Changing Metabolic Signatures of Amino Acids and Lipids During the Prediabetic Period in a Pig Model With Impaired Incretin Function and Reduced β-Cell Mass

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Diabetes is generally diagnosed too late. Therefore, biomarkers indicating early stages of β-cell dysfunction and mass reduction would facilitate timely counteraction. Transgenic pigs expressing a dominant-negative glucose-dependent insulinotropic polypeptide receptor (GIPRdn) reveal progressive deterioration of glucose control and reduction of β-cell mass, providing a unique opportunity to study metabolic changes during the prediabetic period. Plasma samples from intravenous glucose tolerance tests of 2.5- and 5-month-old GIPRdn transgenic and control animals were analyzed for 163 metabolites by targeted mass spectrometry. Analysis of variance revealed that 26 of 163 parameters were influenced by the interaction Genotype × Age (P ≤ 0.0001) and thus are potential markers for progression within the prediabetic state. Among them, the concentrations of seven amino acids (Phe, Orn, Val, xLeu, His, Arg, and Tyr) were increased in 2.5-month-old but decreased in 5-month-old GIPRdn transgenic pigs versus controls. Furthermore, specific sphingomyelins, diacylglycerols, and other phospholipids were decreased in plasma of 5-month-old GIPRdn transgenic pigs. Alterations in plasma metabolite concentrations were associated with liver transcriptome changes in relevant pathways. The concentrations of a number of plasma amino acids and lipids correlated significantly with β-cell mass of 5-month-old pigs. These metabolites represent candidate biomarkers of early phases of β-cell dysfunction and mass reduction.

T he incidence of diabetes and of impaired glucose tolerance, a prestage of type 2 diabetes, is rapidly increasing (1). Changes in insulin secretion, glucose homeostasis, and insulin sensitivity may occur many years before the clinical manifestation of diabetes (2).

While insulin resistance—mostly associated with obesity—often precedes the development of hyperglycemia, overt diabetes develops only with the onset of β-cell dysfunction (3). There is evidence that reductions in both the first and second phases of insulin release occur equally early, precede insulin resistance, and therefore may represent the primary risk factor predisposing individuals to type 2 diabetes (4). An experimental approach to this hypothesis requires an animal model with progressive β-cell dysfunction in the absence of confounding factors such as insulin resistance and obesity.

Transgenic pigs expressing a dominant-negative glucose-dependent insulinotropic polypeptide (GIP) receptor (GIPRdn) develop normally but exhibit reduced insulitropic action of GIP (5), which is typically seen in type 2 diabetic patients (6). In 2.5-month-old GIPRdn transgenic pigs, intravenous glucose tolerance, insulin secretion, and the total β-cell volume do not differ from controls (5). The only overt clue pointing to a prediabetic condition is reduced oral glucose tolerance with a distinct delay in insulin secretion. The insulinogenic index, a parameter of early insulin response (7), is reduced in 2.5-month-old GIPRdn transgenic pigs compared with controls (0.47 vs. 0.96; P < 0.05). With increasing age, the gradual loss of glucose control in GIPRdn transgenic pigs becomes more obvious. Aged 5 months GIPRdn transgenic pigs show a more pronounced reduction in oral glucose tolerance, a distinct reduction of the corresponding insulin secretion (insulinogenic index: 0.44 vs. 2.71 in controls; P < 0.01), a 35% reduced total β-cell mass, and a trend of reduced intravenous glucose tolerance (5).

Comprehensive metabolic profiling of biofluids has been proposed to obtain a deeper understanding of the pathogenesis of prediabetes and also for biomarker discovery (8). Metabolomic approaches depict metabolite homeostasis and the dynamic responses of cells or organisms to environmental challenge, genetic modifications, or disease (9). Because many metabolites are quite universal, the information content of metabolome data from animal studies can be rapidly transferred to human studies (e.g., for biomarker discovery) (10).

The progressive nature of changes in GIPRdn transgenic pigs, together with the opportunity to take serial blood
samples of sufficient volume from unrestrained animals, creates a unique model to screen for metabolomic foot-prints associated with early (2.5-month-old animals) and late (5-month-old animals) prediabetic stages. A targeted metabolomic approach was used to facilitate quantitative discrimination of phenotypes with high resolution (11).

Our study revealed characteristic changes in plasma concentrations of specific amino acids and lipids, signifying the changes in intermediary metabolism during progression within the prediabetic period.

**RESEARCH DESIGN AND METHODS**

**Animal model.** GIPR<sup>dn</sup> transgenic pigs have been generated as described previously (5). GIPR<sup>dn</sup> transgenic pigs and age-matched controls were studied at age 2.5 or 5 months. All animals were housed in single pens under controlled conditions and had free access to water. Animals were fed a commercial diet (Supplementary Table 1) once daily and were fasted for 18 h prior to the glucose tolerance tests (GTTs). Physiological data of GIPR<sup>dn</sup> transgenic pigs and controls are summarized in Table 1.

**Intravenous GTT and recovery of blood samples.** Intravenous GTTs (IVGTTs) were accomplished as described previously (5). Blood was collected at −10, 0, 1, 3, 5, 10, 20, 30, 40, 60, and 90 min relative to the glucose load. Blood samples were placed on ice immediately after collection, centrifuged at 1,500×g for 15 min, and plasma was stored at −80°C. Plasma glucose levels were determined using an AU 400 autoanalyzer (Olympus, Hamburg, Germany). Plasma insulin levels were measured using a porcine insulin radiomunnoassay kit (Millipore, Billerica, MA). The homeostasis model assessment (HOMA) of β-cell function index (HOMA-β) and HOMA of insulin resistance index (HOMA-IR) were calculated as previously described (12). The insulinogenic index was calculated as the ratio of the increment of plasma insulin (μU/mL) to glucose concentration (mg/dL) 30 min relative to the oral glucose load (7).

**Targeted metabolomics.** Metabolites were quantified at the Helmholtz Center Munich using the AbsoluteIDQ p150 Kit (Biocrates Life Sciences AG, Innsbruck, Austria) covering amino acids (14), hexoses (H), free carnitine (C0), acylcarnitines (40), glycerophosphocholines (92), and sphingolipids (15). Leu and Ile are amino acids and lipids, signify-

**RESULTS**

**Plasma amino acid concentrations change with age and progression within the prediabetic state.** Of the 14 plasma amino acids quantified, 6 (His, Trp, Orn, Ser, Phe, and xLeu) were significantly influenced by age and 7 (Phe, Orn, Val, xLeu, His, Arg, and Tyr) by the interaction GT × Age (i.e., the effect of GT was different in younger vs. older animals). In addition, the plasma concentrations of 6 amino acids (Arg, Orn, Phe, Ser, Tyr, and xLeu) were significantly affected by time (Supplementary Table 2) (i.e., their concentrations changed during the course of the IVGTT) (Supplementary Fig. 1). To account for this fact, least squares means (LSMs) and SEs were calculated for GT × Age (Fig. 1). Consistently, the plasma concentrations of Phe, Orn, Val, xLeu, His, Arg, and Tyr were increased in 2.5-month-old but decreased in 5-month-old GIPR<sup>dn</sup> transgenic pigs versus age-matched controls. The concentration of Trp was not different between the younger transgenic and control pigs but was significantly reduced in the older transgenic animals (Fig. 1).

To evaluate previously proposed hypotheses (17) of an increased turnover of glucogenic amino acids during pre-diabetic stages, the sum of the main glucogenic amino acids Ser + Gly was tested. While in 2.5-month-old animals

**TABLE 1**

Physiological characteristics of GIPR<sup>dn</sup> transgenic pigs and controls

<table>
<thead>
<tr>
<th></th>
<th>Aged 2.5 months</th>
<th></th>
<th></th>
<th>P</th>
<th>Aged 5 months</th>
<th></th>
<th></th>
<th>P</th>
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<tbody>
<tr>
<td>n (male/female)</td>
<td>3/2</td>
<td>3/3</td>
<td>6/3</td>
<td>4/5</td>
<td></td>
<td>6/3</td>
<td>4/5</td>
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<td>Age (weeks)</td>
<td>10.69 ± 0.07</td>
<td>10.79 ± 0.52</td>
<td>0.266</td>
<td>21.24 ± 0.55</td>
<td>20.76 ± 0.35</td>
<td>0.775</td>
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<tr>
<td>Body weight (kg)</td>
<td>21.06 ± 1.58</td>
<td>19.72 ± 2.48</td>
<td>0.663</td>
<td>73.56 ± 3.63</td>
<td>72.41 ± 2.47</td>
<td>1.000</td>
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<tr>
<td>Fasting glucose (mg/dL)</td>
<td>83.1 ± 7.54</td>
<td>90.82 ± 5.85</td>
<td>0.214</td>
<td>85.63 ± 3.03</td>
<td>86.85 ± 3.02</td>
<td>0.516</td>
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<tr>
<td>Fasting insulin (μU/mL)</td>
<td>1.738 ± 0.30</td>
<td>2.50 ± 0.33</td>
<td>0.181</td>
<td>3.45 ± 0.60</td>
<td>4.25 ± 0.63</td>
<td>0.150</td>
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<td>HOMA-IR</td>
<td>0.37 ± 0.09</td>
<td>0.58 ± 0.10</td>
<td>0.250</td>
<td>0.76 ± 0.15</td>
<td>0.95 ± 0.17</td>
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<td>HOMA-β</td>
<td>66.80 ± 33.49</td>
<td>40.82 ± 9.09</td>
<td>0.930</td>
<td>64.59 ± 15.60</td>
<td>67.15 ± 10.55</td>
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<tr>
<td>Insulinogenic index (μU/mL)/(mg/dL)</td>
<td>0.47 ± 0.04</td>
<td>0.96 ± 0.17</td>
<td>&lt;0.05</td>
<td>0.44 ± 0.10</td>
<td>2.71 ± 1.24</td>
<td>&lt;0.01</td>
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</table>

**Oral GTT**

AUC glucose (−10 to 120 min) 24928 ± 1519 19248 ± 1402 0.05 23111 ± 1206 18503 ± 799 |<0.05|
AUC insulin (−10 to 120 min) 5155 ± 763 5608 ± 625 0.351 6178 ± 724 13526 ± 1967 |<0.01|
AUC insulin (−10 to 45 min) 1932 ± 187 2783 ± 247 0.05 1715 ± 403 6002 ± 1531 |<0.01|

**IVGTT**

AUC glucose (−10 to 90 min) 13562 ± 359 14315 ± 651 0.663 14252 ± 1262 12733 ± 868 0.165
AUC insulin (−10 to 90 min) 1135 ± 111 1254 ± 269 0.664 1634 ± 191 1982 ± 243 0.106
Total pancreatic β-cell volume (mm<sup>3</sup>) 201.9 ± 22.6 217.6 ± 29.8 0.843 789.2 ± 80.2 1208.6 ± 84.8 |<0.05|

Analyses of age, body weight, fasting glucose, and insulin levels of 5-month-old GIPR<sup>dn</sup> transgenic pigs (tg) and controls (wt) include 9 tg and 9 wt pigs, whereby 5/4 tg and 5/4 wt pigs have been used for oral GTT/IVGTT, respectively. P values <0.05 were considered significant (indicated in bold).
there was no difference between the transgenic and the control group, 5-month-old transgenic pigs displayed a significantly reduced level of these glucogenic amino acids (LSM: 737 vs. 841 μmol/L in controls; GT × Age: P = 0.0043).

Amino acid profiling can potentially provide surrogate information on metabolic aberrations that may contribute to vascular endothelial dysfunction. Increase of reactive oxygen species, as observed in various diabetic conditions and models (18), may lead to consumption of tetrahydrobiopterin (BH4), the cofactor for the enzymes phenylalanine hydroxylase (EC 1.14.16.1) and nitric oxide synthase (EC 1.14.13.39). In addition, increased arginase activity has been reported in diabetic conditions and may contribute to vascular endothelial dysfunction by decreasing L-arginine availability to endothelial nitric oxide synthase (19). Potential aberrations in these enzymes were addressed by calculating Phe/Tyr and Orn/Arg ratios as indirect markers for phenylalanine hydroxylase and arginase activities. While both ratios were significantly affected by GT × Age (P < 0.0001), they did not reveal any sign of endothelial dysfunction in our model (not shown).

Plasma sphingomyelin concentrations are affected in GIPRΔn transgenic pigs. Different sphingolipid metabolites (e.g., ceramide) have been suggested to potentially be important regulators of β-cell survival, proliferation, and function (20,21). Since disturbed cellular sphingomyelin (SM) biosynthesis and metabolism might partially also be reflected in extracellular fluids, we included sphingomyelins in our screening panel. Sums of all SM (Fig. 2A) and hydroxysphingomyelin (SM-OH) (Fig. 2B) concentrations that have been measured (Supplementary Table 3) were calculated. In controls, circulating SM concentrations increased significantly with age (P = 0.0002); this increment was only marginal in GIPRΔn transgenic pigs (GT × Age: P = 0.0393). This was reflected by reduced concentrations of SM C24:0 (−17%), SM C24:1 (−9%), SM C16:0 (−15%), and SM C16:1 (−19%) in 5-month-old GIPRΔn transgenic pigs versus age-matched controls (Supplementary Table 3).

SM-OH concentrations were one order of magnitude lower than SM concentrations and were higher in 2.5-month-old
but lower in 5-month-old GIPR\textsuperscript{dn} transgenic pigs compared with age-matched controls (GT × Age: \( P = 0.0004 \)). The concentrations of SM (OH) C22:1 (−18%) and SM (OH) C22:2 (−10%) were most prominently reduced in 5-month-old GIPR\textsuperscript{dn} transgenic pigs.

**Altered concentrations of specific diacylglycerols, ether phospholipids, and lysophosphatidylcholines.** Aberrant diacylglycerol levels have been reported in the context of prediabetes (22,23). Although the plasma level of PC aa C42:0 was increased in 2.5-month-old GIPR\textsuperscript{dn} transgenic pigs, decreased concentrations of PC aa C42:0, PC aa C42:4, and PC aa C42:5 were observed in 5-month-old transgenic animals when compared with age-matched controls (GT × Age: \( P ≤ 0.0001 \)) (Table 2). In addition, the plasma concentrations of a number of diacylglycerols were age dependent, with higher concentrations in 2.5- versus 5-month-old animals (Supplementary Table 4).

The plasma concentrations of several ether phospholipids were increased in 2.5-month-old GIPR\textsuperscript{dn} transgenic pigs, whereas decreased concentrations of specific ether phospholipids were measured in 5-month-old transgenic pigs compared with age-matched controls (GT × Age: \( P ≤ 0.0001 \)) (Table 2). In addition, a number of ether phospholipid concentrations were affected by age (Supplementary Table 5).

Specific plasma lysophosphatidylcholine concentrations were found to be decreased in 2.5-month-old (lysoPC a C16:1 and lysoPC a C20:3) or 5-month-old (lysoPC a C18:0) GIPR\textsuperscript{dn} transgenic pigs versus age-matched controls (GT × Age: \( P ≤ 0.0001 \)) (Table 2). A significant effect of age was observed for plasma concentrations of several lysophosphatidylcholines (Supplementary Table 6). The plasma concentration of lysoPC a C18:0 was significantly (\( P < 0.0001 \)) affected by the intravenous glucose load (Supplementary Fig. 2A).

**Altered concentrations of specific plasma acylcarnitines.** Accumulation of plasma acylcarnitines was reported to be associated with absolute or relative insulin deficiency (24,25). Irrespective of age, the plasma concentration of glutamoyl-L-carnitine (C5:1-DC) was significantly increased in GIPR\textsuperscript{dn} transgenic pigs compared with controls (GT: \( P < 0.0001 \)). In addition, a trend of increased levels of hydroxypropionyl-L-carnitine (C3-OH; GT: \( P = 0.0003 \)), valeryl-L-carnitine (C5; GT: \( P = 0.0036 \)), hexenoyl-L-carnitine (C6:1; GT: \( P = 0.0006 \)), and octenoyl-L-carnitine (C8:1; GT: \( P = 0.0018 \)) was detected in 2.5- and 5-month-old GIPR\textsuperscript{dn} transgenic pigs compared with age-matched controls (Supplementary Table 7). The plasma concentration of butyryl-L-carnitine (C4) was reduced in 2.5-month-old but increased in 5-month-old GIPR\textsuperscript{dn} transgenic pigs compared with controls (GT × Age: \( P < 0.0001 \)). The plasma concentration of propionyl-L-carnitine (C3) was significantly influenced by age (\( P < 0.0001 \)), with higher levels in 2.5- compared with

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Aged 2.5 months</th>
<th>Aged 5 months</th>
<th>GT × Age</th>
<th>Age P value</th>
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To account for multiple testing, only \( P \) values ≤0.0001 were considered significant (indicated in bold); \( \Delta \% = (tg - wt)/wt \times 100 \); significant differences are indicated in bold. The full dataset of diacylglycerols, ether phospholipids, and lysophosphatidylcholines is provided in Supplementary Tables 4–6.
5-month-old animals. In addition, there was a trend of higher C3 levels in 2.5-month-old GIPR<sup>dn</sup> transgenic pigs versus age-matched controls, while this difference was not present in the 5-month age-group (GT × Age; \( P = 0.0010 \)). The plasma concentration of decanoyl-L-carnitine (C10) was significantly influenced by the glucose infusion during the IVGTT (Supplementary Fig. 2B).

**Altered metabolite concentrations as a result of intravenous glucose infusion versus long-term changes.**

The differential metabolite profiles observed in our study could arise as the result of acute glucose challenge (intravenous glucose infusion) or might represent chronic alterations in glucose metabolism within the prediabetic state. To differentiate between these possibilities, the data analysis was restricted to a subset of metabolites and time points. Only the parameters that were significantly influenced by the interaction GT × Age and only the values from fasted plasma samples (10 min and 0 min before glucose infusion) were taken into account.

It is important that a significant effect of the interaction GT × Age was confirmed for 16 out of 26 metabolites, including all metabolite classes except acylcarnitines. This observation suggests that a significant proportion of the metabolite changes observed in our study are indicative of a chronic alteration of glucose metabolism, rendering them promising candidate biomarkers to monitor the prediabetic period. These metabolites are summarized in Table 3.

**Glucose tolerance, insulin secretion, and total β-cell volume are correlated to specific plasma metabolite concentrations.** Correlations were calculated by using data from the 5-month-old animals, since GIPR<sup>dn</sup> transgenic pigs exhibited a 35% reduction in total β-cell volume at this stage. Metabolic parameters listed in Table 3 were included in the analysis, and all of those depicted in Fig. 3 correlated positively with total β-cell volume. Furthermore, Arg and the majority of plasma lipids selected correlated positively with parameters of insulin release. A negative correlation was revealed between lysoPC a C18:0 and, less clearly, PC ae C40:3, PC aa C42:4, and Val and area under the curve (AUC) glucose, in particular with AUC glucose (10–60).

**Changes in liver transcriptome profiles are consistent with plasma metabolite changes.** To evaluate if some of the plasma metabolite alterations were accompanied by expression changes of targets in corresponding metabolic pathways, we performed a holistic transcriptome study of liver samples from the three male GIPR<sup>dn</sup> transgenic pigs and the three control boars in the 5-month age-group. GSEA was used as a tool, enabling sensitive detection of gene expression alterations in glucose metabolism within the prediabetic state. The plasma concentration of decanoyl-L-carnitine (C10) was significantly influenced by the glucose infusion during the IVGTT (Supplementary Fig. 2B).

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**Glucose tolerance, insulin secretion, and total β-cell volume are correlated to specific plasma metabolite concentrations.** Correlations were calculated by using data from the 5-month-old animals, since GIPR<sup>dn</sup> transgenic pigs exhibited a 35% reduction in total β-cell volume at this stage. Metabolic parameters listed in Table 3 were included in the analysis, and all of those depicted in Fig. 3 correlated positively with total β-cell volume. Furthermore, Arg and the majority of plasma lipids selected correlated positively with parameters of insulin release. A negative correlation was revealed between lysoPC a C18:0 and, less clearly, PC ae C40:3, PC aa C42:4, and Val and area under the curve (AUC) glucose, in particular with AUC glucose (10–60).

**Changes in liver transcriptome profiles are consistent with plasma metabolite changes.** To evaluate if some of the plasma metabolite alterations were accompanied by expression changes of targets in corresponding metabolic pathways, we performed a holistic transcriptome study of liver samples from the three male GIPR<sup>dn</sup> transgenic pigs and the three control boars in the 5-month age-group. GSEA was used as a tool, enabling sensitive detection of gene expression alterations in glucose metabolism within the prediabetic state. The plasma concentration of decanoyl-L-carnitine (C10) was significantly influenced by the glucose infusion during the IVGTT (Supplementary Fig. 2B).

**Altered metabolite concentrations as a result of intravenous glucose infusion versus long-term changes.**

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changes within specific pathways (26). In accordance with several plasma metabolite alterations, GSEA indeed revealed liver transcript changes to be significantly enriched in a number of pathways already predicted by metabolite alterations. Those include Gly, Ser, and Thr metabolism, sphingolipid metabolism, and biosynthesis of unsaturated fatty acids (Table 4). A representative GSEA plot for genes involved in sphingolipid metabolism and the corresponding pathway with the observed transcriptome changes are shown in Fig. 4.

DISCUSSION

A number of targeted and nontargeted metabolomic studies have been performed to address metabolic correlates of clinically manifest diabetes (reviewed in Lucio et al. and Suhre et al.) (27,28). In addition, several studies performed metabolomic screens addressing classical risk factors, such as obesity, or pathogenetic mechanisms, such as insulin resistance. For instance, a monozygotic twin study screened for metabolic correlates of acquired obesity and detected primarily increased levels of lysophosphatidylcholines, while ether phospholipids were decreased (29). Impaired insulin sensitivity in nondiabetic human subjects was found to be associated with altered metabolite profiles in lipid metabolic pathways, steroid hormone biosynthesis, and bile acid metabolism (27). Metabolic profiling of obese versus lean humans revealed that serum concentrations of eight amino acids (Ala, Val, Leu/Ile, Phe, Tyr, Glu/Gln, Asp/Asn, and Arg) were markedly increased, while Gly was decreased, and defined a branched-chain amino acid (BCAA)-related metabolomic signature in obese subjects (30). A recent prospective study found the plasma concentrations of three BCAAs (Leu, Ile, and Val) and two aromatic amino acids (Phe and Tyr) to be significantly associated with the future risk of diabetes (31). However, the patients included in the latter study were characterized by an already increased BMI and HOMA-IR. Human subjects with even earlier stages of prediabetes characterized by β-cell dysfunction are only rarely diagnosed and, thus, difficult to recruit. Our genetically engineered pig model provides a unique time window to investigate the course of gradual β-cell mass reduction in relation to changes of intermediary metabolism. In contrast to human patients, the data on β-cell mass in pigs can also be obtained as quantitative information. A characteristic biochemical feature of this animal model

FIG. 3. Correlations between parameters of glucose tolerance and insulin secretion, total β-cell volume, and fasting plasma metabolite concentrations. AIR, acute insulin response (difference of mean plasma insulin at 1, 3, and 5 min after intravenous glucose load and fasting plasma insulin) (48).
is the progressive reduction of the insulinogenic index with increasing life span. This parameter is correlated with impaired first-phase insulin release (7), which occurs in very early stages of β-cell dysfunction and is an important factor in the development of postprandial hyperglycemia (32).

### Table 4

Selected results of GSEA

<table>
<thead>
<tr>
<th>KEGG pathway (ID)</th>
<th>Genes in the leading edge subset</th>
<th>NES</th>
<th>Nominal P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly, Ser, and Thr metabolism (M766)</td>
<td>SDS, PSPH, PIPOX, PHGDH, AOC2, CBS, ALAS2, GNMT, CTH, ALAS1, GATM, MAOA, AOC3</td>
<td>1.45</td>
<td>0.053</td>
</tr>
<tr>
<td>Sphingolipid metabolism (M15955)</td>
<td>ASAH2, UGT8, SGPP2, SGPL1, ACER3, DEGS2</td>
<td>1.34</td>
<td>0.104</td>
</tr>
<tr>
<td>Glycosphingolipid biosynthesis—lacto and neolacto series (M17377)</td>
<td>FUT2, B3GNT5, B3GALT5, ST3GAL4, GCNT2, B3GALT1, B3GALT2, ST3GAL6, FUT1</td>
<td>−1.66</td>
<td>0.026</td>
</tr>
<tr>
<td>Pentose phosphate pathway (M1386)</td>
<td>RPIA, ALDOC, PGM1, PDF, PGM2, PRPS2, PFKP, TKTL2, FB2</td>
<td>−1.66</td>
<td>0.024</td>
</tr>
<tr>
<td>Trp metabolism (M980)</td>
<td>IL411, CYP1A1, ACMDS, AADAT</td>
<td>−1.65</td>
<td>0.013</td>
</tr>
<tr>
<td>Biosynthesis of unsaturated fatty acids (M11673)</td>
<td>ACOT4, ACOT7, HADHA, SCD, ELOVL2, YOD1</td>
<td>−1.54</td>
<td>0.060</td>
</tr>
<tr>
<td>Lys degradation (M13720)</td>
<td>EHMT1, HADHA, NSD1, TMLHE, PLOD1, AADAT</td>
<td>−1.42</td>
<td>0.064</td>
</tr>
<tr>
<td>Amino sugar and nucleotide sugar metabolism (M8104)</td>
<td>CVB5R1, PGM1, GNPD2, GALK1, PGM2, UGDH, NANS, GNPNAT1, GFT1, NAGK, GCK, FMM1</td>
<td>−1.42</td>
<td>0.048</td>
</tr>
</tbody>
</table>

The enrichment score reflects the degree to which a set of genes belonging to a specific metabolic pathway is overrepresented at the extremes of the entire ranked list of differentially expressed genes. Adjustment for multiple hypothesis testing results in the normalized enrichment score (NES) (15). A positive NES indicates upregulation and a negative NES downregulation in the liver of GIPR<sup>dn</sup> transgenic pigs. The gene lists are ordered according to their rank in the GSEA plot (see Fig. 4A as an example).

**Altered plasma amino acid concentrations as markers of prediabetes.** Changes in plasma amino acid concentrations have been originally described in insulin-resistant obese patients (reviewed in Felig et al. and Muoio and Newgard) (33,34). In particular, elevated levels of BCAAs...
have been confirmed in a number of subsequent studies of obese and/or insulin-resistant patient collectives (30,35,36) and in clinically manifest diabetes (28). Of interest, plasma levels of Val, Leu, Ile, Tyr, and Phe—known to be associated with diabetes risk in human subjects with increased BMI and reduced insulin sensitivity (31)—and those of Orn and Arg were elevated in 2.5-month-old GIPR<sup>dn</sup> transgenic pigs, which show only a reduced incretin effect but are neither obese nor insulin resistant according to HOMA-IR (Table 1). This observation is remarkable because it demonstrates that a mild chronic disturbance of glucose homeostasis caused by reduced GIP function might be sufficient to result in a plasma amino acid profile suggestive for an increased risk to develop diabetes even in the absence of obesity. The elevated levels of seven amino acids in 2.5-month-old GIPR<sup>dn</sup> transgenic pigs could be related to the distinct pattern of insulin secretion in this model. After an oral glucose load, initial insulin secretion is delayed, although the overall amount of insulin secreted during the 120-min period of the test reaches levels comparable with controls (5). In this respect, our model reflects the condition of impaired glucose tolerance preceding type 2 diabetes in human subjects where plasma insulin levels were reduced at 30 min after glucose ingestion but normal or increased at 120 min (37). The bell-shaped curve of insulin secretion in GIPR<sup>dn</sup> transgenic pigs implicates a longer phase of insulin above a level that could inhibit glucoseogenesis and, thus, the use of glucogenic amino acids. A further possible explanation is that in comparison with the steep and fast increase of insulin secretion after oral glucose load, the slower increase in plasma insulin levels seen in GIPR<sup>dn</sup> transgenic pigs could be a weaker anabolic signal, leading to less efficient amino acid uptake in peripheral tissues and clearance from the circulation (38,39).

It is surprising that initially elevated plasma levels of these amino acids (at 2.5 months) were reduced in 5-month-old GIPR<sup>dn</sup> transgenic pigs, which show a more pronounced prediabetic phenotype (Table 1). The reduction in pancreatic β-cell mass and impaired insulin secretion after oral glucose load (5) and probably also after food intake might explain this effect. Of interest, the plasma concentrations of Arg, His, Phe, Val, and xLeu were correlated with total β-cell mass in 5-month-old pigs. During evolution toward diabetes, a continual mitigation of the inhibiting effect of insulin on hepatic glucoseogenesis would be expected to result in a higher demand and consumption of glucogenic amino acids. This hypothesis is indeed supported by the fact that the sum of the major glucogenic amino acids (Gly + Ser) was significantly reduced in plasma of 5-month-old GIPR<sup>dn</sup> transgenic pigs. In addition, the liver transcriptome changes in 5-month-old pigs, showing enrichment of differentially expressed genes in corresponding pathways of amino acid metabolism, might also contribute to the observed alterations in plasma amino acid concentrations.

Specific amino acid ratios (Tyr to Phe and Orn to Arg) did not reveal signs of endothelial dysfunction as expected in view of our model. The GIPR<sup>dn</sup> transgenic pig mimics different stages of the prediabetic period but is not yet manifesting overt clinical signs. Reduced levels of specific SMs are associated with impaired glucose homeostasis and moderately reduced β-cell mass. Sphingolipids are essential components of plasma membranes and endosomes and are critically involved in cell surface protection, protein and lipid transport and sorting, and cellular signaling cascades (40). Reduced plasma SM concentrations were recently observed in cynomolgus monkeys spontaneously developing hyperglycemia and hyperlipidemia (41).

We thus questioned if early stages of altered glucose homeostasis and β-cell dysfunction are associated with altered plasma concentrations of SM. It is interesting that GIPR<sup>dn</sup> transgenic pigs did not show a comparable age-dependent increase in saturated SM species as observed in controls. In the more progressed prediabetic condition of 5-month-old GIPR<sup>dn</sup> transgenic pigs, we found significant reductions in plasma levels of nearly all of the SM metabolites quantified (Supplementary Table 3). This observation is consistent with a recent study exploring specific sphingolipids as potential biomarkers in two different mouse models of insulin-dependent diabetes, streptozotocin-administered mice and Ins2<sup>Δko</sup> mutant mice (42). In this study, plasma SM-C24:1 levels were consistently reduced in both models, and there was a trend of reduced SM C24:0 levels in the streptozotocin-administered animals. While both mouse models are characterized by a massive loss of β-cells, 5-month-old GIPR<sup>dn</sup> transgenic pigs show only a modest disturbance of glucose homeostasis and a 35% reduction in β-cell mass. The concordance of SM changes in prediabetic GIPR<sup>dn</sup> transgenic pigs with two mouse models with clinically manifest diabetes suggests that reductions in specific SMs could signify a proximal event in the cascade toward diabetes rather than being a consequence of manifesting disease. The fact that reduction of SM synthesis increases insulin sensitivity (43) raises the possibility that decreased SM levels in 5-month-old GIPR<sup>dn</sup> transgenic pigs represent an adaptive response to reduced insulin secretion. It is interesting that there was a trend of a smaller HOMA-IR in 5-month-old GIPR<sup>dn</sup> transgenic versus control pigs (see Table 1). The observed liver transcriptome changes related to SM metabolism (see Table 4 and Fig. 4) would support this concept. However, SM synthase 1 deficient mice show impaired insulin secretion (44). Thus, reduced SM levels, if present in β-cells, may also contribute to the impaired insulin secretion observed in 5-month-old GIPR<sup>dn</sup> transgenic pigs.

**Stage-specific changes in plasma phospholipid concentrations.** Since plasma lipid profiles were found to be altered in obesity (29), an important risk factor for diabetes, we quantified the plasma concentrations of 38 diacylglycerols, 39 ether phospholipids, and 15 lysophosphatidylcholines in GIPR<sup>dn</sup> transgenic and control pigs. While ether phospholipids can serve as endogenous antioxidants (45,46), increased levels of lysophosphatidylcholines have been described in the context of proinflammatory and proatherogenic conditions (reviewed in Pietiläinen et al.) (29). Acquired obesity was found to be associated with increases in lysophosphatidylcholines but decreases in ether phospholipids (29). Moreover, the levels of plasmalogens (ether lipids where the first position of glycerol binds a vinyl residue [from a vinyl alcohol] with the double bond next to the ether bond) were found to be decreased by ~50% in LDLs from type 2 diabetic patients (47).

Of interest is the finding that in 2.5-month-old GIPR<sup>dn</sup> transgenic pigs, the concentrations of PC aa C42:0 and several other phospholipids were increased compared with age-matched controls. In contrast, decreased plasma concentrations of specific diacylglycerols and ether phospholipids were observed in 5-month-old GIPR<sup>dn</sup> transgenic pigs. The latter may be interpreted as an early stage of loss of metabolic control associated with reduced antioxidant potential by ether phospholipids. Furthermore, as observed for the plasma amino acid concentrations, the reversed
plasma concentrations of some glycerophospholipids in 2.5- and 5-month-old GIPR<sup>ΔΔ</sup> transgenic pigs are apparently indicative of progression within the prediabetic state. Lysophosphatidylcholines were not elevated in GIPR<sup>ΔΔ</sup> transgenic pigs. This is consistent with the fact that the metabolic alterations observed in 2.5- and 5-month-old GIPR<sup>ΔΔ</sup> transgenic pigs are relatively mild and do not cause increased lipid peroxidation or other changes that might result in increased plasma lysophosphatidylcholine levels as observed in obesity and clinical diabetes (29).

**Altered plasma acylcarnitine levels.** Carnitine and its esters procure fatty acids for β-oxidation and therefore prevent fatty acid accumulation. Absolute or relative insulin deficiency results in increased and incomplete β-oxidation and accumulation of plasma acylcarnitines (24,25). In a recent study exploring metabolic differences between obese and lean humans and their contribution to insulin resistance (30), 4 out of 37 acylcarnitine species measured were found to be increased in the plasma of obese subjects: propionyl-<i>C</i><sub>3</sub>-carnitine (C3), valeryl-<i>C</i><sub>6</sub>-carnitine (C5), hexanoyl-<i>C</i><sub>6</sub>-carnitine (C6), and octanoyl-<i>C</i><sub>8</sub>-carnitine (C8:1). Increased levels of short-chain acylcarnitines (C2, C6, and C8) also were observed in the diabetic state, and a positive correlation with plasma HbA1c suggested a link with the severity of the disease (24). It is interesting that some short-chain acylcarnitines were also increased in GIPR<sup>ΔΔ</sup> transgenic pigs, even at the age of 2.5 months. This observation indicates that in the absence of obesity, insulin resistance, or other severe disturbances of glucose homeostasis, short-chain acylcarnitines are sensitive parameters whose elevation suggests early stages and progression of prediabetes.

**Candidate biomarkers for an early prediabetic state.** Metabolite profiles of 2.5- and 5-month-old GIPR<sup>ΔΔ</sup> transgenic pigs representing an early prediabetic state characterized by a reduced incretin effect and progressive reduction of glucose tolerance, insulin secretion, and pancreatic β-cell mass in part show similar changes as previously detected in obese, insulin-resistant, or diabetic patients. Thus, metabolite concentration alterations detected even in young GIPR<sup>ΔΔ</sup> transgenic pigs appear to be sensitive indicators of very early stages of prediabetes. It is important that >50% of the metabolites altered in GIPR<sup>ΔΔ</sup> transgenic pigs versus controls in an age- and disease progression–specific manner during an IVGTT were also detected if only fasting plasma samples were analyzed. Therefore, these parameters appear to be associated with long-term glucose control and do not arise only as the consequence of an intravenous glucose bolus. This observation also is important in practical terms because time-consuming GTTs are not essential. The age-dependent inverse alteration of specific plasma amino acid and lipid concentrations (elevated in 2.5-month-old and reduced in 5-month-old animals) going along with the progression of phenotypic changes in GIPR<sup>ΔΔ</sup> transgenic pigs strongly argues for repeated metabolomic profiling in regular intervals, which is easily achievable in a large animal model. The fact that metabolic signatures observed in fasting plasma samples of 5-month-old GIPR<sup>ΔΔ</sup> transgenic pigs were J) significantly correlated with total β-cell mass and 2) associated with liver transcriptome changes in corresponding metabolic pathways suggest their potential usage as biomarkers.

In summary, our findings document the potential of a genetically designed large animal model resembling early stages of prediabetes in the absence of confounding obesity or insulin resistance for the discovery of candidate biomarkers that may serve as indicators of early stages of prediabetes.

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S.R. researched data, contributed to discussion, and wrote, reviewed, and edited the manuscript. W.R.-M. and C.P. researched data, contributed to discussion, and reviewed and edited the manuscript. S.K. and H.B. performed the microarray study, including the bioinformatics analysis. J.A., B.G., and K.S. contributed to discussion and reviewed and edited the manuscript. A.A.R. and E.W. contributed to discussion and wrote, reviewed, and edited the manuscript. S.R. and E.W. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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