Original article

Variants within the calpain-10 gene and relationships with type 2 diabetes (T2DM) and T2DM-related traits among Tunisian Arabs

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Abstract

Background. – Common variations in the calpain 10 (CAPN10) gene variants UCSNP-43, UCSNP-19 and UCSNP-63, and the 112/121 diplotype, are associated with an increased risk of type 2 diabetes (T2DM) and T2DM-related traits.

Methods. – The association of UCSNP-43, -19 and -63 CAPN10 SNPs with T2DM was assessed in 917 Tunisian T2DM patients and 748 ethnically matched non-diabetic controls. CAPN10 genotyping was done by PCR-RFLP.

Results. – Significant differences in UCSNP-19 MAF, but not UCSNP-43 or -63, and genotype distribution were seen between patients and controls. Heterogeneity in UCSNP-19, but not UCSNP-43 and -63, genotype distribution was noted according to geographical origin. Obesity was associated with UCSNP-19, while raised fasting glucose was associated with UCSNP-63, and increased HDL was associated with UCSNP-43. Enrichment of homozygous UCSNP-19 2/2 was seen in overweight and obese compared with lean patients; logistic-regression analyses demonstrated a positive association of the 2/2 genotype with overweight [P = 0.003; OR (95% CI) = 2.07 (1.28–3.33)] and obese [P = 0.021; OR (95% CI) = 1.83 (1.10–3.07)] patients. Of the six CAPN10 haplotypes identified, significant enrichment of only haplotype 111 was seen in T2DM patients [Pc = 0.034; OR (95% CI) = 1.22 (1.06–1.41)], while the frequency of all identified CAPN10 diplotypes, including the high-risk 112/121, was comparable between patients and controls.

Conclusion. – While CAPN10 UCSNP-19 SNP and haplotype 111 contribute to the risk of T2DM in Tunisian subjects, no significant association between CAPN10 diplotypes and T2DM was demonstrated.

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Keywords: Calpain-10; Type 2 diabetes; Polymorphism; Haplotypes; Diplotypes; Linkage stdy; Tunisian

Résumé

Association des polymorphismes du gène de la calpaine 10 et diabète de type 2 dans la population tunisienne.

Préambule. – Des variants communs du gène de la calpaine-10 (CAPN10), en particulier UCSNP-4, UCSNP-19 et UCSNP-63 et le diplotype à risque (combinaison haplotypeique) (112/121) ont été associée à l’augmentation du risque du diabète de type 2 (DT2) et les traits associés.

Méthodes. – L’association du DT2 et des SNPs UCSNP-43, UCSNP-19 et UCSNP-63 du gène CAPN10 a été étudiée chez 917 diabétiques tunisiens et 748 témoins non diabétiques de même origine. Le génotypage du gène CAPN10 a été réalisé par PCR-RFLP.

Résultats. – Des différences significatives de la distribution génotypique du UCSNP-19, mais non d’UCSNP-43 et UCSNP-63 ont été observées entre les patients et les témoins. Une hétérogénéité de la distribution génotypique du UCSNP-19 mais non de celle d’UCSNP-43 et UCSNP-63 a été notée selon l’origine géographique. L’obésité était associée avec UCSNP-19, l’hyperglycémie à jeun avec UCSNP-63, alors que l’élévation du HDLc était en corrélation avec UCSNP-43. L’enrichissement d’UCSNP-19 en homozygote 2/2 a été observé chez les patients en surpoids et

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obéses comparés aux patients de poids normal. L’analyse par régression logistique a mis en évidence une corrélation positive entre le génotype 2/2 chez les patients en surpoids \( P = 0.003; \) OR (95 % IC) = 2.07 (1.28–3.33) et obèses \( P = 0.021; \) OR 1.83 (1.10–3.07)]. Parmi les six haplotypes de \textit{CAPN10} identifiés, un enrichissement significatif pour le seul haplotype 111 a été observé chez les DT2 \( P_c = 0.034; \) OR 1.22 (1.06–1.41) alors que les fréquences de tous les diploptypes de \textit{CAPN10} identifiés, dont le diploptyle à risque 112/121 étaient comparables entre les patients et les témoins.

\textit{Conclusions.} – Le variant commun UCSNP-19 SNP de \textit{CAPN10} et l’haplotype 111 contribuent au risque de DT2 chez les sujets tunisiens; aucune association significative n’a été mise en évidence entre les diploptypes de \textit{CAPN10} et le DT2.

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\textit{Mots clés :} Calpaine-10 ; Diabète de type 2 ; Polymorphismes ; Haplotypes ; Diploptypes ; Études d’association ; Tunisiens

1. Introduction

Type 2 diabetes (T2DM) is a heterogeneous metabolic disease defined by an impaired response to insulin stimulation (insulin resistance) coupled with insufficient insulin production [1]. T2DM is multifactorial [1], and a genetic predisposition to T2DM has been confirmed in family and case-control studies [2,3]. Genome-wide association studies have identified a number of genes contributing to T2DM predisposition [2–5], including calpain 10 (\textit{CAPN10}) [6–9], a ubiquitously expressed protease [10] that serves as an intracellular calcium-dependent cysteine protease [11]. \textit{CAPN10} protein also regulates insulin secretion [12–14] and insulin-mediated glucose metabolism. \textit{CAPN10} is expressed at mRNA and protein levels by several tissues, with different mRNA isoforms being reported [12–15].

The \textit{CAPN10} gene, located on chromosome 2q37.3, was the first T2DM candidate gene to be identified [16], and several studies have demonstrated the association of specific \textit{CAPN10} polymorphisms with T2DM development and insulin resistance, specifically in obese patients, and earlier age of disease onset [7,8,12,16]. These polymorphisms include UCSNP-43 G/A (third intron), UCSNP-19 2R (two 32-bp repeats)/3R (three 32-bp repeats; intron 6) and UCSNP-63 C/T (intron 13) variants [15,16]. This was highlighted by the finding that selected UCSNP-43/UCSNP-19/UCSNP-63 haplotypes (112 and 121) are associated with increased T2DM risk in selected ethnic groups [16–18], and that at-risk diploptypes (haplotype pairs) are reported in many populations [8,19–21], exemplified by the association of the 112/121 diploptyle with increased T2DM risk in Mexican-American, German and Finnish populations [16,18].

A number of studies involving various ethnic groups have confirmed the association of \textit{CAPN10} variants and T2DM risk [12,22], while others could find no such association [17,23,24]. Also, some studies showed a trend towards significance, but were insufficiently powered to confirm a definitive effect [17,24–27].

The aim of the present study was to assess the contributions of polymorphisms with T2DM development and insulin resistance, specifically in obese patients, and earlier age of disease onset [7,8,12,16]. These polymorphisms include UCSNP-43/UCSNP-19/UCSNP-63 haplotypes (112 and 121) are associated with increased T2DM risk in ethnic groups [16–18], and that at-risk diploptypes (haplotype pairs) are reported in many populations [8,19–21], exemplified by the association of the 112/121 diploptyle with increased T2DM risk in Mexican-American, German and Finnish populations [16,18].

2. Subjects and methods

2.1. Subjects

Unrelated adult T2DM patients \((n = 917)\) were recruited from endocrinology outpatient clinics in Southern, Central and Northern Tunisia, along with 748 age-, gender- and ethnically matched normoglycaemic control subjects (serum glucose: \( \leq 6.2 \text{ mmol/L} \)). The controls comprised university/hospital staff, volunteers and blood donors recruited from the same geographical areas as the study patients, none of whom were reported positive for a personal or family history of diabetes. Diagnosis of diabetes was based on clinical features, as per World Health Organization (WHO) criteria. T2DM treatment included diet and/or oral antidiabetic drugs and/or insulin; patients who required insulin had been treated with oral drugs for at least two years (Table 1).

Blood pressure (BP) was measured twice, and the mean of the readings (1-min apart) was used in the analyses; hypertension was defined as BP \( \geq 145/90 \text{ mmHg} \) and/or the use of antihypertensive drugs. Demographic details, history of diabetes and biochemical profiles were obtained for all subjects, and verified from clinic records where available. Written informed consent was obtained from all participants, and the study was carried out in accordance with the guidelines of the Helsinki Declaration of 1975, and approved by the University of Monastir ethics committee. Venous blood samples were collected after an overnight fast for measuring plasma glucose, HbA1c and serum lipids.

\begin{table}[h]
\centering
\caption{Demographic, clinical and laboratory characteristics of type 2 diabetes (T2DM) patients and their matched controls.}
\begin{tabular}{lccc}
\hline
 & T2DM patients & Controls & \( P \) \\
& \((n = 917)\) & \((n = 748)\) & \\
\hline
Gender (male:female) & 422:495 & 373:375 & 0.126 \\
Mean age (years) & 59.3 \( \pm \) 10.9 & 58.7 \( \pm \) 8.7 & 0.160 \\
Mean body mass index (kg/m\(^2\)) & 27.7 \( \pm \) 4.3 & 23.5 \( \pm \) 2.2 & \( < 0.001 \) \\
Systolic blood pressure (mmHg) & 140.7 \( \pm \) 27.0 & 121.6 \( \pm \) 14.4 & \( < 0.001 \) \\
Diastolic blood pressure (mmHg) & 81.9 \( \pm \) 12.6 & 77.9 \( \pm \) 10.5 & \( < 0.001 \) \\
Hypertension \(^a\) & 420 (45.8) & 86 (18.0) & \( < 0.001 \) \\
Fasting glucose (mmol/L) & 12.8 \( \pm \) 5.3 & 5.1 \( \pm \) 0.6 & \( < 0.001 \) \\
HbA1c (%) & 9.6 \( \pm \) 3.9 & 4.5 \( \pm \) 1.4 & \( < 0.001 \) \\
Age at disease onset (years) & 46.7 \( \pm \) 10.9 & N/A & N/A \\
T2DM duration (years) & 12.6 \( \pm \) 6.3 & N/A & N/A \\
Total cholesterol (mmol/L) & 5.3 \( \pm \) 1.4 & 4.7 \( \pm \) 1.2 & \( < 0.001 \) \\
Triglycerides (mmol/L) & 1.9 \( \pm \) 1.3 & 1.2 \( \pm \) 0.6 & \( < 0.001 \) \\
HDL (mmol/L) & 1.1 \( \pm \) 0.3 & 1.2 \( \pm \) 0.4 & \( < 0.001 \) \\
LDL (mmol/L) & 3.8 \( \pm \) 1.4 & 2.8 \( \pm \) 1.8 & \( < 0.001 \) \\
\hline
\end{tabular}
\end{table}

Data are expressed as means \( \pm \) SD (Student’s \( t \)-test for continuous variables, \( \chi^2 \) test for categorical variables); N/A: not available; HDL: high-density lipoprotein; LDL: low-density lipoprotein.

\(^a\) Blood pressure >145/90 mmHg and/or the use of antihypertension medication.
2.2. Genotyping

CAPN10 UCSNP-43, -19 and -63 genotyping was carried out as described elsewhere [17,27]. The insertion–deletion UCSNP-19 variant (rs3842570) was genotyped by polymerase chain reaction (PCR) using the primers (forward) 5′-GTTTGGTCTCTTCAAGGTGGAG-3′ and (reverse) 5′-ATGAACCTGGCAAGGTCATG-3′. The two 32-bp-repeat 2R alleles and three 32-bp-repeat 3R alleles were visualized as 155 bp and 187 bp, respectively, on agarose gel. UCSNP-43 (rs3792267) was genotyped by PCR–RFLP (restriction fragment length polymorphism) using the primers (forward) 5′-CAGGCTTGCTTCAGGAATATGC-3′ and (reverse) 5′-AGGGTCTAAGT3TCTGATTCCCATGGTCTCTGATTCCCATGGTCTCTGATTCCCATGG-3′, with digestion by Nsi I (Promega Corporation, Madison, WI, USA), while UCSNP-43, -19 and -63 alleles and genotypes in type 2 diabetes patients and non-diabetic controls.

2.3. Statistical analysis

Statistical analyses were performed using SPSS v. 15.0 (SPSS Inc., Chicago, IL, USA) software. Data were expressed as means ± SD (continuous variables) and as percent totals (categorical variables). Pearson’s χ² test was used to assess intergroup significance, and Student’s t test was used to determine differences in means. Alleles were scored as either ‘1’ (major) or ‘2’ (minor). Linkage disequilibrium among CAPN10 SNPs was calculated by an expectation–maximization algorithm; haplotype frequencies were estimated by LDA v.1.0 software [28]. Univariate and multivariate regression analyses were determined by HPlus 2.5 software, and results were expressed as P value, odds ratio (OR) and 95% confidence intervals (CI). Statistical significance was set at P < 0.05.

3. Results

3.1. Study subjects

The demographic and clinical characteristics of the study T2DM patients and controls are shown in Table 1. Patients had a mean age of 59.3 years (range: 35–86 years), mean body mass index (BMI) score of 29.0 kg/m² (range: 18.4–39.2 kg/m²), and an average T2DM duration of 12.6 years (range: 5–36 years) with an average age at T2DM onset of 46.7 ± 10.9 years; 330 patients (36.0%) reported a positive family history of diabetes. Patients and controls were matched only for gender and age; significant differences were noted for BMI (P < 0.001), BP (P < 0.001), lipid profile (P < 0.001), urea (P < 0.001), and fasting glucose (P < 0.001) and HbA1c levels (P < 0.001).

3.2. UCSNP-43, -19 and -63 genotyping

UCSNP-43, -19 and -63 genotype frequencies were in Hardy–Weinberg equilibrium in the controls. The UCSNP-43 and -63 genotypes and minor allele frequencies (MAF) were not significantly different between patients and controls (Table 2). However, significant differences in UCSNP-19 MAF (P = 0.007) and genotype distribution (P = 0.020) were seen between T2DM patients and controls (Table 2). Heterogeneity in the distribution of UCSNP-19 was noted according to the participants’ geographical origin, and was particularly more so in Northern Tunisia (P = 0.036) and Central (P = 0.015) Tunisia than in the Sahel region (P = 0.246) and Southern Tunisia (P = 0.845). There were no differences in UCSNP-43 and -63 frequencies in different Tunisian strata.

3.3. CAPN10 and T2DM-related quantitative traits

Bivariate analyses revealed that UCSNP-19 was correlated with BMI changes (r² = −0.099; P = 0.003), whereas UCSNP-63 was correlated with fasting glucose changes (r² = 0.087; P = 0.008). UCSNP-43 did not correlate with any of the T2DM-related traits tested. The associations of the UCSNP-43, -19 and -63 CAPN10 genotypes with BMI, fasting glucose, HbA1c and high-density lipoprotein (HDL) levels were also investigated. A significantly higher BMI was associated with the UCSNP-19 2/2 genotype (P = 0.003), while a marked rise in fasting glucose was associated with the UCSNP-63 1/2 and 2/2 genotypes (P = 0.025) and a marked increase in HDL was associated with the UCSNP-43 2/2 genotype (P = 0.008). No effect on HbA1c levels was found with any of the CAPN10 SNPs tested (Table 3).

3.4. CAPN10 and BMI changes

Given the positive association of UCSNP-19 with BMI changes, the T2DM patients were classified as lean (BMI:
≤25 kg/m²), overweight (BMI >25 kg/m² and ≤30 kg/m²) and obese (>30 kg/m²; Table 4). Enrichment of homozygous UCSNP-19 2/2 was seen in the overweight and obese groups compared with lean patients \( (P = 0.041; \text{Table 4}) \), whereas the UCSNP-43 and -63 genotypes were not associated with BMI changes. The association of UCSNP-19 2/2 with an obese state in T2DM patients was confirmed by regression analyses. Using the homozygous wild-type genotype (1/1) as reference, logistic-regression analyses demonstrated a positive association of the 2/2 genotype with both overweight \( [P = 0.003; \text{OR (95% CI)} = 2.07 (1.28–3.33)] \) and obese \( [P = 0.021; \text{OR (95% CI)} = 1.83 (1.10–3.07)] \) patients.

### Table 5

<table>
<thead>
<tr>
<th>SNP</th>
<th>1/1</th>
<th>1/2</th>
<th>2/2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCSNP-43</td>
<td>27.7 ± 4.3</td>
<td>27.5 ± 4.1</td>
<td>25.0 ± 4.3</td>
<td>0.482</td>
</tr>
<tr>
<td>UCSNP-19</td>
<td>27.3 ± 4.5</td>
<td>27.5 ± 4.3</td>
<td>28.6 ± 3.8</td>
<td>0.003</td>
</tr>
<tr>
<td>UCSNP-63</td>
<td>27.6 ± 4.3</td>
<td>27.8 ± 4.2</td>
<td>28.4 ± 4.3</td>
<td>0.556</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>SNP</th>
<th>1/1</th>
<th>1/2</th>
<th>2/2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCSNP-43</td>
<td>252 (82.1)</td>
<td>293 (82.8)</td>
<td>214 (83.6)</td>
<td>0.746</td>
</tr>
<tr>
<td>UCSNP-19</td>
<td>53 (17.3)</td>
<td>60 (16.9)</td>
<td>42 (16.4)</td>
<td>0.010</td>
</tr>
<tr>
<td>UCSNP-63</td>
<td>97 (31.6)</td>
<td>86 (24.3)</td>
<td>64 (25.0)</td>
<td>0.041</td>
</tr>
</tbody>
</table>

Lean: BMI <25 kg/m²; overweight: BMI >25 kg/m² and ≤30 kg/m²; obese: BMI >30 kg/m².

### 3.5. Haplotype and diplotype distributions

Only six of the possible eight CAPN10 haplotypes were identified, and neither haplotype 211 nor 212 was observed. Of the six haplotypes, significant enrichment of only haplotype 111 was seen in T2DM patients \( [Pc = 0.034; \text{OR (95% CI)} = 1.22 (1.06–1.41)] \), and the distribution of the remaining five haplotypes was similar between patients and controls (Table 5).

### Table 5

<table>
<thead>
<tr>
<th>Haplotypea</th>
<th>Patients</th>
<th>Controls</th>
<th>P</th>
<th>( P_c^b )</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>111</td>
<td>0.392</td>
<td>0.345</td>
<td>0.006</td>
<td>0.034</td>
<td>1.22 (1.06–1.41)</td>
</tr>
<tr>
<td>112</td>
<td>0.072</td>
<td>0.059</td>
<td>0.147</td>
<td>0.615</td>
<td>1.24 (0.94–1.64)</td>
</tr>
<tr>
<td>121</td>
<td>0.368</td>
<td>0.399</td>
<td>0.072</td>
<td>0.361</td>
<td>0.88 (0.76–1.01)</td>
</tr>
<tr>
<td>122</td>
<td>0.080</td>
<td>0.096</td>
<td>0.115</td>
<td>0.520</td>
<td>0.82 (0.64–1.04)</td>
</tr>
<tr>
<td>211</td>
<td>0.078</td>
<td>0.086</td>
<td>0.422</td>
<td>0.963</td>
<td>0.90 (0.70–1.15)</td>
</tr>
<tr>
<td>221</td>
<td>0.010</td>
<td>0.015</td>
<td>0.259</td>
<td>0.834</td>
<td>0.66 (0.36–1.24)</td>
</tr>
</tbody>
</table>

Note: Haplotypes are coded according to the allele at each locus (major = 1, minor = 2); the first refers to UCSNP-43, the second to UCSNP-19 and the third to UCSNP-63.

\( ^a \) Frequency determined by the maximum-likelihood method.

\( ^b \) Corrected \( P \) according to the Bonferroni method \( [Pc = 1–(1–P)^n] \), where \( n \) = number of comparisons.

The frequency of all identified CAPN10 diplotypes, including the 112/121 high-risk diplotype reported in Mexican-Americans and Northern Europeans, was comparable between patients and controls, even before applying the Bonferroni correction (data not shown).

### 4. Discussion

While CAPN10 is a confirmed T2DM candidate gene \([3,4]\), the contribution of CAPN10 variants to T2DM risk has been confirmed in some \([8,16,21]\), but not all, populations studied so far \([8,9,21,23,26]\). The main findings of the present study were that: (1) UCSNP-19 was associated with T2DM and obesity in T2DM patients; (2) UCSNP-63 was associated with raised glucose levels; (3) UCSNP-43 was associated with higher HDL levels in T2DM patients; (4) haplotype 111 was positively associated with T2DM in Tunisians; and (5) the 112/121 high-risk diplotype, reported in Mexican-Americans and northern Europeans \([8,9,16]\), was not associated with T2DM in Tunisians. Insofar as ethnic/geographical heterogeneity in the contribution of CAPN10 variants to T2DM risk has been demonstrated \([12,16,18,23,24,26]\), only Tunisian subjects of Arab origin were...
UCSNP-19 was positively associated with increased T2DM risk and altered BMI, as evidenced by increased MAF and homozygous 2/2 genotype frequency in T2DM patients, and by enrichment of the UCSNP-19 homozygous variant in overweight and obese T2DM patients. UCSNP-19 has also been implicated in insulin sensitivity in Finnish [15], Northern European [25], Scandinavian [23] and Spanish [29] subjects. However, although the present study findings are consistent with a study in Southern Tunisia of mixed Arab and Berber (non-Arab) subjects, where the association of UCSNP-19 with T2DM was seen only in Tunisian Arabs [30], it is apparently in disagreement with a Central Tunisia study that found that UCSNP-43 (not UCSNP-19) was enriched in T2DM patients [31]. These discrepancies are most likely due to inadequate statistical power and differences in the selection of subjects [31] that may have oversimplified the association of UCSNP-43 with T2DM.

Non-obese T2DM subjects carrying the UCSNP-43 2/2 genotype had higher HDL levels than did 1/2 or 1/1 genotype carriers (P = 0.032) and obese patients (P = 0.427). As such a difference was not seen among the controls, it suggests that the genotype effect depends on the presence of specific conditions (obesity), as it has been suggested elsewhere [15,32]. In agreement with previous studies, there was no association between UCSNP-43 and -63 and obesity [32–34], indicating that UCSNP-43 and -63 have no effect on obesity, but may influence the development of T2DM-related traits, such as insulin resistance and fat metabolism [15,20,32]. Other studies have found no [33] or marginal [29] associations between CAPN10 variants and obesity-related parameters, thereby underscoring the need for population-based and functional studies to confirm (or not) the association of a particular CAPN10 variant with T2DM-related metabolic traits.

The association of haplotype 111 with T2DM in Tunisians was similar to reports linking haplotype 111 with increased T2DM risk in Koreans [33,35] and altered insulin sensitivity in Spanish subjects [29]. Whereas haplotypes 111 and 121 (commonly seen in Europeans [7,36]) were frequently identified in our present patients and controls, respectively, haplotype 112 (common in Africans and Asians) was found at lower frequencies in our present study. The greater similarity of (North African) Tunisians in the distribution of CAPN10 haplotypes to Europeans than to Africans may be explained by the admixture of the original Tunisians (Berbers) with Phoenicians (ancestors of present-day Lebanese) and, later, with Muslims (Arabian Peninsula) and Turks (Ottoman rule) and, most recently, Europeans.

The association of CAPN10 diplotypes with T2DM risk has been previously reported, but are inconsistent [8,18,35], as exemplified by the association of 111/121 in Koreans [37], 111/221 in Northern Europeans [25], 112/221 in Chinese [19] and 121/121 in pan-European [8] populations. However, in agreement with Scandinavian [23], Korean [35,37] and Mexican [18] studies, we found that the 112/121 diplotype had no influence on the risk of T2DM, but failed to identify any specific T2DM at-risk CAPN10 diplotypes. The present results are also inconsistent with a recent study in Central Tunisia, in which the 121/221 diplotype was associated with increased T2DM risk [31]. These discrepancies may be explained by differences in sample size, patients’ characteristics (BMI, hypertension status and T2DM duration) and data presentation.

5. Conclusion

The present study failed to replicate the associations between CAPN10 alleles, haplotypes, diplotypes and T2DM in Tunisian Arabs, as identified in earlier studies. Differences in the association between specific CAPN10 variants and increased T2DM risk could be due to the presence of multiple alleles at the CAPN10 locus, different linkage disequilibrium patterns, racial/ethnic differences in the distribution of CAPN10 variants, multiple hypotheses in testing and insufficient statistical power leading to overestimation of the association. Further research, including large-scale, population-based, case-control studies, is needed to better understand the contribution of CAPN10 to T2DM risk.

Conflicts of interests

None to declare.

References
