A common haplotype of carnitine palmitoyltransferase 1b is associated with the metabolic syndrome

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(Submitted 30 November 2011 – Final revision received 3 April 2012 – Accepted 4 May 2012 – First published online 19 July 2012)

Abstract
The carnitine palmitoyltransferase (CPT) enzyme system facilitates the transport of long-chain fatty acids into mitochondria to provide substrates for β-oxidation. We performed an analysis including three coding SNP in the muscle isoform of the CPT1b gene (rs3213445, rs2269383 and rs470117) and one coding SNP in the CPT2 gene (rs1799821) to find associations with traits of the metabolic syndrome (MetS). Male participants (n 755) from the Metabolic Intervention Cohort Kiel were genotyped and phenotyped for features of the MetS. Participants underwent a glucose tolerance test and a postprandial assessment of metabolic variables after a standardised mixed meal. Carriers of the rare CPT1b 66V (rs3213445) allele had significantly higher γ-glutamyl transpeptidase (GGT), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvate transaminase (GPT) activities (P = 0.0001, 0.03 and 0.048, respectively) and a higher fatty liver index (FLI, P = 0.026). Fasting and postprandial TAG (P = 0.007 and 0.009, respectively) and fasting glucose (P = 0.012) were significantly higher in 66V-allele carriers. The insulin sensitivity index determined after a glucose load was lower in those subjects (P = 0.005). Total cholesterol (P = 0.051) and LDL-cholesterol (P = 0.062) tended to be higher in 66V-allele carriers when compared with 66I homozygotes. Homozygosity of the rare K531E allele presented with lower GOT and GPT activities (P = 0.011 and 0.027, respectively). E531E homozygotes tended to have lower GPT and FLI (P = 0.078 and 0.052, respectively). CPT2 V368I (rs1799821) genotypic groups did not differ in the investigated anthropometric and metabolic parameters. The present results confirm the association of CPT1b coding polymorphisms with the MetS, with a deleterious effect of the CPT1b I66V and a protective impact of the CPT1b K531E SNP, whereas haplotype analysis indicates a relevance of the E531K polymorphism only.

Key words: Carnitine palmitoyltransferase; Polymorphisms; TAG; Cholesterol; Metabolic syndrome

Mitochondrial oxidation of long-chain fatty acids provides an important source of energy for the heart as well as for skeletal muscle during prolonged aerobic work and for hepatic ketogenesis during long-term fasting. The carnitine shuttle is responsible for transferring long-chain fatty acids across the barrier of the inner mitochondrial membrane to gain access to the enzymes of β-oxidation. The shuttle consists of three enzymes (carnitine palmitoyltransferase 1 (CPT1), carnitine acylcarnitine translocase and carnitine palmitoyltransferase 2 (CPT2)) and a small, soluble molecule, carnitine, to transport fatty acids as their long-chain fatty acylcarnitine esters1. CPT1b is the muscle isoform of CPT1 but also expressed in adipocytes and testes and CPT2 is expressed ubiquitously with a predominant expression in the liver and intestine.

Abbreviations: CPT, carnitine palmitoyltransferase; MetS, metabolic syndrome.

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The CPT1b (muscle) gene is located on the human chromosome 22q13.33; the CPT2 gene on chromosome 1p32.

In a French-Canadian population, an interaction of CPT1b genetic variants with fat intake to modulate obesity has been shown. Furthermore, in African ancestry men, CPT1b coding polymorphisms were associated with lower intermuscular adipose tissue but higher subcutaneous adipose tissue. In human hypertension, several haplotypes in the CPT1b and CPT2 genes were identified to modify the left ventricular mass index, which is independently associated with the incidence of cardiovascular and all-cause mortality.

Based on these findings, we investigated the contribution of CPT1b and CPT2 coding polymorphisms to traits of the metabolic syndrome (MetS), including parameters of TAG metabolism and susceptibility to the MetS by employing the Metabolic Intervention Cohort Kiel. The investigated SNP are non-synonymous coding SNP: rs470017 (E531K), rs3213445 (I66V) and rs2269383 (D320G). The coding polymorphism in CPT2 rs1799821 (V368I) has not been investigated in association studies so far.

**Experimental methods**

**Study population**

The Metabolic Intervention Cohort Kiel is a prospective population-based cohort study on natural incidence and risk factors of the MetS. A total of 755 men aged 45–65 years were randomly recruited via the registration register of the town of Kiel, Germany. The majority (83%) of subjects were older than 55 years. Exclusion criteria were known diabetes, liver diseases, intestinal absorption disorders, renal diseases, intestinal surgery within the last 3 months, thyroid disorders and hormone therapy. A group of 714 volunteers underwent a clinical examination including measurement of pulse, blood pressure, weight, height, waist circumference and hip circumference. At inclusion in the study, all participants were guided by a physician to complete a standardised questionnaire concerning individual and family history, including lifestyle data such as physical activity, dietary habits (frequency of fish, meat, vegetable, fruit consumption per week, intake of low-fat and fibre-rich products), smoking and alcohol consumption. Participants were instructed not to change their eating habits and not to use any dietary supplements of vitamins and minerals, or special oil preparations, and not to follow a special diet at least 3 d before and during the test meals. Furthermore, participants were instructed to restrain from abnormally high physical activity and excessive alcohol consumption (the day before the study). Assessment of the MetS was based on the criteria of the International Diabetes Federation. Volunteers underwent an oral metabolic tolerance test and an oral glucose tolerance test at different days with a minimum of 3 d in between. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of the Medical Faculty, Christian-Albrechts University of Kiel. Written informed consent was obtained from all subjects. The detailed design and methodology of the study and characteristics of the cohort have been described elsewhere.

**Oral glucose tolerance test**

A 75 g oral glucose tolerance test was performed, following a 12 h overnight fast and after dietary advice was given to ensure a carbohydrate intake of >150 g/d over the previous 3 d. Blood samples for glucose and insulin were taken before and at 30, 60, 120, 180 and 240 min after the glucose load.

**Oral metabolic tolerance test**

An oral metabolic tolerance test provided by Nutricia diät + pharma GmbH was performed as described elsewhere. In brief, after a 12 h fasting period and withdrawal of the fasting blood sample, the subjects drank 500 ml of the oral metabolic tolerance test containing the following ingredients: 30 g protein (11.9% energy), 75 g carbohydrate (29.6% energy; 93% sucrose and 7% lactose), 58 g fat (51.6% energy; 65% SFA and 35% unsaturated fatty acids), 10 g alcohol (6.9% energy), 600 mg cholesterol and 9 mg retinol (31.5 μmol) in the form of retinyl palmitate. The total energy content was 4255 kJ. Before and at 0.5, 1, 2, 3, 4 and 5 h after ingestion, blood samples were drawn for the analysis of insulin, glucose and TAG, and at 6, 7, 8 and 9 h after ingestion for the analysis of TAG and NEFA.

**Laboratory analyses**

After the meals, blood samples were taken on ice and centrifuged, and plasma and serum were deep frozen for later analysis. Serum was used for the determination of insulin, TAG and NEFA and fluoride plasma for glucose determination. Leucocytes and Hb were assessed in EDTA blood. Other parameters were assessed in lithium-heparin plasma.

Lipid, glucose and insulin measurements have been described previously. Serum NEFA were analysed enzymatically (Wako). Homeostatic model assessment-insulin resistance was calculated as glucose (mmol/l) × insulin (mU/l)/135. Postprandial insulin sensitivity was assessed by the oral glucose insulin sensitivity test according to Mari et al.; this index correlated strongly with insulin sensitivity assessed by glucose clamping, the ‘gold standard’ method. The fatty liver index (FLI) was calculated as follows:

\[
FLI = (e^{0.953\log (e)(TAG)} + 0.139\text{BMI} + 0.7186\log (e)(GGT)
+ 0.053\text{waist circumference} - 15.745) \\
(1 + e^{0.953\log (e)(TAG)} + 0.139\text{BMI} + 0.7186\log (e)(GGT)
+ 0.053\text{waist circumference} - 15.745) \times 100.
\]

**Genetic analysis**

SNP selection was based on literature reports and on the dbSNP database. We selected all polymorphic missense variants, which were catalogued in the dbSNP database within the CPT1b and CPT2 genes at this time. Eventually, five SNP were genotyped,
of which four SNP that had a minor allele frequency (MAF) > 1% were included in the analyses (Table 1).

DNA was isolated from buffy coat (100 µl) using E.Z.N.A.® Blood DNA MiniKits (Peqlab Biotechnologie) according to the manufacturer's instructions. Genotyping of CPT1b I66V (rs3213445), CPT1b G320D (rs2269383), CPT1b K531E (rs470117) and CPT2 V368I (rs1799821) polymorphisms was performed with the TaqMan system (ABI). TaqMan analysis was performed as described elsewhere(10).

Statistical analysis

Allele and genotype frequencies were determined by gene counting. The study populations were tested for the distribution of genotypes according to the Hardy–Weinberg equilibrium with a χ² test. As obesity has been shown to have an impact on the outcome of CPT1b polymorphisms(2), metabolic parameters were adjusted for BMI. Between-group comparisons were analysed in a crude model using the Mann–Whitney U test since the probability plot of standardised residuals was not normally distributed. SPSS (PASW Statistics 18, version 18.0.0; SPSS, Inc.) was used for statistical analyses. Linkage disequilibrium (D²) was calculated using the HPlus program(12). Differences were considered significant at P<0.05.

Results

Subject characteristics and CPT1b and CPT2 gene variants

The genotype distributions of genetic variations in the CPT1b and CPT2 genes were in compliance with the Hardy–Weinberg equilibrium. It was observed that two coding SNP (I66V and E531K) in CPT1b and one coding SNP (V368I) in CPT2 had a minor allele frequency above 5%, and therefore they were selected for subsequent genetic analysis (Table 1). Only two subjects were homozygous for the rare V66V allele and were combined for statistical analysis with I66V heterozygotes. There was no difference between the genotypes regarding their dietary habits (data not shown).

Association of carnitine palmitoyltransferase variants with traits of the metabolic syndrome

None of the investigated SNP was associated with BMI or waist circumference. There also was no association with blood pressure values after adjustment for BMI (Table 2). Carriers of the rare 66V allele had higher γ-glutamyl transpeptidase (GGT) (P<0.001), glutamic oxaloacetic transaminase (GOT) (P=0.030) and glutamic pyruvate transaminase (GPT) activity (P=0.048) concomitant with an elevated FLI (P=0.026) in comparison with I66I homozygotes. Total cholesterol (P=0.051) and LDL-cholesterol (P=0.062) tended to be higher in 66V-allele carriers.

Subjects carrying the 66V allele showed higher TAG concentrations in the fasted state (P=0.007) and after ingestion of a mixed meal (Table 2, Fig. 1). Incremental TAG AUC was not significantly different (P=0.383). Fasting glucose concentrations (P=0.045) differed between the genotypic groups, with higher levels in CPT1b 66V carriers. The oral glucose tolerance test-based index of insulin sensitivity (oral glucose insulin sensitivity) was significantly lower in 66V carriers (P=0.005), indicating increased insulin resistance in these subjects. Other lipid parameters, postprandial glucose or insulin were not different between the groups.

Concerning the CPT1b E531K polymorphism, K531K homozygotes presented with significantly lower GGT (P=0.011), GOT (P=0.027) and marginal but not significantly lower GPT activity (P=0.078). NEFA concentrations differed significantly between the genotypes, with lowest levels for E531E (P=0.043). The FLI was lower in K531K homozygosity, although with borderline significance (P=0.052). LDL-cholesterol concentrations tended to be lower in K531K homozygotes when compared with homozygotes and heterozygotes carrying the common E531 allele (P=0.057). Other lipid parameters, fasting glucose and insulin concentrations, and postprandial TAG, glucose and insulin were not changed.

As CPT1b K531E and I66V are in linkage disequilibrium, an additional analysis based on haplotypes was performed (Fig. 2). The polymorphisms in the CPT1b gene (rs470017 and rs3213445) showed intermediate concentrations of gametic linkage disequilibrium (D² = 1.0 (confidence bound 0.82–1), r² = 0.047). Moreover, three haplotypes with a frequency of more than 1% were detected (CPT1b-H1-K/I (47.9 %), CPT1b-H2-E/I (47.2 %) and CPT1b-H3-E/V (4.9 %)) and the results of the haplotype analysis confirmed the genotype-based comparison. Haplotype H1-K/I was significantly less prevalent in subjects with the MetS compared with the controls (P=0.023) under a log-additive model and controlled for BMI (Table 3). In agreement with these results, the association was significant, applying the dominant model (OR 0.642, 95% CI 0.429, 0.962, P=0.053). On the other hand, haplotype H2-E/I tended to be associated with MetS risk under a log-additive model (P=0.052; Table 3). The dominant model for an association between the H2-E/I haplotype and MetS risk was not significant (OR 1.299, 95% CI 0.853, 1.978, P=0.22). Haplotype H3-E/V was not significantly associated with the MetS under either model (data not shown).

Concerning the CPT2 V368I SNP, the genotypic groups did not differ in the investigated anthropometric and metabolic parameters (data not shown).
Discussion

In the present study, we performed an examination of polymorphisms of the CPT gene family. CPT1 and CPT2 are the rate-limiting enzymes of long-chain fatty acid β-oxidation in the mitochondria. The induction of fatty acid oxidation has been shown to improve insulin resistance and obesity phenotypes. The present data suggest that genetic variants in the CPT1b gene were associated with traits of the MetS and fatty liver disease. In our group of middle-aged men, the CPT2 V368I polymorphism had no impact on the parameters of the MetS.

In accordance with the results from French-Canadian males and females, there were no associations between BMI, weight and waist girth, and the CPT1b I66V SNP in the present study group (Table 2). Yet, while obesity was associated with the CPT1b coding SNP K531E in those French-Canadians, the present findings did not confirm the earlier results. Interestingly, the differences in obesity phenotypes between the K531E genotypic groups in the French-Canadian study population were particularly evident when fat intake was high, suggesting a gene–diet interaction. Thus, the discrepancy might be explained by a lower fat intake in the present study group.

Table 2. Anthropometric, fasting and postprandial metabolic variables according to CPT1b 66I/V and CPT1b 531G/L polymorphisms* (Mean values with their standard errors)

<table>
<thead>
<tr>
<th></th>
<th>rs3213445 CPT1b 66I/V</th>
<th>rs470117 CPT1b 531E/K</th>
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<tr>
<td></td>
<td>II</td>
<td>IV + V</td>
</tr>
<tr>
<td></td>
<td>Mean SEM</td>
<td>Mean SEM</td>
</tr>
<tr>
<td>n</td>
<td>613</td>
<td>66</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58.73 ± 0.26</td>
<td>60.02 ± 0.61</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>100.20 ± 0.49</td>
<td>100.64 ± 1.18</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.42 ± 0.17</td>
<td>27.55 ± 0.43</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>128.94 ± 0.75</td>
<td>132.95 ± 2.38</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80.38 ± 0.45</td>
<td>80.08 ± 1.47</td>
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</table>
| Fasting and postprandial TAG concentrations after an oral metabolic tolerance test in II-homozygotes (—) compared with V-allele carriers (—) of the 66I/V polymorphism. Values are means, with their standard errors represented by vertical bars. * Mean values were significantly different from those of II-homozygotes (P < 0.05) after controlling for BMI.

Fig. 1. Fasting and postprandial TAG concentrations after an oral metabolic tolerance test in II-homozygotes (—) compared with V-allele carriers (—) of the 66I/V polymorphism. Values are means, with their standard errors represented by vertical bars. * Mean values were significantly different from those of II-homozygotes (P < 0.05) after controlling for BMI.
Unfortunately, data on dietary fat intake in Metabolic Intervention Cohort Kiel participants have not been collected. We further addressed the associations of genetic variations in the CPT1b gene with traits of the MetS other than obesity. Thus, the coding polymorphism I66V presented with higher liver enzymes and FLI, suggesting this SNP to be involved in the development of hepatic steatosis. This is further supported by higher fasting and postprandial lipid parameters and insulin resistance (Table 2). Moreover, the lacking difference obtained when TAG incremental AUC was calculated (Table 2) indicates an accelerated clearance of TAG and a shortened residence time in the circulation(14) or lower VLDL synthesis in the fasting state. In contrast, the minor allele of K531E was associated with lower liver enzymes and FLI concomitant with lower total cholesterol and LDL-cholesterol concentrations (Table 3). These associations were independent of underlying obesity for which the results were adjusted for. Strong evidence is accumulating that β-oxidation, which is dependent on fatty acid transport by CPT1, plays a crucial role in the development of insulin resistance and fatty liver. Inhibition of CPT1b has been reported to induce intramyocellular lipid accumulation and insulin resistance(15), while CPT1b overexpression in rats ameliorated lipid-induced, muscle-specific insulin resistance via a reduction of lipid intermediates(16). Indeed, elevated oxidative capacity is able to inhibit the generation of lipid intermediates such as diacylglycerol and ceramides, resulting in improved insulin signalling(16,17). Muscle-specific insulin resistance in mice leads to the redistribution of substrates to the adipose tissue, which resulted in obesity and an increase in plasma TAG concentrations(18).

Petersen et al.(19) demonstrated in young, lean insulin-resistant subjects that skeletal muscle insulin resistance caused atherogenic dyslipidaemia and hepatic steatosis by redirected ingested carbohydrates away from muscle glycogen synthesis into hepatic de novo lipogenesis. Accordingly, inhibited muscle fatty acid oxidation induced by a potential loss-of-function of CPT1b in subjects carrying the rare 66V allele might explain the higher risk for fatty liver, elevated lipid parameters and insulin resistance which was most pronounced after a glucose load. Altogether, a deleterious role of the rare 66V allele for the MetS might be suggested. The opposite might apply for E531E homozygosity compared with carriers of the 531K allele (Table 2). The haplotype analysis suggested that haplotype H1-K/I protects from the MetS when compared with the less frequent haplotype (Table 3). H1 and H2 include both variants of I66, but are associated with contrasting OR (0·737 v. 1·300). This does not support a functional role of this I66V polymorphism. A low OR for H1 and a high OR for H2 and H3, however, may indicate a relevance of the E531K polymorphism. The biological relevance for the present findings might be interpreted with the help of the corresponding CI. Hence, H1 and H2 presented with a very narrow CI and therefore might prove the biological relevance of these haplotypes.

Yet, this is in contrast to another study showing decreased skeletal muscle fat and increased subcutaneous fat in Caribbeans with minor CPT1b 531K but also the 66V allele(3). According to this study, the allelic and genotypic distribution of the CPT1b SNP was considerably different between European and African ancestry populations. Moreover, linkage disequilibrium between the two SNP in African ancestry is not known. This might explain the somewhat conflicting results. The functional impact of CPT1b genetic variants on CPT1b enzyme activity has been investigated(20); however, there

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Control genotype frequency (n 403)</th>
<th>Case genotype frequency (n 276)</th>
<th>OR</th>
<th>95 % CI</th>
<th>P</th>
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<tr>
<td>H1-K/I</td>
<td>0·505</td>
<td>0·442</td>
<td>0·737</td>
<td>0·566, 0·958</td>
<td>0·023</td>
</tr>
<tr>
<td>H2-E/I</td>
<td>0·450</td>
<td>0·504</td>
<td>1·300</td>
<td>0·998, 1·693</td>
<td>0·052</td>
</tr>
<tr>
<td>H3-E/V</td>
<td>0·045</td>
<td>0·054</td>
<td>1·252</td>
<td>0·725, 2·296</td>
<td>0·404</td>
</tr>
</tbody>
</table>

CPT, carnitine palmitoyltransferase.
* Only haplotypes with a frequency more than 1 % are considered.
was no remarkable alteration in enzymatic properties due to the substitution of amino acids. Thus, the associations found in the present study group might be reflected by the associations of other not yet identified variations. Recently, two genome-wide association studies were performed for the MetS\textsuperscript{21,22}. CPT1b or CPT2 has not been reported as significant gene loci to be associated with the MetS. However, genome-wide association studies can only be successful if the so-called ‘common-disease common-variant’ hypothesis is valid. This hypothesis assumes that most of the genetic risk for common complex diseases is caused by a small to medium number of disease loci that have common variants. This means that many rare variants cannot be found with genome-wide studies, although of biological relevance\textsuperscript{23}.

In summary, the present study confirms the association of genetic variants in the muscle-specific CPT1b gene with traits of the MetS. Particularly, haplotype H1-KI seems to be relevant in terms of insulin sensitivity and hepatic steatosis. Haplotype analysis suggests a relevance of the CPT1b E531K polymorphism or a gene in linkage with this SNP in their pathogenesis.

Acknowledgements
This study was supported by a grant of the German Federal Ministry of Education and Research (BMBF) ‘Dietary fat and metabolism, gene variability, regulation and function’ (AZ 031282 A/B). Nutrichem supported the studies of the BMBF-supported cluster and was partner in this cluster. A. A., D. R., U. H. and J. S. were responsible for the conception and design of the study; A. A., D. R., M. S., U. H., A. R., S. S., U. R. F., F. D. and J. S. were responsible for conducting the experiments and data collection; A. A. was responsible for the statistical analysis; A. A., D. R., M. S., F. D. and J. S. were responsible for the data interpretation; A. A. and D. R. were responsible for drafting the manuscript; J. S. was responsible for raising the funds and supervision. All authors contributed to the final version of the manuscript and did a critical revision, and evaluated and approved the final version of the manuscript. The authors declare that there are no conflicts of interest.

References