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Mild maternal hyperglycemia in *INS*^{C93S} transgenic pigs causes impaired glucose tolerance and metabolic alterations in neonatal offspring

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Summary statement: Mild maternal hyperglycemia causes impaired glucose tolerance and metabolic alterations in wild-type neonatal offspring of *INS*^{C93S} transgenic pigs, a novel large animal model for mutant *INS* gene induced diabetes of youth (MIDY).

ABSTRACT

Alongside with the obesity epidemic the prevalence of maternal diabetes is rising worldwide and adverse effects on fetal development and metabolic disturbances in the offspring's later life have been described. To clarify if metabolic programming effects occur due to mild maternal hyperglycemia without confounding obesity, we investigated wild-type offspring of *INS*^{C93S} transgenic pigs, a novel genetically-modified large animal model expressing the mutant insulin (*INS*) C93S in the pancreatic beta cells, resulting in impaired glucose tolerance, mild fasting hyperglycemia and insulin resistance during late pregnancy. Compared with offspring from wild-type sows, piglets from hyperglycemic mothers showed impaired glucose tolerance and insulin resistance (HOMA-IR: +3-fold in males; +4.4-fold in females) prior to colostrum uptake. Targeted metabolomics in the fasting and insulin-stimulated state revealed distinct alterations in the plasma metabolic profile of piglets from hyperglycemic mothers, such as increased levels of fatty acid mitochondrial transport (e.g. acylcarnitines), gluconeogenic precursors as alanine, phospholipids, in particular lyso-phosphatidylcholines, and alpha-aminoadipic acid, a potential biomarker for type 2 diabetes. These observations indicate that mild gestational hyperglycemia can cause impaired glucose tolerance, insulin resistance and associated metabolic alterations in neonatal offspring of a large animal model born at a developmental maturation status comparable to human babies.

INTRODUCTION

The prevalence of maternal diabetes, in particular of gestational diabetes mellitus (GDM), is rapidly increasing worldwide, primarily due to the increased prevalence of obesity. To date, the mean global prevalence of GDM in women aged 20-49 years is estimated 16.9% (Guariguata et al., 2014) and more than 50% of pregnant women are overweight or obese (Friedman, 2018). The physiological insulin resistance (IR) in the second half of pregnancy is exacerbated in obese women and can further impair a pre-existing hyperglycemic condition or lead to GDM if insulin secretion cannot meet the increased demand (Kautzky-Willer et al., 1997).

Maternal diabetes is associated with an increased risk of adverse outcomes for mother and offspring (Wendland et al., 2012). In addition to clinical/epidemiological studies, diet-induced obese nonhuman primate (NHP) models were used to dissect the underlying mechanisms (reviewed in (Friedman, 2018)). However, since these NHP models represent the entire spectrum of the metabolic syndrome it is difficult to differentiate consequences of hyperglycemia from those of obesity (Thompson et al., 2017).

Mouse models are widely used for studies of developmental programming, but their pups are born at a relatively immature stage compared to humans. Programming effects of maternal diabetes in the second half of pregnancy are therefore difficult to model in rodents. In contrast, fetal maturation in pigs is similar to humans (reviewed in (Litten-Brown et al., 2010)). To study developmental consequences of gestational hyperglycemia, we generated *INS*^{C93S} transgenic pigs expressing the mutant insulin (*INS*) C93S in the pancreatic beta cells as a new model for mutant *INS* gene induced diabetes of youth (MIDY) (Liu et al., 2015) and characterized their glucose homeostasis during pregnancy. The mutant insulin C93S corresponds to the Munich *Ins2*C95S mutant mouse model (Herbach et al., 2007) and is similar to the human C95Y mutation leading to permanent neonatal diabetes (Colombo et al., 2008; Stoy et al., 2010). The progression of MIDY is attributed to a so called gain of toxic function (Colombo et al., 2008).

In all three models, disruption of the A6-A11 disulfide bond within the A-chain of the insulin leads to misfolding of the insulin, entrapment of the insulin in the endoplasmic reticulum (ER), also affecting endogenous insulin processing and exit from the ER, endoplasmic reticulum stress and finally beta-cell death (Cunningham et al., 2017; Hodish et al., 2010). *INS*^{C93S} transgenic pigs revealed impaired glucose tolerance (IGT), reduced insulin secretion and mild fasting hyperglycemia. During pregnancy they developed insulin resistance, but maintained a mild hyperglycemic state. Nevertheless, profound changes of carbohydrate and lipid metabolism were observed in their neonatal offspring.

MATERIALS AND METHODS

Generation of *INS*^{C93S} transgenic pigs. All experiments were performed according to the German Animal Welfare Act with permission from the responsible authority (Government of Upper Bavaria), following the ARRIVE guidelines and Directive 2010/63/EU. The *INS*^{C93S}, i.e. the mutant insulin C93S, expression vector consists of a 1.0 kb coding region of the porcine *INS* gene sequence including three exons with a T → A transition in exon 3 resulting in a Cys → Ser amino acid exchange at position 93, its essential regulatory elements and a neomycin selection cassette (*neo*) (Fig. 1*A*). Pools of stable transfected cell clones were used for somatic cell nuclear transfer (SCNT). SCNT and embryo transfer were performed as previously described (Kurome et al., 2015). Genotyping was carried out by PCR using the transgene-specific primers 5' TGATTCCCACTTTGTGGTTC 3' and 5' GTGGATGTGGAATGTGTGC 3', and Southern blot analysis using PvuII-digested genomic DNA and a ^{32}P-CTP-labeled probe specific for the neomycin resistance cassette (Fig. 2*A*). For expression analysis of the *INS*^{C93S} transgene, total RNA was extracted from pancreatic tissue (RNeasy® total RNA isolation Kit; Qiagen), digested with DNaseI (Roche), and reverse-transcribed with SuperScript™ II Reverse Transcriptase (Invitrogen) using random hexamer primers. *INS*^{C93S} and wild-type *INS* cDNAs

were amplified using the primers 5' CGGGAGGCGGAGAACCCTCA 3' and 5' CCCTCAGGGGCGGCCTAGTT 3' and the ratio of the products was determined by next-generation sequencing (Genome Analyzer IIx; Illumina; ≥ 10.000 reads per sample).

Animals and study design during the pregnant state. Animals (Landrace-Swabian-Hall background) were housed under controlled conditions, fed a commercial pig diet once daily with free access to water. The study outline is shown in Fig. 3*A*. Briefly, female primiparous transgenic (TG) and wild-type (WT) littermate sows were estrus synchronized as described previously (Kurome et al., 2015) and artificially inseminated with semen of the same boar. WT non-pregnant sows served as controls. Within the third trimester hyperinsulinemic-euglycemic clamps (HIC) and mixed meal glucose tolerance tests (MMGTT) were performed. Blood glucose concentrations of sows were determined on a weekly basis during pregnancy. Birth was induced by a single injection of a prostaglandin-$F_{2\alpha}$-agonist (Estrumate®, MSD). At the day of birth oral glucose tolerance tests (OGTT) were performed in newborn piglets prior to the first colostrum uptake. Piglets were euthanized within 24 hours after birth for organ sampling and determination of absolute and relative organ weights.

Blood parameters. Blood samples for clinical-chemical analyses were taken after an overnight fasting period of 18-20 hours and at time point 120 minutes during a MMGTT from sows within the third trimester (gestation day 92 ± 0.9) and from piglets at the day of birth prior to first colostrum uptake as well as at time point 120 minutes during an OGTT. Blood was collected into EDTA-coated tubes and processed as previously described (Renner et al., 2010). Plasma insulin concentrations were determined using a species-specific RIA (Merck Millipore) or ELISA (Mercodia). Clinical-chemical parameters (Suppl. table 1 and 4) were determined from EDTA-plasma using an AU480 autoanalyzer (Beckman-Coulter) and adapted reagent kits from

Beckman-Coulter, Randox or Wako Chemicals. Blood glucose of sows during pregnancy was determined weekly using a Precision Xceed® glucometer (Abbott).

Intravenous, oral and mixed meal glucose tolerance tests. IVGTTs and MMGTTs were performed as previously described (Renner et al., 2010). Briefly, animals were fitted with central venous catheters prior to the tests. For the IVGTT a 0.5g/kg body weight (BW) bolus of 50% glucose solution and for the MMGTT 2 g/kg BW glucose and 150-400 g of a commercial pig diet dependent on the BW of the respective age group was administered. In neonatal pigs OGTTs were performed prior to first colostrum uptake. Therefore, a bolus of 50% glucose solution (2g/kg BW) was administered via a nasogastric tube. Blood samples were taken at indicated time points relative to the glucose load from the catheter (sows) or directly from the jugular vein (piglets).

Hyperinsulinemic-euglycemic clamp. For stress-free, frequent blood sampling in unrestrained animals, animals were fitted with central venous (glucose and insulin infusion) and arterial (blood sampling) catheters. After an 18-hour fasting period, insulin (Insuman® rapid) was infused as a prime dose (0.5 mU/kg BW) for 2.5 minutes followed by a constant infusion rate of 1 mU/kg BW/minute (Koopmans et al., 2006) for a total period of 180 minutes. Blood glucose concentrations were clamped at 75 mg/dl. Blood glucose was determined every five minutes and glucose infusion rate adjusted accordingly. Blood samples during steady-state for determination of glucose infusion rate (GIR, mg/kg*min) were collected every ten minutes starting at 130 minutes. Calculated GIR was normalized to plasma insulin concentrations of the respective time point.

Targeted metabolomics. The targeted metabolomics approach was based on LC-ESI-MS/MS and FIA-ESI-MS/MS measurements by AbsoluteIDQ™ p180 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). The assay allows simultaneous quantification of 188 metabolites out of 10 µL plasma, and includes free carnitine, 39 acylcarnitines (Cx:y), 21 amino acids (19 proteinogenic + citrulline + ornithine), 21 biogenic amines, hexoses (sum of hexoses – about 90-95 % glucose), 90 glycerophospholipids, 14 lysophosphatidylcholines (lysoPC), 76 phosphatidylcholines (PC)), and 15 sphingolipids (SMx:y) (Suppl. table 2). The abbreviations Cx:y are used to describe the total number of carbons and double bonds of all chains, respectively (for more details see (Römisch-Margl et al., 2012). The method of AbsoluteIDQ™ p180 kit has been proven to be in conformance with FDA-Guideline "Guidance for Industry - Bioanalytical Method Validation (May 2001") (U.S. Department of Health and Human Services, 2001), which implies proof of reproducibility within a given error range. Measurements were performed as described in the manufacturer in manual UM-P180. Analytical specifications for LOD and evaluated quantification ranges, further LOD for semiquantitative measurements, identities of quantitative and semiquantitative metabolites, specificity, potential interferences, linearity, precision and accuracy, reproducibility and stability were described in the Biocrates manual AS-P180. The LODs were set to three times the values of the zero samples (PBS). The LLOQ and ULOQ were determined experimentally by Biocrates. The assay procedures of the AbsoluteIDQ™ p180 kit have been described in detail previously (Römisch-Margl et al., 2012; Zukunft et al., 2013). Metabolite nomenclature is provided in Suppl. table 2. Sample handling was performed by a Hamilton Microlab STARTM robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, U.K.), beside standard laboratory equipment. Mass spectrometric analyses were done on an API 4000 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL auto sampler (CTC Analytics,

Zwingen, Switzerland) controlled by the software Analyst 1.6.1. Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the MetIDQ™ software package, which is an integral part of the AbsoluteIDQ™ kit. Internal standards serve as reference for the calculation of metabolite concentrations [µM].

Necropsy, pancreas sampling, immunohistochemistry and quantitative stereology. Pigs were anesthetized by injection of ketamine (Ursotamin®, Serumwerke Bernburg) and azaperone (Stresnil®, Elanco) and euthanized under anesthesia by intravenous injection of T61® (Intervet) and immediately subjected to necropsy. The pancreas was explanted *in toto*, weighed, and samples were taken by a systematic random sampling procedure (Renner et al., 2010). Samples were routinely processed for paraffin histology. Insulin containing cells were identified by immunohistochemistry, and the volume density of beta cells within the pancreas [$Vv_{(beta\text{-}cell/Pan)}$] and the total beta-cell volume [$V_{(beta\text{-}cell,\ Pan)}$] were quantified (Streckel et al., 2015). Piglets born to TG and WT sows were necropsied within 24 hours after birth and selected organs as well as the carcass were weighed.

Statistics. All data are presented as means ± SEM if not otherwise indicated. The results of glucose tolerance tests (IVGTT, MMGTT, OGTT) and glucose infusion rate (GIR) during the hyperinsulinemic-euglycemic clamp as well as blood glucose concentrations during pregnancy were statistically evaluated by ANOVA (Linear Mixed Models; SAS 8.2; PROC MIXED), taking the fixed effects of Group (NG vs. HG for neonates, wt vs. tg for all others), the status (non-pregnant vs. pregnant) for sows, Sex (male vs female) if applicable, Time (relative to glucose application or glucose/insulin infusion) and the interaction Group*Time or Group*Status*Time or Group*Sex*Time as well as the random effect of Animal into account. Body weight, absolute and relative organ weights as well as clinical chemical parameters

were statistically evaluated by ANOVA (General Linear Models; SAS 8.2) taking the fixed effects of Group, Sex if applicable, Time, and the interaction Group*Time, Group*Sex and Group*Sex*Time into account. AUC insulin/glucose was calculated using GraphPad Prism® software (version 5.02). AUCs and all data from quantitative-stereological analyses were evaluated by Mann-Whitney-U-test using GraphPad Prism® software. P values less than 0.05 were considered significant. For metabolomics data, missing values in the metabolomics measurements were imputed using half the value of the minimum measurement for that metabolite. Metabolites with more than 50% missing values were excluded. Normalization and batch effect removal was performed by calculating a plate factor using the means of individual metabolites per plate and multiplying each metabolite with the corresponding value. Student's two-tailed t-test were used on log transformed and Pareto scaled (centered and divided by square root of standard deviation) metabolites for calculating significance. For multiple testing correction the Benjamini Hochberg procedure was employed, an FDR below 0.05 was considered significant.

RESULTS

Generation of *INS*^{C93S} transgenic pigs

In total, nine male *INS*^{C93S} transgenic founder animals were obtained (Landrace-Swabian Hall background) whereof four died shortly after birth. Founder boars 9776 and 9748 and their respective F1-offspring revealed the highest *INS*^{C93S}-to-*INS* transcript ratios in the pancreas (Fig. 1*B*) going along with reduced intravenous glucose tolerance (Fig. 1*C*) and highly reduced insulin secretion (Fig. 1*D*). Nevertheless, they showed normal growth and development as all other founders (data not shown). Boars 9748 and 9776 were mated with WT sows. Southern blot analysis of DNA from F1-offspring revealed the same integration pattern as their respective sire (Fig. 2*A*), suggesting only one transgene integration site in each line.

Reduced glucose control in *INS*^{C93S} transgenic pigs

IVGTTs and MMGTTs in offspring of founder boars 9748 and 9776 were performed at three to four months of age and revealed reduced glucose tolerance and insulin secretion (Fig. 2 *B,C*, Suppl. Fig. 1 and 2) to a similar extent in male and female *INS*^{C93S} transgenic pigs. In addition, fasting blood glucose concentrations were significantly elevated [+56% (141.0 ± 6.6 vs. 90.2 ± 2.6 mg/dL; $p<0.0001$) and +19% (100.8 ± 2.6 vs. 84.5 ± 0.4 mg/dL; $p<0.001$) for offspring of 9776 and 9748 vs. age-matched controls, respectively], while fasting insulin concentrations were not altered. Offspring of founder 9776 only were used for further analyses.

At seven months of age, i.e. when pigs had reached sexual maturity, glucose control of TG pigs had further deteriorated with significantly ($p = 0.02$) decreased fasting plasma insulin (FPI) concentrations (7.0 ± 0.6 vs. 10.3 ± 1.1 µU/ml in WT littermates; Suppl. Fig. 1). Female TG pigs revealed elevated fasting glucose concentrations and showed a further deteriorated glucose tolerance with less insulin secretion compared to their male counterparts (Suppl. Figure 1*C-F*).

Volume density of beta cells in the pancreas and total beta-cell volume of 1-year-old TG pigs were as a tendency, but not significantly lower than in WT littermates (Fig. 2*D*).

Reduced insulin sensitivity and beta-cell function during late gestation

HICs and MMGTTs were performed in pregnant (P) TG (TG-P) and age-matched WT sows (WT-P) in the third trimester (Fig. 3*A*). Non-pregnant (NP) WT sows (WT-NP) served as controls. TG-P sows did not differ from WT-P sows in any clinical-chemical and metabolomic parameters except for significantly elevated methionine sulfoxide concentrations in the fasting and elevated glucose concentrations in the fasting and postprandial state (Suppl. Table 1,3). During HIC, the steady state glucose concentrations (130 - 180 minutes) were not significantly different between WT-NP, WT-P and TG-P sows, but insulin concentrations between 140 and 160 minutes were significantly higher in WT-P vs. WT-NP sows (Fig. 3*B*). The glucose infusion rate (GIR) was significantly reduced in both WT-P and TG-P sows (AUC GIR: -56% and -63% compared with WT-NP sows), indicating an insulin resistant state (Fig. 3*C*). During MMGTT, WT-P sows showed a small but significant impairment of glucose tolerance associated with a distinctly increased insulin secretion (AUC glucose: +29%; AUC insulin: +62% compared with WT-NP sows; Fig. 3*D,E*). In TG-P sows, glucose tolerance was severely impaired (AUC glucose: +129%; AUC insulin: -3% compared with WT-NP sows; Fig. 3*D,E*). FPG was significantly elevated in TG-P sows, but unaltered in WT-P vs. WT-NP sows (Fig. 3*F*). During the entire course of pregnancy, FPG of TG-P sows did not increase significantly compared to non-pregnant TG sows (Fig. 3*G*).

Impaired glucose tolerance and metabolic alterations in neonatal offspring of hyperglycemic sows

OGTTs were performed in neonatal piglets of normoglycemic WT sows (NG piglets) and hyperglycemic TG sows (HG piglets) prior to the first colostrum uptake. The latter group included only the wild-type, but not the transgenic offspring. Plasma samples from time points 0 min. and 120 min. relative to glucose load (representing the fasting and insulin-stimulated state) were analyzed by clinical chemistry and targeted metabolomics.

Compared with NG piglets, male and female HG piglets revealed a significantly impaired glucose tolerance to a similar extent (Fig. 4*A*) associated with increased insulin secretion (Fig. 4*B*) and fasting hyperinsulinemia (Fig. 4*C*) in female but not male HG piglets. HOMA-IR was increased by 3-fold in male and 4.4-fold in female HG piglets compared to their male and female NG counterparts (Fig. 4*D*).

Plasma lactate, lipase, glycerol, NEFA, cholesterol, urea and bilirubin-D concentrations were significantly elevated in HG piglets (Figure 4*E-J*, Suppl. Table 4). A sex effect was observed for urea levels with a pronounced elevation in male HG piglets (Fig. 4*J*).

Targeted metabolomics of fasting plasma samples revealed significantly increased concentrations of hexoses and glycerophospholipids (lyso-phosphatidylcholines and total phosphatidylcholines (PC) with mainly diacylglycerols (PC aa) and containing a higher proportion of mono- and polyunsaturated PCs) in samples from HG vs. NG piglets. The ratio of total sphingomyelins (SM) to total phosphatidylcholins was significantly reduced. Additionally, HG piglets exhibited changes in their amino acid metabolism characterized by a distinct elevation in lysine (160.5 vs. 97.2 µM, p=0.002) going along with increased concentrations of alpha-aminoadipic acid (alpha-AAA). Furthermore, plasma concentrations of alanine and asymmetric dimethylarginine (ADMA) were increased, while those of citrulline and trans-4-hydroxyproline (t4-OH-Pro) were reduced in HG vs. NG piglets (Fig. 4*K*).

After the oral glucose bolus, the ratio of total and short-chain acylcarnitines (C2+C3) to carnitine was significantly increased in HG piglets. Total lyso-phosphatidylcholines and the concentrations of lysine, alanine and alpha-AAA were significantly increased, while those of tyrosine and acetyl-ornithine (Ac-Orn) were decreased in HG vs. NG piglets (Fig. 4 *L*). The complete metabolomic data set is presented in Suppl. Table 5.

Body and organ weights of neonatal HG and NG piglets were not significantly different (Suppl. Table 6).

Discussion

INS^{C93S} transgenic pigs represent a novel large animal model for Mutant Insulin Diabetes of Youth (MIDY) (Liu et al., 2015). The C93S mutation was chosen on the basis of the analogous Munich *Ins2*C95S mutant mouse model (Herbach et al., 2007) and the human C95Y mutation (Colombo et al., 2008; Stoy et al., 2010) leading to an early diabetic phenotype, i.e. permanent neonatal diabetes. The phenotype of *INS*^{C93S} transgenic pigs was less severe than in a line of *INS*^{C94Y} transgenic pigs (Renner et al., 2013), most probably due to a lower level of mutant *INS* transgene expression (mutant *INS*:WT *INS* transcript ratio 55% vs. 78% in *INS*^{C93S} and *INS*^{C94Y} transgenic pigs).

To establish a genetically modified large animal model for maternal diabetes we have initially characterized the metabolic status of *INS*^{C93S} transgenic pigs during late gestation. A genetically modified model is superior to already existing surgically and chemically induced large animal models in this field, as it is less invasive, has no direct impact on the exocrine pancreas and a minor phenotypic inter-individual variance (Renner et al., 2016).

Pigs are of translational value for the evaluation of metabolic programming effects as they are very similar to humans, e.g. considering their nutrition, digestive and pancreas physiology (Renner et al., 2016) as well as their propensity for the development of obesity and the metabolic syndrome (Renner et al., 2018). Genetic engineering in combination with dietary intervention allows the evaluation of metabolic programming effects due to maternal diabetes, obesity or a combination of both. Also, evaluation of glucose homeostasis by e.g. glucose tolerance tests and more sophisticated tests as glucose clamps can be evaluated in the unrestrained and unstressed animal due to long-term catheterization techniques and animal training. Despite differences in pig versus human placenta (epithelial-chorial versus hemochorial) transfer of relevant nutrients like glucose, amino acids and fatty acids to some

extent take place in both species (Litten-Brown et al., 2010). Decisive phases of early embryonic development are more similar in pigs as e.g. major genome activation takes place between 4-cell and 8-cell stage in pig and human versus 2-cell stage in mouse (Simmet et al., 2018). Also, in rodents late phases of embryonic development are generally not exposed to an altered maternal metabolism as their pups are born at a more immature state compared to pigs and humans (Litten-Brown et al., 2010).

Mild maternal hyperglycemia as present in *INS*^{C93S} transgenic sows closely represents the human situation as glucose concentrations of diabetic mothers are very tightly regulated due to current recommendations from professional societies (ADA, 2018).

During late pregnancy, insulin sensitivity of WT and TG sows was reduced by 50%, a similar degree as observed in pregnant women (Catalano et al., 1992b). In the MMGTT, insulin secretion in TG-P sows raised to the level of WT-NP sows, but did not meet the elevated insulin demand.

Male and female newborns of mildly hyperglycemic sows exhibited IGT, while only females showed fasting hyperinsulinemia and increased insulin secretion. HOMA-IR was increased in male and female piglets, however in females to a higher extent. Sex differences in metabolic programming have been reported in rodents, non-human primates and humans (Dearden et al., 2018). In accordance with the here described pig model, maternal diabetes was associated with pronounced glucose intolerance, hyperinsulinemia and elevated HOMA-IR in rodent (Samuelsson et al., 2013; Vickers et al., 2011) and human (Krishnaveni et al., 2010) female compared to male offspring. Female human offspring revealed an increased sensitivity to even mildly elevated maternal glucose levels compared to males (Regnault et al., 2013).

Mild maternal hyperglycemia even in the absence of maternal and neonatal increased fat mass/obesity is sufficient for metabolic derangements in the offspring. In humans, the development of fetal IR *in utero* was associated with increased fetal fat mass and had a strong correlation with maternal pre-gravid BMI and degree of insulin resistance (Catalano et al., 2009).

Early neonatal metabolism in humans is characterized by a drop in insulin due to adrenergic stimulation associated with delivery, increased lipolytic activity and switch from maternal glucose supply to absolute dependence on active gluconeogenesis as glucose supply from milk covers only 40-50% of glucose utilization rate (Girard, 1989). Increased availability of typical gluconeogenic precursors as lactate, alanine, glycerol and NEFAs provided by increased lipase activity in HG piglets might be explained by the reduced insulin sensitivity allowing higher rates of gluconeogenesis. Further, increased lactate concentrations in HG piglets might be caused by an earlier switch to energy supply from anaerobic glycolysis due to fetal IR and increased metabolic rates (Taricco et al., 2009). Elevated plasma urea concentration in HG piglets are in contrast to human studies showing no effect of maternal diabetes on urea synthesis (Kalhan, 1993).

The most pronounced metabolic changes of HG piglets were increased concentrations of lysine (+65%) and alpha-aminoadipic acid (2-fold), a product of lysine metabolism/breakdown (Requena et al., 2001) and potential biomarker for type 2 diabetes (Wang et al., 2013), associated with increased insulin secretion as a compensatory mechanism for early insulin resistance in cells and rodents (Wang et al., 2013).

HG piglets revealed also profound differences in their lipid metabolism. Similar to HG piglets, increased lyso-PC concentrations were reported in fetuses from lean mothers with

GDM (Lu et al., 2018) as well as a positive correlation to maternal glycemia in obese mother-offspring pairs (Patel et al., 2018). In contrast, decreased lyso-PC concentrations were associated with obesity, insulin resistance (Lehmann et al., 2013) and diabetes (Barber et al., 2012) in humans.

Apart from differences present in the fasting state, HG piglets revealed an increased ratio of total and short chain (C2+C3) acylcarnitines to free carnitine indicative of an increased import of fatty acids into mitochondria and increased β-oxidation rate. Increased plasma acylcarnitine concentrations have been observed in association with IR in humans (Schooneman et al., 2013). Enhanced β-oxidation in an insulin-stimulated state normally dominated by glucose oxidation can occur due to lack of insulin's inhibiting effect on fat oxidation in the IR state as accumulation of acetyl-CoA from β-oxidation inhibits pyruvate-dehydrogenase, glycolysis and finally glucose uptake into the cell (Kelley and Mandarino, 2000). Moreover, increased abundance of C2 might be part of a compensatory mechanism restoring glucose uptake into the cell by enabling export of acetyl-CoA as membrane-permeable C2. Increased concentrations of C2 contraindicative of acylcarnitine deacylation in the kidney (Guder and Wagner, 1990).

Mild maternal hyperglycemia did not alter birth weight in HG piglets. In humans, maternal obesity and increased neonatal fat mass has emerged as major determinant of variations in neonatal body weight. Maternal BMI was strongly associated with fetal overgrowth and increased fat mass independent of glycemia in the HAPO study (Catalano et al., 2012). Due to a comparatively high body fat content of 16% differences in fat mass can account for ~50% of variations in human birth weight (Catalano et al., 1992a). Unlike humans, rodents and pigs have a lower body fat content of 1% and 2% making them more sensitive for hypoglycemia-

associated starvation shortly after birth, but possibly less sensitive for the development of increased birth weight in the absence of obesity.

Mild maternal hyperglycemia in *INS*^{C93S} transgenic pigs leads to IGT, IR and profound associated metabolic alterations in the absence of maternal obesity. Therefore, *INS*^{C93S} transgenic pigs represent a valuable model for the dissection of developmental programming originating from maternal hyperglycemia, obesity and their cumulative effects in a highly standardized manner.

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Author contributions. SR, BA and EW designed the study. SR, SM and MB analyzed data. NK and CLS generated the genetic construct and Southern blot. AW, BK, MK and HN performed nucleofection, SCNT and embryo transfer. SR, CBR and AB performed PCR analyses. SK and HB performed next generation sequencing. SR, SM, CBR, ES, AH and SJK performed the metabolic characterization of *INS*^{C93S} transgenic pigs and controls. ES did morphometric analysis. SR, SM, ABl and RW performed necropsies. CP and JA performed targeted metabolomics. BR and MHdA performed clinical chemical analyses. MR coordinated health control of study animals. SR and EW wrote the manuscript with contributions from all authors. All authors contributed to discussion, read and approved the final manuscript. SR and EW 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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Figures

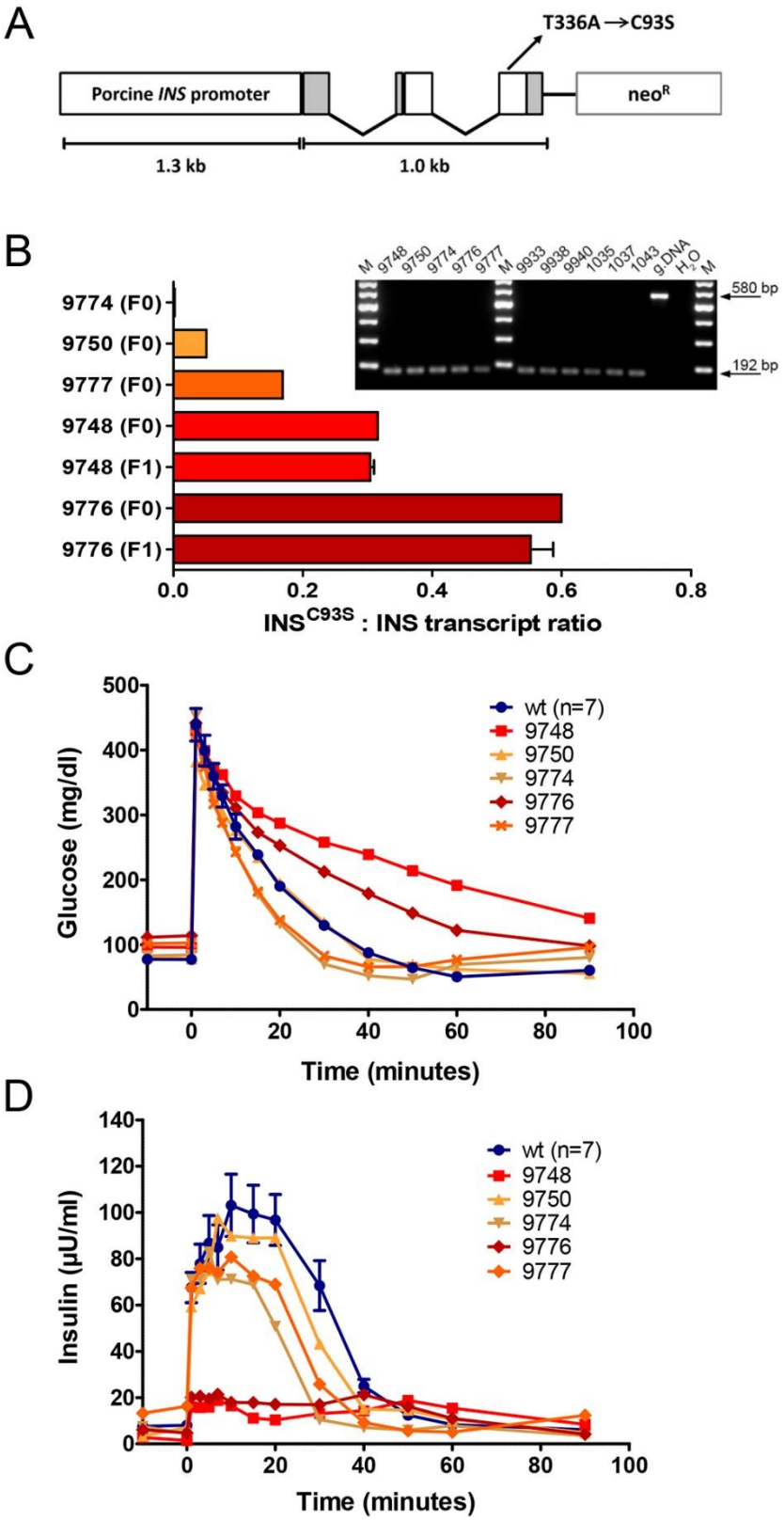

Figure 1: Generation of *INS*^{C93S} transgenic pigs. (A) Expression vector consisting of a 1.0 kb coding region of the porcine *INS* gene sequence including three exons with a T → A transition in exon 3 resulting in a Cys → Ser amino acid exchange at position 93, its essential regulatory elements and a neomycin selection cassette *(neo)*. **(B)** Quantification of *INS*^{C93S} and wild-type INS transcripts in pancreatic tissue of *INS*^{C93S} transgenic pigs by next generation sequencing of RT-PCR amplicons. Founder 9748 and 9776 show at least 1.9-fold higher expression of the mutant *INS*^{C93S} compared to the other three founders (F0) and similar expression compared to their F1-offspring (F1; n=3); insert: RT-PCR products of *INS*^{C93S}/*INS* transcripts in pancreatic tissue of all *INS*^{C93S} transgenic founders (left panel) and offspring from founder 9748 (9933, 9938, 9940) and founder 9776 (1035, 1037, 1043) (right panel); M: pUC Mix Marker; gDNA: genomic DNA; H_2O: Aqua bidest. **(C,D)** Intravenous glucose tolerance test (IVGTT) of *INS*^{C93S} transgenic founder boars (9748, 50, 74, 76, 77) and age-matched controls at eight months of age, **(C)** Glucose and **(D)** insulin levels. Data are means ± SEM.

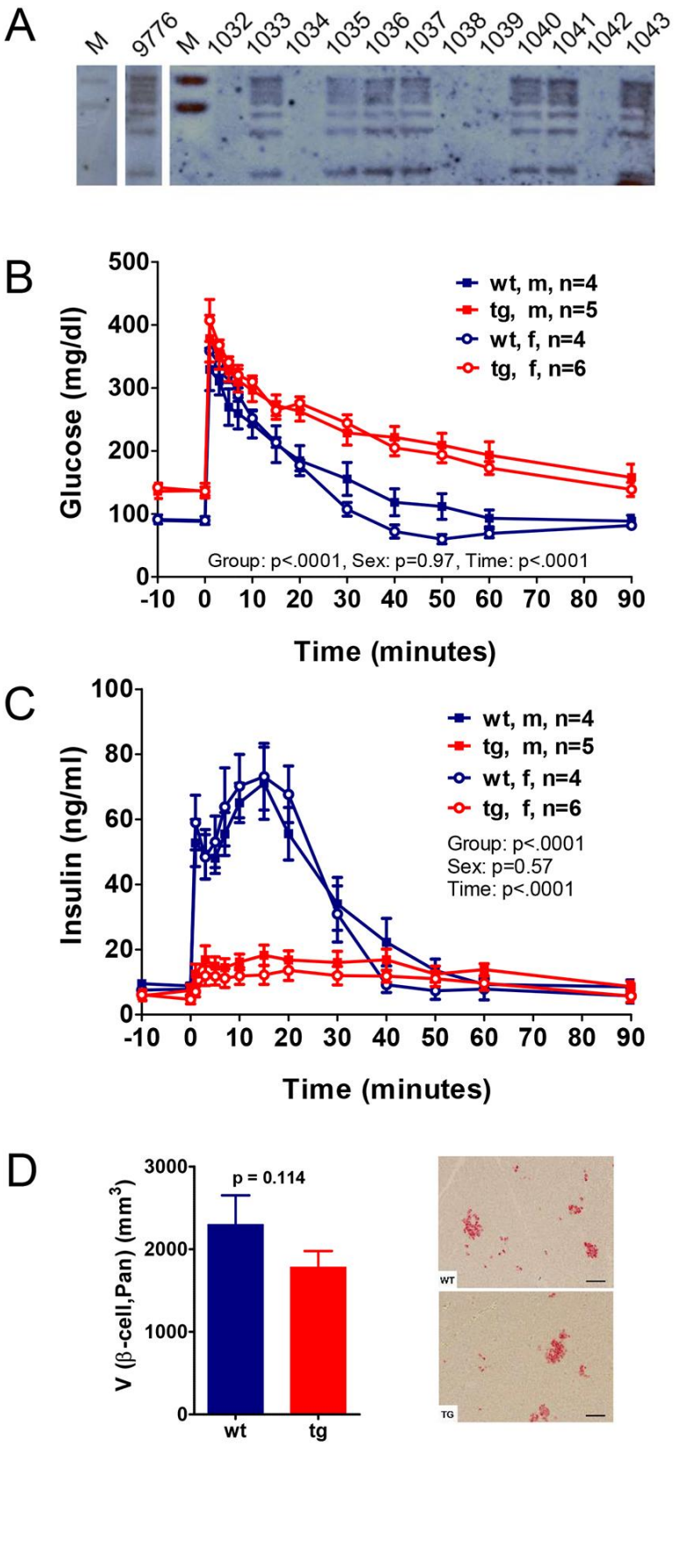

Figure 2: Reduced glucose tolerance and insulin secretion in *INS*^{C93S} transgenic pigs

(A) Southern blot analysis of PvuII-digested genomic DNA from *INS*^{C93S} transgenic pigs (1033, 1035, 1036, 1037, 1040, 1041, 1043) and littermate control animals (1032, 1034, 1038, 1039, 1042) using a ^{32}P-CTP-labeled probe specific for the neomycin resistance cassette. Founder 9776 and its transgenic offspring show the same pattern, demonstrating a single integration site. **(B,C)** Intravenous glucose tolerance test (IVGTT) of offspring (male & female as effect was gender independent) from *INS*^{C93S} transgenic founder boar 9776 and non-transgenic littermates at three-four months of age, **(B)** Glucose and **(C)** insulin levels. Data are means ± SEM; *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. **(D)** Quantitative-stereological analyses of pancreatic tissue from *INS*^{C93S} transgenic pigs and non-transgenic littermates at one year of age, V (b-cell,Pan): total beta-cell volume in the pancreas; representative histological sections of pancreatic tissue stained with an α-insulin antibody from a control (wt) and an *INS*^{C93S} transgenic pig (tg); Scale bar = 50 µm.

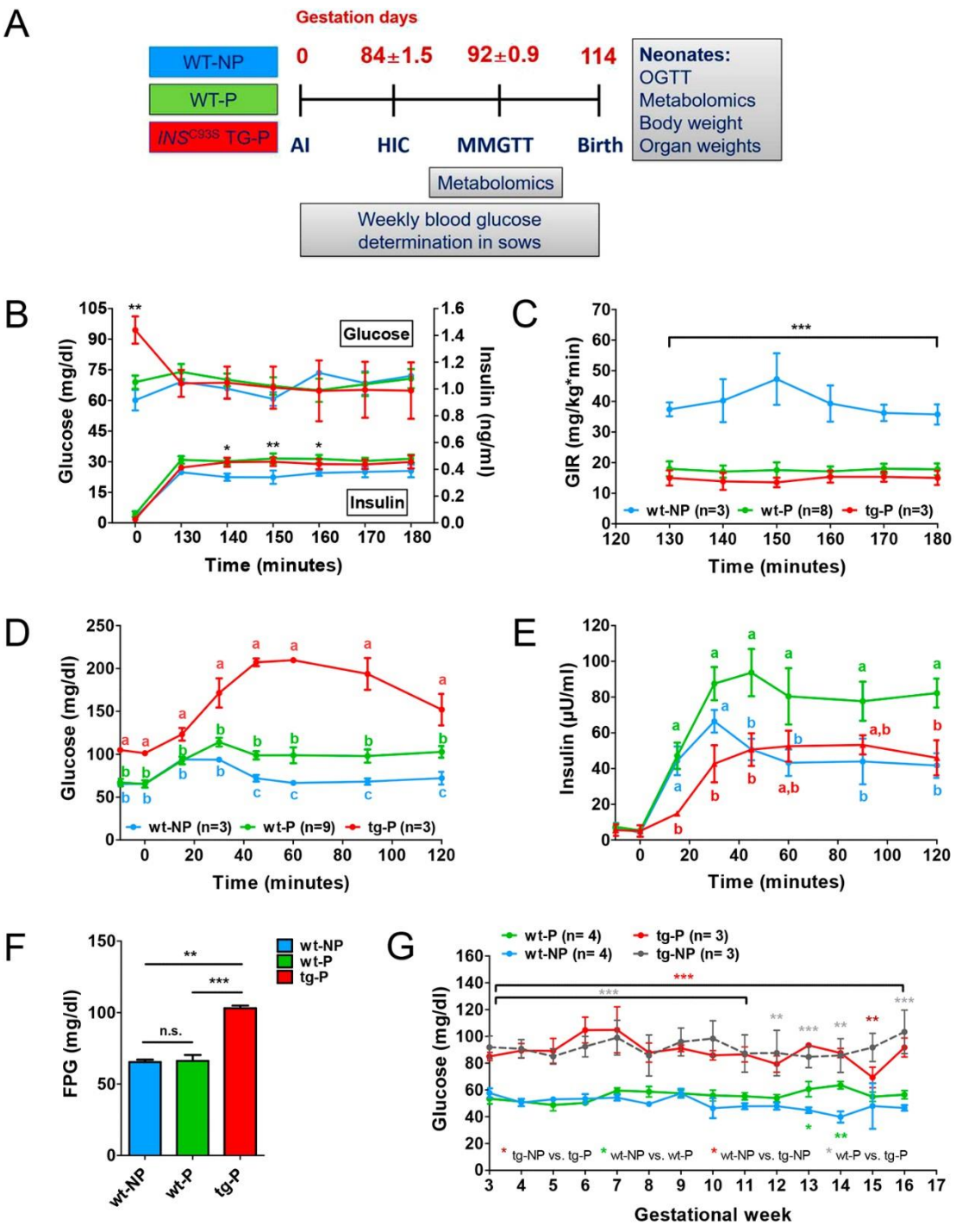

Figure 3: Reduced insulin sensitivity and insufficient compensatory capacity to maintain glucose control in *INS*^{C93S} transgenic pigs during pregnancy.

(A) Study outline of the evaluation of glucose control in sows during pregnancy and in neonates after birth. **(B,C)** Hyperinsulinemic-euglycemic clamp (HIC) of pregnant *INS*^{C93S} transgenic

(TG-P) and non-transgenic (WT-P) sows as well as of non-pregnant controls (WT-NP), **(B)** Glucose and insulin levels, **(C)** Glucose infusion rate (GIR). **(D,E)** Mixed-meal glucose tolerance test (MMGTT) in TG-P, WT-P and WT-NP sows, **(D)** glucose and **(E)** insulin concentrations. **(F,G)** Fasting blood glucose concentrations within the third trimester (time-point of the MMGTT) **(F)** and throughout pregnancy **(G)**. Data are means ± SEM; *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$; a, b, c: labelling indicates comparison between all groups; different letters indicate significant difference between groups.

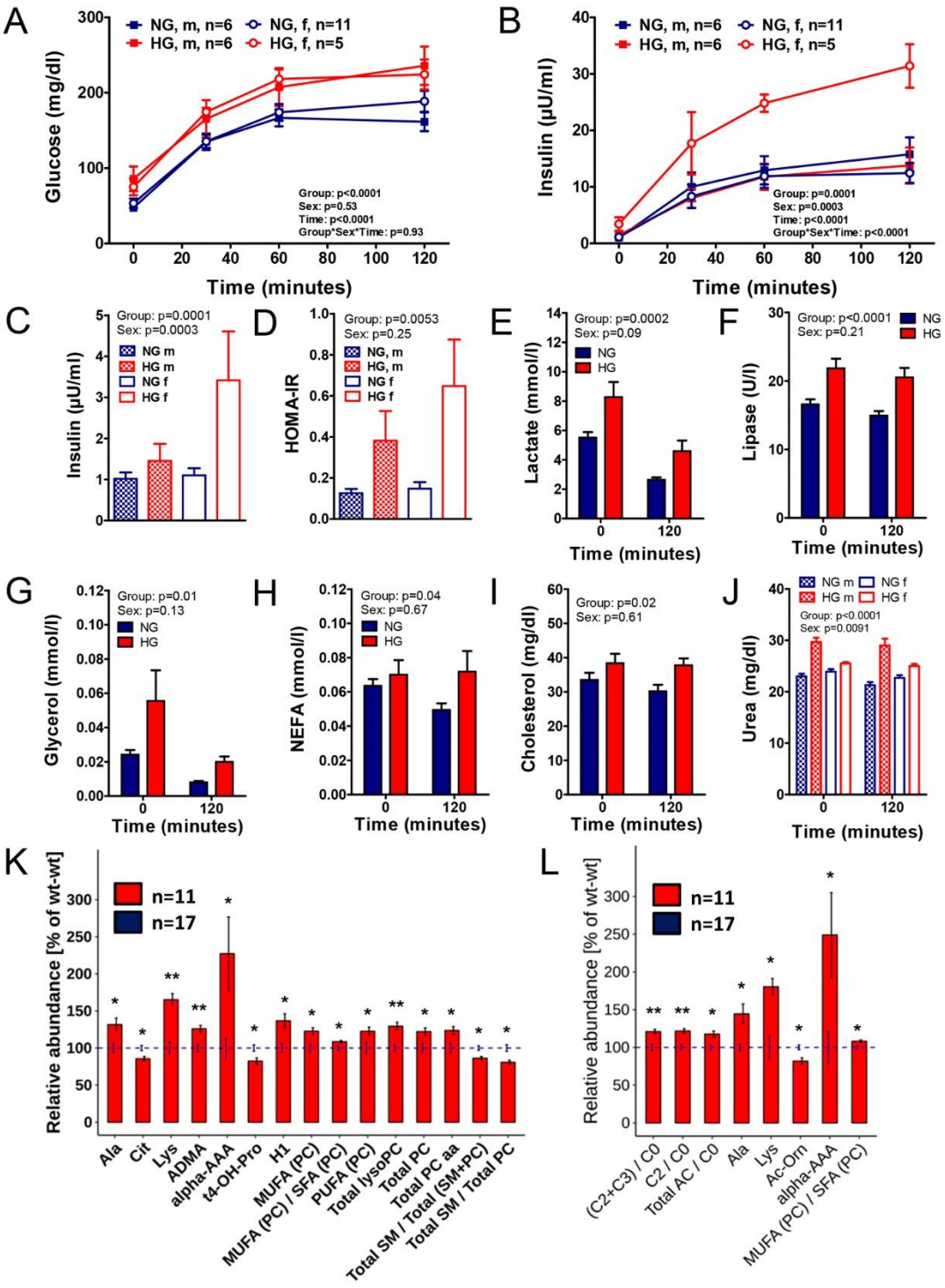

Figure 4: Reduced glucose tolerance and increased insulin secretion in neonatal piglets born to *INS*^{C93S} transgenic sows

(A,B) Oral glucose tolerance test in piglets prior to first colostrum uptake born to normoglycemic wild-type sows (NG) or to hyperglycemic *INS*^{C93S} transgenic sows (HG), **(A)**

glucose, **(B)** insulin, **(C)** fasting insulin, **(D)** HOMA-IR. **(E-J)** Clinical chemical parameters in NG and HG piglets in the fasting (0 minutes) and insulin-stimulated (120 minutes relative to an oral glucose load) state, **(E)** lactate, **(F)** lipase, **(G)** glycerol, **(H)** NEFA, **(I)** cholesterol, **(J)** urea; **(K,L)** Significantly different abundance of metabolites in plasma samples of NG compared to HG piglets in the **(K)** fasting state and **(L)** insulin-stimulated (120 minutes relative to an oral glucose load) state. *: $p<0.05$, **: $p<0.01$.

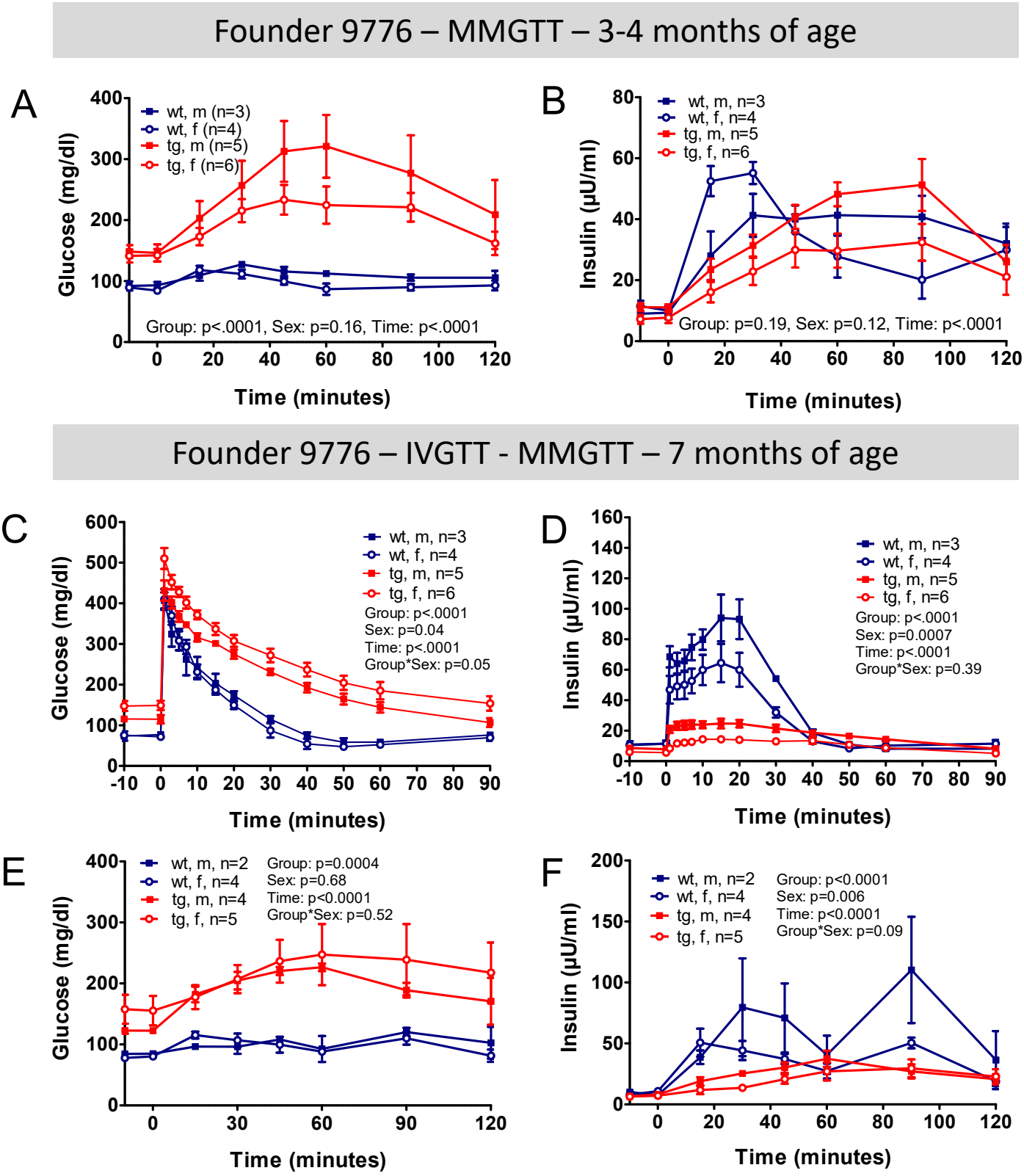

Figure S1: MMGTT from male and female offspring of founder boar 9776 at three to four months of age and IVGTT and MMGTT at seven months of age. Data are means ± SEM.

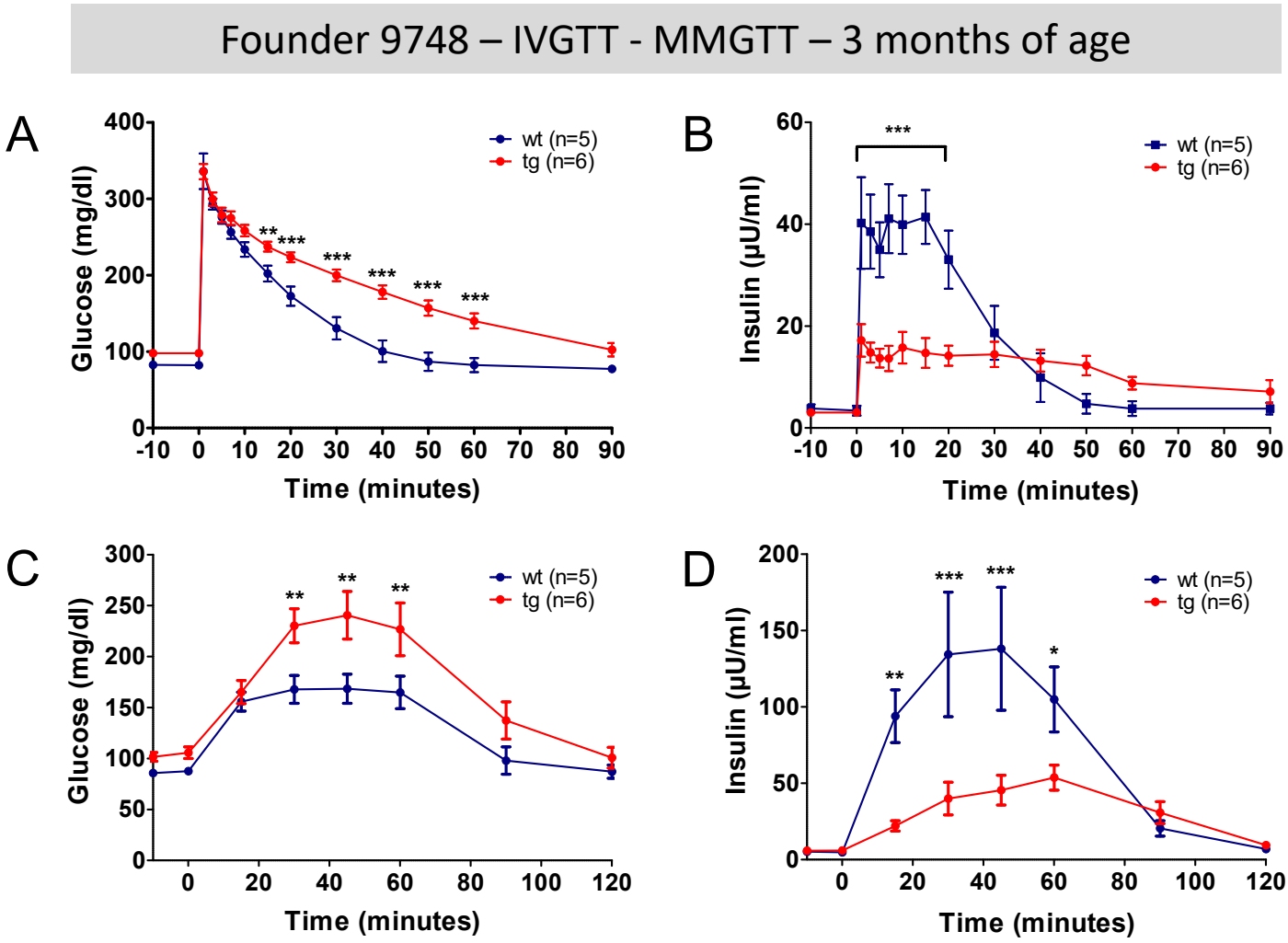

Figure S2: IVGTT and MMGTT from male and female offspring of founder boar 9748 at three months of age. Data are means ± SEM; *: p<0.05, **: p<0.01, ***: p<0.001.

Table S1: Clinical-chemical parameters in pregnant wild-type (WT-P) and pregnant *INS*^{C93S} transgenic (TG-P) sows.
Clinical-chemical parameters in pregnant wild-type (WT-P, n=6) and pregnant *INS*^{C93S} transgenic (TG-P, n=3) sows during late gestation (gestation day 92 ± 1.1) at time-points 0 and 120 minutes of a MMGTT. Longitudinal data were evaluated by ANOVA (General Linear Models) taking the fixed effects of Group, Time and the interaction Group*Time into account.

Parameter	Time	Group		Analysis of variance		
		WT-P	TG-P	Group	Time	Group x Time
Glucose [mg/dl]	**0**	71.3 ± 5.7	109.5 ± 3.1	0.0004	0.0002	0.82
	120	111.7 ± 7.6	154.0 ± 15.2			
Lactate [mmol/l]	**0**	0.89 ± 0.05	0.87 ± 0.06	0.94	0.0002	0.99
	120	1.9 ± 0.2	1.9 ± 0.4			
LDH [U/l]	**0**	333.8 ± 14.1	520.3 ± 176.7	0.06	0.95	0.70
	120	359.2 ± 11.4	485.6 ± 141.4			
Bicarbonate [mmol/l]	**0**	20.2 ± 0.5	21.2 ± 1.1	0.90	0.89	0.21
	120	21.2 ± 0.7	20.4 ± 0.3			
Cholesterol [mg/dl]	**0**	67.0 ± 4.2	74.5 ± 4.8	0.19	0.52	0.90
	120	64.3 ± 4.5	70.5 ± 4.0			
LDL [mg/dl]	**0**	43.3 ± 2.7	49.4 ± 3.5	0.19	0.51	0.68
	120	42.4 ± 3.1	45.7 ± 3.4			
HDL [mg/dl]	**0**	25.7 ± 1.9	28.0 ± 1.82	0.54	0.48	0.66
	120	25.2 ± 2.0	25.5 ± 1.3			
Triglycerides [mg/dl]	**0**	28.2 ± 2.9	27.9 ± 2.7	0.47	0.31	0.42
	120	22.1 ± 2.9	27.2 ± 2.4			
Glycerol [mmol/l]	**0**	0.05 ± 0.03	0.02 ± 0.006	0.4	0.16	0.39
	120	0.004 ± 0.0003	0.004 ± 0.0009			
NEFA [mmol/l]	**0**	0.6 ± 0.3	0.3 ± 0.06	0.49	0.12	0.49
	120	0.04 ± 0.005	0.04 ± 0			
ASAT [U/l]	**0**	18.8 ± 1.8	26.3 ± 9.9	0.15	0.78	0.87
	120	18.3 ± 1.6	24.3 ± 7.5			
ALAT [U/l]	**0**	34.0 ± 3.2	41.3 ± 5.8	0.04	0.65	0.82
	120	33.3 ± 3.4	39.3 ± 5.0			
GGT [U/l]	**0**	33.3 ± 1.5	31.7 ± 8.3	0.22	0.97	0.40
	120	36.7 ± 2.9	28.0 ± 5.7			
Bilirubin [mg/dl]	**0**	0.2 ± 0.02	0.1 ± 0.01	0.64	0.26	0.88
	120	0.1 ± 0.003	0.1 ± 0			
Lipase [U/l]	**0**	2.1 ± 0.2	4.2 ± 1.0	0.03	0.32	0.12
	120	2.4 ± 0.2	2.8 ± 1.1			
Albumin [g/dl]	**0**	3.8 ± 0.07	3.9 ± 0.04	0.67	0.12	0.16
	120	3.8 ± 0.04	3.7 ± 0.1			
Total protein [g/dl]	**0**	6.7 ± 0.1	7.0 ± 0.1	0.49	0.05	0.14
	120	6.8 ± 0.08	6.6 ± 0.08			
Creatinine [mg/dl]	**0**	1.6 ± 0.04	1.7 ± 0.06	0.42	0.94	0.43
	120	1.6 ± 0.14	1.6 ± 0.08			
Urea [mg/dl]	**0**	23.3 ± 1.9	28.3 ± 2.3	0.07	0.88	0.79
	120	23.6 ± 2.0	27.4 ± 1.1			
CRP [mg/l]	**0**	11.2 ± 1.7	12.0 ± 7.8	0.79	0.95	0.94
	120	11.1 ± 1.5	12.6 ± 8.9			

Table S2: List of metabolites measured with the AbsoluteIDQ® p180 Kit GAC, Helmholtz Zentrum München

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Table S3: ***A.*** Targeted metabolomics findings in pregnant wild-type **(WT-P, n=9)** and *INS*^{C93S} transgenic **(TG-P, n=3)** sows in the fasting condition. ***B.*** Targeted metabolomics findings in pregnant wild-type **(WT-P, n=9)** and *INS*^{C93S} transgenic **(TG-P, n=3)** sows in the insulin-stimulated condition (120 minutes time-point of the mixed-meal glucose tolerance test, MMGTT).

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Table S4: Clinical-chemical parameters in piglets born to wild-type (NG) and *INS*^{C93S} transgenic sows (HG). Clinical-chemical parameters in wild-type piglets born to wild-type sows (NG, n=17) and wild-type piglets born to *INS*^{C93S} transgenic sows (HG, n=11) at the day of birth prior to and 120 minutes following an oral glucose load (2g / kg body weight). Longitudinal data were evaluated by ANOVA (General Linear Models; SAS 8.2) taking the fixed effects of Group, Sex, Time, and the interaction Group*Sex and Group*Sex*Time into account.

Parameter	Time	Group		Analysis of variance				
		wt-wt	wt-tg	Group	Sex	Time	Group x Sex	Group x Sex x Time
Glucose [mg/dl]	**0**	53.7 ± 4.33	81.2 ± 12.8	0.0015	0.84	<0.0001	0.15	0.72
	120	187.3 ± 10.8	239.0 ± 17.6					
Lactat [mmol/l]	**0**	5.51 ± 0.37	8.27 ± 1.03	0.0002	0.09	<0.0001	0.03	0.75
	120	2.64 ± 0.16	4.6 ± 0.7					
LDH [U/l]	**0**	312.7 ± 9.8	310.8 ± 27.0	0.43	0.16	<0.0001	0.77	0.49
	120	430.6 ± 18.9	447.8 ± 25.8					
Cholesterol [mg/dl]	**0**	33.5 ± 2.1	38.4 ± 2.7	0.02	0.61	0.45	0.56	0.77
	120	30.2 ± 1.9	37.8 ± 2.0					
LDL [mg/dl]	**0**	25.8 1.41	28.3 3.3	0.14	0.65	0.53	0.31	0.96
	120	23.6 1.37	28.0 2.1					
HDL [mg/dl]	**0**	12.7 ± 0.9	17.2 ± 1.3	0.001	0.07	0.19	0.33	0.95
	120	11.7 ± 0.8	15.5 ± 0.9					
Triglycerides [mg/dl]	**0**	11.7 ± 0.53	14.4 ± 1.7	0.11	0.24	0.0002	0.07	0.32
	120	9.36 ± 0.42	9.8 ± 0.5					
Glycerol [mmol/l]	**0**	0.02 ± 0.003	0.06 ± 0.02	0.01	0.13	0.002	0.11	0.34
	120	0.008 ± 0.0009	0.02 ± 0.003					
NEFA [mmol/l]	**0**	0.06 ± 0.004	0.07 ± 0.008	0.04	0.67	0.32	0.91	0.48
	120	0.05 ± 0.004	0.07 ± 0.01					
ASAT [U/l]	**0**	13.6 ± 0.94	15.8 ± 1.2	0.56	0.27	0.0003	0.67	0.71
	120	21.8 ± 2.07	22.4 ± 2.2					
GGT [U/l]	**0**	89.9 ± 3.0	92.6 ± 2.5	0.28	0.2	0.02	0.25	0.78
	120	78.2 ± 2.7	86.0 ± 3.8					
Bilirubin [mg/dl]	**0**	0.06 ± 0.005	0.06 ± 0.005	0.05	0.17	0.06	0.36	0.75
	120	0.07 ± 0.008	0.1 ± 0.01					
Bilirubin-D [mg/dl]	**0**	0.017 0.006	0.02 0.008	0.01	0.05	0.02	0.41	0.73
	120	0.025 0.003	0.04 0.02					
Lipase [U/l]	**0**	16.6 ± 0.7	21.8 ± 1.4	<0.0001	0.21	0.27	0.11	0.89
	120	14.9 ± 0.7	20.5 ± 1.4					
Albumin [g/dl]	**0**	0.75 ± 0.02	0.7 ± 0.06	0.26	0.61	0.19	0.75	0.67
	120	0.66 ± 0.02	0.7 ± 0.05					
Total protein [g/dl]	**0**	2.26 ± 0.03	2.3 ± 0.1	0.18	0.82	0.004	0.80	0.55
	120	1.98 ± 0.04	2.2 ± 0.08					
Creatinine [mg/dl]	**0**	1.08 ± 0.06	1.0 ± 0.04	0.91	0.06	0.90	0.01	0.60
	120	1.04 ± 0.06	1.1 ± 0.04					
Urea [mg/dl]	**0**	23.6 ± 0.6	28.3 ± 0.9	<0.0001	0.0091	0.06	<0.0001	0.88
	120	22.2 ± 0.4	27.2 ± 1.0					
Bicarbonate [mmol/l]	**0**	19.4 ± 1.46	21.6 ± 3.3	0.28	0.56	0.14	0.13	0.88
	120	21.9 ± 1.1	25.6 ± 2.6					

Table S5: *A*. Targeted metabolomics findings in wild-type piglets born to normoglycemic wild-type sows **(NG, n=17)** and wild-type piglets born to hyperglycemic *INS*^{C93S} transgenic sows **(HG, n=11)** in the fasting condition. ***B*.** Targeted metabolomics findings in wild-type piglets born to normoglycemic wild-type sows **(NG, n=17)** and wild-type piglets born to hyperglycemic *INS*^{C93S} transgenic sows **(HG, n=11)** in the insulin-stimulated condition (120 minutes relative to an oral glucose load).

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Table S6: Body and organ weights in piglets born to wild-type and *INS*^{C93S} transgenic sows. Body and (relative) organ weights in wild-type piglets born to wild-type sows **(NG)** and wild-type piglets born to *INS*^{C93S} transgenic sows **(HG)** at day one of age. Data were evaluated by ANOVA (General Linear Models; SAS 8.2) taking the fixed effect of Group into account.

Parameter	LS Means		P value
	HG	NG	
Body weight (g)	1473.806	1476.686	0.974
CRL (cm)	28.223	28.559	0.785
*rel*CRL (%)	2.550	2.583	0.676
Pancreas (g)	2.109	1.998	0.549
*rel*Pancreas (%)	0.141	0.138	0.649
Liver (g)	44.338	40.929	0.429
*rel*Liver (%)	3.001	2.762	0.067
Heart (g)	12.593	11.536	0.349
*rel*Heart (%)	0.851	0.789	0.066
Kidneys (g)	5.505	5.815	0.572
*rel*Kidneys (%)	0.371	0.395	0.265
Lungs (g)	43.024	46.475	0.280
*rel*Lungs (%)	2.982	3.196	0.222
Spleen (g)	2.130	2.093	0.888
*rel*Speen (%)	0.142	0.141	0.980
Carcass (g)	1149.384	1142.208	0.936
*rel*Carcass (%)	78.267	77.783	0.758

rel: relative; CRL: crown-rump length