A GENETIC VARIANT IN SULF2 AND METABOLIC RESPONSES IN A POPULATION-BASED COHORT

Hassing HC, Bernelot Moens SJ, Mooij HL, Dekker JM, Stroes ESG, Dallinga-Thie GM, Nieuwdorp M

Manuscript in preparation
ABSTRACT

Background - Genome-Wide Association Studies have associated the SULF2 locus with the presence of type 2 diabetes mellitus (T2DM), whereas in T2DM the SULF2 rs2281279 has also been associated with lower fasting glucose and postprandial plasma triglycerides levels. The exact relation between SULF2 and glucose homeostasis or lipid metabolism is, however, to date unclear. Therefore, we evaluated the impact of the SULF2 rs2281279 on metabolic responses in non-diabetic healthy individuals.

Methods - A 75g oral glucose tolerance test (OGTT) and a standardized meal tolerance test (MTT) was performed in 165 non-diabetic individuals stratified according to SULF2 rs2281279.

Results - SULF2 rs2281279 genotype was associated with a significant decrease in postprandial glycaemic excursions following OGTT in a stepwise manner with highest excursion in the homozygote carriers of the major allele (AA) and the lowest excursions in the homozygote carriers of the minor allele (GG)(p<0.05). Insulin sensitivity also revealed a stepwise improvement from AA to GG phenotype (p<0.05). Postprandial triglyceride levels were similar between the groups.

Conclusion - These results imply that SULF2 is involved in insulin-glucose homeostasis in humans. Further studies are needed to unravel the mechanism by which SULF2 affects insulin sensitivity.
INTRODUCTION

The prevalence of the metabolic syndrome and type 2 diabetes mellitus (T2DM) is increasing worldwide. (1) This cardiometabolic syndrome is characterized by impaired glucose metabolism and metabolic dyslipidemia, including delayed clearance of postprandial triglyceride-rich lipoprotein (TRL) remnants. Heparan sulfate proteoglycans (HSPGs) are cell-membrane bound core proteins with 2-3 sulfated HS chains attached enabling the binding of various ligands, including lipoproteins and growth hormones. (2) HSPGs are involved in the regulation of cell growth, apoptosis and lipid metabolism. Syndecan-1, the primary form of HSPGs in the liver, has been implicated in type 2 diabetes mellitus (T2DM) as well as diabetes associated hypertriglyceridemia. (3, 4) In T2DM, the HSPGs are characterized by a decreased negative charge, most likely reflecting decreased heparan sulfation, leading to impaired binding of remnant lipoproteins. (4, 7) Thus, perturbation of HSPG metabolism may contribute to the development of the metabolic syndrome.

Genome-Wide Association Studies (GWAS) have associated the SULF2 locus (20q13.1) with the presence of T2DM. (8) SULF2, a member of the Sulfatase family, encodes for glucosamine-6-O-endosulfatase-2, known to be a HSPG degrading enzyme. We previously showed in murine diabetic model that hepatic Sulf2 expression was positively related to plasma TG levels. Inhibition of hepatic Sulf2 expression by targeted allele-specific antisense administration resulted in normalization of plasma TG levels, reduced postprandial hypertriglyceridemia and lower fasting glucose levels in db/db mice. (7) In humans, genetic variation at the SULF2 locus was associated with lower fasting glucose and plasma TG levels in T2DM as well as accelerated postprandial hepatic TG clearance (Hassing, submitted). These data suggest a direct effect of SULF2 on both triglyceride metabolism and glycemic control per se in T2DM. The relative contribution of SULF2 on postprandial glucose and lipid metabolism in non-diabetic individuals, however, has not been reported yet.

In the present study we evaluated the metabolic effects of genetic variation in SULF2 in a cohort of non-diabetic otherwise healthy individuals.

METHODS

Study design

Patients were included from a population based-cohort drawn from the municipal registry of Hoorn consisting of 208 subjects for which in- and exclusion criteria have been described previously. (9) For the present analyses, we included patients of whom DNA was available. Patients with known diabetes mellitus type 2 (defined as those using oral antihyperglycaemic agents) were excluded. Participants received a 75g-OGTT and a standardized mixed meal test after a 10h-overnight fast, on separate days, in random order, within 2 weeks. To reduce the impact of diurnal variation, all tests started between 7:30 and 9:00 a.m. Apart from the OGTT or test meal and small amounts of water, participants refrained from food, drinks and physical activity during the test. The study was
approved by the Institutional Review Board of the VU University Medical Center in accordance with the Declaration of Helsinki (updated version 2008).

Genotyping
We genotyped a single predetermined rs2281279 [c.2494+267A>G] by allelic discrimination using Tagman mastermix on a BioRad CFX system.

Oral glucose tolerance test (OGTT)
Blood samples were drawn from the antecubital vein in the fasting state and at 15, 30, 60, 90, and 120 min following 75 gram glucose ingestion. Prior to the test, blood pressure (Collin Press-mate BP-8800, Colin, Komaki-City, Japan), weight, height, waist and hip circumference were measured.

Meal Tolerance Test (MTT)
Subjects were served a standardized mixed breakfast consisting of 2 croissants (90 g), 10 g butter, 40 g cheese, 150 g full-fat milk, and 100 g yoghurt drink enriched with 10 g of soluble carbohydrates (maltose). The approximate total nutrient content was 3487 kJ (74 g [36 Energy%] carbohydrates, 49 g [52 Energy%] fat of which 28.2 g was saturated and 24 g [12 Energy%] proteins). Blood samples were collected in fasting state and at 15, 30, 60, 90, 120, 180, and 240 min after meal ingestion.

Laboratory analysis
Plasma glucose levels were determined with a glucose hexokinase method (Gluco-quant; Roche Diagnostics, Mannheim, Germany); serum insulin and C-peptide, with immunometric assays (ACS Centaur; Bayer Diagnostics, Mijdrecht, the Netherlands), and TG, total cholesterol, and high-density lipoprotein cholesterol, with enzymatic colorimetric assays (Roche, Basel, Switzerland). Low-density lipoprotein cholesterol was calculated according to the Friedewald-formula except when fasting TG levels exceeded 5.0 mmol/L. Free fatty acid was measured by enzymatic colorimetric assays (WAKO Chemicals, Neuss, Germany). Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated by the following formula: \([\text{glucose (mmol/L)} \times \text{insulin (mU/L)}}] / 22.5\).

Beta-cell function parameters and insulin sensitivity during OGTT
The insulinogenic index (as an estimation of early insulin secretion) was calculated by dividing the increment in insulin during the first 30 min by the increment in glucose over the same period \((\Delta I_{30}/\Delta G_{30})\). Negative or infinite insulinogenic indexes were excluded (n=10). Overall glucose-stimulated insulin secretion was calculated as AUC_\text{insulin}/AUC_\text{glucose} ratio.

Insulin sensitivity was estimated from glucose and insulin values according to methods described by Mari et al. (OGIS: oral glucose insulin sensitivity) (10), Matsuda and DeFronso (ISIcomp: index of composite whole-body insulin sensitivity) (11) and Stumvoll et al. (MCRest: metabolic clearance rate of glucose) (12).
Statistical analysis
Descriptive statistics are presented as mean ± standard deviation (SD) or median and interquartile range. Differences between genotypes are calculated by Pearson chi-square for categorical variables. Continuous variables are calculated by one-way analysis of variances (ANOVA) with Bonferroni post hoc test for normally distributed variables. Natural log-transformed variables were used in case of non-normally distributions. Total areas under the curve (AUC) of insulin, glucose, c-peptide and triglycerides were calculated by the trapezoid rule. Area under the incremental curve (iAUC) was obtained by subtracting the baseline (t=0) levels from each point. Comparisons between groups were analysed using univariate ANOVA. Two-sided probability values of less than 0.05 were considered statistically significant.

RESULTS
Population characteristics
Of the 208 patients, 28 patients were excluded because of missing DNA analysis and additionally 15 patients were excluded because of known type 2 diabetes mellitus. Thus, a total of 165 patients were included in the current analysis. Stratification according SULF2 rs2281279 genotype, resulted in 87 (53%) homozygous carriers of the major allele (AA), 63 (38%) heterozygous carriers (AG) and 15 (9%) homozygous carriers of the minor allele (GG). Carriers of the minor G allele were more often female, displayed a lower waist-hip ratio, a lower HOMA-IR and had higher HDL-cholesterol levels. Other baseline characteristics did not differ significantly between the genotypes (Table 1).

Metabolic responses following OGTT and MTT
The presence of SULF2 rs2281279 genotype was associated with a significant decrease in glycaemic excursions following OGTT in a stepwise manner with highest excursion in the homozygote carriers of the major allele (AA) and the lowest excursions in the homozygote carriers of the minor allele (GG) (Figure 1). Areas under the glucose curves differed significantly between genotypes [AUC 804 (689-941) (mmol/L)*min, 793 (669-875) (mmol/L)*min and 650 (618-771) (mmol/L)*min for AA, AG and GG respectively, p<0.05] which remained significant following correction for baseline values [(iAUC 147 (53-274) (mmol/L)*min for AA, 148 (40-228) (mmol/L)*min for AG and 71 (0-159) for GG (mmol/L)*min, p<0.05]. In line, insulin and C-peptide responses (Figure 1) revealed a comparable stepwise trend by SULF2 genotype. Glucose, insulin, C-peptide and triglyceride excursions following mixed meal tolerance test are displayed in Figure 2. There were no significant differences in postprandial triglyceride values.
### Table 1 - Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>AA (n=87)</th>
<th>AG (n=63)</th>
<th>GG (n=15)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, n (%)</td>
<td>50 (57%)</td>
<td>25 (40%)</td>
<td>3 (20%)</td>
<td>0.008</td>
</tr>
<tr>
<td>Age, years</td>
<td>52.9 (±6.5)</td>
<td>54.6 (±6.6)</td>
<td>50.4 (±5.9)</td>
<td>0.061</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.5 (±4.1)</td>
<td>26.5 (±3.5)</td>
<td>25.8 (±4.0)</td>
<td>0.153</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.93 (±0.08)</td>
<td>0.89 (±0.07)</td>
<td>0.86 (±0.07)</td>
<td>&lt;0.001&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Systolic blood pressure mmHg</td>
<td>133.9 (±14.0)</td>
<td>136.6 (±17.3)</td>
<td>126.8 (±16.4)</td>
<td>0.087</td>
</tr>
<tr>
<td>Diastolic blood pressure mmHg</td>
<td>76.9 (±9.1)</td>
<td>77.5 (±10.5)</td>
<td>72.3 (±9.3)</td>
<td>0.172</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>4.8 (±0.6)</td>
<td>4.9 (±1.0)</td>
<td>4.7 (±0.3)</td>
<td>0.587</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.08 (±1.88)</td>
<td>1.44 (±0.89)</td>
<td>1.69 (±1.27)</td>
<td>0.099&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.05 (±0.98)</td>
<td>5.16 (±0.85)</td>
<td>5.05 (±1.08)</td>
<td>0.825</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.09 (±0.89)</td>
<td>3.12 (±0.81)</td>
<td>2.87 (±0.68)</td>
<td>0.592</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.33 (±0.35)</td>
<td>1.46 (±0.37)</td>
<td>1.52 (±0.48)</td>
<td>0.032&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.20 (0.80-1.70)</td>
<td>1.10 (0.90-1.50)</td>
<td>1.10 (0.70-1.40)</td>
<td>0.525</td>
</tr>
</tbody>
</table>

Data are presented as mean (±SD), number (percentage) or median (IQR).

Abbreviations: LDL = low-density lipoprotein; HDL = high-density lipoprotein; BMI = body mass index.

<sup>1</sup>Significant P-values for Bonferroni post hoc tests: AA vs AG p=0.005, AA vs GG p=0.002.

<sup>2</sup>Significant P-values for Bonferroni post hoc tests: AA vs AG p=0.035

<sup>3</sup>Not significant following Bonferroni post hoc tests.
Figure 1 - Oral Glucose Tolerance Tests. Glucose, insulin and C-peptide responses (mean ± S.E.) following OGTT in subjects with different SULF2 Rs2281279 genotypes (AA, AG or GG). * = p value for incremental AUC.
Beta-cell function parameters and insulin sensitivity
Following OGTT there were no differences in beta-cell function as measured by insulinogenic index (early insulin secretion) nor by AUC_{insulin}/AUC_{glucose} ratio (overall glucose-stimulated insulin secretion, Table 4). Interestingly, insulin sensitivity differed significantly between genotypes by all three estimates of insulin sensitivity used (OGIS, ISI_{comp} and MCR_{est}) in these non-diabetic subjects. Subject with heterozygote or homozygote carriership of the minor G allele displayed significant higher insulin sensitivity in a stepwise manner compare to homozygote carriers of the major allele (Table 2).

**DISCUSSION**

This study evaluated the effect of genetic variation in SULF2 on metabolic responses following OGTT and MTT in a healthy non-diabetic population. We found that carriership of the rs2281279 minor G allele is already associated with higher insulin sensitivity Further studies are needed to dissect the underlying mechanisms by which SULF2 regulates insulin-glucose homeostasis and whether this can be pursued as therapeutic target for glucose control.
It has previously been shown that SULF2 is associated with elevated plasma triglyceride concentration and an impaired postprandial clearance of triglyceride-rich lipoprotein remnants in T2DM. (4, 7) In contrast to T2DM subjects, we did not find any effects of the rs2281279 genotype on postprandial triglyceride levels. It is recognized that SULF2 modifies the sulfation grade of the HSPG chain thereby affecting its affinity for binding of TG-rich lipoproteins. However, other ligand receptors beyond the HSPG system are present at the hepatocyte membrane, including the LDL-receptor, which also binds TRLs. (13, 14) The present study was performed in healthy normo-triglyceridemic subjects. In these subjects a normal capacity of the liver for TRL clearance is expected to suffice. Thus other receptors can compensate for the loss of HSPG capacity in the clearance of TRLs. In line, it was previously shown that both baseline TG levels and the presence of T2DM are independent predictors of postprandial TG curves. (15, 16) Therefore, in a diabetic and hypertriglyceridemic state this capacity might become limited leading to impaired TRL clearance due to a lower HSPG clearance capacity as suggested by our experimental models. (7)

Interestingly, we did find significant higher insulin sensitivity in non-diabetic subjects who were carriers of the rs2281279 minor G allele. Also, the SULF2 locus (20q13.1) was identified previously in a GWAS study to be associated with the presence of T2DM. (8) Although proteoglycans and the endothelial glycocalyx are recognized as being essential for optimal endothelial function, the role of SULF2 in peripheral insulin sensitivity is unknown. Interestingly, glypican-4 (Gpc4), which belongs to the HSPG family, acts as an insulin sensitizer. (17) In this study, Gpc4 was found to interact with the insulin receptor, enhance insulin receptor signalling and enhance adipocyte differentiation. Furthermore, serum Gpc4 levels were positively correlated with body fat content.

### Table 2 - Beta-cell function parameters and insulin sensitivity during OGGT

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rs2281279 genotypes</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA (n=87)</td>
<td>AG (n=63)</td>
</tr>
<tr>
<td>Insulinogenic index (pmol/mmol)</td>
<td>105.4 (63.2-181.1)</td>
<td>117 (63.7-158.2)</td>
</tr>
<tr>
<td>AUCinsulin/AUCglucose ratio (pmol/mmol)</td>
<td>41.1 (30.1-63.2)</td>
<td>37.2 (28.2-53.2)</td>
</tr>
<tr>
<td>OGIS (ml/(min m²))</td>
<td>408 (357-450)</td>
<td>422 (380-456)</td>
</tr>
<tr>
<td>ISIcomp (µmol/(kg min pmol L))</td>
<td>16.4 (11.1-25.7)</td>
<td>21.4 (14.5-28.9)</td>
</tr>
<tr>
<td>MCR index (ml/(min kg))</td>
<td>8.9 (7.6-10.1)</td>
<td>9.2 (8.5-10.1)</td>
</tr>
</tbody>
</table>

Values are presented as median (interquartile range). AUC = area under the curve, OGIS = oral glucose insulin sensitivity, ISIcomp = index of composite whole-body insulin sensitivity, MCR = metabolic clearance rate of glucose. *Significant p-values for Bonferroni post hoc test: AA vs GG; p<0.007. †Not significant following Bonferroni post hoc tests. ‡No t significant following Bonferroni post hoc tests.
insulin resistance and BMI. For the latter this was also true for non-diabetic subjects. In line, our findings imply a protective effect of SULF2 against impaired insulin sensitivity. As Sulf2 is strongly upregulated in diabetic liver tissue (4), it is tempting to speculate on a potential role in hepatic gluconeogenesis. Since these data are currently lacking, further studies on the role of SULF2 on hepatic gluconeogenesis and peripheral insulin sensitivity are warranted.

In summary, the present data provide the first evidence that SULF2 might be involved in human glycaemic control. Regarding the GWAS association of SULF2 with T2DM, previous experimental findings linking SULF2 to diabetic dyslipidemia and current results implicating an insulin sensitizing role for SULF2 in a pre-diabetic state, these finding indicate that SULF2 is involved in insulin-glucose metabolism. Although not significant in healthy subjects, SULF2 might also be pivotal for lipid metabolism in metabolically challenged subjects. Therefore, SULF2 might play an essential role in several processes at the core of the metabolic syndrome. Future studies are warranted to evaluate whether HSPG can serve as a target in metabolic disturbances.
SULF2 IN A POPULATION-BASED COHORT

REFERENCE LIST


