on a detailed case report form, in accordance with standardized procedures. In each center, trained physicians carried out complete physical examinations, including weight, height, and blood pressure measurements. For both studies, the protocol was approved by each investigating center’s independent ethics committee (19). Written, informed consent was obtained from each adolescent and both of his/her parents or legal representatives. Participation in the studies was voluntary.

Anthropometry
Skinfold thicknesses were measured at six sites (biceps, triceps, subscapular, suprailiac, thigh, and calf) using a Holtain Caliper (20). The reliability of skinfold thickness measurements is known to be adequate for epidemiological surveys (21). BF% was derived from the skinfold measurements according to equations published by Parizková et al. (22). The BMI was calculated.

Blood analysis
Blood for DNA extraction was collected in EDTA K3 tubes, stored at the Analytical Laboratory at the University of Bonn and then sent to the Genomic Analysis Laboratory at the Institut Pasteur de Lille in Lille, France (23). DNA was extracted from white blood cells with the Puregene kit (QIAGEN, Courtaboeuf, France) and stored at −20 °C.

Gene SNP selection and genotyping
With the criterion used in our SNP selection procedure (a minor allele frequency above 0.1 and tag SNPs with an r² value >0.8), the HapMap database (2007 release; http://www.hapmap.org) describes five haplotype blocks and two independent SNPs that span the whole gene. In this study, we selected one SNP from each of the five haplotype blocks (block 1: rs152779, block 2: rs3211816, block 3: rs3211867, block 4: rs3211883, and block 5: rs3211931) and the two independent SNPS (rs3211908 and rs1527483). We also selected three other SNPs (rs1984112, rs1761667, and rs1049673) from the literature (12) in order to cover the whole range of genetic variability. Altogether, subjects were genotyped on an Illumina chip, one SNP (rs1761667) using the VeraCode technology and the nine other SNPs using GoldenGate technology (http://illumina.com/). Genotyping was performed once for each sample.

Statistical methods
Statistical analyses were performed with SAS software (SAS Institute, Cary, NC). Departure from Hardy–Weinberg equilibrium was tested using a χ²-test. Interlocus linkage disequilibrium (LD) was assessed using Haplovie. Allele frequencies were estimated by gene-counting. In the Pécs case–control study, multivariate logistic regression was used to calculate the odds ratios (ORs) for obesity for different allele exposures, using various genetic models. Adjustment variables were age and gender. A general linear model was used to compare mean values of anthropometric markers. In this case, the adjustment variables were age, gender, and center. Dominant and recessive models were tested. Given the rarity of some minor allele only the dominant models are presented. The statistical significance threshold was set to P ≤ 0.007 (after Bonferroni correction, i.e., 0.05/7) for the Pécs case–control study and to P ≤ 0.05 for the HELENA-CSS (validation study). Haplotype analyses were based on a maximum likelihood model (24) linked to the s.e.m. algorithm (25) and were performed using the THESIAS software package developed by INSERM unit U525, Paris, France (http://ecgene.net/genecanvas) (26). All variables were normally distributed.

RESULTS

Table 1 shows the clinical characteristics of the subjects from the case–control and the cross-sectional studies. The mean age (s.d.) of the obese and normal-weight adolescents in the case–control study and the adolescents in the cross-sectional study was 15.0 (1.1), 14.6 (1.1), and 14.8 (1.4), respectively. There was a higher proportion of girls in the normal-weight control group and in the cross-sectional study than in the group of obese adolescents. As expected, obese adolescents had significantly higher BMI and BF% values than the normal-weight adolescents in the case–control study (all P < 0.001).

Table 1 Characteristics of the subjects

<table>
<thead>
<tr>
<th></th>
<th>Obesity case–control study</th>
<th>HELENA cross-sectional study</th>
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<tbody>
<tr>
<td></td>
<td>Normal weight</td>
<td>Obese</td>
</tr>
<tr>
<td>n</td>
<td>339</td>
<td>307</td>
</tr>
<tr>
<td>Boys/girls</td>
<td>164/175</td>
<td>165/142</td>
</tr>
<tr>
<td>Age (years)</td>
<td>15.0 (1.1)</td>
<td>14.6 (1.1)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>56.4 (8.9)</td>
<td>91.4 (18.6)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168.4 (8.7)</td>
<td>166.8 (8.8)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.1 (2.5)</td>
<td>32.7 (5.5)</td>
</tr>
<tr>
<td>BF (%)</td>
<td>25.1 (6.4)</td>
<td>39.4 (6.0)</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>118.6 (13.5)</td>
<td>127.6 (11.0)</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>66.6 (8.6)</td>
<td>72.4 (8.4)</td>
</tr>
</tbody>
</table>

Data are means (s.d.). BF, body fat; BP, blood pressure.
DNA samples were genotyped for the 10 selected CD36 SNPs. The genotyping success rate varied between 97.1 and 100%. In each study, all the observed SNP genotype frequencies conformed to Hardy–Weinberg proportions. The LD pattern for the SNPs was assessed in the HELENA-CSS using both the $r^2$ and $D^2$ values (Figure 1). The three SNPs selected from literature data (rs1984112, rs1761667, and rs1049673) were in strong LD with three haplotype blocks described by the HapMap database (rs1761667 with rs1527479 (block 1) ($D^2 = 0.98$, $r^2 = 0.93$), rs1984112 with rs3211816 (block 2) ($D^2 = 0.97$, $r^2 = 0.89$), and rs1049673 with rs3211931 (block 5) ($D^2 = 1$, $r^2 = 0.98$)). Thus, further analyses were performed with the seven following SNPs: rs1527479, rs3211816, rs3211867, rs3211883, rs3211908, rs3211931, and rs1527483.

Table 2 shows the genotype distribution of the seven CD36 SNPs in obese and normal-weight subjects, the age- and gender-adjusted OR and the 95% confidence interval for obesity from the case–control study. Using a dominant model, seven SNPs were associated with a higher risk of obesity (rs3211867: OR (95% confidence interval) = 1.96 (1.26–3.04), $P = 0.003$; rs3211883: OR = 1.73 (1.16–2.59), $P = 0.007$; rs3211908: OR = 2.42 (1.47–4.01), $P = 0.0005$ and rs1527483: OR = 1.95 (1.25–3.05), $P = 0.003$). After Bonferroni correction, the rs3211867, rs3211883, rs3211908, and rs1527483 SNPs were still significantly associated with a greater risk of obesity.

Table 3 shows the BF% and BMI as a function of the CD36 genotypes in the cross-sectional study. Multivariate analyses (adjusted for age, gender, and center) revealed that the mean BMI and BF% were significantly higher in carriers of at least one minor allele of rs3211867 (BMI: $P = 0.03$, BF%: $P = 0.02$), rs3211883 (BMI: $P = 0.03$, BF%: $P = 0.05$), rs3211908 (BMI: $P = 0.04$, BF%: $P = 0.02$), and rs1527483 (BMI: $P = 0.05$, BF%: $P = 0.04$) compared with individuals who were homozygous for the common allele. These associations were not modified by further adjustment for pubertal status (data not shown).

Haplotype analyses using the seven SNPs (order: rs1527479, rs3211816, rs3211867, rs3211883, rs3211908, rs3211931, and rs1527483) were performed to assess the relationship with obesity in the case–control study and the association with BMI and BF% in the HELENA-CSS. Nine haplotypes had an estimated frequency of $>1\%$ (Tables 4 and 5). Compared with the most common haplotype (GGCTGGG; estimated frequency: 0.44), 1 haplotype: AGAAAAA (estimated frequency: 0.05, minor alleles underlined) was significantly associated with a higher risk of obesity (OR: 2.28 for obesity; $P = 0.0008$). This haplotype was also associated with a higher BF% ($P = 0.03$) and BMI ($P = 0.04$) in the cross-sectional study.

**DISCUSSION**

The goal of this study was to assess whether common polymorphisms at the CD36 locus could affect body-weight regulation in adolescents. The results showed that four SNPs were associated with a higher risk of obesity in the case–control study and excess adiposity in the cross-sectional study. Further analyses identified a haplotype carrying the minor allele of these SNP as being linked with obesity and a higher BF in the two studies. These findings, however, must be interpreted with caution and replication in other population samples are necessary before a link between CD36 gene variability and body-weight metabolism can definitely be established.

The rs3211908, rs3211867, rs3211883, and rs1527483 SNPs were related to the risk of obesity and the BF although the strength of the association was weaker for the latter three SNPs. These SNPs were in partial LD with rs3211908 (0.43 < $r^2 < 0.64$), suggesting that the results reflect a single signal.

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### Table 2 Genotype distribution in obese and normal-weight subjects of the Pécs case–control study

<table>
<thead>
<tr>
<th></th>
<th>Normal weight</th>
<th></th>
<th>Obese</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
<td>12</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td>rs1527479</td>
<td>98 (0.29)</td>
<td>174 (0.52)</td>
<td>66 (0.19)</td>
<td>64 (0.21)</td>
</tr>
<tr>
<td>rs3211816</td>
<td>134 (0.40)</td>
<td>162 (0.48)</td>
<td>40 (0.12)</td>
<td>111 (0.36)</td>
</tr>
<tr>
<td>rs3211867</td>
<td>299 (0.882)</td>
<td>40 (0.118)</td>
<td>0</td>
<td>244 (0.797)</td>
</tr>
<tr>
<td>rs3211883</td>
<td>284 (0.840)</td>
<td>53 (0.157)</td>
<td>1 (0.003)</td>
<td>233 (0.764)</td>
</tr>
<tr>
<td>rs3211908</td>
<td>311 (0.917)</td>
<td>28 (0.083)</td>
<td>0</td>
<td>255 (0.831)</td>
</tr>
<tr>
<td>rs3211931</td>
<td>108 (0.32)</td>
<td>163 (0.48)</td>
<td>66 (0.20)</td>
<td>84 (0.27)</td>
</tr>
<tr>
<td>rs1527483</td>
<td>299 (0.885)</td>
<td>39 (0.115)</td>
<td>0</td>
<td>248 (0.808)</td>
</tr>
</tbody>
</table>

Data are n (freq). Frequent allele: 1; minor allele: 2.
CI, confidence interval; OR, odds ratio.
$^*$P value is for $\chi^2$-test. ORs. **P values are for dominant model. $P$ values are adjusted for age and gender.
This hypothesis is further supported by haplotype analysis, which showed that only one haplotype (AGAAAAA; frequency ~5%) carrying the minor allele of the four SNPs was associated with obesity and BF in the case–control and cross-sectional studies, respectively. All other haplotypes were not consistently linked to excess BF suggesting that the genetic variant responsible for these finding is in LD with the AGAAAAA haplotype block.

Several CD36 variants are known to be associated with metabolic disorders related to excess BF, such as high plasma...
free fatty acid and triglyceride levels, low high-density lipoprotein-cholesterol (10–12), insulin resistance, type 2 diabetes mellitus (13,14), and metabolic syndrome (10). Although some of these SNPs were genotyped or were in high LD with our SNPs, we found no significant association with fasting plasma triglycerides, high-density lipoprotein-cholesterol, glucose and insulin levels in the cross-sectional study (data not shown). In Korean coronary heart disease patients, a common CD36 (TG)-repeat in intron 3 has been linked to high BMI (15). The LD existing between this dinucleotide repeat polymorphism and our SNPs is not known. To the best of our knowledge, the relation between CD36 gene variability and body-weight regulation has not previously been investigated in adolescents. An advantage of working with adolescents is that their body weight is less likely influenced than adults by important lifestyle determinants, such as smoking or alcohol intake, which could limit the statistical power to detect genetic associations in adults. Our results support the original findings in transgenic mice that showed altered BF metabolism, but not metabolic disorders. The reasons for these discrepancies warrant further investigations.

Previous genome-wide scans (GWAS) for children and adolescents with extreme obesity have not linked CD36 SNPs to obesity suggesting that CD36 variability is not a major determinant of obesity risk (27,28). Other reasons may explain the lack of association. First, it is possible that no CD36 SNP was present on the chips or could be imputed from other SNPs. In this study some CD36 tag SNPs were not associated with obesity so, if the associated haplotype block was not appropriately tagged by genotyping or imputation, the association with obesity could have been missed. Second, the GWAS were performed in children with extreme obesity who may suffer from different genetic defects than adolescents with mild obesity (27). Finally, in the GWAS with population samples including <700 obese children, the P value of the association between CD36 tag SNPs and obesity might not reach the required P value threshold in GWAS (at least P < 10 −7), and thus CD36 associations could have been missed.

The identity of the responsible variant(s) for our finding is still unknown. Rs3211908 is located 100 base pairs downstream of the donor site of exon 7 that codes for the CD36 amino acids, which interacts with long-chain polyunsaturated fatty acids (29). Although the Genomatix MatInspector analysis software (www.genomatix.de) (30) indicates that the T allele of rs3211908 abolishes a predicted glucocorticoid receptor binding site (i.e., a response element), the likelihood that this mutation may affect CD36 gene expression is uncertain owing to its intrinsic position in the middle of the gene. Alternatively, the AGAAAAA haplotype spans a locus that include exon 5 to exon 11, which could be in LD with an as yet undetected functional SNP located in this region or elsewhere within the CD36 gene.

Several hypotheses might explain the link between CD36 genetic variability and BF depots. First, CD36 is a cell membrane long-chain polyunsaturated fatty acid transporter in a wide variety of metabolically active tissues, including heart, muscle, and adipocytes (31). Impaired CD36 function could decrease the intramuscular fatty acid oxidation rate and thus increase the availability of fatty acids for storage in adipocytes (32,33). Second, cell coculture experiments have shown that activated macrophages secrete various factors that inhibit the formation of mature adipocytes (34). A CD36 dysregulation in infiltrated macrophages within adipose tissue could alter the signaling pathway that feeds back to control adipocyte expansion (34). Third, by supplying long-chain polyunsaturated fatty acids and oxidized low-density lipoprotein-cholesterol, CD36 plays a key role in the activation of peroxisome proliferator–activated receptor γ (PPARγ) (35,36), a nuclear receptor responsible for adipocyte differentiation and adipogenesis (37). Thus, an alteration in CD36 may impact on PPARγ-mediated adipocyte differentiation (38).

This study had several strengths and several limitations. The analyses were carried out in two independent samples of European adolescents, an approach that reduces, but does not eliminate, the likelihood of a spurious finding. However, even if two independent samples were used to assess the association this might still be insufficient to definitely conclude on the possible link between CD36 and obesity given the limited sample size of our surveys. Replication in other samples, drawn from different population background, is necessary to confirm our hypothesis-generating finding. Indeed, a posteriori statistical power calculation indicates that using a threshold of P < 0.007, the case–control study had a statistical power of 80% to detect ORs ranging between 1.80 and 2.25 for allele frequencies ranging from 0.30 to 0.10. Similarly, using a P value at 0.05 and a common variance of 3.7 kg/m2 for BMI (6.7% for BF%), the HELENA study had a statistical power of 80% to detect a BMI (BF%) difference of 0.7–1 kg/m2 (1.3–1.8 BF%) for allele frequencies ranging from 0.30 to 0.10, respectively. Thus, only large associations could be detected in the case–control study. It is therefore likely that the effect size observed in the case–control study is overestimated. With no doubt, additional studies are necessary to confirm the results. Another limitation concerns the characterization of the haplotype structure of the CD36 locus. Although seven SNPs were used to tag the shorter isoform of CD36, the spacing may not be narrow enough to identify all common haplotypes. Thus, further fine mapping of the CD36 locus is needed to understand the relation between CD36 and body-weight metabolism. Finally, we used simple anthropometric and metabolic traits that may reduce the ability to detect more subtle associations.

In conclusion, we have assessed the relation between CD36 gene variations and obesity and identified a haplotype that was associated with obesity and BF% in two independent populations of adolescents. Further studies are necessary to confirm the results and to identify the molecular mechanism relating this haplotype with a functional disorder of the CD36 gene that could affect body-weight homeostasis.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/ob
ACKNOWLEDGMENTS
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DISCLOSURE
The authors declared no conflict of interest.

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REFERENCES