Glycobiology in cardiometabolic homeostasis
Hassing, H.C.

Citation for published version (APA):
Hassing, H. C. (2013). Glycobiology in cardiometabolic homeostasis

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
CARRIERS OF LOSS-OF-FUNCTION MUTATIONS IN EXT
DISPLAY IMPAIRED PANCREATIC BETA-CELL RESERVE DUE TO
SMALLER PANCREAS VOLUME

Mooij HL, Bernelot Moens SJ, Hassing HC, Kruit JK, Witjes JJ, van de Sande MAJ, Stoker
J, Nederveen AJ, Xu D, Esko JD, Dallinga-Thie GM, Stroes ESG, Nieuwdorp M
ABSTRACT

Aim - A genome wide association study identified exostosin 2 (EXT2) as a novel risk factor for the development of type 2 diabetes mellitus. As EXT genes, involved in the chain elongation step of heparan sulfate (or HSPG) biosynthesis, are intricately involved in organ development, we hypothesized that mutations in these genes might affect pancreatic islet mass and insulin secretion capacity. Here we used a translational approach to study the effect of EXT mutations on pancreatic development, insulin secretion and glucose metabolism in mice and humans with heterozygous EXT mutations causing hereditary multiple exostoses (HME).

Methods - In heterozygous Ext1 or Ext2 knock-out mice we performed oral glucose tolerance tests (OGTT), insulin tolerance tests (ITT) and harvested each mouse pancreas for extraction of islets (insulin secretion) and immunohistochemistry (beta cell mass). In HME subjects and family-based non-carriers (similar age, sex, and BMI) we repeated OGTT followed by hyperglycemic clamps to investigate first-phase insulin secretion (GSIS). Finally, abdominal MRI was assessed to quantify total pancreas volume.

Results - No differences in oral glucose tolerance and insulin resistance were found in mice and humans with EXT mutations compared to controls. No effects on insulin signalling were found in isolated islets challenged with hyperglycaemia. However, glucose stimulated insulin secretion (hyperglycaemic clamp) showed that HME subjects had a significantly altered GSIS as well as an impaired beta cell reserve (upon arginine bolus). In line with these finding, Magnetic Resonance Imaging showed a significantly smaller pancreas volume in HME subjects compared to controls.

Conclusions - Carriers of loss-of-function mutations in EXT showed impaired GSIS without insulin resistance due to reduced functional beta cell mass and decreased anatomic pancreas volume. Our data provide evidence that heparansulfates are important for normal beta-cell function in humans.
INTRODUCTION

Although the worldwide rising incidence of type 2 diabetes and obesity is largely attributed to environmental factors, the existence of a genetic predisposition has long been recognized. In this respect, genome wide association studies (GWAS) have rendered conflicting results on the role of SNPs in EXT2 (exostosin 2) as a novel genetic risk factor for the development of type 2 diabetes mellitus (DM2). Also, SNPs in EXT2 have been associated with impaired glucose clearance in DM2 as assessed by an oral glucose tolerance test. A recent meta-analysis of Liu et al showed a significant association between the EXT2 SNP variants and the risk of developing DM2. However, despite these epidemiological associations, little is known about the pathophysiological role of EXT in glucose metabolism.

EXT1 and 2 genes encode for an endoplasmic reticulum-resident type II transmembrane glycosyltransferase involved in the chain elongation step of heparan sulfate biosynthesis. Heparansulfate proteoglycans (HSPG) play a role in many biological processes including fine-tuning most of the (patho)physiological processes such as (fetal) organ development, lipid metabolism and inflammatory pathways. More specific, a subset of the Ext genes, Ext2 and Ext3, were reported to be involved in pancreatic β cells development in mice. Whereas in humans, the role of EXT3 is not involved in the initiation of HS Biosynthesis, the role of EXT1 and EXT2 genes has been widely recognized.

Heterozygous EXT mutations are known to be involved in the development of hereditary multiple exostoses (HME) syndrome, a disorder in which disrupted HS synthesis induces growth of multiple bony tumors (eg. Exostoses or osteochondromas). However, metabolic derangements have never been studied in this patient group. In the present study we designed a dedicated series of experiments in both mice and humans to unravel the effect of EXT mutations on beta cell mass and function, as well as insulin-glucose homeostasis in mice and humans with heterozygous EXT mutations.

METHODS

Oral glucose tolerance test and insulin tolerance tests in Ext1 and Ext2 heterozygous knockout mice and wild type mice

All animals were housed in barrier conditions in vivaria of the AMC-UvA and all protocols were approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Moreover, standards and procedures approved by the local Institutional Animal Care and Use Committee were followed. Mice were weaned at 3 weeks, were maintained on a 12-hour light–dark cycle and fed water and standard rodent chow (Harlan-TekLad City Country) ad libitum. All animals were fully backcrossed on a C57Bl/6 background. Genotyping for Ext1 and Ext2 heterozygosity was performed using primers as described earlier. All investigations were performed in 4h fasted male mice (25-30g, aged 9-12 weeks). After baseline blood sampling, glucose was administered as oral gavage (2g/
kg body weight) followed by tail vein blood sampling at 15, 30, 45, 60, 90 and 120 min. For the insulin tolerance test (ITT) mice were fasted for 4 hours before the study. After baseline blood sampling, insulin (Actrapid 1U/kg bodyweight) was administered intraperitoneally and subsequent tail vein blood sampling was performed at 15, 30, 45, 60, 90 and 120 min. Plasma glucose concentrations were determined with a glucose meter (Lifescan One Touch, Johnson and Johnson Company).

**Insulin secretion in isolated mice islets**

At 12 weeks of age, mice were sacrificed using and pancreas was extracted after collagenase injection for islet isolation as described previously. Islets were cultured overnight in RPMI 1640 medium (Sigma Aldrich). The following day, batches of 10 hand picked islets were preincubated in Krebs Ringer bicarbonate buffer (KRB) containing 1.67 mM glucose for 1 h at 37 °C in 95% O₂/5% CO₂. Thereafter, islets were incubated for 1 hour in 0.1 ml of fresh KRB containing either 1.67 mM or 20mM of glucose. Subsequently, media was removed and islets were lysed in 1M acidic acid. Insulin levels in both media and islets were determined using an ultrasensitive Mouse Insulin ELISA (Mercodia, Uppsala, Sweden).

**Human studies**

We enrolled HME subjects and family-based non-carriers over 18 years of age, without pre-existent type 1 or 2 diabetes. As redundancy between EXT1 and EXT2 exists we tested both subjects with either EXT1 or EXT2 heterozygous mutations for alterations in glucose metabolism and pancreatic reserve. For all tests, subjects were requested to arrive in a fasting state and written informed consent was obtained after explanation of the study. The study was approved by the institutional review board of the Academic Medical Center of the University of Amsterdam and carried out according to the Declaration of Helsinki.

**Oral Glucose Tolerance Test (OGTT) and hyperglycaemic normoinsulinemic clamp**

After an overnight fast, a standardized OGTT was performed. After baseline venous sampling subjects were asked to ingest 75 g glucose. At t = 30, 60, 90 and 120 minutes an 4.5 ml blood sample was obtained for assessment of blood glucose, insulin and C-peptide. On a separate study day, a hyperglycemic clamp was performed. On the day of study antecubital veins of both arms were canulated for blood sampling and infusion of fluids. All bedside glucose measurements were performed using a bedside calibrated glucose sensor (YSI 2300 STAT S; YSI, Yellow Springs, OH). Based on the fasting plasma glucose level and the subject’s bodyweight first phase insulin secretion was determined using a 20% glucose bolus (weight/70 x 10 – plasma glucose = millilitres required) given over 1 min, with the aim of reaching a plasma glucose level of 14 mmol/L. Subsequently, blood glucose levels were kept at 14 mmol/L by continuous glucose infusion. Pump settings (glucose infusion rate) were adapted based on blood glucose levels at t = 0, 2.5, 5, 7.5, 10 and 20. Simultaneously, blood samples were collected for insulin and C-peptide determination. After 120 minutes an arginine bolus (5 gram arginine hydrochloride, 50 ml per 100mg/ml solution) was given, followed by measurement of plasma insulin levels at t = 125, 130, 140 and 150 minutes. Basal
fasting glucose, HbA1c, total cholesterol, HDL, and LDL cholesterol and triglycerides were assessed in fasting plasma using standard laboratory procedures within 1 h after sampling. For the OGTT and hyperglycaemic clamps, samples were centrifuged at 4ºC, 3000 RPM for 20 minutes and plasma was stored at -80 ºC until analysis. Glucose was determined by the hexokinase method (Hitachi), Insulin was determined on an Immulite 2000 system (Diagnostic Products, Los Angeles, CA). C-peptide was measured by RIA (RIA-coat C-peptide; Byk-Sangtec Diagnostica, Dietzenbach, Germany).

Homeostasis model assessment (HOMA) indexes were calculated for insulin sensitivity (HOMA-ir = insulin (picomoles)/6.945*glucose (millimoles)/22.5) and insulin secretion (HOMA-%β = 20* fasting insulin (picomoles)/6.954/glucose (millimoles-3.5). In line, in the OGTT insulin sensitivity was estimated using the metabolic clearance rate (MCR) of glucose and the insulin sensitivity index (ISI), both as described previously. Overall glucose-stimulated insulin secretion was calculated as AUCinsulin/AUCglucose ratio.

MRI
In a subset of previous participants (both HME-subjects and healthy controls) we performed abdominal imaging preceding the OGTT, using a 3-T MR imaging unit (Intera, Philips Healthcare, Best, The Netherlands). A Single Shot Fast Spin Echo (SSFSE) sequence was performed and a T1-weighted high-resolution axial anatomical scan obtained in breath hold (matrix 320X320, field of-view [FOV] ((450x450mm) was used to investigate the morphology of the pancreas. Images were analyzed by 2 independent, blinded investigators using ITK Snap software version 2.4 (University of Pennsylvania). Pancreatic area was labelled and number of voxels in this area was determined, subsequently this number was transcribed to volume in cubic millimetres as previously published. The mean area of separate measurements was used.

Statistical analysis
Data are presented as mean ± SD or medians with interquartile range ([IQR] unless stated otherwise. When normally distributed baseline characteristics where compared using a student’s t test (all but triglycerides). Differences in triglyceride levels, known not to be normally distributed, and continues outcome variables were assessed using the nonparametric Mann-Whitney U test. For all outcomes P<0.05 was used to indicate significant differences. All analyses were performed with SPSS software version 19.0.0.1.

RESULTS
Metabolic parameters and islets function in Ext1 and Ext2 heterozygous versus wild type mice
To investigate the effects of Ext on pancreas anatomy and function (insulin secretion) we performed oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance tests (ITT) in mice with heterozygous mutations in Ext1+/−, Ext2+/− as well as wild type mice (Table 1A and Table 1B). As compared to wild type mice, heterozygous Ext1+/− and Ext2+/− mice were characterized by normal oral
glucose disposal (IAUC Ext1+/- 579 [494-707], Ext2+/- 680 [636-756] and WT 688 [501-794] mmol·l⁻¹·min⁻¹, ns, see Figure 1A). Moreover, insulin sensitivity as assessed by ITT was comparable between Ext1+/-, Ext2+/- and WT mice (IAUC Ext1+/- 472 [453-492], Ext2+/- 381 [316-453], WT 362 [345-361] mmol·l⁻¹·min⁻¹, ns, see Figure 1B). Next, we investigated islets insulin secretion in isolated islets from all phenotypes. Insulin secretion was not impaired in Ext1+/- or Ext2+/- mice, also total islet insulin content was comparable between all groups (Insulin secretion after 24mmol glucose stimulation for Ext1+/-: 0.23 [0.19-0.37] μg/L, Ext2+/-: 0.30 [0.17-0.48] μg/L and wild type mice 0.24 [0.18-0.38] μg/L, ns see Figure 2).

**Beta cell function and glucose metabolism in human EXT carriers versus controls**

Age, BMI, fasting glucose, HbA1C and insulin levels did not differ significantly between carriers and control subjects (see Table 2). Moreover, no differences between EXT carriers and controls were found upon ingestion of 75 g of glucose with respect to plasma glucose (iAUC: carriers; 233 [2174-299] vs controls; 183 [100-286] nmol·l⁻¹·min⁻¹, p=0.46, Figure 3A). No differences between the two groups were found with respect to plasma insulin levels (iAUC: carriers; 13.2 [8.5-18.6,0] vs controls; 16.5 [11.1-21.7] nmol·l⁻¹·min⁻¹, p=0.46, Figure 3B). Several markers of insulin resistance and beta cell function were calculated yet showed no significant differences between the two groups (summarized in Table 3).

To evaluate the potential effect on GSIS, we subsequently performed a hyperglycaemic normoinsulinemic clamp, followed by arginine infusion. During this hyperglycaemic clamp, first phase insulin response to a glucose bolus (as determined by incremental AUC) was lower in carriers than control subjects (0.72 [0.46-1.16] vs. 1.53 [0.69-3.36] nmol·l⁻¹·min⁻¹, P=0.046) (Figure 4A). In addition, C-peptide responses were also lower in carriers (3.57 [2.26,5-0,0] vs. 6.62 [4.48-9,84] nmol·l⁻¹·min⁻¹, p=0.006) (Figure 4B). Of note, in line with our HOMA findings, glucose infusion rates were similar between the groups (iAUC carriers vs. controls 11.1 [8.88-19.00] vs. 14.5 [11.98-23.98] mg·kg⁻¹·min⁻¹, p=0.3, Figure 4C), suggesting that differences found in insulin and C-peptide secretion are not due to differences in glucose tolerance. Finally, upon intravenous arginine bolus the peak-insulin response was impaired in EXT carriers compared to controls (IAUC 7.14 [4.22-10,95] vs. 10.32 [7.91-12,70] nmol·l⁻¹·min⁻¹, p=0.041) (Figure 4D and E).

Based on these findings of functional decreased beta cell insulin secretory capacity we hypothesized whether a difference in anatomical pancreas volume could be detected in a subset of the previously tested EXT carriers compared to controls (see Table 4). Subsequent abdominal MRI imaging revealed a significantly smaller pancreas volume in EXT carriers compared to control subjects (74 [63-86] cm³ vs.87 [82-105] cm³, p=0.016) (Figure 5).
EXT MUTATIONS AND BETA-CELL FUNCTION IN HUMANS

Table 1 - Baseline mouse characteristics

<table>
<thead>
<tr>
<th></th>
<th>WT (N=8)</th>
<th>Ext1&lt;sup&gt;−/−&lt;/sup&gt; (N=8)</th>
<th>Ext2&lt;sup&gt;−/−&lt;/sup&gt; (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>8.5±0.5</td>
<td>8.6±0.5</td>
<td>8.5±0.5</td>
</tr>
<tr>
<td>Bodyweight (gr)</td>
<td>27.8±1.1</td>
<td>26.8±1.3</td>
<td>27.8±1.6</td>
</tr>
<tr>
<td>Fasting triglycerides</td>
<td>1.1 [0.91-1.2]</td>
<td>1.0 [0.96-1.1]</td>
<td>1.1 [1.1-1.2]</td>
</tr>
</tbody>
</table>

Table 1B

<table>
<thead>
<tr>
<th></th>
<th>WT (N=8)</th>
<th>Ext2&lt;sup&gt;−/−&lt;/sup&gt; (N=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>10.3±0.5</td>
<td>10.0±0.0</td>
</tr>
<tr>
<td>Bodyweight (gr)</td>
<td>30.0±0.69</td>
<td>29.2±0.82</td>
</tr>
</tbody>
</table>

Baseline mouse characteristics for (A) oral glucose tolerance test and (B) intraperitoneal insulin tolerance test. Data are means ± SD or median [IQR].

Figure 1A: glucose levels during OGTT in mice

Figure 1B: glucose levels during IPITT in mice

Figure 1 - Glucose homeostasis in WT (▲) versus Ext1<sup>−/−</sup> (■) vs Ext2<sup>−/−</sup> (○) mice. (A) Plasma glucose after oral glucose tolerance test. (B) Plasma glucose after intraperitoneal insulin tolerance test.
Figure 2 - No difference in insulin secretion in isolated islets from each genotype. (A) Insulin secretion from isolated islets, value represent data from 2 separate experiments, each consisting of 3 mice per genotype. Values are expressed as percent of islet content relative to basal secretion (set at 1) (B) Total islets insulin content.

Table 2 - Baseline characteristics of participants

<table>
<thead>
<tr>
<th></th>
<th>Noncarriers (N=13)</th>
<th>Carriers (N=14)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49±12</td>
<td>39±10</td>
<td>0,6</td>
</tr>
<tr>
<td>Men</td>
<td>8 (40)</td>
<td>7 (30)</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>25,6±3,4</td>
<td>25,8±4,9</td>
<td>0,18</td>
</tr>
<tr>
<td>BSA</td>
<td>1,8±0,16</td>
<td>1,8±0,19</td>
<td>0,16</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5,44±1,38</td>
<td>4,83±1,15</td>
<td>0,24</td>
</tr>
<tr>
<td>LDL</td>
<td>3,38±1,28</td>
<td>2,99±1,05</td>
<td>0,16</td>
</tr>
<tr>
<td>HDL</td>
<td>1,49±0,44</td>
<td>1,32±0,40</td>
<td>0,40</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0,92[0,68-1,38]</td>
<td>0,87[0,56-1,32]</td>
<td>0,96</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4,9±0,60</td>
<td>4,8±0,53</td>
<td>0,63</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>44±19</td>
<td>30±15</td>
<td>0,40</td>
</tr>
</tbody>
</table>
Table 3 - Beta-cell function and insulin sensitivity parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Noncarriers</th>
<th>Carriers</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline HOMA index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1,2[0,79-1,79]</td>
<td>0,97[0,60-1,67]</td>
<td>0,30</td>
</tr>
<tr>
<td>HOMA-B</td>
<td>78[47-126]</td>
<td>78[50-144]</td>
<td>0,85</td>
</tr>
<tr>
<td>Insulinogenic index (pmol/mmol)</td>
<td>41,9[36,4-182]</td>
<td>62,6[32,2-107,5]</td>
<td>0,76</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;glucose&lt;/sub&gt;/AUC&lt;sub&gt;insulin&lt;/sub&gt; ratio (pmol/mmol)</td>
<td>86,2[71,5-254,8]</td>
<td>52,2[43,1-93,4]</td>
<td>0,23</td>
</tr>
<tr>
<td>ISIcomp(μmol/(kg min pmol L))</td>
<td>24,5[22,5-48,3]</td>
<td>43,1[30,0-51,0]</td>
<td>0,23</td>
</tr>
<tr>
<td>MCR (ml/(min kg))</td>
<td>9,9[9,4-10,6]</td>
<td>10,1[9,8-10,6]</td>
<td>0,40</td>
</tr>
</tbody>
</table>

Figure 3 - OGTT results in HME subjects (■) versus controls (○) Plasma glucose and insulin curves after 75g orally ingested glucose.
Figure 4 - Functional (GSIS) pancreas reserve in HME subjects (■) versus controls (○). (A and B) The first phase insulin and C-peptide response to a hyperglycaemic clamp was lower in HME subjects compared to controls. (C) The Glucose infusion rate (GIR), an estimation of the amount of glucose being metabolized, was not different between groups. (D and E) Insulin secretion after an intravenous bolus of arginine was lower in carriers vs controls.
Table 4 - Baseline characteristics of participants in MRI

<table>
<thead>
<tr>
<th></th>
<th>Noncarriers (N=12)</th>
<th>Carriers (N=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>42±14</td>
<td>41±10</td>
<td>0.95</td>
</tr>
<tr>
<td>Men</td>
<td>5 (41)</td>
<td>3 (37.5)</td>
<td></td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.72±0.10</td>
<td>1.72±0.10</td>
<td>0.9</td>
</tr>
<tr>
<td>Weight</td>
<td>69.5</td>
<td>78±12</td>
<td>0.1</td>
</tr>
<tr>
<td>BMI</td>
<td>23.8±1.9</td>
<td>22.9±10</td>
<td>0.75</td>
</tr>
<tr>
<td>BSA</td>
<td>1.8±0.16</td>
<td>1.8±0.19</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Data are means ± SD, n (%), or median [IQR]

Figure 5 - Image of pancreas in HME subjects and controls on volume. Pancreas volume, assessed with 3T MRI, was smaller in HME subjects versus controls. (A) Labelled pancreatic area and 3D composition. (B) Pancreatic volume.
DISCUSSION

Following the reports on genetic associations between EXT-SNPs and type 2 diabetes mellitus, we now provide the first evidence that carriers of loss-of-function mutations in EXT show an impaired GSIS in hyperglycaemic clamping studies. Combined without any evidence for beta cell dysfunction or peripheral insulin resistance in mice, the significant reduction in total pancreas volume implies a structural rather than a functional beta cell defect in carriers of loss-of-function mutations in EXT.

Functional and structural beta cell defects in heterozygous carriers of EXT mutations

In contrast to the previous reports in type 2 diabetes subjects(9), both murine and human displayed no signs of hyperglycemia but a small (non significant) difference in insulin response upon an oral glucose challenge. Using hyperglycemic clamps, we subsequently found that EXT heterozygotes show a reduced first-phase insulin response to hyperglycemia (GSIS) and normal insulin sensitivity, resulting in a markedly decreased disposition index compared with noncarriers of similar age, sex, and BMI. Collectively, these findings point to a defect in β-cell function in EXT heterozygotes.

The specific means by which EXT dysfunction impairs β-cell function remain incompletely understood. Glucose enters the β-cell via GLUT, whereupon it is modified by glucokinase in the rate-limiting step in glucose sensing. The subsequent glucose metabolism pathway results in closing of the ATP-sensitive potassium channel, membrane depolarization, calcium influx into the cell via the L-type calcium channel, and exocytosis of insulin-containing granules. This first-phase secretory response is augmented by a potassium channel-independent pathway, which is largely responsible for the second-phase insulin response. Arginine is known to stimulate insulin secretion by directly inducing membrane depolarization independent of potassium channels and thus largely independent of glucose sensing and glucose metabolism pathways. In EXT heterozygotes, the first-phase insulin response and secretory response to arginine were significantly impaired, whereas insulin secretion during steady hyperglycemia (between t = 90 and t = 120) was not statistically different between groups. These findings are in contrast with our previous report in ABCA1 carriers showing decreased GSIS with an intact maximal insulin release capacity following arginine. Indeed, MRI based pancreas volume measurements underscore the presence of an absolute diminished beta-cell mass suggesting that EXT dysfunction reflects a latent insulin deficiency due to an absolutely decreased beta cell mass.

Pathophysiological mechanisms linking EXT to beta cell mass and function

After the first reports on EXT in GWAS studies performed in type 2 diabetes subjects, several studies have rendered conflicting results. Recently however, Liu et al. performed a pooled analyses using all existing GWAS data available for EXT2 gene and showed a small but significant effect (OR 1.06-1.07). Recent genetic studies in Drosophila and mice have provided compelling evidence that HS plays an essential role in embryonic development by interacting with several signalling molecules including Wnts, Hedgehogs, and fibroblast growth factors (FGFs). Our findings of normal insulin
sensitivity combined with an impaired pancreatic beta cell architecture in Ext heterozygous knockout mice thus provide a pathophysiological substrate attributing to the role for the EXT gene in the pathogenesis of type 2 diabetes mellitus. Most likely via decreased pancreas beta cell development, which corroborate with findings in conditional Ext3 knockout mice. Previous data have suggested that HS plays an important role in organ development since HS is required as a co-receptor for FGF growth factor signalling in β-cells. In this respect, it has been suggested that hedgehog (hh) signalling via heparansulfates is of pivotal importance in murine organ development and function in particular. Moreover, it is reported that mutations in the EXT protein family in this gene lead to impaired distribution of hh signalling remains important throughout life in beta cell function.

On the other hand, it has been recognized that GSIS reflects the available previously synthesized formed and stored insulin that can be secreted upon glucose stimulation. In line, previous in vitro studies using both genetic and enzymatic approaches to induce decreased beta cell HS have all resulted in impaired GSIS. In this regard, it has been reported that Wnts are involved in GSIS in adult mouse islets. However as we found no effect on in vitro GSIS in isolated islets of Ext heterozygous mice, we believe that defective Wnt signalling does not provide an additional explanation for impaired GSIS reported in our study. In contrast, a recently published paper implicated that in mice pancreas HSPGs are involved in beta cell survival providing a buffer mechanism against reactive oxygen species (ROS). Thus HSPGs may have several roles in beta cell homeostasis via either regulation of postnatal islet and pancreas development, whereas on the other hand HSPGs might protect the beta cell against destruction later in life. Thus, the inadvertent depletion of pancreas heparansulfates in EXT heterozygous subjects might render the already decreased amount of beta cells vulnerable for exogenous pathogenic stimuli including obesity. Indeed, it has been previously noted that β-cell failure precedes the development of impaired glucose tolerance (IGT) in insulin resistant subjects due to ROS induced exhaustion of the normal beta cell capacity to adjust for increased insulin demand.

**Study limitations**

Our study also has several limitations. First, it should be noted that in contrast to the intronic SNPs in the EXT2 gene identified in the different GWAS studies that may have rather small effects on impaired (first phase) insulin secretion and smaller pancreas volume, the EXT1 and EXT2 genes encode homologous proteins with significant sequence identity and the majority of mutations are nonsense or frameshift, leading to complete loss of function of the protein. Thus, further studies are needed to address whether a similar mechanism of decreased pancreas volume might be responsible for the genetic association between EXT2 and development of type 2 diabetes mellitus. Second, based on the small numbers of available carriers of loss-of-function mutations in EXT our study does not allow us to analyse the individual effects of these specific genes on beta cell function. Finally, as large well genotyped clinical cohort of HME subjects are not available, we are currently
unable to investigate the incidence of overt type 2 diabetes in HME subjects with ranging BMI which could underscore clinical validity of our findings.

In conclusion, we now provide the first evidence on the relation between genetic defects in heparan sulfates and smaller pancreas anatomic volume with ensuing impaired betacell reserve capacity in human carriers of loss-of-function mutations in EXT. Our findings will hopefully provide novel pathophysiological clues and potential therapeutic targets to prevent betacell failure in obese humans.

Acknowledgments
We are grateful to all participating HME subjects and Jan de Lange from the Dutch HME Foundation (www.hme-mo.nl) for their help with inclusion and being able to perform this study.
REFERENCES

EXT MUTATIONS AND BETA-CELL FUNCTION IN HUMANS


