The role of ghrelin and ghrelin-receptor gene variants and promoter activity in type 2 diabetes

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Abstract

Background: Ghrelin and its receptor play an important role in glucose metabolism and energy homeostasis, and therefore they are functional candidates for genes carrying susceptibility alleles for type 2 diabetes.

Methods: We assessed common genetic variation of the ghrelin (GHRL; five single nucleotide polymorphisms (SNP)) and the ghrelin-receptor (GHSR) genes (four SNPs) in 610 Caucasian patients with type 2 diabetes and 820 controls. In addition, promoter reporter assays were conducted to model the regulatory regions of both genes.

Results: Neither GHRL nor GHSR gene SNPs were associated with type 2 diabetes. One of the ghrelin haplotypes showed a marginal protective role in type 2 diabetes. We observed profound differences in the regulation of the GHRL gene according to promoter sequence variants. There are three different GHRL promoter haplotypes represented in the studied cohort causing up to 45% difference in the level of gene expression, while the promoter region of GHSR gene is primarily represented by a single haplotype.

Conclusion: The GHRL and GHSR gene variants are not associated with type 2 diabetes, although GHRL promoter variants have significantly different activities.

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Introduction

Type 2 diabetes is a disorder of glucose metabolism characterized by a spectrum of insulin resistance, obesity, and energy disturbances whose etiology resides in the interaction of genetic and non-genetic (environmental and lifestyle) factors (1). The ghrelin pathway has emerged over the last decade as a novel endocrine pathway involved in the control of feeding behavior and energy homeostasis, operating in parallel to the leptin and adiponectin circuits (2–5). Ghrelin is a 28 amino-acid peptide with a fatty acid chain modification on the N-terminal third amino acid by the ghrelin O-acyltransferase enzyme (6). It has a widespread tissue expression but the main source of circulating ghrelin is the gastric mucosa (7). Ghrelin has a role in GH regulation (8) but in the recent years the majority of the data have shown its effects on appetite regulation via the hypothalamus (9) and the brain stem and influence on the peripheral metabolism, especially the carbohydrate and lipid metabolism (10). Ghrelin is a factor involved in meal initiation which acts on the hypothalamus via the ghrelin receptor (11). Ghrelin and obestatin are encoded by the same gene and propeptide, but different post-translational processes generate two peptides with opposing functions; obestatin was originally suggested to counteract the effects of ghrelin on food intake (5), although controversy has recently arisen regarding the function of obestatin (12).

Ghrelin and its receptor are functional candidates for type 2 diabetes: i) ghrelin influences glucose homeostasis, hyperglycemia caused by 50 g glucose administrated either orally or i.v. suppressed ghrelin levels and ghrelin is lowered by a euglycemic hyperinsulinemic clamp suggesting that insulin responsiveness is a major regulator of ghrelin levels in both human and rodents (13), ii) it is expressed in the pancreatic islets and ghrelin cells replace insulin cells in case of abnormal β-cell development (14), and iii) lack of ghrelin can rescue the diabetic phenotype of the leptin-deficient mouse (15). The ghrelin and ghrelin-receptor genes have been studied in the context of...
obesity and type 2 diabetes, but any putative associations have been controversial (16–22). It has been difficult to tease out these conditions due to the fact that obesity is an independent risk factor for type 2 diabetes, and it is likely that different pathophysiological mechanisms occur in non-obese and obese subjects with type 2 diabetes (23).

We have assessed the genetic variation of the ghrelin and ghrelin-receptor genes in a French population in order to study the role of these genes in type 2 diabetes, and aimed to model the impact of promoter variations in both genes using luciferase reporter assays in two well-established cell models, GH3 somatolactotroph rat cell line for the ghrelin receptor promoter (24) and human medullary thyroid tumor (MTT) cell line for the ghrelin promoters (25), under different hormonal challenges previously reported to modulate gene expression (24, 25). We identified a protective haplotype for type 2 diabetes in the ghrelin gene and established that this haplotype is linked to a promoter region with reduced activity.

Materials and methods

Subjects

A total of 610 unrelated diabetic individuals were drawn from families with at least two affected probands who participated in a French public campaign in 1990 entitled ‘200 families to overcome diabetes’ (see details in (26)). The diabetic phenotype was defined in the light of the clinical report and the results of the latest measurements for fasting and/or oral glucose-tolerance test according to the established American Diabetes Association criteria for type 2 diabetes (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997; 27): ‘diabetic’ (DM) when receiving oral hypoglycemic agents or insulin 1 year after the diagnosis or when fasting glycaemia was 7 mmol/l or glycaemia 11.1 mmol/l 2 h after an oral glucose load. The 820 controls were selected from the SUVIMAX study, an 8-year prospective primary prevention trial assessing the impact of anti-oxidant and minerals in cardiovascular disease and cancer (28) and from the Fleurbaix Laventie Ville Santé study, a cross-sectional survey of the nutritional and behavioral characteristics of the free-living population (29). Clinical details are shown in Table 1.

Genotyping

The single nucleotide polymorphisms (SNPs) A-604G (rs27647), C-501A (rs26802), C247A (rs696217), A265T (rs4684677), and G62T (rs35683) in the ghrelin gene (GHRL) and the SNPs Ins/Del (rs10618418a), A477G (rs572169), C607G (rs2232169), and C658T (rs2232165) in the ghrelin-receptor gene (GHSR) were genotyped with matrix-assisted laser desorption/ionization time of flight mass spectrometry following the manufacturer’s recommendations (Sequenom, San Diego, CA, USA). A small PCR fragment of not more than 100 bp flanking the variant loci becomes the template of a minisequence reaction where a third primer adjacent to the variant loci becomes the template of a minisequence and the assay has been validated in our previous studies by fluorescence sequence in both directions (30). These variants are haplotype tagging SNPs (default $r^2 > 0.8$) following the algorithms implemented in Haplovie (32) and TAGGER (33).

Plasmids

The wild-type (WT) GHRL promoter cloned into pGL3 plasmid (Promega) has been developed and previously characterized in MTT cells (25) and the C-604G, C-501A, T-227C, and C-36T variations (34) were incorporated into the pGL3–GHRL plasmid using site-directed mutagenesis with QuikChange (Stratagene, La Jolla, CA, USA). The WT GHSR promoter cloned into pGL3 has been previously characterized in pituitary cells (24), and the A-959C, A-794T, C-786T, A-756G, and C-498T variations were incorporated into the pGL3–GHSR plasmid (34). These promoter variants were determined in 70 obese children for ghrelin and 60 short children for GHSR as part of a SNP discovery study (Table 2). Sequence confirmation using an ABI3700 (Perkin Elmer, Warrington, UK) was performed on both strands for each variant. Some of these SNPs, two in the GHRL and five in the GHSR receptor have not been annotated in HAPMAP and they are highlighted in Fig. 1.

### Table 1 Clinical characteristics.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n=820)</th>
<th>Case (n=610)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>333/487</td>
<td>342/268</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.2 (3.6)</td>
<td>26.8 (3.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>47–58</td>
<td>52–57</td>
<td></td>
</tr>
<tr>
<td>Age of diagnosis</td>
<td>–</td>
<td>45.2 (11.5)</td>
<td></td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>83</td>
<td>96</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median</td>
<td>72–92</td>
<td>87–103</td>
<td></td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>99</td>
<td>102</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median</td>
<td>95–103</td>
<td>96–109</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Basal glucose (mM/l)</td>
<td>5.3</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>5.0–5.7</td>
<td>7.0–11.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Clinical characteristics.
Cell culture  Cells were seeded and maintained in 75 cm² flasks in medium as follows: GH3 somatolactotroph mouse cells in DMEM (Gibco) and human MTT cell line in a 50:50 admixture DMEM/F12K, supplemented with 10% serum and antibiotics (penicillin/streptomycin) at 37 °C, 5% CO₂ humidified atmosphere.

Transient transfection and luciferase assay analysis Fifty thousand cells were plated in 96-well white-wall clear-bottom plates (Costar 3610, Fisher Scientific, Loughborough, UK) and were transfected with 12 ng test plasmid and 1.2 ng pRL renilla control plasmid (Promega) using 0.5 ml Lipofectamine 2000 (Invitrogen) in a volume of 50 μl unsupplemented media. After 3 h, media was changed to 20% FBS/antibiotic free media and the cells were incubated for 24 h. Basal luciferase activity was measured at 24 h.

Cells were hormone treated 24 h later after liposome transfection with estradiol (E₂; 10⁻⁹ M), triiodothyronine (T₃; 10⁻⁹ M), and hydrocortisone (10⁻⁷ M) and harvested 8 h later in 20 μl passive lysis buffer (Promega) for luciferase experiments. The control cells were treated in parallel with the appropriate vehicle: ethanol (1 mM) for hydrocortisone and E₂ and ethanol/HCl (6 × 10⁻⁹ M/1.1 × 10⁻² M) for T₃. Luciferase and renilla activities were measured using the Dual Luciferase assay (Promega) in a plate-reader fitted with two injectors (Victor 1420, Perkin Elmer). Background luminescence was measured for 5 s and the luciferase and renilla activities were measured for 10 s. Each experiment was performed in duplicate. Data are expressed as the luciferase/renilla activity ratio (25).

Statistical analysis Normal distributed variables are shown as mean and s.d. The χ²-test applied for categorical variables. Non-normal distributed variables are presented as the median and interquantile range and the non-parametric Kruskal–Wallis test applied when testing quantitative variables using the platform R version 2.6.2. Hardy–Weinberg equilibrium (HWE) and association analysis were performed with the software Thesias (Paris, France) (35). For the functional assay data, ANOVA was used to test the differences between haplotypes and Tukey’s post hoc test (GraphPad Prism, La Jolla, CA, USA).

Table 2  Haplotypes in the promoter of the ghrelin and ghrelin-receptor gene observed in 70 French obese children for GHRL and 60 idiopathic short children for GHSR.

<table>
<thead>
<tr>
<th>GHRL promoters</th>
<th>A-604G</th>
<th>C-501A</th>
<th>T-227C</th>
<th>C-36T</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>G</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>0.39</td>
</tr>
<tr>
<td>GHRL2</td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>0.28</td>
</tr>
<tr>
<td>GHRL3</td>
<td>A</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>0.26</td>
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<tr>
<td>GHRL4</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>0.04</td>
</tr>
<tr>
<td>GHRL5</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>0.02</td>
</tr>
<tr>
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<td>A</td>
<td>C</td>
<td>T</td>
<td>T</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GHSR promoters</th>
<th>A-959C</th>
<th>A-794T</th>
<th>C-786T</th>
<th>A-756G</th>
<th>C-498T</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>0.96</td>
</tr>
<tr>
<td>GHSR2</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>0.007</td>
</tr>
<tr>
<td>GHSR3</td>
<td>A</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>0.01</td>
</tr>
<tr>
<td>GHSR4</td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Underlined characters indicates mutated site to get the haplotype.
Results

Case-control study results

A French cohort comprising 610 subjects with type 2 diabetes and 820 controls was studied (Table 1) and genotyped for five SNPs in the ghrelin gene and four SNPs in the ghrelin-receptor gene. All of the SNPs were in HWE except the rs27647 that was not included in the haplotype analysis. No significant differences were observed in allele frequencies between the type 2 diabetes and the control group either for the ghrelin gene (Table 3) or for the ghrelin-receptor gene (Table 4). No significant differences were found under recessive, dominant, multiplicative, and general models for both genes. The SNP combination within the GHRL rs26802 – rs696217 – rs4684677 – rs35683 was marginally associated and the haplotype AAAG was found to be protective for type 2 diabetes after adjustment for gender, age, and body mass index (BMI) 0.64 (0.41–0.98) odds ratio (OR) 95% confidence interval (CI) P=0.044 (Table 5).

No association was observed between haplotypes of the ghrelin-receptor gene in the entire cohort with type 2 diabetes (Table 5).

Basal luciferase activity of the ghrelin and GHSR promoters in GH3 cells

SNPs in the promoter region of ghrelin and ghrelin-receptor gene were studied with luciferase assay.

Ghrelin

The promoter of the ghrelin gene comprises six haplotypes, and we created constructs according to each of these haplotypes (the WT-GHRL1 and GHRL2–6). Three of these six haplotypes (WT-GHRL1, GHRL2 and GHRL3) were observed with a frequency O5%. The GHRL3 promoter haplotype is linked with the AAAG GHRL gene haplotype. The luciferase activity of the different promoter variants significantly differed from the most common WT-GHRL1 haplotype (Fig. 2). The WT-GHRL1 promoter-driven basal luciferase activity was 0.054±0.0002 (S.E.M.); GHRL2 showed

Table 3 Single nucleotide polymorphism association analysis of the ghrelin gene.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Allele/ genotype</th>
<th>Control</th>
<th>Case</th>
<th>HWE</th>
<th>Control genotype frequencies</th>
<th>Case genotype frequencies</th>
<th>OR 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-604G</td>
<td>AA</td>
<td>198</td>
<td>187</td>
<td>0.02</td>
<td>0.36</td>
<td>0.35</td>
<td>0.94 (0.80–1.11)</td>
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<tr>
<td>rs27647</td>
<td>AG</td>
<td>235</td>
<td>248</td>
<td>0.02</td>
<td>0.43</td>
<td>0.46</td>
<td>0.89 (0.76–1.03)</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>112</td>
<td>104</td>
<td></td>
<td>0.21</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>C-501A</td>
<td>CC</td>
<td>87</td>
<td>59</td>
<td>0.98</td>
<td>0.11</td>
<td>0.10</td>
<td>1.15 (0.87–1.51)</td>
</tr>
<tr>
<td>rs26802</td>
<td>CA</td>
<td>353</td>
<td>249</td>
<td></td>
<td>0.45</td>
<td>0.43</td>
<td>1.07 (0.79–1.45)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>338</td>
<td>265</td>
<td></td>
<td>0.43</td>
<td>0.46</td>
<td>1.02 (0.88–1.18)</td>
</tr>
<tr>
<td>C247A</td>
<td>CC</td>
<td>633</td>
<td>523</td>
<td>0.48</td>
<td>0.15</td>
<td>0.13</td>
<td>1.02 (0.88–1.18)</td>
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<tr>
<td>rs696217</td>
<td>CA</td>
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<td>75</td>
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<td>0.48</td>
<td>0.46</td>
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<td>Leu72Met</td>
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<td>2</td>
<td>0.9</td>
<td>0.10</td>
<td>0.11</td>
<td>1.11 (0.73–1.70)</td>
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<tr>
<td>A265T</td>
<td>TT</td>
<td>2</td>
<td>3</td>
<td></td>
<td>0.25</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>rs4684677</td>
<td>TA</td>
<td>81</td>
<td>65</td>
<td>0.08</td>
<td>0.48</td>
<td>0.46</td>
<td>1.02 (0.88–1.18)</td>
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<tr>
<td>Gln90Leu</td>
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<td>509</td>
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<td>0.27</td>
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<td>rs35683</td>
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<td>0.48</td>
<td>1.02 (0.88–1.18)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>187</td>
<td>156</td>
<td></td>
<td>0.27</td>
<td>0.27</td>
<td></td>
</tr>
</tbody>
</table>

Table 4 Single nucleotide polymorphism association analysis of the ghrelin receptor gene.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Allele/ genotype</th>
<th>Control</th>
<th>Case</th>
<th>HWE</th>
<th>Control genotype frequencies</th>
<th>Case genotype frequencies</th>
<th>OR 95% CI</th>
</tr>
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<tbody>
<tr>
<td>In/Del</td>
<td>II</td>
<td>37</td>
<td>40</td>
<td>0.82</td>
<td>0.05</td>
<td>0.06</td>
<td>0.93 (0.78–1.10)</td>
</tr>
<tr>
<td>rs10618418</td>
<td>ID</td>
<td>294</td>
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<td>0.33</td>
<td>1.01 (0.85–1.20)</td>
</tr>
<tr>
<td></td>
<td>DD</td>
<td>440</td>
<td>419</td>
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<td>0.57</td>
<td>0.61</td>
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<tr>
<td>A577G</td>
<td>GG</td>
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<td>324</td>
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<td>0.54</td>
<td>0.53</td>
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<td>0.54</td>
<td>0.53</td>
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</tr>
<tr>
<td></td>
<td>AA</td>
<td>61</td>
<td>43</td>
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<td>0.94</td>
<td>0.94</td>
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<td>C658T</td>
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<td>605</td>
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<td>0.94</td>
<td>0.94</td>
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<td>rs2232165</td>
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<td>0.64</td>
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<td>1</td>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>
36% increased activity (0.074 ± 0.0005, P < 0.01), and GHRL3 45% decreased activity (0.0029 ± 0.0007, P < 0.001; Fig. 2; overall P < 0.0001).

Ghrelin receptor The haplotypes of the ghrelin-receptor gene displayed less variability due to the high level of linkage disequilibrium. One common haplotype (WT-GHSR1) with a frequency of 96% and three other minor haplotypes were studied with luciferase assay. Statistical significant differences were observed in the basal luciferase activity driven by the promoter variants of the GHSR gene. The basal luciferase activity of the WT-GHSR1 promoter was 0.048 ± 0.003; GHSR2 0.110 ± 0.007, (P < 0.01, compared with WT-GHSR1); GHSR3 0.013 ± 0.002, (P < 0.01) and GHSR4 0.083 ± 0.003, (P < 0.01, Fig. 2, overall P < 0.0001).

Ghrelin and ghrelin receptor promoter activity in response to hormonal stimulation

Ghrelin Hydrocortisone treatment of GH3 cells bearing the six different ghrelin promoter haplotypes showed that GHRL5 had the lowest level of luciferase activity (Fig. 3). E2 caused no significant difference in the luciferase activity of the six promoter haplotypes, while T3 stimulated the activity of GHRL6 significantly more than the activity of GHRL1. In MTT cells hydrocortisone and T3 did not cause a difference in the activity of the promoters, while E2 was found to induce differential expression in luciferase activity, and the post hoc analysis reveals that the GHRL2 promoter differs significantly with GHRL3, GHRL4, and GHRL6.

Ghrelin receptor In GH3 cells hydrocortisone and T3 had similar effect on the four different GHSR promoter haplotypes, while T3 had a significantly stronger effect on the WT-GHSR1 than on GHSR3 and GHSR4 (Fig. 4). In MTT cells hydrocortisone stimulated GHSR2 more than the WT GHSR1, while E2 and T3 caused no difference in their activation.

Discussion

Polymorphisms of the ghrelin or the ghrelin-receptor gene were not associated with type 2 diabetes in a French population using a case–control approach comprising 610 type 2 diabetic subjects and 820 controls. A borderline protective association was observed with one of the ghrelin haplotypes. Nevertheless, when the regulatory regions of these genes were modeled in a luciferase reporter assay significant differences were observed in the promoter activities of the ghrelin gene. The relevance of these findings in the

Table 5 Haplotype analysis of the ghrelin and GHSR gene. There is no value for the haplotype ACAT/DACC as it is taken as reference.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency in controls</th>
<th>Frequency in cases</th>
<th>Adjusted OR 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHRL</td>
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<tr>
<td>rs26802; rs696217; rs4684677; rs35683</td>
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<td>0.42</td>
<td>–</td>
</tr>
<tr>
<td>ACAT</td>
<td>0.23</td>
<td>0.22</td>
<td>0.97 (0.78–1.12)</td>
</tr>
<tr>
<td>ACAG</td>
<td>0.15</td>
<td>0.15</td>
<td>0.98 (0.75–1.28)</td>
</tr>
<tr>
<td>CCAT</td>
<td>0.09</td>
<td>0.07</td>
<td>0.71 (0.47–1.08)</td>
</tr>
<tr>
<td>AAAG</td>
<td>0.06</td>
<td>0.04</td>
<td>0.64 (0.41–0.98)</td>
</tr>
<tr>
<td>ACTG</td>
<td>0.05</td>
<td>0.05</td>
<td>1.08 (0.71–1.64)</td>
</tr>
<tr>
<td>GHSR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs10618418; rs572169; rs2232169; rs2232165</td>
<td>0.45</td>
<td>0.46</td>
<td>–</td>
</tr>
<tr>
<td>DACC</td>
<td>0.29</td>
<td>0.29</td>
<td>0.97 (0.78–1.22)</td>
</tr>
<tr>
<td>IACC</td>
<td>0.24</td>
<td>0.23</td>
<td>0.96 (0.75–1.23)</td>
</tr>
<tr>
<td>DAGT</td>
<td>0.02</td>
<td>0.02</td>
<td>0.63 (0.31–1.25)</td>
</tr>
</tbody>
</table>

*Adjusted by gender, age, and BMI.
context of type 2 diabetes is not clear due to the fact that our population study did not identify a genetic association with this trait.

The luciferase activity driven by one of the GHRL gene promoter haplotypes, assigned in this study as GHRL3, was found to be 45% less active when compared with the most common regulatory haplotype (WT-GHRL1) under basal conditions in pituitary cells. This suggests that carrying the GHRL3 promoter will lead to reduced ghrelin activity, which supports data from leptin/ghrelin double-knockout animals where the lack of ghrelin rescued the diabetic phenotype of the leptin-deficient mice (15). The 247A/72Met allele has been associated with protection against fat accumulation and associated metabolic disturbances (18). However, other studies found an earlier onset of obesity and early blunted response to insulin after a glucose tolerance test in carriers of the 72Met allele (16, 17). In the context of the present study, no association was found for the C247A polymorphism and type 2 diabetes. Therefore, clinical studies have been contradictory regarding the role of the 247A/72Met in type 2 diabetes-associated abnormalities.

The ghrelin-receptor gene was not associated with type 2 diabetes; based on the observation that neither differences in both allele and genotype nor differences in haplotype frequencies between cases and controls were identified. This gene has been found associated with
obesity in a study using the transmission disequilibrium test and a case–control approach in subjects with a BMI > 32 kg/m² and non-obese with a BMI < 28 kg/m² (21). After applying this BMI definition to our population, we cannot replicate this association with obesity (21), although it needs to be emphasized that our population was selected based on the presence of type 2 diabetes and not obesity. Furthermore, evidence of the GHSR gene role in obesity was presented recently (36): a variation ~ 3.5 kb downstream in the promoter of GHSR assisted in the identification of subjects that could benefit from dietary interventions (this SNP is boxed in Fig. 1). Homozygotes carrying the CC allele, 10% of the cohort studied, benefited most in terms of weight loss after 3 years dieting/exercise intervention. However, it is uncertain what the impact is of this variant in the etiology of type 2 diabetes. It would be attractive to genotype this variant in a group of diabetic subjects that benefited most of dietary interventions; this is an issue that can be explored in prospective studies. Our results should be interpreted with caution due to the fact that binary outcomes, presence or absence of the disease methodologically differ from those studies using quantitative intermediate phenotypes as proxies for disease status. Efforts were made in the current study to translate our findings in the context of previous studies using their definitions, and this is a valid approach to justify replication and the role of a gene in a disease of complex nature; however, non-statistical significant findings were found.

The WT GHRL and GHSR promoters have been previously described (24, 25). The aim of these experiments was to test if common variations found in the promoter regions of these genes have an impact on gene expression. Basal activity of the GHRL promoter was characterized in MTT cells and in human hepatoma cell lines (HepG2) (25) and we now present data on its hormonal modulation in GH3 and MTT cells. The GHRL gene has three common haplotypes observed in human populations with a frequency of 0.39 for GHRL1, 0.28 for GHRL2, and 0.26 for GHRL3 respectively. A significantly lower activity was observed for the GHRL3 promoter haplotype (AATC) and this haplotype drives the AAAG gene haplotype observed with a frequency of 6% in type 2 diabetes and 4% in the control cohort. While basal GHRL promoter haplotype luciferase activities were different (Fig. 2), they all responded similarly to hormonal challenge (Fig. 3). For example, the GHRL3 and GHRL6 haplotypes have low basal activity but normal response to hormonal stimulation in both cell-type models, suggesting that the two loci T-501A and C-36T, where they differ from WT-GHRL1 are crucial in gene expression regulation profiles in vivo. The luciferase activity values for both genes were higher in MTT than in GH3 cells suggesting a differential regulation of the WT promoter depending on the cell line.

In our populations, GHSR has one unique promoter haplotype commonly observed while the others run in a lower frequency below 5%. The GHSR WT promoter has been characterized in GH4 cells with thyroid hormone and E2 (stimulatory) and glucocorticoids (inhibitory) effects on luciferase activity (24). In this study using the GH3 cell line and the MTT cells, we reproduced this observation for E2 and T3 but we observed stimulatory effects for hydrocortisone. The discrepancy between the two studies can be explained by different cell lines and experimental design. The rare GHSR2 and GHSR4 promoters were shown to be inhibited by hydrocortisone. Our findings are concordant with a tissue-specific regulation of ghrelin and its receptor that can be influenced by common genetic variation in the regulatory regions of these genes. The functional assessment of SNPs on transcriptional regulation in vivo is at present a difficult task; in vitro assay of protein–DNA interaction and plasmid reporter gene expression are not suitable for a genome wide approach, and new approaches have also been proposed for this aim (37). We speculate that SNPs in the regulatory region of a gene can be one of the underlying modulators linking the association between a gene and a complex trait.

A limitation in this study is the number of subjects that would affect statistical power; therefore we attained for a genetic association and the role of genetic variation in the regulatory regions of these genes with haplotypes and not with single genetic variants. Power calculations for differences in proportion in a biallelic system (SNPs) indicates that with the cohorts studied a difference of 0.06 in allele frequencies can be identified with an α value of 0.05 and a β value of 0.2. While this study shows minor evidence for the genetic role of these genes in type 2 diabetes, the functional consequences conferred by nucleotide variations on these genes are very deterministic. The likelihood of a false negative because of low statistical power is plausible nevertheless, neither GHRL nor the GHSR have been identified as hits for type 2 diabetes in genome-wide association studies or have been included into the genetic risk score (38, 39) recently proposed for prediction models of type 2 diabetes risk.

In summary, this study found no association of the ghrelin and ghrelin-receptor gene with type 2 diabetes, but variants in the regulatory region of these genes cause profound differences in gene expressions.

Declaration of interest
There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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