Impaired glucose control in newborn piglets exposed to mild hyperglycemia *in utero*: study in a novel transgenic pig model for mild maternal diabetes

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To my family

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I. INTRODUCTION

Maternal diabetes is one of the most common metabolic disorders complicating pregnancy. Prevalence estimates in 2015 assumed that 20.9 million (16.2 %) of 129.4 million live births were affected by hyperglycemia (IDF, 2015). Maternal diabetes can either result from preexisting type 1 or type 2 diabetes, or occurs during late pregnancy and hence is defined gestational diabetes mellitus (GDM) (ADA, 2016). Human epidemiological studies demonstrate that intra-uterine exposure to hyperglycemia, regardless of the type of maternal diabetes, has short- and long-term negative consequences for both the mother and the offspring (FETITA et al., 2006; FRASER & LAWLOR, 2014; MCCANCE, 2015). Even more alarming is the evidence from a large multicenter, multicultural, observational study involving 25.000 pregnant women, revealing that less severe degrees of hyperglycemia as such in overt diabetes mellitus are also associated with maternal/fetal and neonatal negative outcomes (GROUP et al., 2008).

The impact of maternal diabetes on the offspring is extremely difficult to study in humans. Conversely, experimental animal models offer the possibility to investigate the consequences of maternal hyperglycemia and the underlying molecular mechanisms at different developmental stages. To date, animal models of maternal diabetes were mainly established in rodents (JAWERBAUM & WHITE, 2010; PASEK & GANNON, 2013), with considerable numbers using chemical diabetes induction with clear disadvantages. Only few models represent clinically-relevant hyperglycemic levels (DAMASCENO et al., 2013) that are comparable to the tightly regulated glycemic control observed in pregnant diabetic women. Therefore, there is a need to develop clinically relevant experimental models of maternal diabetes. Pigs are an excellent and well-established animal model in diabetes research, closely resembling aspects of human glucose metabolism, including physiological metabolic alterations during pregnancy (PÈRE & ETIENNE, 2007; WOLF et al., 2014; RENNER et al., 2016a).

The aim of present study was to first generate and characterize a novel genetically engineered porcine model of mild diabetes, characterize glucose control and insulin sensitivity of this model during the pregnant state and evaluate effects of *in utero* exposure to mild maternal hyperglycemia on the offspring.

II. REVIEW OF THE LITERATURE

1. Metabolic and morphological adaptations during pregnancy in humans

Metabolism encompasses complex networks of tightly regulated cellular processes that sustain a physiological balance in biological systems. Within these networks, maintenance of energy balance is a fundamental task that mainly involves i) maintenance of physiological glucose levels; ii) adequate insulin production and secretion; iii) adequate insulin response in peripheral insulin-sensitive tissues; and iv) maintenance of optimal glycogen and lipid storage to be catabolized at times of caloric restriction (BERG et al., 2007). Pregnancy is a transitional state that accounts for tremendous metabolic adaptations compared to the non-pregnant state. Those include changes in glucose metabolism, whole body insulin sensitivity, insulin secretion, lipid metabolism and, less studied, changes in protein metabolism (HADDEN & MCLAUGHLIN, 2009). In the context of pregnancy, glucose and energy metabolism are of particular relevance since the mother must meet the needs of a progressively increasing energy demand to allow a sufficient supply of nutrients for fetal development. In addition, these metabolic changes are critical to prepare the maternal organism for delivery and lactation as well as to provide adequate energy storages for the growing fetus upon birth (HADDEN & MCLAUGHLIN, 2009). In the first part of the literature review, the main metabolic changes in glucose and lipid metabolism that occur during normal pregnancy in humans will be described and compared to adaptations observed in large animal models, particularly in the pig. In the second part, pregnancy-associated metabolic changes in the context of a (pre-) diabetic state in humans and large animal models are in focus.

1.1. Changes in glucose metabolism

Glucose homeostasis involves a network of metabolic processes that maintain circulating glucose levels within a physiological range. In humans, this range comprises fasting plasma glucose concentrations between 70 mg/dl to 90 mg/dl, in a physiological non-pregnant state (ADA, 2016). Maintenance of normoglycemia is therefore a balance between the rates of glucose entering the circulation and glucose disposal to peripheral tissues. Circulating plasma glucose is derived from external sources like nutrients absorbed through the small intestine, or from endogenous

sources such as the breakdown of glycogen stores via glycogenolysis as a primary energy source or by endogenous hepatic glucose production via gluconeogenesis, e.g. during longer fasting periods (GAGLIARDINO, 2005). Glucose removal from the blood stream is a strictly regulated process involving the key gluco-regulatory hormone insulin that stimulates glucose uptake into peripheral tissues and inhibits hepatic glucose production (DEFRONZO et al., 1983; DEFRONZO & FERRANNINI, 1987). In the fasting state, when glucose is only provided by endogenous sources (mainly by the liver), the rate of hepatic glucose production is equivalent to the rate of basal glucose-uptake. Thus, circulating glucose concentrations mainly reflect hepatic glucose production (DEFRONZO et al., 1989). Upon glucose load, plasma glucose levels increase and stimulate insulin secretion as well as insulin biosynthesis in the pancreas. Insulin inhibits hepatic glucose production and enhances glucose uptake in insulin sensitive tissues, mainly skeletal muscle, liver and fat (DEFRONZO et al., 1983; DEFRONZO & FERRANNINI, 1987).

Generally, fasting glucose levels are decreased by 10 to 20 % in healthy pregnant women compared to healthy non-pregnant women (BLACKBURN, 2013). The lower glucose concentrations during pregnancy are attributed to dilution effects in volume distribution of glucose (KALHAN & ADAM, 1980), as well as to continuous increased demand in glucose utilization by the fetal-placental unit, especially during the last trimester (KALHAN et al., 1979; HADDEN & MCLAUGHLIN, 2009; ANGUEIRA et al., 2015). As to compensate fasting plasma glucose (FPG) decay endogenous glucose production is increased by 16 to 30 % in pregnant women with the progression of pregnancy (KALHAN et al., 1979; CATALANO et al., 1992; ASSEL et al., 1993), and gluconeogenesis is the preferred pathway contributing to glucose production during the fasting state in pregnant women (KALHAN et al., 1997). Contrary to the fasting state, postprandial glucose levels during pregnancy are increased, and plasma glucose concentrations remain elevated for longer period following a meal, as compared to the non-pregnant state. Elevated postprandial glucose levels result from a minor impairment in glucose tolerance mainly due to changes in insulin sensitivity as explained below (DI CIANNI et al., 2003; HADDEN & MCLAUGHLIN, 2009; ANGUEIRA et al., 2015).

1.1.1. Insulin sensitivity

In order to understand glucose homeostasis, primary physiological variables such as ß-cell response to glucose and tissue insulin sensitivity are pivotal parameters that can

be assessed by a variety of methods in diabetes research. The hyperinsulinemic-euglycemic clamp (HIC) is the gold-standard method to determine *in vivo* insulin sensitivity (DEFRONZO et al., 1979). Contrary to the standard glucose and insulin stimulation tests (including oral and intravenous), where insulin sensitivity is mainly estimated through surrogated indexes, in the HIC, insulin sensitivity can be directly assessed by glucose infusion rate. In a HIC setup, after an overnight fasting, insulin is infused at a constant rate, as to achieve a steady state of hyperinsulinemia. This suppresses endogenous production of insulin by ß-cells and glucose by the liver, as well as stimulates glucose uptake by peripheral tissues, mainly in skeletal muscle and adipose tissue. Simultaneously, glucose is infused at a variable rate, as to maintain a defined fasting plasma glucose concentration within the physiological range (also defined as euglycemic state). Therefore, the glucose amount infused throughout the steady state equals the glucose amount taken up by insulin-sensitive peripheral tissues. Consequently, the glucose infusion rate during steady state provides an absolute index of whole body insulin sensitivity. In insulin sensitive subjects, higher glucose infusion rates are required to maintain euglycemia as glucose is rapidly taken up and utilized by insulin sensitive tissues during the hyperinsulinemic condition. In contrast, in insulin resistant subjects, lower glucose infusion rates are necessary to maintain euglycemia as glucose uptake and utilization is reduced proportionally to the degree of insulin resistance in these subjects (DEFRONZO et al., 1979; MUNIYAPPA et al., 2008; KIM, 2009).

Studies using the hyperinsulinemic-euglycemic clamp have demonstrated that in the first 10-12 weeks of gestation insulin sensitivity is normal, compared to the non-pregnant state (CATALANO et al., 1991; CATALANO et al., 1992). With progression of pregnancy, insulin sensitivity is reduced by 50 to 70 % in the third trimester in healthy pregnant women (RYAN et al., 1985; BUCHANAN et al., 1990; CATALANO et al., 1991; CATALANO et al., 1992; CATALANO et al., 1993; SIVAN et al., 1997). Reduced insulin sensitivity during pregnancy results from a marked reduction in insulin action in maternal peripheral tissues, especially in skeletal muscle and adipose tissue (BUTTE, 2000; DI CIANNI et al., 2003; HADDEN & MCLAUGHLIN, 2009; ANGUEIRA et al., 2015) and is the main event contributing to increased nutrient provision for the fetus (BUTTE, 2000; DI CIANNI et al., 2003). Although the molecular mechanisms underlying physiological insulin resistance in pregnancy are not completely understood, they involve impairment of insulin signaling to

downstream targets within the insulin signaling cascade (BARBOUR et al., 2007). Insulin-mediated glucose uptake is initiated by binding of insulin to the α-subunit of the insulin receptor tyrosine kinase (IR) in insulin-sensitive tissues. This induces a conformational change in the two subunits of the IR, resulting in autophosphorylation of several tyrosine kinase substrates in the IR. The active IR, subsequently phosphorylates downstream signaling molecules, including insulin-receptor substrate (IRS) proteins (IRS-1 and IRS-2 are important mediators in muscle and adipose tissue), which act as scaffolds to recruit and mediate signaling complexes (BOUCHER et al., 2014). Among them, recruitment of type 1A PI3-kinase (PI3K) is known to be directly involved in facilitating translocation of intracellular glucose receptors (GLUT) into the plasma membrane (BACKER et al., 1992; ALESSI & DOWNES, 1998). Although the molecular mechanisms are not fully understood, it involves activation of AKT and subsequent phosphorylation cascade of PH-domain containing proteins including the serine/threonine protein kinase B (PKB)/AKT and the atypical protein kinase C (PKC) ζ isoform (PKCζ) that ultimately are recruited to the plasma membrane and facilitate intracellular-membrane-vesicles-containing GLUT receptors to translocate into the plasma cell membrane (BRYANT et al., 2002; ROWLAND et al., 2011). Reduced phosphorylation levels on the tyrosine kinase of IR, indicating IR impairment activity, were demonstrated in purified IR from skeletal muscle of pregnant women (SHAO et al., 2000). Moreover, IRS1 phosphorylation is reduced in muscle and liver of an experimental rat model of pregnancy, as well as in skeletal muscle of healthy pregnant women (DAMM et al., 1993; SAAD et al., 1997). In addition, reduced translocation of GLUT receptors has been identified. GLUT4 protein expression is significantly reduced in subcutaneous adipose tissue of healthy pregnant as compared to non-pregnant women (OKUNO et al., 1995). Friedman et al. demonstrated that insulin-induced GLUT4 translocation was reduced by 32 % in skeletal muscle of healthy pregnant women as compared to non-pregnant controls (FRIEDMAN et al., 1999). On the basis of understanding the underlying molecular mechanisms of insulin resistance, pregnancy-related endocrine changes have been intensively investigated (RYAN & ENNS, 1988; HANDWERGER & FREEMARK, 2000; NEWBERN & FREEMARK, 2011). Progesterone has *in vivo* insulin desensitization effects particularly on hepatic glucose production in pregnant rats (NELSON et al., 1994). Plasma cortisol concentrations are greatly increased in human pregnancy (KIRWAN et al., 2002), and glucocorticoid treatment interfered with IR

signaling and IRS-1 expression *in vivo* in rats (GIORGINO et al., 1993). Lactogen hormones including human placental lactogen (hPL) and prolactin (PRL) stimulate maternal food intake by targeting *in vivo* leptin resistance in the rat brain (AUGUSTINE & GRATTAN, 2008). In addition, hPL stimulates *in vitro* insulin secretion in rat, mouse and human islets (BRELJE et al., 1993). Transgenic mice overexpressing human placental growth hormone (hPGH), demonstrated that hPGH enhances p85α subunit of PI3K which, in turn, impairs IRS1 and PI3K complex activity, thus blocking downstream insulin signaling in skeletal muscle (BANDYOPADHYAY et al., 2005; BARBOUR et al., 2005). In addition to pregnancy-associated hormones, others have investigated the role of adipose tissue-specific cytokines, known as adipokines, and their contribution to insulin resistance. During pregnancy adipose tissue expansion takes place (CLAPP et al., 1988; ROJAS-RODRIGUEZ et al., 2015) and adipokines like leptin, adiponectin and tumor necrosis factor alpha (TNF-α) have been implicated with increased insulin resistance in pregnancy (BARBOUR et al., 2007). Plasma leptin concentrations increase throughout gestation (BUTTE et al., 1997) and they correlate with maternal insulin and increase maternal fat mass (HIGHMAN et al., 1998; VIRKAMAKI et al., 1999). TNF-α is related with obesity-induced insulin resistance by inhibiting the activity of the IR (HOTAMISLIGIL et al., 1996) and so far is the only signaling factor that directly correlates with progressive changes in insulin sensitivity *in vivo* in humans (KIRWAN et al., 2002). The role of adiponectin as an insulin-sensitizing hormone in obese and type 2 diabetes patients is well described (ARITA et al., 1999; HOTTA et al., 2000; WEYER et al., 2001). Also, adiponectin levels were found reduced during the 3rd trimester of pregnancy compared to the non-gravid state, in agreement with reduced adiponectin mRNA expression in white adipose tissue of lean pregnant women (CATALANO et al., 2006).

1.1.2. Insulin secretion

In addition to insulin sensitivity, β-cell function is the other pivotal variable impacting glucose metabolism. Glucose challenging tests are standard methods used to determine *in vivo* insulin secretion and therefore to assess ß-cell function and response. They are mainly performed with an oral or intravenous administration of a glucose bolus, and the effects of endogenous insulin secretion on systemic glucose clearance are evaluated based on frequently blood sampling (CERSOSIMO et al., 2014). Alternatively, ß-cell function can be assessed by the hyperglycemic clamp (HGC) test,

where ß-cell function is assessed under maximal stimulatory conditions (DEFRONZO et al., 1979). After an overnight fasting, plasma glucose concentration is acutely raised by intravenous infusion of glucose, and hyperglycemia is constantly held by continuous glucose infusion. To maintain the desired hyperglycemic plateau, the glucose infusion is adjusted based on frequent plasma glucose measurements. Because the plasma glucose concentration is held constant, the glucose infusion rate is a measure of insulin secretion capacity and β-cell function. Under this constant hyperglycemia setup, non-diabetic subjects reveal a biphasic pattern of plasma insulin response with a first phase insulin response (approximately the first 10 min) characterized by an early burst of insulin, followed by a gradually progressive increase in plasma insulin concentrations (second phase) (DEFRONZO et al., 1979; CERSOSIMO et al., 2014).

Longitudinal studies on glucose tolerance upon intravenous glucose challenge indicate that insulin response is increased within the first weeks of pregnancy (12-14 week), achieving maximum levels during the third trimester, and returning to normal values after term (CATALANO et al., 1991; CATALANO et al., 1993). By the third trimester, both basal and postprandial insulin secretion levels are increased compared to non-pregnant state. This is accompanied by increased insulin secretion both in the 1st phase (time 0 to 5 min.) and 2nd phase (from 5 until end test) (CATALANO et al., 1991). Similarly, increased insulin response was observed during oral glucose tolerance tests in pregnant women, with a 120 % increased 1st phase insulin response during weeks 12-14 of gestation (BOWES et al., 1996). The capacity to compensate with increased insulin secretion during pregnancy is therefore an adaptive mechanism to maintain normoglycemia in pregnant women (BUTTE, 2000; DI CIANNI et al., 2003). Despite development of insulin resistance in healthy pregnant women, glucose tolerance is only slightly reduced and associated with moderately elevated postprandial glucose levels (CATALANO et al., 1991; CATALANO et al., 1993; BOWES et al., 1996). These are essential to provide sufficient glucose to the fetus during interrupted periods of energy intake (BUTTE, 2000; DI CIANNI et al., 2003; ANGUEIRA et al., 2015). The inability to compensate the increased demand for insulin during pregnancy underlies the pathophysiological mechanisms of development of GDM (BUCHANAN et al., 2007). Hence, the insulin producing β-cells must undergo several morphological and functional adaptions that are further discussed in 1.3.

1.2. Changes in lipid metabolism

Like glucose metabolism, lipid metabolism is also target of many physiological alterations during pregnancy. There is a great effort during the first two trimesters of pregnancy to promote accumulation of maternal lipid storages that can be later mobilized as energy source during the last trimester (BUTTE, 2000). Thus, morphological and functional changes occur at the level of adipocytes within the adipose tissue. There is an increase in adipose tissue expansion which is promoted by hypertrophy of fat cells (HERRERA, 2000; ROJAS-RODRIGUEZ et al., 2015). It is estimated that 3.3 kg of additional fat is stored within the first 15 weeks of normal, non-obese pregnancy (CLAPP et al., 1988). Concomitantly increased levels of pregnancy-related hormones and insulin are thought to facilitate the increased fat deposition in adipocytes by favoring lipogenesis and inhibiting lipolysis (HERRERA & DESOYE, 2016). In fact, the number of insulin receptors as well as insulin-mediated lipogenesis are increased in adipocytes in the first trimester of pregnancy (BAIRD, 1986) which is then reduced by the development of insulin resistance (SIVAN et al., 1999). Similar to glucose concentrations, circulating levels of triglycerides, non-esterified fatty acids, cholesterol and phospholipids are reduced within the first eight weeks of pregnancy. Cholesterol is mainly canalized to the placenta for the synthesis of steroid hormones while fatty acids serve as building blocks e.g. for the synthesis of new cell membranes (BUTTE, 2000). Upon initial reduction, circulating lipid metabolites are found elevated following the first eight weeks of pregnancy. This is mainly due to increased lipid synthesis in the liver and reduced clearance of lipids from the circulation due to a decreased activity of lipases, particularly hepatic lipase (SATTAR et al., 1997). In parallel with increased levels of total cholesterol and triglycerides, levels of lipoproteins are also found elevated with progression of pregnancy and remain elevated until delivery (HERRERA & DESOYE, 2016). There is an overall increase in circulating high-density lipoproteins (HDL), low density lipoproteins (LDL), very low density lipoproteins (VLDL) and triacylglycerols (SATTAR et al., 1997; BUTTE, 2000). Elevation of the lipoprotein content is in line with increased levels of cholesterol ester transfer protein (CETP) found by several studies (SILLIMAN et al., 1993; IGLESIAS et al., 1994; ALVAREZ et al., 1996). CETP catalyzes the transfer of triglycerides in exchange of cholesterol esters to HDL and LDL lipoproteins.

In the 3rd trimester of pregnancy, where most of glucose is shuttled to the fetal-placental unit to meet fetal energy demand, maternal metabolism relies on lipid content as a primary energy source. Thus, a switch from an anabolic state of building and storing lipid mass is replaced by a catabolic state of activation and utilization of the lipid reservoir (BUTTE, 2000; DI CIANNI et al., 2003). Hence, lipolysis and fat mobilization takes place, which is consistent with increased circulating free fatty acids (FFA), triglycerides and lipoproteins (HERRERA & DESOYE, 2016). Furthermore, in periods of prolonged (48 h) and shorter fasting (18 h), where most intermediates of carbohydrate metabolism are utilized for glucose production by gluconeogenesis, lipid oxidation is highly increased with accelerated production of ketone bodies. In fact, fatty acids and β-hydroxybutyrate products are increased in the circulation during fasting periods and are generated faster in pregnant women as compared to the non-pregnant state (METZGER et al., 1980; METZGER, 1991).

1.3. Pancreas morphology, β-cell mass and mechanisms of β-cell adaptation to pregnancy

During pregnancy, there is a continuous increase in insulin demand caused by the physiological endocrine-metabolic "insult" intrinsic to pregnancy. In order to respond to increased insulin secretion, the endocrine fraction of the pancreas is subject of major morphological and functional adaptions that contribute to enhanced insulin responsiveness. These major adaptions will be reviewed here mainly based on animal studies (especially mice) due to the obvious scarcity and heterogeneity among human samples.

The pancreas is the organ mainly responsible for glucose homeostasis, since it produces the hormone insulin which is necessary to regulate circulating glucose levels. Insulin is produced exclusively by the β-cells, those comprising one out of five endocrine cell types in the pancreas (α-cells secrete glucagon; PP-cells secrete pancreatic polypeptide; δ-cells secrete somatostatin and ε-cells secrete ghrelin (MARICHAL, 2010). β-cells can either be organized in cell clusters of pancreatic islets or in small numbers of dispersed β-cells within the pancreas known as isolated β-cells (BOUWENS & PIPELEERS, 1998; DOLENŠEK et al., 2015). β-cells are the most abundant cell type within the islets (with exception for birds), however, with great differences in cell type proportion distribution and cytoarchitecture among species (KIM et al., 2009; STEINER et al., 2010). Human islets have reduced β-cell content (60 % β-cells) and increased α-content (30 % α-cells) as compared to 70 % β-

cells and 20 % α-cells in murine islets, respectively (CABRERA et al., 2006). The cytoarchitecture of human islets is characterized by a heterogeneous β-cell distribution, while murine islets have a core of β-cells surrounded by the other cell types (BRISSOVA et al., 2005; CABRERA et al., 2006). Porcine islets resemble more human islets, where small β-cell clusters are scattered among the other endocrine cells types (CABRERA et al., 2006; STEINER et al., 2010).

In order to compensate for the increased insulin demand due to reduced insulin sensitivity, the endocrine pancreas must enhance insulin synthesis and secretion capacity by expanding β-cell mass. It is known that β-cells can change in number (hyperplasia) and size (hypertrophy) during periods of increased insulin demand such as growth, pregnancy, or obesity (RIECK & KAESTNER, 2010). A great number of studies indicate that murine islets show a 3 to 4-fold increase in β-cell mass during pregnancy and proliferation of existing β-cells is the predominant underlying mechanism. (PARSONS et al., 1992; SORENSON & BRELJE, 1997; RIECK et al., 2009; RETNAKARAN et al., 2016). Placental lactogens (SORENSON et al., 1993) and other regulatory hormones like serotonin (KIM et al., 2010) and menin (KARNIK et al., 2007) are involved in the regulation of β-cell mass during pregnancy in rodents. In humans, there is also evidence for an increased β-cell mass during pregnancy, although to a much smaller extent. Van Assche and co-workers reported a 2-fold increase in β-cell mass in five pregnant women (with an increase in number and size of β-cells) (VAN ASSCHE et al., 1978), whereas Butler et al. reported a smaller (1.4-fold) increase in β-cell mass in 18 pregnant women around gestational week 25 (BUTLER et al., 2010). The Butler study reported an increased number of small islets instead of enlargement of pre-existing islets with no changes in replication. Contrary to mice, where proliferation (PARSONS et al., 1992; RIECK et al., 2009) and hypertrophy (SORENSON & BRELJE, 1997; RIECK et al., 2009) of pre-existing islets were shown to be the preferential mechanisms of β-cell mass expansion, the data are contradictory in humans. The study by the Butler team shed light on the generation of new β-cells via neogenesis (generation of β-cells from non-differentiated precursors) since the increase in β-cell numbers resulted from newly formed islets as well as increase in insulin positive duct cells (BUTLER et al., 2010). In line with this finding a study conducted by Kou et al. on 72 pancreata obtained from autopsies of individuals without a history of pancreatitis or any type of diabetes (not involving pregnancy) observed that islet number rather than islet size was the main parameter

determining β-cell mass (KOU et al., 2014). Regulation of β-cell mass during pregnancy in the pig remains to be investigated.

1.4. Metabolic adaptions to pregnancy in large animal models with focus on the pig

Experimental animal models that naturally depict the most common metabolic features of human pregnancy are of great value. They allow not only a comprehensive understanding of the physiological metabolic processes during pregnancy, but also the study of pregnancy complicated by metabolic disorders such as diabetes. Like humans, pigs use glucose as the major energy substrate for the fetus (FORD et al., 1984; PERE, 1995, 2001). This is also true for other large animals like cows (FERRELL et al., 1983) and sheep (CHRISTENSON & PRIOR, 1978; MESCHIA et al., 1980). Blood samples, collected at the end of gestation revealed fetal glucose extractions of 0.3 mmol/L (PERE, 1995, 2001), 0.2 mmol/L (FOWDEN et al., 1997) and 0.32 mmol/L (COMLINE et al., 1979) in pigs. These values are comparable with fetal glucose extractions found in two human studies: 0.38 mmol/L (HOLME et al., 2015) and 0.34 mmol/L (KUO, 1991), but lower than one report 0.6 mmol/L in humans (METZGER et al., 1985).

Regarding fasting glucose levels during pregnancy in sows, most studies report no alterations in FPG between the pregnant and non-pregnant state (AHERNE et al., 1969; REYNOLDS et al., 1985; DUEE et al., 1987; SIMOES NUNES et al., 1987; PÈRE et al., 2000; PÈRE & ETIENNE, 2007). Glucose-lowering effects of insulin, measured upon ingestion of a meal, were progressively reduced in primiparous sows, at mid pregnancy (59 days) and the end of pregnancy (106 days) resulting in significantly increased postprandial glucose levels, as compared to non-pregnant controls (PÈRE & ETIENNE, 2007). Pregnancy-related reduced glucose tolerance in primiparous sows is similar to observations in healthy pregnant women (KUHL, 1991; PIVA et al., 1991; HOMKO et al., 2001). Fasting insulin concentrations were reported to be unaltered in pregnant sows vs. non-pregnant controls (BOUILLON HAUSMAN, 1986; SCHAEFER et al., 1991), while some studies observed decreased fasting insulin during pregnancy (SIMOES NUNES et al., 1987; PÈRE et al., 2000). Insulin sensitivity is also altered during pregnancy in pigs. Studies in pregnant sows at term, using hyperinsulinemic-euglycemic clamps (PÈRE & ETIENNE, 2007), oral glucose tolerance tests (PÈRE & ETIENNE, 2007) and intravenous glucose tolerance tests (GEORGE et al., 1978; SCHAEFER et al., 1991; PÈRE & ETIENNE, 2007),

demonstrated that, like in humans, pigs showed a slight reduction in glucose tolerance (more pronounced at end of pregnancy), together with a decreased insulin sensitivity. In healthy pregnant women insulin resistance and elevated insulin secretion immediately return to the normal state postpartum (HOMKO et al., 2001; MAZAKI-TOVI et al., 2011), whereas in pigs, insulin resistance and impaired glucose tolerance are still present throughout lactation and normalize after weaning (PÈRE & ETIENNE, 2007). In fact, development of insulin resistance in pregnancy seems to be a conserved metabolic adaptation among species occurring not only in pigs, but also in sheep (DUEHLMEIER et al., 2013), goats (DEBRAS et al., 1989), dogs (CONNOLLY et al., 2004), rats (LETURQUE et al., 1984; LETURQUE et al., 1986; ROSSI et al., 1993) and mice (MUSIAL et al., 2016). Of relevance, the degree of glucose tolerance in pregnant pigs can be influenced by the type of diet (VAN DER PEET-SCHWERING et al., 2004; CORSON et al., 2008b; METGES et al., 2014). Sows fed from day 85 of gestation a standard diet containing non-starch polysaccharides supplemented with 164 g of fat (soybean oil) revealed increased body mass, back fat gain and decreased glucose tolerance during lactation as compared with pregnant controls fed a standard diet (VAN DER PEET-SCHWERING et al., 2004). In addition, Metges et al. demonstrated that pregnant sows (gestation day 84) fed a high protein/low carbohydrate diet were more insulin resistant compared with sows fed an isoenergetic low protein/ high carbohydrate diet and sows fed an isoenergetic standard diet. It was suggested that low carbohydrate fed sows, adapted to a dietary deficit in glucose by increasing insulin resistance, glucagon concentrations, decreasing glucose oxidation, and stimulating gluconeogenesis (METGES et al., 2014). Thus, diet supplementation in the pig during pregnancy can be used to manipulate maternal glucose metabolism towards an aggravated phenotype of impaired glucose tolerance, as seen in obese pregnant women (CATALANO et al., 1999).

Similar to humans, increased lipid mobilization towards the end of pregnancy is also observed in pregnant sows which revealed elevated circulating FFA near term (day 110) (PÈRE et al., 2000; PÈRE & ETIENNE, 2007). However, compared to humans, hyperlipidemia appears later in pigs, and is greater during the lactation period coinciding with the more pronounced insulin resistance phenotype (PÈRE & ETIENNE, 2007). Altogether, despite some differences, physiological metabolic adaptations to pregnancy in pigs resemble those in humans, making the pig a relevant animal model of human pregnancy that can be used to investigate molecular

mechanisms underlying pregnancy complicated by diabetes and its deleterious consequences for the offspring.

2. Maternal diabetes: preconceptional and gestational diabetes mellitus

2.1. Definition, prevalence and diagnostic criteria

Pregnancy is often defined as a transitory "diabetogenic" event (DI CIANNI et al., 2003). This is due to its intrinsic physiological alterations in glucose-insulin metabolism which resemble complications seen in diabetes. However, contrary to the diabetic state, healthy pregnant women still maintain circulating glucose levels within physiological ranges that are defined by specific glycemic threshold values (BUTTE, 2000; HADDEN & MCLAUGHLIN, 2009). Two forms of maternal diabetes are known: (1) preconceptional diabetes mellitus (PCDM) that includes pregnant women previously diagnosed either with type 1 or type 2 diabetes and (2) gestational diabetes mellitus (GDM), defined as "any degree of glucose intolerance with an onset or first recognition during pregnancy," particularly arising around the 24th week of gestation (ADA, 2016). Both types of maternal diabetes are considered as a high-risk condition for the mother and the child. The International Diabetes Federation (IDF) reported that over the year 2015, 20.9 million (16.2 %) of 129.4 million live births (to women aged 20-49 years) were affected by hyperglycemia. Of those, 85.1 % were due to GDM, 7.4 % due to other types of diabetes first detected during pregnancy and 7.5 % due to diabetes detected prior to pregnancy (IDF, 2015). The prevalence of maternal diabetes is related to ethnicity, with the highest prevalence in South-East Asia (24 %), Middle East and North Africa (21.8 %), and much influenced by the socioeconomic status, with 87.6 % prevalence in low and middle income countries. In Europe the prevalence is 13.7 % with 1.7 million live births affected in 2015 (IDF, 2015).

The same diagnostic criteria used to identify individuals in the population with diabetes (either type 1 or type 2) are applied to diagnose women with PCDM. Those consist of defined threshold values for specific diagnostic criteria. Thus, diabetes mellitus, is diagnosed when one of the following criteria is met: (1) FPG (with fasting being defined as non-caloric intake for a period of at least 8 h) $\geq$ 126 mg/dl, or (2) postprandial glucose (PG) levels two hours after a standardized oral glucose tolerance test (in humans a glucose load using 75 g anhydrous glucose dissolved in water) $\geq$ 200

mg/dl, or (3) glycated hemoglobin (HbA_{1c}), a monitoring parameter of long-term blood glucose is ≥ 6.5 % (ADA, 2016).

For decades there has been an extensive debate on defining clear cutoff criteria for GDM (HOUSHMAND et al., 2013; MCINTYRE et al., 2015). This was due to, heterogeneous criteria applied to define glucose tolerance in pregnancy (which initially focused primarily on maternal risk to develop type 2 diabetes rather than poor pregnancy outcomes), and second, to some uncertainty to which extent milder degrees of hyperglycemia during pregnancy impact maternal and neonatal outcomes. As a result of the current epidemic rise in obesity that has led to more cases of women in childbearing age with undiagnosed type 2 diabetes (LAWRENCE et al., 2008), it became mandatory to redefine GDM criteria. Furthermore, a large multinational cohort study, the Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) study, was performed to clarify the impact of minor degrees of hyperglycemia (FPG 75 to 100 mg/dL; 1h PG 106-212 mg/dL; 2h PG 90-178 mg/dL) during pregnancy on maternal/offspring adverse outcomes (GROUP et al., 2008). The study was conducted on a large group of 25.000 pregnant women undergoing a 75-g oral glucose tolerance test at 24-28 weeks of gestation. From this study, a continuous association between the degree of maternal glycemia and the risk of adverse maternal, fetal and neonatal outcomes was demonstrated (GROUP et al., 2008). Importantly, the risk association of maternal hyperglycemia with adverse neonatal outcomes was reported in maternal glycemia degrees less severe than overt diabetes mellitus (GROUP et al., 2008), and was independent of maternal obesity (which in combination with GDM aggravated the risk for deleterious outcomes) (CATALANO et al., 2012). Taking these considerations into account, the American Diabetes Association (ADA) redefined the diagnostic criteria for GDM as follows. If women at their initial prenatal visit present risk factors for type 2 diabetes, (those including Body Mass Index (BMI) ≥ 25 kg/m^2 with additional risk factors like physical inactivity, family history of diabetes, high risk race/ethnicity, hypertension ≥ 140/90 mmHg or undergoing hypertension therapy, HDL cholesterol levels < 35 mg/dL and/or triglyceride levels ≥ 250 mg/dL, history of cardiovascular diseases, polycystic syndrome, HbA_{1c} levels ≥ 5.7 %, or any other clinical condition associated with insulin resistance previously diagnosed) they should be tested for diabetes mellitus using the standard diagnostic criteria. If diabetes is confirmed during the 1st trimester, women should be classified with type 2 diabetes. If women do not present any of the risk factors above, they should be diagnosed for GDM

within weeks 24-28 of gestation (ADA, 2016). However, as a result of the HAPO study, the GDM diagnostic criteria differ and are stricter as compared to the standard type 1 and 2 criteria. Thus, GDM diagnostic criteria can be accomplished following either a one-step or a two-step screening approach. A one-step approach involves performing a 75-g oral glucose tolerance test within weeks 24-28 of gestation with samples collected at fasting, 1-h and 2-h following the glucose load. Women are diagnosed with GDM when any of the following parameters are met: FPG ≥ 92 mg/dL, or 1-h PG ≥ 180 mg/dL or 2-h PG ≥ 153 mg/dL (threshold values defined according to the International Association of the Diabetes Pregnancy Study Groups) (INTERNATIONAL ASSOCIATION OF et al., 2010; ADA, 2016). In a two-step approach, step one consists of a 50 g glucose load test (non-fasting), with plasma measurements at 1-h post glucose load. If plasma glucose levels are ≥ 140 mg/dL, they proceed to step two with a 100 g oral glucose tolerance test (OGTT) (plasma measurements at fasting, 1-h, 2-h and 3-h after OGTT). The diagnosis of GDM is confirmed if at least two of the following parameters are met: FPG ≥ 95 mg/dL, 1-h PG ≥ 180 mg/dL, 2-h PG ≥ 155 mg/dl and 3-h PG ≥ 140 mg/dL (threshold values defined by Carpenter/Coustand) (CARPENTER & COUSTAN, 1982; ADA, 2016) or FPG ≥ 105 mg/dL, 1-h PG ≥ 190 mg/dL, 2-h PG ≥ 165 mg/dl and 3-h PG ≥ 145 mg/dL (threshold values defined by the National Diabetes Data Groups) (GROUP, 1979; ADA, 2016). The decision for the one-step or two-step procedure is of debate (VANDORSTEN et al., 2013). The one-step procedure using the threshold criteria from the IADPSG has been adopted internationally and therefore, it is the preferential approach (DURAN et al., 2014). Nevertheless, it has been under discussion, since these criteria raise the prevalence of women diagnosed with GDM, and it is not yet clear if this results in an overestimation of the number GDM cases with need for medical intervention and costs (VANDORSTEN et al., 2013). The two-step approach is the preferential route in the US which has updated its guidelines in 2013 by The American College of Obstetricians and Gynecologists (COMMITTEE ON PRACTICE, 2013). Long-term outcome studies using both approaches are needed and are currently ongoing to commit to an internationally applicable diagnostic regimen (ADA, 2016).

2.2. Consequences of maternal diabetes

Human epidemiological and experimental animal studies demonstrate that intra-uterine exposure to hyperglycemia, regardless of which type of maternal diabetes, has

short- and long-term negative consequences for both the mother and the offspring (FETITA et al., 2006; FRASER & LAWLOR, 2014; MCCANCE, 2015). Thus, maintenance of normoglycemia in pregnancy is imperative for reducing adverse maternal, fetal and neonatal outcomes. In PCDM, the onset of diabetes is prior to gestation and therefore, poorly maternal glycemic control (especially within the 1st trimester) has great impact on early embryonic development (ORNOY et al., 2015). A systematic meta-analysis review, involving 14.099 women with type 1 diabetes and 4.035.373 women from background population revealed a 2-to 5-fold increased risk for the development of congenital malformations, perinatal mortality, preterm delivery and large for gestational age births in women with type 1 diabetes (COLSTRUP et al., 2013). Moreover, despite generally milder glycemic impairment in women with type 2 compared with type 1 diabetes, both diabetes types showed poor pregnancy outcomes in a systematic meta-analysis (BALSELLS et al., 2009). In fact, women with type 2 diabetes showed higher risk of perinatal mortality compared to type 1 diabetic women and were not significantly different from type 1 women in rates of congenital malformations, stillbirth and neonatal mortality (BALSELLS et al., 2009). In the case of GDM, the rate of fetal congenital malformations is not increased compared with normal pregnancy (SCHAEFER et al., 1997; FARRELL et al., 2002). Nevertheless, despite lower impairment in glucose control compared to type 1 and type 2 diabetic women, several deleterious consequences for both mother and offspring are known. Macrosomia, defined as weight at birth >4.000 g (LUBCHENCO, 1970; KC et al., 2015), occurs in 30 % of the neonates of diabetic mothers (UVENA-CELEBREZZE & CATALANO, 2000) and a linear relationship between this outcome and the degree of maternal hyperglycemia has been supported by different studies, including pregnancies complicated by GDM (HILL et al., 2005; YOGEV et al., 2005; GROUP et al., 2008). Macrosomia is usually accompanied by disproportional growth of shoulders and abdomen in comparison to the head, thus contributing to serious obstetric complications such as bone injuries, shoulder dystocia, increased emergency cesarean section and assisted deliveries (KC et al., 2015). As a result of an intrauterine hyperglycemic environment, fetal hyperinsulinemia was long hypothesized (PEDERSEN, 1954) and is in line with findings of elevated cord blood C-peptide levels in GDM offspring (GROUP et al., 2008) and increased insulin levels in the amniotic fluid of both PCDM and GDM fetuses (SILVERMAN et al., 1995).

In addition to deleterious neonatal outcomes, both PCDM and GDM have an impact beyond the perinatal period. Studies with Pima Indians, a population with the highest prevalence of type 2 diabetes (KNOWLER et al., 1978), were the first to demonstrate an increased incidence of type 2 diabetes in the offspring as a consequence of exposure to a hyperglycemic intrauterine environment (DABELEA et al., 2000; LINDSAY et al., 2000). In the Pima Indian population, offspring of diabetic mothers had an increased incidence of type 2 diabetes compared to the offspring of type 2 diabetic fathers (LINDSAY et al., 2000). Another study conducted in siblings of Pima Indians, in which one of the siblings was born before maternal development of type 2 diabetes, revealed 45 % higher incidence of diabetes in the offspring whose mothers were diabetic during pregnancy as compared to 9% incidence for those siblings where the mother became diabetic after pregnancy (DABELEA et al., 2000). Because the Pima Indian population is particular in terms of small genetic variability (WILLIAMS et al., 1992) and since the development of type 2 diabetes has partially a genetic contribution (MORRIS et al., 2012), the impact of hyperglycemia *in utero* could still be argued. Using individuals from populations with higher genetic variability, increased impairment of glucose tolerance and reduced insulin secretion was demonstrated in adult offspring from type 1 diabetic mothers (without any presence of islet-autoantibodies of type 1 diabetes) as compared to offspring of diabetic type 1 fathers (SOBNGWI et al., 2003). Like in PCDM, offspring of GDM mothers also have a greater risk to develop metabolic complications during childhood and adult life. A follow-up study in offspring (age 18-27) of GDM, revealed reduced insulin sensitivity, β-cell function and glucose tolerance compared with the offspring of background population (KELSTRUP et al., 2013). Moreover, the prevalence of IGT and type 2 diabetes in offspring (age 18-27) of diet-treated GDM mothers was 26 % compared with 4 % in the offspring from the background population (CLAUSEN et al., 2008). Earlier reports also support an increased prevalence of IGT (CLAUSEN et al., 2009), or of IGT and type 2 diabetes (SILVERMAN et al., 1995; PLAGEMANN et al., 1997) in offspring of GDM mothers.

2.3. Animals models of maternal diabetes

Diabetes in pregnancy results in an unfavorable hyperglycemic intrauterine environment for the growing fetus, thus supporting the onset of maternal, fetal, neonatal and perinatal complications. With the current epidemic rise in obesity in both economically favorable and unfavorable countries, and the increased number of

women developing GDM and type 2 diabetes it is imperative to understand the pathophysiological consequences of maternal hyperglycemia and to develop appropriate disease prevention/treatment strategies. Due to obvious ethical reasons, human studies, don´t allow to fully explore the molecular mechanisms underlying maternal diabetes and poor offspring outcomes. Therefore, animal models, in particular large animal models, are of great value as they can bridge the gap between rodent models and humans (WOLF et al., 2014; RENNER et al., 2016a). Different methods can be applied to induce hyperglycemia prior/during pregnancy, and according to the onset, they intended to mimic PDCM (hyperglycemia before or during early pregnancy with either features of type 1 or type 2 diabetes) or GDM (hyperglycemia in late phase of pregnancy with mild maternal glycemia) (JAWERBAUM & WHITE, 2010; PASEK & GANNON, 2013). Pancreatectomy was the first method used to induce maternal diabetes. This method involves partial or total removal of the pancreas by surgery, resulting in corresponding degrees of diabetes. As a highly invasive procedure, it generates a high degree of inflammation with high post-surgical mortality rates and is therefore not commonly used anymore (PASEK & GANNON, 2013). More recently, other methods such as chemical targeted ablation of β-cells (either with streptozotocin or alloxan), diet-induced diabetes and genetically engineered animal models became preferential strategies. Chemically induced maternal diabetes is described in multiple species including rats, mice, rabbits, sheep and pigs (JAWERBAUM & WHITE, 2010; PASEK & GANNON, 2013). This strategy has been extensively used with dose administrations either before pregnancy or right after mating in rodents as to evaluate congenital malformations potentially induced by hyperglycemia (SIMAN et al., 2000; HIGA et al., 2007; MORGAN et al., 2008; SUGIMURA et al., 2009). In addition, dose titration of these compounds, type and route of administration, animal strain and age are all variables that play a role in inducing either mild maternal hyperglycemia (FPG levels within 117 – 176 mg/dl) or severely maternal hyperglycemia (FPG levels > 200-360 mg/dl) during pregnancy (DAMASCENO et al., 2013). Rodent studies using chemically-induced-mild-maternal diabetes resulted in β-cell hyperplasia, increased pancreatic insulin content and elevated *in vivo* insulin secretion in fetuses evaluated at term (KERVRAN et al., 1978; BIHOREAU et al., 1986a). However, adult animals exposed to mild maternal hyperglycemia were glucose intolerant due to a reduced glucose-induced insulin secretion (BIHOREAU et al., 1986b; AERTS et al., 1988; GAUGUIER et al., 1991).

Distinct effects were observed in neonates from chemically-induced severe maternal hyperglycemia. In these animals, although β-cell mass was increased, the islets contained few insulin granules suggesting insulin secretion exhaustion (AERTS et al., 1990) In adulthood, β-cells were hyperactive and secreted more insulin however, insulin sensitivity was reduced (AERTS et al., 1990). In a study of chemically-induced maternal diabetes in pigs, hyperglycemia in the late phase of pregnancy impacted the energy status of the offspring. Piglets born to diabetic sows showed increased liver weight together with increased liver protein content as well as higher glycogen and lipid content. No differences were reported for litter size and birth weights of the piglets (EZEKWE et al., 1984). Chemical induction of maternal diabetes has been widely used, particularly in mice, and these experiments reflected important proof of principle studies in which impaired maternal glucose metabolism may be transmitted to the offspring by exposure to hyperglycemia *in utero*. Nevertheless, it is a very artificial method in which the natural etiology of the disease is omitted and side effects with cytotoxicity in other organs cannot be excluded (LENZEN, 2008).

Genetic engineering of important genes involved in glucose metabolism is a strategy that has generated a great number of diabetic animal models (type 1 and 2), particularly in rodents. However, only a small number of studies used this strategy in the context of maternal diabetes and until present, all these studies were performed in rodents. Genetically engineered strains like the non-obese diabetic (NOD) mice (KOLB, 1987) and Akita mouse (YOSHIOKA et al., 1997) are examples of type 1-like diabetes models, hence being used to investigate consequences of PCDM in the offspring. Wild-type embryos transferred into diabetic NOD mice show an increased rate of congenital embryo malformations and oocytes of diabetic NOD mice show a higher number of chromosomal abnormalities (OTANI et al., 1991). Macrosomia and increased insulin content have been reported in the offspring of NOD mice (FORMBY et al., 1987). Wild-type offspring born to Akita mice show metabolic alterations including reduced glucose tolerance, decreased body weight (BW) and bone mineral density, and those alterations are more pronounced in male offspring (GRASEMANN et al., 2012). Other mouse models were generated by targeting genes which play important roles in β-cell expansion during pregnancy. Those include the global heterozygous prolactin receptor deficient mice (*Prlr*$^{+/-}$) (HUANG et al., 2009), the conditional knockout c-Met in the pancreas (PancMet KO) (DEMIRCI et al., 2012), the global conditional serotonin receptor knockout (*Htr2b*$^{-/-}$) mice (KIM et al., 2010),

the conditionally overexpressed menin in β-cells (βMen1) (KARNIK et al., 2007) and the depleted Forkhead box D3 (FOXD3) transcriptional factor (*Foxd3*$^{fl/-}$) mice (PLANK et al., 2011). These animals maintain normal glucose levels before pregnancy and β-cell mass is preserved (with exception for the *Prlr*$^{+/-}$ and *Foxd3*$^{fl/-}$ mice in which β-cell mass deterioration occurs already before pregnancy). With progression of pregnancy, β-cell expansion as a compensatory mechanism is impaired, so the animals render diabetic during pregnancy. Although these models have contributed to a better understanding of the role of specific factors and signaling pathways in β-cell expansion in murine pregnancy, most have not explored consequences of maternal diabetes in offspring. A recent study using a mouse model of maternal insulin resistance that develops transient hyperglycemia during pregnancy due to a liver-specific knockout of the insulin-receptor (LIRKO mouse model), showed metabolic alterations in the wild-type offspring of diabetic mothers during post-natal life. Those included low birth weight followed by rapid weight gain. Plasma concentrations of glucose and insulin were increased. In addition, the offspring developed alterations in β-cell mass and increased fat deposition as shown by an increase in adipocyte size (KAHRAMAN et al., 2014).

Finally, nutrition manipulation can be used to alter maternal metabolism during pregnancy. In humans, obesity is a well described risk factor of type 2 diabetes and GDM (DABELEA & CRUME, 2011). In the context of maternal diabetes, nutrition manipulation has been extensively used in rodent models, and to a smaller extent in large animal models, to describe the impact of environmental factors on metabolic changes in the offspring and the development of type 2 diabetes later in life (WILLIAMS et al., 2014). In mice, high fat diet (HFD) has been shown to impair maternal food intake, body composition, glucose and lipid metabolism, leptin concentrations and placental nutrient transport (WILLIAMS et al., 2014). In the offspring, HFD leads to hypertension, insulin resistance, dyslipidemia and hepatic steatosis and even the “transmission” to type 2 diabetes in later offspring generations as well as behavioral and mental changes (WILLIAMS et al., 2014). Dietary interventions using HFD have also been used in large animal models. HFD before and throughout pregnancy in ewes induced maternal insulin resistance and increased plasma glucose levels at mid gestation (FORD et al., 2009). Offspring from these pregnancies were evaluated at fetal stage (75 days of gestation) and exhibited increased pancreas weight and β-cell mass with increased β-cell proliferation (FORD

et al., 2009). Another study with diet-induced obesity in sheep reported early placental inflammation with changes in fetal lipid metabolism like increased circulating fatty acids, triglycerides and cholesterol (ZHU et al., 2010). Due to their value in the food chain, dietary studies were performed in sows during pregnancy as to improve piglet birth performance and to optimize the natural variance in birth weights in piglets within the same litter (ANDERSON et al., 1971; AVERETTE et al., 1999). A study using diet supplementation with different oil types during gestation in sows demonstrated larger birth weights, when the diet was supplemented with palm oil or olive oil (LAWS et al., 2007). Using the same oil supplementation principle, Corson et al. showed that maternal glucose tolerance is decreased upon HFD-feeding during gestation, however these effects were seen only in the group feed in the first half of gestation (CORSON et al., 2008b). Reduced glucose tolerance as a consequence of HFD-feeding was also observed by others (VAN DER PEET-SCHWERING et al., 2004).

In summary, different approaches in different animal species have been used to induce diabetes prior to/during pregnancy proving that experimental animals are valid and valuable tools to understand the mechanisms by which maternal hyperglycemia modulates fetal metabolism and perpetuates metabolic dysfunctions in the offspring. Nevertheless, the majority of the understanding comes from rodent studies, which cannot be directly translated into human patients. The pig is an excellent and well-established model in diabetes research, as several aspects of the human glucose metabolism, including physiological alterations in pregnancy are depicted in pigs (GEORGE et al., 1978; PERE, 1995; FOWDEN et al., 1997; PÈRE & ETIENNE, 2007; WOLF et al., 2014). In addition, for evaluating the impact of maternal hyperglycemia at a phenotypical and molecular level in the offspring, pigs become a much more suitable model compared to mice. Not only the fetal developmental stages in pigs resemble more the developmental stages in humans, the last fetal developmental stage (which accounts for period of great maternal metabolic changes impacting the fetus), occurs *in utero* in both pigs and humans, whereas this phase occurs postnatally in mice (LITTEN-BROWN et al., 2010).

The aim of present study was to first generate and characterize a novel genetically engineered porcine model of mild diabetes, characterize glucose control and insulin

sensitivity of this model during the pregnant state and evaluate effects of *in utero* exposure to mild maternal hyperglycemia on the offspring.

III. ANIMALS, MATERIAL AND METHODS

1. Animals

Animals included in this study were hemizygous transgenic pigs expressing the mutant insulin C93S as well as age-matched non-transgenic controls on a German Landrace-Swabian Hall background. In most cases littermate controls were used. During the whole study pigs were housed under controlled conditions in planar pens covered with straw, had *ad libitum* access to water and were fed a commercial diet Table 1. All animal experiments were approved by the responsible animal welfare authority (Regierung von Oberbayern, AZ 55.2-1-54-2531-26-06 and 55.2-1-54-2532-68-11).

Table 1: Diet composition

	Piglets up to 25 kg	Juvenile and adult pigs
MJ ME/kg	14.6	13.8
Crude protein %	21.7	21.5
Crude fat %	3.2	3.3
Crude ash %	6.3	6.6
Crude fiber %	5.6	7.4
Calcium %	9.2	8.0
Phosphorus %	5.8	5.4
Sodium %	2.4	1.8
Magnesium %	3.1	2.4

ME: metabolized energy

2. Materials

2.1. Chemicals

Acetic Acid (glacial)	Roth, Karlsruhe
Agarose UltraPure™	Invitrogen, Karlsruhe
Braunol®	B. Braun, Melsungen
Bromophenol Blue	Roth, Karlsruhe
1.4-Dithiothreitol (DTT)	Biomol GmbH, Hamburg
EDTA (Ethylenediaminetetraacetic acid)	Roth, Karlsruhe and VWR, Darmstadt

Ethanol	Roth, Karlsruhe
Ethidium bromide (1mg/ dL)	Merck, Darmstadt
Glycerin (Glycerol)	Roth, Karlsruhe
Glucosteril® 50% Glucose solution	Fresenius Kabi, Standort Neufahrn
H_2O_2 (Hydrogen peroxide)	Roth, Karlsruhe
HCl (Hydrochloric acid)	VWR, Darmstadt
Histokitt	Glaswarenfabrik Karl Hecht
$MgCl_2$ (Magnesium chloride)	Fluka Chemie, Schweiz
Mayer‘s Hemalum solution	Merck, Darmstadt
$Na_3C_6H_5O_7$ (Sodium citrate)	Merck, Darmstadt
NaCl (Sodium chloride)	Merck, Darmstadt
Na_2HPO_4 (Di-sodiumhydrogenphosphate-2-hydrate)	Merck, Darmstadt
NaOH (Sodium hydroxide)	Roth, Karlsruhe and VWR, Darmstadt
Roti-Histofix 4 %	Roth, Karlsruhe
Sodium chloride solution (0.9%)	B. Braun, Melsungen
Tris (Tris-(hydroxymethyl) aminomethane)	Roth, Karlsruhe
Xylene	Applichem GmbH, Darmstadt

2.2. Consumables

Adhesive tape	Tesa SE, Hamburg
Adhesive tissue tape	Henry Schein® Vet GmbH, Hamburg
Aluminium spray	CP – Pharma, Burgdorf
Careflow® 3 Fr, 200 mm	Argon Medical Devices, USA
Catheter stopper with injection cap	Fresenius Kabi, Standort Neufahrn
CBAS® Heparin Coated Clear PU Cath 7Fr	Access Technologies, USA
Combitips® plus (2.5 mL, 10 mL)	Eppendorf, Hamburg
Cover slips (24x40 mm)	VWR International GmbH, Darmstadt
Discofix® multi-way cock system	B. Braun, Melsungen

Discofix® 3-way stop-cock with connection line (10 cm)	B. Braun, Melsungen
Disposable syringes (2, 5, 10, 20 mL)	Henry Schein® Vet GmbH, Hamburg
Gauze pads (7.5 cm x 7.5 cm)	Hartmann, Austria
Neolus® hypodermic needles (30 G)	Terumo, Eschborn
Falcon® centrifuge tubes (15, 50 mL)	Becton Dickinson, Heidelberg
Fixomull stretch	BSN medical GmbH, Hamburg
FreeStyle Precision® glucose stripes	Abbott, USA
Hypodermic needles (18 G, 20 G)	Henry Schein® Vet GmbH, Hamburg
Monovette® blood collection system (Plasma, EDTA, 9 mL)	Sarstedt, Nümbrecht
Nylon membrane (Nylon-N+)	GE Healthcare, UK
OP-Cover (60 x 90 cm)	A. Albrecht, Aulendorf
Original Perfusor® Line (50cm)	B. Braun, Melsungen
Original Perfusor® syringes (50 mL)	B. Braun, Melsungen
Parafilm® M	American Can Company, USA
PCR reaction tubes (0.2 mL)	Braun, Wertheim
Pipette tips with filter	Axygen Inc., USA
SafeGrip® latex gloves	SLG, Munich
Scalpel blade sterile No.36	Medicon eG, Tuttlingen
Sempermed® supreme latex OP gloves	Sempermed, USA
Sephadex G-50 columns	GE Healthcare limited, Munich
Skin adhesive spray	A. Albrecht, Aulendorf
Star Frost® microscope slides	Engelbrecht, Edermünde
3-way-stopcock Variostop®	Clinico GmbH, Bad Hersfeld
Surgibond tissue glue (SMI)	SMI AG, Belgium
Surgicryl® suture material (USP 2-0 without needle)	SMI AG, Belgium
Surgicryl suture (USP 2/0 with needle DS30)	SMI AG, Belgium
Surgicryl suture (USP 2/0 with needle DS36)	SMI AG, Belgium

Test tube peg wrack	Polylab, India
Uni-Link embedding cassettes	Engelbrecht, Edermünde

2.3. Devices

Agarose gel electrophoresis chamber	OWL Inc., USA
ART-Micra D-8 tissue-homogenizer	ART, Müllheim
AU 480 analyzer	Beckman & Coulter, USA
Benchtop 96 tube working rack	Stratagene, USA
BX41 light microscope	Olympus, Hamburg
DP72 video camera	Olympus, Hamburg
FreeStyle Precision® neo glucometer	Abbott, USA
Gel documentation system	Bio Rad, Munich
Genome Analyzer IIx, Illumina®	Illumina®, USA
HemoCue® Glucose 201+ Glucometer	Radiometer GmbH, Willich
Hybrid mini 38 hybridisation oven	H. Saur, Reutlingen
HM 315 microtome	Microm, Walldorf
Injectomat® MC Agilia, infusion pumps	Fresenius Kabi, Bad Homburg
Incubator 37 °C	Wagner + Munz, Munich
Incubator 60 °C	Memmert, Schwabach
inoLab® pH meter 7110	WTW, Weilheim
LB 2111 γ-counter	Berthold, Bad Wildbad
Object micrometer	Zeiss, Oberkochen
Mastercycler® gradient	Eppendorf, Hamburg
Microwave	DAEWOO, Korea
Multichannel pipette mLine® (300 µl)	Sartorius, USA
Multipipette® plus	Eppendorf, Hamburg
NanoDrop ND-1000 spectrophotometer	NanoDrop Technologies, USA
Pipettes (1000 µl, 200 µl, 100 µl, 10 µl, 2 µl)	Gilson Inc., USA
Power Pac 300 gel electrophoresis unit	Bio Rad, Munich
RH Basic heating plate with magnetic stirrer	IKA, Staufen
Shandon Citadel tissue processor 1000	Thermo Fisher Scientific, Schwerte
Select vortexer	Select BioProducts, USA
Sony video graphic printer UP-895CE	Sony, USA

TBS 88 tissue embedding system	Medite, Burgdorf
Tecan infinite M200Pro ELISA reader	Tecan, Swizerland
Thermomixer 5436	Eppendorf, Hamburg
WB 6 water bath	Preiss-Daimler Group, Puschwitz
X-ray cassette	Rego, Augsburg

Centrifuges:

Eppendorf Centrifuge 5430 R	Eppendorf, Hamburg
Eppendorf Centrifuge 5810 R	Eppendorf, Hamburg
Heraeus Sepatech Megafuge 1.0R	Heraeus, Munich
Rotanta 460R	Hettich, Tuttlingen

Scales:

Analytic balance	Sartorius, Göttingen
Analytic balance MS 100	Schippers GmbH, Kerken
Kern EOB 15K5, animal balance	Kern und Sohn GmbH, Barlingen-Frommern

2.4. Antibodies and drugs

2.4.1. Antibodies

Polyclonal guinea pig anti-porcine insulin	Dako Cytomation, Hamburg
AP-conjugated goat anti-guinea pig IgG	Southern Biotech, USA

2.4.2. Drugs

Altrenogest (Regumate®)	Serumwerke Bernburg, Bernburg
Azaperon (Stresnil®)	Jansen Pharmaceutica, Belgium
Cefquinom (Cobactan® 2.5%)	Intervet, Unterschleißheim
Choriongonadotropine (hCG) (Ovogest®)	Intervet, Unterschleißheim
Cloprostenol (Estrumate-Schwein®)	Intervet, Unterschleißheim
Embutramid, Mebezoniumiodid, Tetracainhydrochlorid (T61®)	Intervet, Unterschleißheim
Insulin (Insuman® rapid)	Sanofi-Aventis Deutschland GmbH, Frankfurt
Isobar® Isoflurane	Intervet, Unterschleißheim

Ketamine hydrochloride (Ursotamin®)	Serumwerke Bernburg, Bernburg
Leukase® N Kegel	Dermapharm AG
Meloxicam (Metacam®)	Boehringer Ingelheim, Ingelheim
Pregnant Mare Serum Gonadotropin (PMSG) (Intergonan®)	Intervet, Unterschleißheim
Taurolock™ Hep 500, Catheter lock solution	TauroPharm GmbH, Winsen
Xylazine 2 %	WDT, Garbsen

2.5. Buffers and solutions

DNA loading buffer (10×):

10 % glycerol in distilled water

1 spatula tip of Bromophenol Blue

0.5 M NaOH until color turns blue

Aliquoted and stored at 4 °C

dNTP-mix:

2 mM dATP, dCTP, dGTP, dTTP

Mixed in distilled water

Aliquoted and stored at -20 °C

Southern blot church buffer:

1% BSA

1 mM EDTA, pH 8.0

500 mM sodium-phosphate buffer pH 7.2

7% SDS

Southern blot high stringency buffer:

1 mM EDTA, pH 8.0

40 mM sodium-phosphate buffer pH 7.2

1% SDS

Southern blot low stringency buffer:

0.5% BSA

1 mM EDTA

40 mM sodium-phosphate buffer pH 7.2

5% SDS

Southern blot neutralisation solution:

0.5 M Tris

1.5 M NaCl

pH 7.5

Southern blot strand break solution:

0.5 M NaOH

1.5 M NaCl

Southern blot 20x SSC:

0.3 M Na-Citrate

3 M NaCl

TAE buffer (50×):

242 g 2 M Tris

100 mL 0.5 M EDTA (pH 8.0)

57 mL glacial acetic acid

1000 mL distilled water

Filtrated and autoclaved for storage

Before usage diluted to single concentration

TBS buffer:

90 g NaCl

60.5 g Tris

1000 mL distilled water

pH adjusted to 7.6, autoclaved and diluted to single concentration before use

100 mM Tris HCl (pH 8.5):

12.114 g Tris

1000 mL distilled water

pH adjusted to 8.5, autoclaved

2.6. Kits

QiaexII Gel Extraction kit	Qiagen, Hilden
Porcine C-peptide ELISA kit	Mercodia, Sweden
Nexttec™ Genomic DNA Isolation Kit	Nexttec GmbH, Leverkusen
Porcine Insulin RIA Kit	Millipore, USA
RNeasy® Mini total RNA isolation Kit	Qiagen, Hilden
Vector® Red Substrate Kit (AP)	Biozol, Eching
Wizard genomic DNA purification Kit®	Promega, USA

2.7. Other reagents

BamHI restriction enzyme	Fermentas, St. Leon Roth
Bovine serum albumin (BSA)	Roth, Karlsruhe
DNA labeling α-[^{32}P]-dCTP	PerkinElmer, Netherlands
DNase I, RNase-free (1 U/µL)	Thermo Scientific, Schwerten
dNTPs (dATP, dCTP, dGTP, dATP)	MBI Fermentas, St. Leon Roth
Gene Ruler™ (1 kb DNA ladder)	MBI Fermentas, St. Leon Roth
Goat serum	MP Biomedicals, France
Klenow fragment exo$^-$ (5 U/µL)	Fermentas, St. Leon Roth
Lambda DNA *E*coRI + *Hind*III	Fermentas, St. Leon Roth
10 x PCR buffer	Qiagen GmbH, Hilden
Pig serum	MP Biomedicals, France
SuperScript® III Reverse Transcriptase (200 U/µL)	Thermo Scientific, Schwerten
Taq DNA Polymerase (5 U/mL)	Agrobiogen, Hilgertshausen

2.8. Software

Graph Pad Prism® version 5.02	GraphPad Software Inc., USA
Magellan™ data analysis version 7.2	Tecan, Swizerland
Olympus Visiomorph™ image analysis	Visiopharm, Denmark
LBIS immunoassay software version 3.3.0.0	Berthold Technologies GmbH, Bad Wildbad

3. Methods

3.1. Generation of *INS*^{C93S} transgenic pigs

3.1.1. Expression construct, SCNT and embryo transfer

INS^{C93S} transgenic pigs were generated by additive gene transfer into somatic cells followed by somatic cell nuclear transfer (SCNT) and embryo transfer. The expression vector designed by Dr. Nikolai Klymiuk and Dr. Christina Landbrecht-Schessl (Chair for Molecular Animal Breeding and Biotechnology, LMU Munich) consists of three major fragments: a 1.3-kb fragment of the porcine insulin promoter, a 1.0-kb fragment of the porcine full-length insulin gene sequence including the three exons and a neomycin resistance cassette (Figure 1). In exon 3 of the insulin gene, a T→A point mutation at nucleotide position 336 was inserted, leading to an amino acid exchange from cysteine to serine at position 93 in the amino acid sequence of the insulin protein and subsequently a loss of an intra-insulin-A-chain disulfide bond. The porcine insulin (*INS)* promoter is active exclusively in the β-cells of the pancreas (GRZECH et al., 2010). The expression vector was transfected into male porcine fetal fibroblasts of both the German Landrace and the Swabian-Hall pig breed. Selection of positive cell clones was performed in a G418 containing culture medium. Pools of stable transfected cell clones were used for SCNT using *in vitro* maturated oocytes as described in (KUROME et al., 2015). Briefly, a single donor cell was inserted into the perivitelline space of an enucleated oocyte, followed by fusion with electrical pulses. After culturing (1 to 2 days), the cloned embryos were transferred laparoscopically into the oviduct of estrus-synchronized surrogate gilts. In total, 503 cloned embryos were transferred into five recipients, of which two went to full term. Cell culture experiments were executed by Dr. Annegret Wünsch, SCNT and embryo transfer experiments were performed by Dr. Mayuko Kurome and Dr. Barbara Kessler (Chair for Molecular Animal Breeding and Biotechnology, LMU Munich).

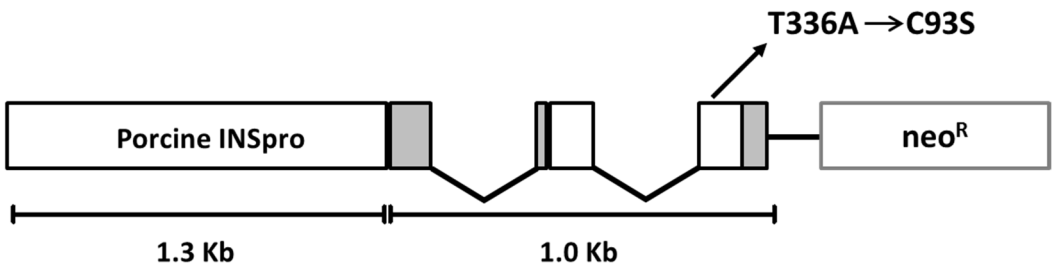

Figure 1: *INS*^{C93S} expression construct

The insulin gene (1.0-kb) is flanked by the porcine insulin promoter (1.3-kb) and the removable neomycin resistance cassette. Within the insulin gene fragment, boxes represent exons whereas connective lines between boxes represent introns. Unfilled boxes depict coding sequence regions (cds) whereas filled boxes represent untranslated regions (UTR) on the message RNA sequence. In the last exon the point mutation at nucleotide position 366 is indicated.

3.2. Identification of *INS*^{C93S} transgenic animals

Both polymerase chain reaction (PCR) and Southern blot analyses were used to identify *INS*^{C93S} transgenic animals. In addition, Southern blot analysis allowed to determine the number of integration sites of the *INS*^{C93S} transgene in the F0-generation and possible segregation events in the F1-generation.

3.2.1. Polymerase chain reaction (PCR)

3.2.1.1. Genomic DNA isolation from tails

Tail punches were obtained and stored at -20 °C. Genomic DNA was isolated using the "nexttec^{TM} Genomic DNA Isolation Kit from Tissue and Cells" (nexttec GmbH, Leverkusen) according to the manufacturer's instructions. Briefly, samples were cut in small pieces of a diameter < 1 mm and were incubated with an appropriate lysis buffer. Samples were incubated overnight at 60°C in a thermomixer. Lysates were then purified using nexttec^{TM} clean columns to elute purified DNA.

3.2.1.2. PCR reaction

Previous purified DNA was amplified in a PCR reaction. The following specific primers directed to the sequence of the neomycin resistance cassette were used to differentiate between transgenic and non-transgenic pigs:

NeoP(f): 5′-CTG TGC TCG ACG TTG TCA C-3′
NeoS(r): 5′-GAA GAA CTC GTC AAG AAG GCG ATA G-3′

In addition, a control PCR using ß-actin (ACTB) was run in parallel with the same samples as loading and DNA integrity control. ACTB specific primers are listed below:

ACTB (f): 5′-TGG ACT TCG AGC AGA GAT GG-3′
ACTB (r): 5′-CAC CGT GTT GGC GTA GAG G-3′

PCR components were mixed on ice to a final volume of 25 µL in 0.2 mL reaction tubes. Genomic DNA from wildtype (WT) pigs served as control and distilled water was used as a non-template control. Details for master mix ingredients and PCR conditions are listed in Table 2 and Table 3.

Table 2: Master mix components per PCR reaction

Master Mix components	***INS*^{C93S}**	**ACTB**
	µL	**µL**
10× PCR buffer	2.5	2.5
$MgCl_2$ (15 mM)	2.5	2.5
dNTPs (2 mM)	5	5
Primer (f) (10 µM)	0.4	0.5
Primer (r)(10 µM)	0.4	0.5
Taq Polymerase (5 U/µL)	0.2	0.2
Aqua dest.	13	12.8
DNA template	1	1

Table 3: PCR reaction conditions for both *INS*^{C93S} and ACTB

Denaturation	95 °C	4 min.	
Denaturation	95 °C	30 sec.	
Annealing	62 °C	30 sec.	**35x**
Elongation	72 °C	30 sec.	
Final elongation	72 °C	5 min.	
Termination	4 °C	5 min.	

3.2.1.3. Agarose gel electrophoresis

An agarose gel electrophoresis was run to visualize the final PCR products. Therefore, a 2 % agarose gel was prepared by heating 1 × TAE buffer with 1 g/100 mL universal

agarose in the microwave. After cooling down to about 55 °C, ethidium bromide was added to the mixture in a concentration of 0.5 µg/mL and the gel was decanted into an electrophoresis chamber (OWL Inc., USA) for polymerization.

Samples were mixed with 2.5 µL 10× DNA loading buffer and pipetted individually into the slot chambers of the gel. Six µL of Gene Ruler^{TM} 1 kb DNA molecular weight marker (MBI Fermentas) was included for the determination of DNA fragment sizes. An electric field with a voltage of 130 V was applied so that DNA samples migrate according to their size. After separation DNA samples were visualized under ultraviolet (UV) light.

3.2.2. Southern blot

3.2.2.1. Isolation of genomic DNA from ear punches

Tissue was obtained from ear punches and genomic DNA was isolated with the Wizard genomic DNA purification Kit® (Promega) according to the manufacturer's instructions. Briefly, tissue pieces of 3-5 mm were lysed overnight by incubation at 55°C in a thermomixer. After overnight digestion, stepwise purification was performed to remove larger non-digestible components, RNA and proteins. Purified samples were mixed with isopropanol for DNA precipitation. DNA was washed with 70 % ethanol, air-dried and reconstituted with rehydration solution. Genomic DNA concentrations were measured using a NanoDrop ND-1000 (NanoDrop Technologies) spectrophotometer.

3.2.2.2. Restriction enzyme digestion and gel electrophoresis

Genomic DNA was digested into smaller fragments by overnight incubation at 37°C with the restriction enzyme BamH1 (MBI Fermentas). Fragments were loaded into a 1 % agarose gel and separated by electrophoresis as described in 3.2.1.3. Following electrophoresis, the gel was treated with 0.3M hydrochloric acid for 45 min. to further digest larger fragments (larger than 15 kb) and subsequently incubated with a DNA double-strand break solution for 45 min. Thereafter, the gel was incubated with neutralization solution for 20 min. Finally, genomic DNA was transferred to a positive loaded Nylon membrane (Nylon-N+, GE Healthcare) by capillary transfer for 24 hours. After the transfer the membrane was soaked with neutralization solution for 5

min. and air-dried. Transferred DNA was cross-linked to the membrane by UV-light irradiation at a dose of 120 J/cm^2. Then the membrane was stored at room temperature.

3.2.2.3. Radioactive labeling of the probe

A specific probe to the neomycin resistance cassette of the *INS*^{C93S} expression vector was generated for detection of the integrated *INS*^{C93S} transgene. Probes were amplified by PCR using the same transgene-specific primers and protocol as described in 3.2.1.2. Plasmid DNA containing the *INS*^{C93S} construct served as template. After electrophoresis, bands were removed from the gel and the amplified probe DNA was eluted with QiaexII Gel Extraction kit (Qiagen) according to manufacturer's instructions. The DNA concentration was estimated by comparison of DNA band intensity to the band intensity of the known molecular weight standard Lambda DNA *E*coRI + *Hind*III (MBI Fermentas) on an agarose gel. Probes were radioactively labeled with α-[^{32}P]-dCTP (Perkin-Elmer, Netherlands), using a fragment of Polymerase I (Klenow exo$^-$), which integrates radiolabeled nucleotides. Next, probes were denatured at 97 °C for 10 min. and directly placed on ice. Subsequently, single strand DNA probes were hybridized in a mixture with random primers (3 µg/µl), an appropriate buffer, a 0.33 M mix of dTTs, α-[^{32}P]-dCTP (3000 Ci/mmol) and distilled water for 1 hour at 37 °C. Unincorporated nucleotides were removed by centrifugation through Sephadex G-50 columns. Finally, radioactive labeled probes were denaturized at 97°C for five min. a second time and stored on ice.

3.2.2.4. Hybridization and signal detection

Previously, blotted Hybond-N+ Nylon membranes were pre-wetted with 5 x SSC and pre-hybridized in 30 mL of Southern blot church buffer for one hour at 58°C in a hybridization oven. Previously labelled probes were diluted with Southern blot church buffer and were allowed to hybridize to the nylon membranes overnight at 58°C under permanent rotation in the hybridization oven. After hybridization washing steps were performed as follows: 2 x with low-stringency buffer at room temperature and 2 x with high-stringency buffer at 58°C. For signal detection, membranes were exposed to X-ray films in an X-ray cassette for at least 24 hours at -80°C. X-ray films were developed in developing and fixing solutions according to the manufacturer's protocol and subsequently analyzed.

3.2.3. Quantification of expression levels of the endogenous and mutant insulin by next generation sequencing

Expression levels of *INS* and *INS*^{C93S} transcripts in pancreas samples from five founder boars and from F1 offspring of founder boars 9748 and 9776 respectively were quantified by next generation sequencing of RT-PCR products as described in (RENNER et al., 2013).

3.2.3.1. RNA isolation from pancreas

Total RNA was extracted from pancreatic tissue using RNeasy® Mini total RNA isolation Kit (Qiagen) according to the manufacturer´s instructions. Briefly, frozen pancreatic tissue (30-50 mg) was homogenized with recommended buffer using an ART-Micra D-8 tissue-homogenizer (23,500 rpm). Lysates were centrifuged (3 min., 14,000 rpm, 4°C) and supernatants were mixed 1:1 with ethanol (70%). Total RNA was eluted with clean-up columns in 50 µl RNase-free water. After RNA elution, RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA quality was evaluated by agarose gel electrophoresis.

3.2.3.2. DNaseI digest and reverse transcription

Purified RNA was digested with DNaseI to eliminate possible contaminants of genomic DNA. Therefore, 800 ng of total RNA from each sample were incubated together with DNaseI (10 U/µl) at a final concentration of 1 U/µl and an appropriate lysis buffer for 30 min. at 37°C on a thermomixer. DNaseI was inactivated by incubation at 75°C for ten min. followed by cooling down on ice for several min. Ten µl DNaseI digested RNA were reverse transcribed using random hexamer primers and SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Complementary DNA was stored at -20 °C until further processing.

3.2.3.3. PCR

Complementary DNA (cDNA) samples were then amplified by PCR. **Table 4** and **Table** 5 indicate the used PCR reagents and PCR reaction conditions respectively.

The following primers were used for cDNA amplification of insulin (INS):

Insulin(f): 5´-CGGGAGGCGGAGAACCCTCA-3‘

Insulin (r): 5‘-CCCTCAGGGGCGGCCTAGTT-3‘

Table 4 Master mix components per PCR reaction

Master Mix components	Insulin µL
10× PCR buffer (Qiagen)	2
$MgCl_2$ (25 mM) (Qiagen)	1.25
Q-solution (Qiagen)	4
dNTPs (2 mM)	2
Primer (f)(10 µM)	0.4
Primer (r) (10 µM)	0.4
Taq Polymerase (5 U/µL)	8.75
Distilled water	1
cDNA template	1

Table 5: PCR reaction conditions for INS.

Denaturation	95 °C	4 min	
Denaturation	95 °C	30 sec	
Annealing	62 °C	30 sec	**35x**
Elongation	72 °C	45 sec	
Final elongation	72 °C	10 min	
Termination			

Agarose gel electrophoresis was performed as described in 3.2.1.3.

3.2.3.4. Next generation sequencing

Next generation sequencing was used to determine the expression level of the mutant insulin C93S and endogenous insulin transcripts respectively using an Illumina Genome Analyzer IIx (>10,000 reads per sample). Next generation sequencing analysis was performed by Dr. Stefan Krebs, Laboratory of Functional Genome Analysis, Gene Center, LMU Munich.

3.3. Phenotypic characterization of *INS*^{C93S} transgenic pigs before pregnancy

After generation and genotypic characterization, the metabolic phenotype of *INS*^{C93S} transgenic animals was further characterized. Fasting and non-fasting plasma glucose levels, and glucose tolerance was investigated in five *INS*^{C93S} founder boars. From those, founders 9776 and 9748, with the most pronounced phenotype, were chosen for further investigations and were bred to wild-type sows. Data of F1 offspring of founder 9776 is presented while results of F1 offspring of founder 9748 are only mentioned in

the text. BW gain, (fasting) glucose concentrations, glucose tolerance and insulin secretion as well as total β-cell volume were evaluated. The phenotype of offspring from founder 9776 was followed until the F4 generation as well as during pregnancy.

3.3.1. Body weight (BW) gain and (fasting) blood glucose levels (FBG)

BW was recorded using a standard large animal scale when animals were 57, 72, 153 and 175 days of age.

For determination of (non-)fasting blood glucose levels, a blood drop was taken from a superficial ear vein using a blood lancet. Samples were directly measured with a FreeStyle Precision® neo glucometer. Blood glucose was regularly evaluated in all founder boars from 15 to 207 days of age.

3.3.2. Metabolic tests

To assess *in vivo* glucose clearance and therefore, investigate glucose tolerance and insulin secretion, mixed meal glucose tolerance tests (MMGTT) and intravenous glucose tolerance tests (IVGTT) were performed in *INS*^{C93S} transgenic pigs and non-transgenic littermates at four and seven months of age.

3.3.2.1. Surgical implantation of marginal ear vein catheters

Three days prior to the glucose challenge marginal ear vein catheters were placed under general anesthesia. This assured stress-free, frequent blood sampling in unrestrained animals during the tests. Anesthesia was induced by intramuscular injection of azaperone (2 mg / kg BW) and ketamine hydrochloride (20 mg / kg BW) and maintained by ketamine (20 mg / kg BW) and xylazine (0.05 mg / kg BW). Under anesthesia, animals were positioned in dorsal recumbence and the internal and external area of the ear was shaved, washed with soap and finally aseptically prepared using a 7.5% povidon-iodide containing solution (Braunol®) and an alcohol-based disinfectant (Kodan®). A marginal ear vein was punctured with a cannula (Figure 2: Catheter placement into a marginal ear vein

(**A**) Ear vein punctured with cannula, (**B**) Guide wire insertion, (**C**) Skin expansion with dilatator, (**D**) Catheter placement, (**E**) Blood collection and (**F**) Catheter suture.

A) followed by straight insertion of a guide wire through the cannula ~ 20 cm upstream into the vein (Figure 2 B). Subsequently, the needle was gently removed without pulling the wire. A dilator was placed over the wire and was pushed forward several millimeters to expand the skin opening and facilitate the insertion of the catheter 1 mm

in diameter (Figure 2 C). The dilator was removed and the catheter (Careflow® 3 Fr, 200 mm) was inserted over the wire into the vein (Figure 2 D). Once the catheter was fully inserted the guide wire was removed. To assess proper function of the catheter a blood sample was withdrawn using a 2 mL syringe. (Figure 2 E). An inject stopper was placed to lock the catheter followed by catheter flushing with 0.9% NaCl. The catheter was fixed to the ear with one suture and secured with adhesive tape (Figure 2 F). Finally, the catheter was filled with TauroLock® solution containing heparin to preserve its patency. Catheter placement was performed by Dr. Simone Renner and Dr. Istvan Novak (Chair for Molecular Animal Breeding and Biotechnology, LMU Munich).

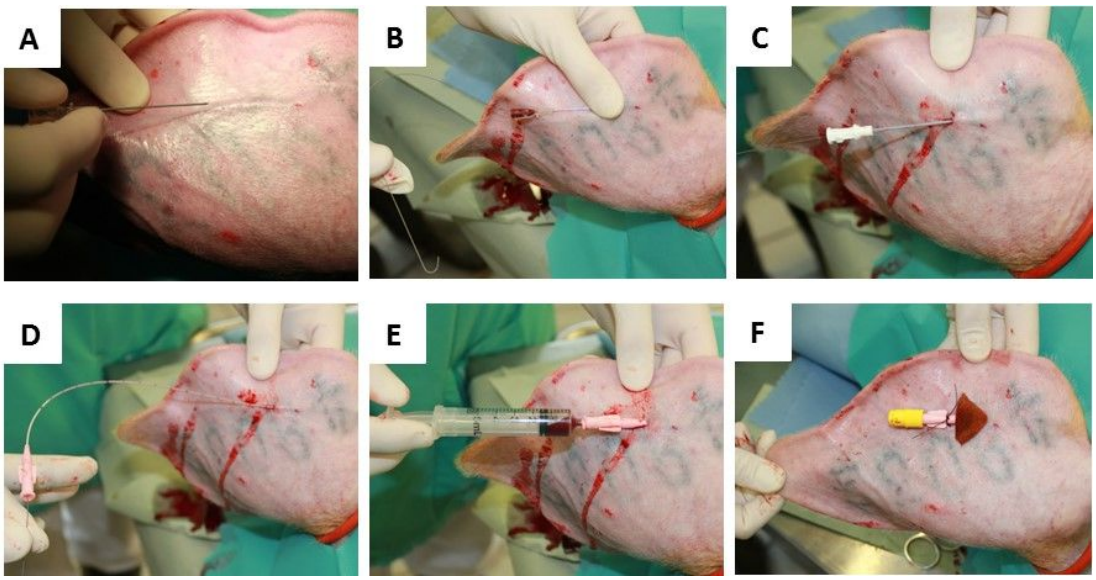

Figure 2: Catheter placement into a marginal ear vein

(**A**) Ear vein punctured with cannula, (**B**) Guide wire insertion, (**C**) Skin expansion with dilatator, (**D**) Catheter placement, (**E**) Blood collection and (**F**) Catheter suture.

3.3.2.2. Mixed meal oral glucose tolerance test (MMGTT)

Six days prior to the metabolic tests animals were housed in individual pens with straw bedding and free access to water. During the adaption period animals were normally fed with standard pig food according to age and BW. One day prior to the MMGTT BWs were recorded using a standard large animal scale. Animals were fasted for 18 hours prior to the MMGTT. A mixed meal-glucose mixture was prepared by mixing 50% glucose (2 g/kg BW) with commercial pig food (90 g for 4-month-old and 400 g for 7-month-old animals). Extension tubes (50 cm) were connected to the catheters

and secured on the back of the pigs with one stripe of tape. The mixed meal was given at the time point zero and the time until the meal was fully eaten was recorded. Blood samples were taken at -10, 0, 15, 30, 45, 60, 90 and 120 min. relative to meal administration and processed as described in 3.3.3. Also, pig behavior was recorded throughout the test.

3.3.2.3. Intravenous glucose tolerance test (IVGTT)

For IVGTTs the same standard housing conditions were applied as previously described in 3.3.2.2. Animals were fasted for 18 hours prior to the test. At time point 0, a glucose bolus (0.5 g/kg BW) was injected into the ear vein catheter within the minimum time possible and the injection duration was recorded. Immediately afterwards, the catheter was flushed with 20 mL of 0.9% isotonic NaCl solution. Blood samples were collected at time points -10, 0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50, 60 and 90 min. relative to the glucose load. Samples were processed as described in 3.3.3. Throughout the test the pig behavior was recorded.

3.3.3. Sample processing

Immediately after collection in nine mL plasma EDTA collection tubes, samples were placed on ice, centrifuged (1,500 x g for 15 min. at 4°C) and stored at -80°C for further analyses.

3.3.3.1. Determination of plasma glucose concentrations by spectrophotometry

Glucose was analyzed with an automated analyzer (Beckman & Coulter AU480 Analyzer). Optical density was measured with a photometric lamp. Determination of plasma glucose was performed by Dr. Birgit Rathkolb, Helmholtz Zentrum Muenchen.

3.3.3.2. Determinations of plasma insulin concentration by radioimmunoassay (RIA)

Insulin concentrations were assessed using a porcine insulin RIA kit (Millipore) according to the manufacturer's instructions. In this assay a fixed concentration of ^{125}I-labeled insulin is mixed with an unknown concentration of unlabeled insulin present in the porcine plasma samples. Both substrates compete for a constant but limited concentration of binding sites of anti-insulin antibodies. Subsequently, the antibody-bound tracer is separated from the unbound radioactive tracer by precipitation. The antibody-bound fraction is measured in a γ-counter. Insulin concentrations of unknown samples were calculated with LBIS immunoassay software (version 3.3.0.0). All

samples were measured in duplicates and only values with a coefficient of variance (CV) less than 10% were accepted. The detection limit of this assay is 1.611 µU/mL.

3.3.4. Quantitative-stereological analyses of the pancreas

Quantitative stereological analyses of the pancreas were performed to investigate differences in volume density and total volume of β-cell between *INS*^{C93S} transgenic pigs and non-transgenic littermate controls.

3.3.4.1. Pancreas preparation and random systematic sampling

Pancreata from F1 offspring of founder 9776 were collected at an age of 13 months (388.57± 19.5 days). Following euthanasia, the entire pancreas was explanted. Then connective tissue was removed and the pancreas was weighed. The pancreatic ring was cut between the *lobus pancreatis sinister* and the connective lobe and brought into a straight position. For subsampling the length of the pancreas was determined and tissue slices (thickness 0.5 cm) were cut out every 2.5 cm over the whole length of the organ, tilted to the left side and prefixed in 4% neutral buffered formalin for 8 hours. Then tissue samples were selected according to a random systematic sampling procedure as described in (ALBL et al., 2016). Briefly, tissue slices were covered by a 1 cm^2 point-counting grid. All points of the counting grid hitting pancreatic tissue were marked, counted and their sum was divided by 20 and defined the quotient Y. Then a random number X between one and quotient Y was chosen. Finally, tissue samples of about 1 cm^2 at position X, X+Y, X+2*Y, X+3*Y until X+20*Y were selected, placed in embedding cassettes and routinely processed with a tissue processor (Shandon Citadel tissue processor 1000). Following paraffin embedding with the TBS 88 Paraffin Embedding System, sections of 3 µm thickness were cut with a HM 315 microtome, mounted on 3-aminopropyltriethoxysilane-treated glass slides and placed in an incubator at 37 °C to dry until immunohistochemical staining.

3.3.4.2. Immunohistochemical staining of insulin-positive cells

The immunohistochemistry technique was used to stain insulin-positive cells. Pancreatic tissue sections were deparaffinized in xylene for 15 min. and rehydrated in gradually decreasing alcohol concentrations (99%, 96%, 70% alcohol and distilled water). The indirect alkaline phosphatase (AP) immunohistochemistry method was used. The individual steps of the protocol are indicated in Table 6. Finally, slides were dehydrated in gradually increasing alcohol concentrations (distilled water, 70%, 96%,

99% alcohol), cleared in xylene and mounted with cover slips using the quick-hardening mounting medium histokitt.

Table 6: Immunohistochemistry staining protocol for insulin

	Protocol step	Purpose	Incubation time
1.	Hydrogen peroxide (1%)	Blocking of endogenous peroxidase	15 min
2.	TBS buffer	Washing	10 min
3.	Goat serum 1:10 in TBS	Blocking unspecific binding	30 min
4.	Primary antibody (Polyclonal guinea pig anti-porcine insulin) 1:1000 in TBS	Primary antibody	60 min
5.	TBS buffer	Washing	10 min
6.	Secondary antibody (AP-conjugated goat anti-guinea pig IgG) 1:100 in TBS + 5 % porcine serum	Secondary antibody	60 min
7.	TBS buffer	Washing	10 min
8.	Vector® Red chromogen diluted in 100 mM Tris HCl (pH 8.5)	Color reaction	20 min
9.	Distilled water	Washing	5 sec
10.	Mayer´s hemalum solution	Counterstaining	10 min
11.	Running tap water	Washing	5 min

3.3.4.3. Quantitative stereological analyses

Volume density of β-cells in the pancreas and total β-cell volume was determined using the computer-assisted Olympus Visiomorph™ image analysis system with the NEWCast software coupled to a light microscope (Olympus, BX41) and a color video camera (Olympus DP72). With the 20x objective selected (total magnification 200x), an automated-random-image-sampling of 50 % of the total tissue area in each slide was performed. For each image generated, the software automatically recognized red-stained insulin positive cells and blue-stained pancreatic tissue and attributed a numeric score per region identified. Thus, total β-cell area $A_{(\beta\text{-cell},50\%)}$ and total area of pancreatic tissue $A_{(Pan,50\%)}$ was calculated from the sum of all scores. From these areas,

the volume density of β-cells in the pancreas ($\mathbf{Vv_{(\beta\text{-cell/Pan})}}$) was determined by dividing the total β-cell by the total pancreas area [$\mathbf{Vv_{(\beta\text{-cell/Pan})}} = A_{(\beta\text{-cell},50\%)} / A_{(Pan,50\%)}$]. The total β-cell volume $\mathbf{V_{(\beta\text{-cell,Pan})}}$ was then calculated by multiplying $\mathbf{Vv_{(\beta\text{-cell/Pan})}}$ with the total pancreas volume $\mathbf{(V_{(Pan)})}$. $\mathbf{V_{(Pan)}}$ is defined by the quotient of the pancreas weight and the specific weight of the pig pancreas (sp. $W._{(Pan)}$= 1.07 g/cm^3). The specific weight of the pig pancreas was determined by the submersion method as previously described (Scherle 1970).

3.4. Phenotypic characterization of *INS*^{C93S} transgenic sows and wild-type littermates during pregnancy

Effects of the expression of the mutant insulin C93S were investigated during pregnancy in pregnant *INS*^{C93S} transgenic sows (*INS*^{C93S}-p) and pregnant wild-type sows (wt-p) and these animals were named 'study group' in the following chapters. As a control group for physiological adaptations during pregnancy in wt sows non-pregnant wt sows (wt-np) were included.

3.4.1. Reproduction management of sows

3.4.1.1. Estrus synchronization and artificial insemination

Wild-type (n=9) and *INS*^{C93S} transgenic sows (n=3) aged 9 ± 0.84 months were estrus synchronized by a 16-day once daily oral application of Altrenogest (Regumate®, 20 mg/animal per day). Ovulation was induced 48 hours after the last Regumate® administration by a single intramuscular injection of Pregnant Mare Serum Gonadotropin (Intergonan®; 750 I.U. per animal) followed by a single intramuscular injection of Choriongonadotropin (Ovogest®, 750 I.U. per animal) 76 hours later. Twenty-four and 48 hours after Ovogest® injection, sows were artificially inseminated with semen from the same wt boar. Pregnancy was confirmed by ultrasonography 21 days after insemination. Sows were group-housed until 107 of pregnancy.

3.4.1.2. Birth induction

At day 107 of pregnancy sows were placed in individual maternity pens for adaption. On day 112 of pregnancy birth was induced by a single intramuscular injection of cloprostenol (Estrumate®, 0.175 mg per animal). Farrowing took place between 24 to 36 hours after birth induction.

3.4.2. Fasting blood glucose levels throughout gestation

To assess effects of pregnancy on fasting glucose homeostasis, blood glucose levels were measured between gestational weeks three and sixteen in regular intervals. Four wt-p and three *INS*^{C93S}-p sows as well as four wt-np and three *INS*^{C93S}-np sows were included in the measurements. Following an 18-hour fasting period blood was collected and blood glucose measured as described in 3.3.3.1

3.4.3. Glucose clamps

Two types of glucose clamps, the hyperinsulinemic-euglycemic clamp (HIC) and the hyperglycemic clamp (HGC), were performed during pregnancy. Hence *in vivo* insulin sensitivity and β-cell function were characterized. Sows included in the HIC and HGC were 13 ± 0.15 months of age. The HIC and HGC were performed at gestational week 80 ± 2.30 gestational week 86 ± 2.82 respectively.

3.4.3.1. Surgical implantation of arterial and central venous catheters

At gestational day 71 ± 2.5 catheters were inserted into the carotic artery and external jugular vein under general anesthesia.

Anesthesia was induced by intramuscular injection of azaperone (2 mg / kg BW) and ketamine hydrochloride (20 mg / kg BW) and maintained by inhalation of 1% isoflurane. Peri-surgical analgesia was provided by an intravenous injection of Metamizol (50 mg per kg BW). Under anesthesia, animals were positioned in dorsal recumbence and the jugular groove was shaved, washed with soap and finally aseptically prepared using a 7.5% povidon-iodide containing solution (Braunol®) and an alcohol-based disinfectant (Kodan®). A skin incision of about five centimeters length was made in the sulcus jugularis. The carotic artery and the external jugular vein were exposed, surrounding connective tissue was removed and two fixation sutures were placed proximally and distally around the artery and vein respectively. Following arteriotomy/venotomy a CBAS® Heparin Coated catheter was inserted 15 cm into the artery and vein respectively. A proximal and distal ligature was applied to inhibit blood reflux and hold catheters in place. Subsequently, the operation wound was sutured in two layers and Leukase® N Kegel (Dermapharm AG) were placed between the tissue layers to provide local analgesia. For external fixation the catheters were tunneled separately through the skin up to the back of the neck using a tunneling instrument and were fixed with single-sutures to the skin, covered with gauze and secured with tape. Post-surgical analgesia was provided by intravenous injection of

Metamizol (50 mg/kg BW) for two days and cefquinom 2.5% was administered intramuscularly once daily for three days (2 mL per 25 kg BW) to prevent infections of the surgical site. Both catheters were flushed once daily with 50 IU heparin/mL 0.9% isotonic sodium chloride solution. Glucose clamps were performed following a recovery period of one week. Catheter placement was performed by Dr. Simone Renner (Chair for Molecular Animal Breeding and Biotechnology, LMU Munich).

3.4.3.2. Hyperinsulinemic-euglycemic clamp (HIC)

HICs were performed to directly assess insulin sensitivity *in vivo* and to uncover insulin resistant states (DEFRONZO et al., 1979). During HIC, insulin is infused at a constant rate while variable rates of glucose are infused in parallel in order to maintain euglycemia. Once steady state conditions are achieved, the glucose infusion rate is a direct measure of glucose uptake by insulin-sensitive peripheral tissues and therefore a direct measure of whole body insulin sensitivity. Prior to the HIC, sows were fasted overnight. At the day of the HIC sows were placed in individual and space-restricted pens to avoid free movement. Plasma insulin concentration was raised by insulin infusion (Insuman® rapid) at a rate of 1 mU/kg BW/min into the jugular vein. Blood glucose was clamped at a level of 75 mg/dl. This euglycemic glucose level was maintained by variable glucose infusion rates using a 40% glucose solution. For determination of the glucose infusion rate blood glucose concentration was measured from arterial blood every five min. using a FreeStyle Precision® neo Glucometer (Abbott) until the end of test. Total clamp duration was 180 min. and steady-state was considered to be reached two hours following the start of the insulin infusion. During steady-state blood samples were collected in nine mL plasma EDTA collection tubes every ten min., i.e.at 130, 140, 150, 160, 170 and 180 min. relative to the start of the insulin infusion for further analyses. The glucose infusion rate (GIR) a measurement of whole body insulin sensitivity was expressed as mg of infused glucose per kg BW per minute. Blood samples were processed as described in 3.3.3. Plasma glucose and insulin levels were measured as described in 3.3.3.1and 3.3.3.2.

3.4.3.3. Hyperglycemic clamp (HGC)

HGCs were performed to evaluate β-cell function (DEFRONZO et al., 1979). During the HGC plasma glucose concentration was acutely raised to a certain hyperglycemic level by administration of a glucose bolus followed by a variable glucose infusion rate. Sows were placed in individual and space restricted pens following an 18-hour

overnight fasting period. Plasma glucose concentration was raised to 300 mg/dl by a glucose bolus followed by variable glucose infusion. To adjust glucose infusion rate blood glucose concentration was measured every five min. until the end of the assay at 90 min., using the HemoCue® Glucose 201^{+} Glucometer (Radiometer GmbH). Blood samples were collected in nine mL plasma EDTA collection tubes every ten min. throughout the entire test period. Fifty min. after the start of the glucose infusion an arginine bolus (5 g per animal) was injected into the jugular vein to determine maximal insulin secretion capacity. Following collection blood samples were processed as described in 3.3.3. Plasma glucose and insulin levels were measured as described in 3.3.3.1and 3.3.3.2.

3.4.4. Mixed-meal oral glucose tolerance test (MMGTT)

MMGTTs during pregnancy were performed in three wt-np, nine wt-p and three *INS*^{C93S}-p sows at gestational day 92 ± 3.61. The procedure was performed as previously described in 3.3.2.2. and samples were processed as described in 3.3.3. Plasma glucose and insulin levels were measured as described in 3.3.3.1and 3.3.3.2.

3.5. Phenotypic characterization of neonatal piglets

The effects of chronic exposure to mild hyperglycemia during gestation were investigated in neonatal wt piglets born to wt and *INS*^{C93S} tg sows. In the following chapters wt piglets born to *INS*^{C93S} tg sows are referred to as wt/tg and wt piglets born to wt sows are referred to as wt/wt respectively.

3.5.1. Glucose challenge tests in newborn piglets

3.5.1.1. Oral glucose tolerance test (OGTT)

OGTT were performed in neonatal piglets (wt/wt: n=18, and wt/tg: n=13) born to three tg and two littermate wt sows. The principle of an OGTT is the same as of an MMGTT with the exception that during an OGTT solely a glucose bolus is administrated through a nasogastric tube. Therefore, this procedure is suitable for suckling piglets which cannot yet digest a meal. Piglets were separated from the sows immediately after birth to avoid first colostrum uptake. Subsequently, animals were weighed. Following a recovery period of 15-30 min. after birth a bolus of 50 % glucose solution (2 g/kg body weight) was administrated via a nasogastric tube. Blood glucose concentrations were measured at time points 0, 15, 30, 45, 60, 90 and 120 min. relative to the glucose load from blood drops taken from a superficial ear vein with a FreeStyle

Precision® neo Glucometer. In addition, one mL blood samples were collected at time points 0, 30, 60 and 120 min. in nine mL plasma EDTA collection tubes from the jugular vein and processed as described in 3.3.3. Plasma glucose and insulin levels were measured as described in 3.3.3.1and 3.3.3.2.

3.5.1.2. Assessment of plasma metabolites by spectrophotometry.

EDTA-plasma samples collected during the OGTT (time point 0 and 120 min. relative to the glucose load) were assessed for different clinical chemical parameters (Table 7) of six wt/tg and six wt/wt piglets that were randomly selected from all five sows (two wt/tg piglets from each of the three *INS*^{C93S} transgenic sows and three wt piglets from each of the two wt sows). Analyses were performed by spectrophotometry (Beckman & Coulter AU480 Analyzer) as previously described in 3.3.3.1

Table 7: Clinical chemical parameters

• **Carbohydrate metabolism**	Glucose
	Lactate
	LDH
• **Lipid metabolism**	Cholesterol
	HDL
	LDL
	Lipase
	NEFA
	Triglycerides

Non esterified fatty acids (NEFA)

3.6. Necropsy

At day one of age 12 wt/wt and 13 wt/tg piglets underwent necropsy. Anesthesia was induced by intramuscular injection of ketamine (20 mg/kg BW) and azaperone (2 mg/kg BW) followed by euthanasia via intra-cardial injection of Embutramid, Mebezonium, Tetracain (T61, 4 - 6 mL T 61 / 50 kg BW). Subsequently, BW, crown-rump length (CRL) and organ weights (heart, kidneys, liver, lung, pancreas, spleen) and the weight of the carcass defined as the whole body excluding all organs but including head and brain were recorded. Relative CRL and relative organ weights were determined by dividing the absolute organ weights by the BW of respective piglet.

3.7. Statistical analysis

All data are presented as means ± standard error of the mean (SEM). The results of oral, mixed-meal and intravenous glucose tolerance tests as well as glucose clamps (GIR) were statistically evaluated by analysis of variance (PROC MIXED; SAS 8.2) taking the fixed effects of Group (*INS*^{C93S} vs. wt control), Time (relative to glucose administration, steady-state during HIC) and interaction of Group*Time into account. Statistical differences regarding clinical-chemical parameters were evaluated by analysis of variance (General Linear Models; SAS 8.2) taking the fixed effects of Group (wt/tg vs. wt/wt), Time (0 min. and 120 min.) and the interaction Group*Time into account. Absolute and relative BW, CRL and organ weights were statistically evaluated by analysis of variance (PROC GLM; SAS 8.2) taking the fixed effect of Group (wt/tg vs. wt/wt) into account. Differences between two groups regarding quantitative-stereological analyses, expression levels and areas under the glucose/insulin curve were evaluated by Mann-Whitney-U test in combination with an exact test procedure (SPSS 21.0). Area under the curve (AUC) for insulin and glucose were calculated using Graph Pad Prism® software (version 5.02). P values less than 0.05 were considered to be significant.

IV. RESULTS

1. Generation of *INS*^{C93S} transgenic pigs by SCNT

Transgenic pigs were established by additive gene transfer and SCNT technology. As a result of the amino acid exchange in the coding sequence of the insulin gene, the disulfide bond within the A-chain is disrupted and consequently a misfolded proinsulin protein is generated Figure 3). The mutant insulin accumulates in the endoplasmic reticulum by formation of high-molecular-weight complexes possibly interfering with the secretion of the endogenous insulin (Liu et al. 2010).

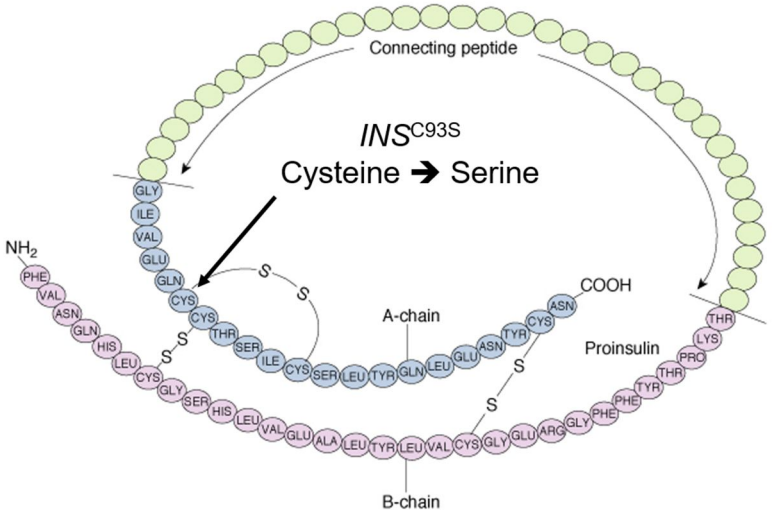

Figure 3: Proinsulin amino acid sequence

Proinsulin amino acid sequence composed of the A-chain, B-chain and the connecting peptide (C-peptide). The A- and B-chain of the insulin are connected by two interchain and one intrachain disulphide bonds. The arrow depicts the amino acid exchange cysteine to serine at position 93 (C93S) within the mutant insulin.

A total of 503 *INS*^{C93S} transgenic reconstructed embryos were transferred into the oviducts of five cycle-synchronized recipient gilts from which two pregnancies went to term (Table 8). Five out of ten piglets were born alive. The overall efficiency of nuclear transfer experiments from the two established pregnancies was 2.42 % (calculated by the total number of alive piglets of pregnancy 1 and 3 per respective number of transferred embryos).

Table 8: Overview of NT experiments

Embryo Transfer	Somatic cells	Transferred embryos	Pregnancy established	Delivered piglets	Alive piglets
1	PFF 14	95	+	3 (9748-9750)	2 (9748, 9750)
2	PFF 14	95	-	-	-
3	PFF 26	112	+	7 (9772-9778	3 (9774, 9776, 9777)
4	PFF 26	101	-	-	-
5	PFF 26	100	-	-	-
Total	-	503	2/5	10	5

Porcine fetal fibroblasts (PFF)

1.1. Identification of *INS*^{C93S} transgenic pigs by PCR and Southern blot

PCR experiments using transgene specific primers were used to identify *INS*^{C93S} transgenic animals. Figure 4 shows a representative example of an *INS*^{C93S} genotyping PCR, in which four out of nine animals were identified as *INS*^{C93S} transgenic pigs. In addition, Southern blot experiments were performed to prove correct identification of transgenic animals by PCR, as well as to identify the number of integration sites of the *INS*^{C93S} transgene.

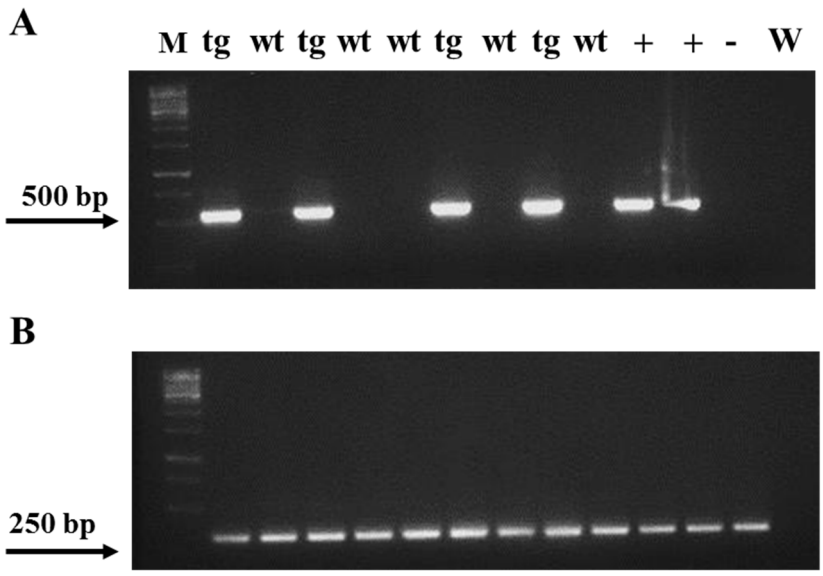

Figure 4: Identification of *INS*^{C93S} transgenic pigs by PCR

(**A**) *INS*^{C93S} PCR for detection of the *INS*^{C93S} transgene; (**B**) ACTB PCR using the same genomic DNA samples for the control of DNA integrity; (tg): *INS*^{C93S} transgenic pigs; (wt): non-transgenic littermate control pigs; M: 1kb DNA ladder marker; +: primer positive control (genomic DNA of a previously confirmed *INS*^{C93S} tg pig); -: primer negative control (genomic DNA of a previously confirmed wt pig); W: internal negative control (distilled water).

Figure 5 depicts an example of Southern blot experiments of all founder boars from embryo transfer n°3 and F1 offspring of founder 9776. Integration of the *INS*^{C93S} transgene could be detected in founders (9773 – 9778) (Figure 5 A). According to the expression level and results from the glucose tolerance test (see 1.2 and 2.1) founders 9748 and 9776 were selected for further characterization and mated to wildtype sows. Southern blot analyses of F1 offspring from founder 9748 (data not shown) and 9776 (Figure 5 B) showed the same transgene integration pattern as the respective founder boar, confirming germline transmission of the *INS*^{C93S} transgene and a single integration site in the genome in these two lines.

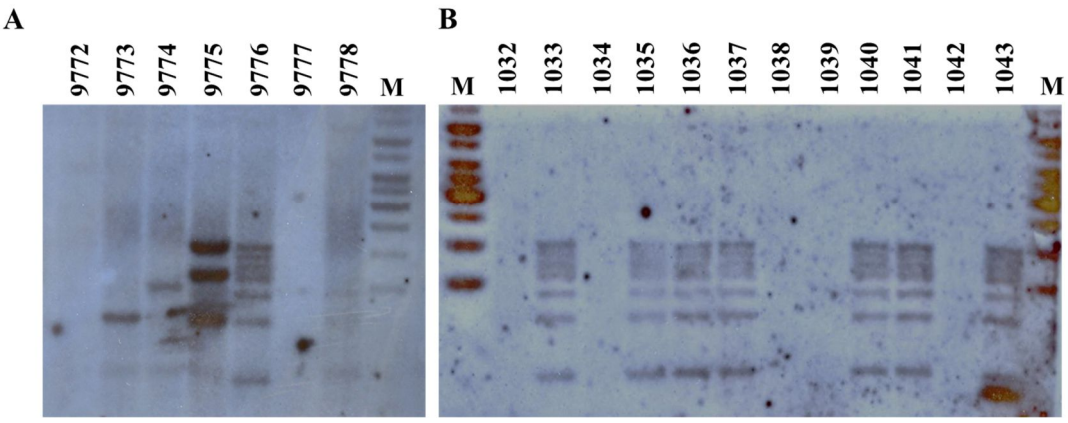

Figure 5: Southern blot analysis of *INS*^{C93S} founders and F1 offspring of founder 9776

X-ray exposed membranes, labeled with a transgene-specific radioactive probe for the detection of the *INS*^{C93S} transgene in (**A**) seven *INS*^{C93S} transgenic founder boars and (**B**) F1 offspring of founder 9776. The identical transgene integration pattern in F1 offspring from founder 9776 argues for one single integration site of the *INS*^{C93S} transgene.

1.2. Founders 9776 and 9748 exhibited the highest *INS*^{C93S} expression level

Next, gene expression analyses of pancreatic tissue from the five living founder boars (9776, 9748, 9777, 9750 and 9774) as well as from F1-offspring of founder boar 9748 and 9776 were performed. Good quality RNA according to the presence of distinct 28S and 18S ribosomal RNA bands without considerable RNA degradation was obtained from all animals and was reverse transcribed. RT-PCR using insulin-specific primers was run to determine the presence of insulin transcripts. Insulin cDNA transcripts were detected in all five founders as well as in F1 offspring from founder 9748 and 9776 (Figure 6 A). RT-PCR with ACTB primers was run on the same cDNA samples as internal positive control. A signal of equal intensity was detected in all animals indicating that RNA was successfully reverse transcribed into cDNA (Figure 6 B). Minus RT control PCR revealed no signal (Figure 6 C) indicating complete DNase digest and excluding genomic DNA contamination of cDNA samples.

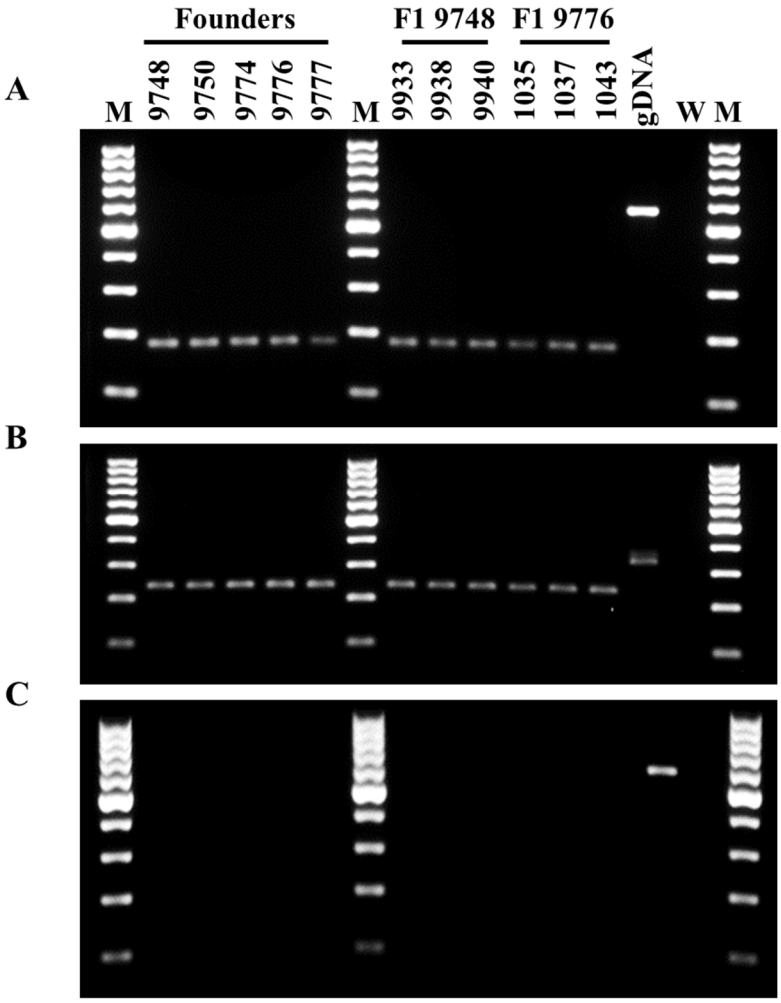

Figure 6: RT-PCR products

(**A, B, C**) PCR products from cDNA samples of five founder boars and F1-offspring from founder 9748 and 9776. (**A**) *INS*^{C93S} PCR, (**B**) ACTB PCR and (**C**) DNaseI digested RNA products proving no genomic DNA contamination; M: pUC Mix molecular weight marker; gDNA: positive control (genomic DNA); W: distillated water as non-template control.

Next, *INS*^{C93S}/*INS* ratios were determined from cDNA amplicons by next generation sequencing. Founder 9776 exhibited the highest *INS*^{C93S}/*INS* ratio (0.599), followed by founders 9748 (0.315), 9777 (0.169), 9750 (0.051) and 9774 (0.002). Transgenic F1 piglets exhibited similar *INS*^{C93S}/*INS* ratios as their respective founders: F1 offspring of founder 9776 (0.552 ± 0.035, n=3) and F1 offspring of founder 9748 (0.304 ± 0.006, n=3) (Figure 7).

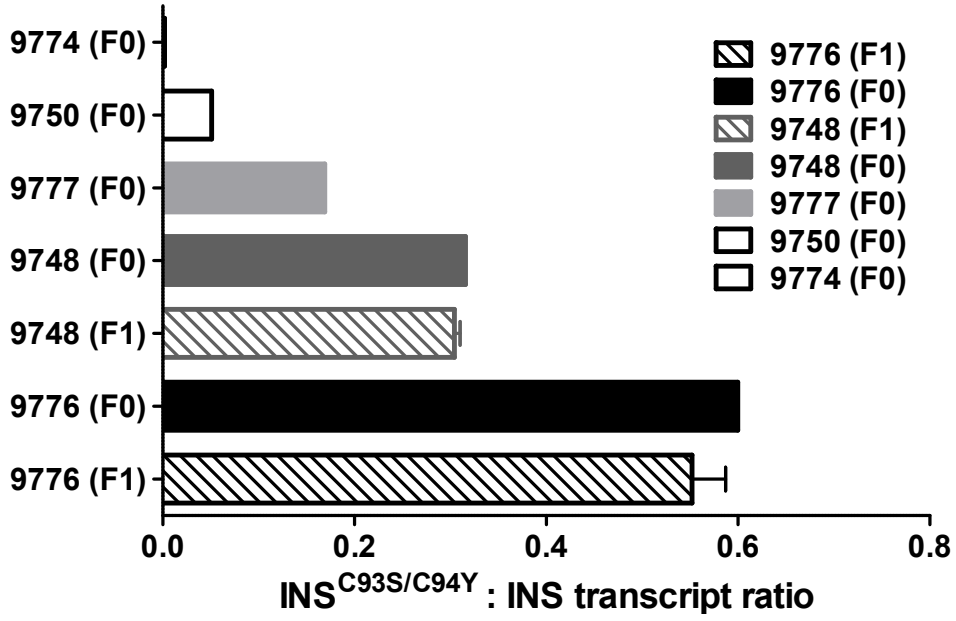

Figure 7: Expression levels of the *INS*^{C93S} transgene

Quantification of *INS*^{C93S}/*INS* transcript ratios by next-generation sequencing of the five living founder boars as well as of F1-offspring from founder 9748 and 9776. Founders 9776 and 9748 exhibited the highest expression levels of the *INS*^{C93S} transgene. F1 offspring revealed similar *INS*^{C93S}/*INS* ratios as their respective founders.

2. Phenotypic characterization of *INS*^{C93S} transgenic pigs

2.1. *INS*^{C93S} transgenic founders exhibited unaltered (fasting) blood glucose levels but impaired intravenous glucose tolerance

Following expression analysis, effects of *INS*^{C93S} expression on non-fasting and fasting blood glucose levels were assessed in the five *INS*^{C93S} transgenic founder boars. Data were collected from fed animals between 15 to 46 days of age (prior to weaning) and after an overnight fasting period between 52 and 207 days of age (after weaning). All founders exhibited (fasting) glucose concentrations within the normal range for pigs (Figure 8). Therefore, *INS*^{C93S} transgenic founders have unaltered fasting blood glucose levels.

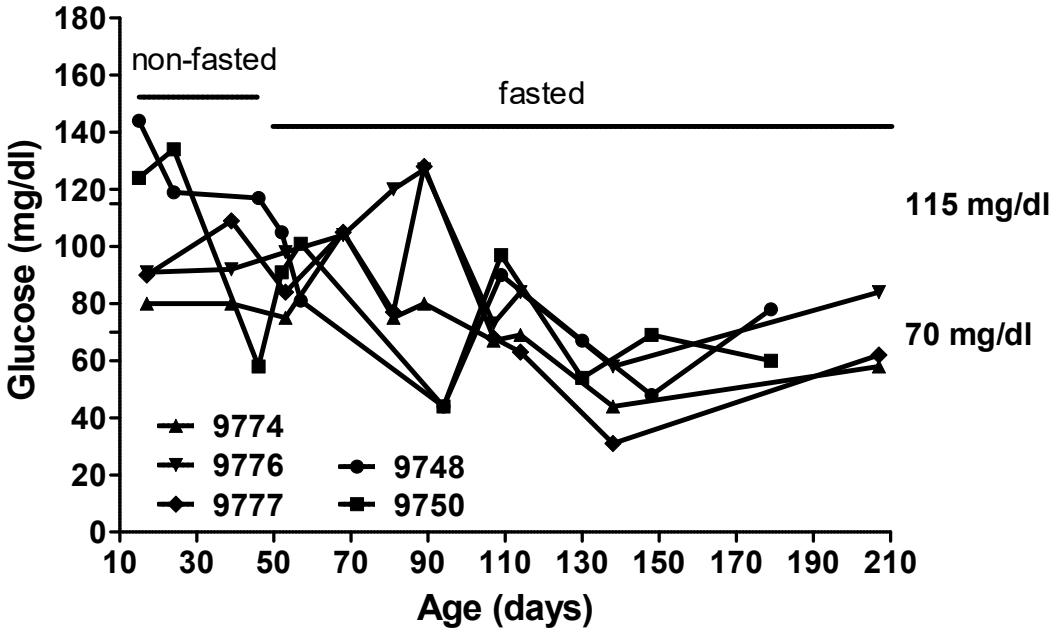

Figure 8: (Fasting) blood glucose levels in *INS*^{C93S} transgenic founder boars
Non-fasted values represent measurements before weaning. After weaning, glucose levels were determined after an 18-hour fasting period. Highlighted in grey is the reference range of blood glucose for pigs.

In addition, effects of *INS*^{C93S} expression on glucose tolerance were investigated in an IVGTT in the five living *INS*^{C93S} transgenic founders and two age-matched wt animals. At the time of the IVGTT the animals were 237 ± 3.67 days old. After intravenous glucose bolus (0.5 g/kg body weight), transgenic founders 9776 and 9748 revealed decelerated decline of blood glucose and distinctly reduced insulin secretion compared to transgenic founders 9750, 9774, 9777 and wt controls (Figure 9A and B). These findings are in line with gene expression data. Founders 9776 and 9748, which exhibited the highest *INS*^{C93S} expression levels (shown previously in Figure 7D), revealed an aggravated phenotype in terms of glucose control as compared to founders 9750, 9774 and 9777.

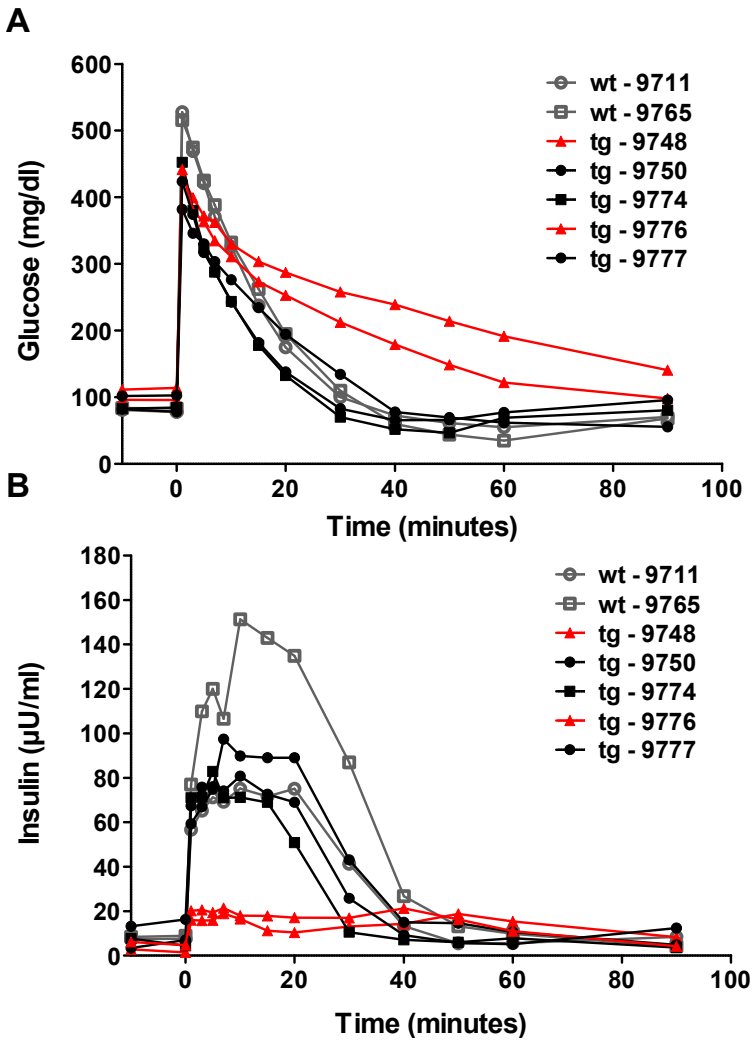

Figure 9: Intravenous glucose tolerance in *INS*^{C93S} tg founder boars

IVGTTs of 8-months-old *INS*^{C93S} transgenic founders depict impaired intravenous glucose tolerance of founders 9776 and 9748. **(A)** Plasma glucose concentration; **(B)** plasma insulin concentration. *INS*^{C93S} transgenic (tg) and age-matched wt controls (wt).

According to the gene expression and IVGTTs results, founders 9776 and 9748 revealed the highest expression levels of the mutant insulin C93S, as well as an impaired glucose clearance and insulin secretion phenotype therefore, they were mated with wt sow and effects on F1 offspring were evaluated. Data from F1 offspring from founder 9766 is presented. The data from 9748 is mentioned in the text.

2.2. Body weight is unaltered in *INS*^{C93S} transgenic pigs

BW gain was assessed in F1 offspring from founder boar 9748 and 9776. Data were collected in regular intervals from 57 to 175 days of age in F1 offspring from founder 9776 and eight to 168 days of-age in offspring from founder 9748. There were no

significant differences observed in body weight gain between *INS*^{C93S} transgenic and wt animals of both lines (Figure 10).

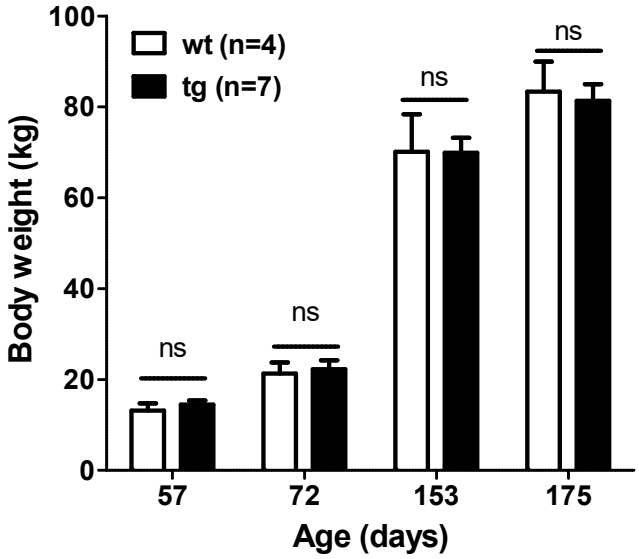

Figure 10: Unaltered body weight gain in *INS*^{C93S} transgenic pigs.
Body weight measurement in F1-offspring of founder 9776. There were no significant differences between the two groups at any time point. Data are means ± SEM.

To evaluate the effect of *INS*^{C93S} expression on glucose metabolism, IVGTTs and MMGTTs were performed at four and seven months of age. *INS*^{C93S} transgenic and wt animals from the F1 and F4 generation were included in the tests.

2.3. *INS*^{C93S} transgenic pigs showed reduced oral and intravenous glucose tolerance at four and seven months of age

IVGTTs were performed in 4- and 7-month-old transgenic pigs (n=11) and eight littermate controls. Fasting plasma glucose (FPG) levels at four and seven months were significantly elevated in *INS*^{C93S} transgenic pigs compared to wt controls ($FPG_{4\ months}$ = 136.4 ± 6.108 vs. 88.8 ± 4.010, p= 0.006) (Figure 11 A) and ($FPG_{7\ months}$ = 133.4 ± 9.173 vs. 73.9 ± 4.430, p= 0.0014) (Figure 12 A). Fasting plasma insulin was reduced, but not significantly, in *INS*^{C93S} transgenic animals compared to controls at both ages ($Insulin_{4\ months}$ = 5.939 ± 1.139 vs. 8.348 ± 0.929, p=0.5842) (Figure 11 C) and ($Insulin_{7\ months}$ = 6.629 ± 0.712 vs. 11.471 ± 1.050, p=0.348) (Figure 12 C). After an intravenous glucose challenge (0.5 g/kg body weight), *INS*^{C93S} transgenic animals exhibited significantly elevated plasma glucose levels and reduced insulin secretion

compared to non-transgenic littermates both at four and seven months of age (Figure 11 A, C and Figure 12 A, C). Glucose tolerance was significantly reduced in *INS*^{C93S} transgenic pigs at both ages tested. This was demonstrated by a reduced glucose clearance resulting in an increased area under the glucose curve (AUC glucose) at four months (AUC glucose $_{4\ months}$ = 21156 ± 879 vs. 12812 ± 841, $p<0.001$) (Figure 11 A, B), as well as at seven months of age (AUC glucose $_{7\ months}$ = 21766 ± 1126 vs. 10841 ± 286, $p<0.001$) (Figure 12 A, B). In parallel, the AUC of insulin was significantly reduced in *INS*^{C93S} transgenic pigs as compared to wt littermates at four and seven months of age, respectively (AUC insulin $_{4\ months}$ = 1132 ± 150 vs. 2479 ± 248, $p<0.001$) (Figure 11 C, D) and (AUC insulin $_{7\ months}$ = 1301 ± 138 vs. 2797 ± 251, $p=0.001$) (Figure 12 C, D). Although there were no significant gender-related differences in glucose tolerance at four months of age (data not shown), *INS*^{C93S} transgenic females revealed an aggravated impairment of glucose tolerance compared to *INS*^{C93S} tg males at seven months of age (AUC glucose $_{7\ months}$ = 23694 ± 1576 vs. 19453 ± 892, $p<0.05$) (Figure 13 A, B). In line with glucose levels, impaired insulin secretion was aggravated in *INS*^{C93S} tg females as compared to *INS*^{C93S} tg males (AUC insulin $_{7\ months}$ = 1011 ± 112 vs. 1650 ± 174, $p<0.05$) (Figure 13 C, D). These results demonstrate that *INS*^{C93S} transgenic pigs showed significantly reduced FPG levels as well as a distinct impairment of intravenous glucose tolerance and reduced insulin secretion that was stable in its manifestation between four and seven months of age. Moreover, *INS*^{C93S} transgenic females developed a more pronounced impairment of glucose tolerance compared to *INS*^{C93S} transgenic males only at seven months of age.

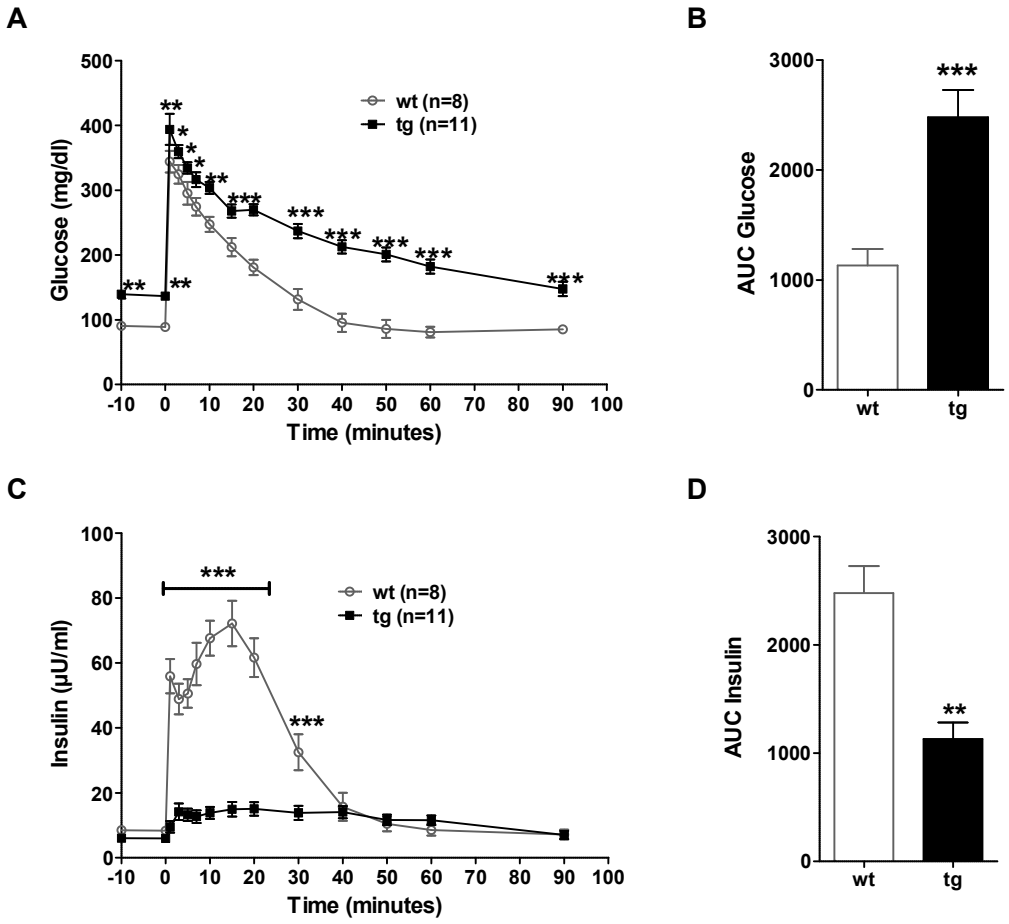

Figure 11: Intravenous glucose tolerance in 4-month-old *INS*^{C93S} transgenic pigs

(**A**) Plasma glucose levels; (**B**) AUC glucose: area under the glucose curve; (**C**) Plasma insulin levels; (**D**) AUC insulin: area under the insulin curve; *INS*^{C93S} transgenic pigs (tg) and non-transgenic controls (wt) of the F1 and F4 generation; data are means ± SEM; (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$.

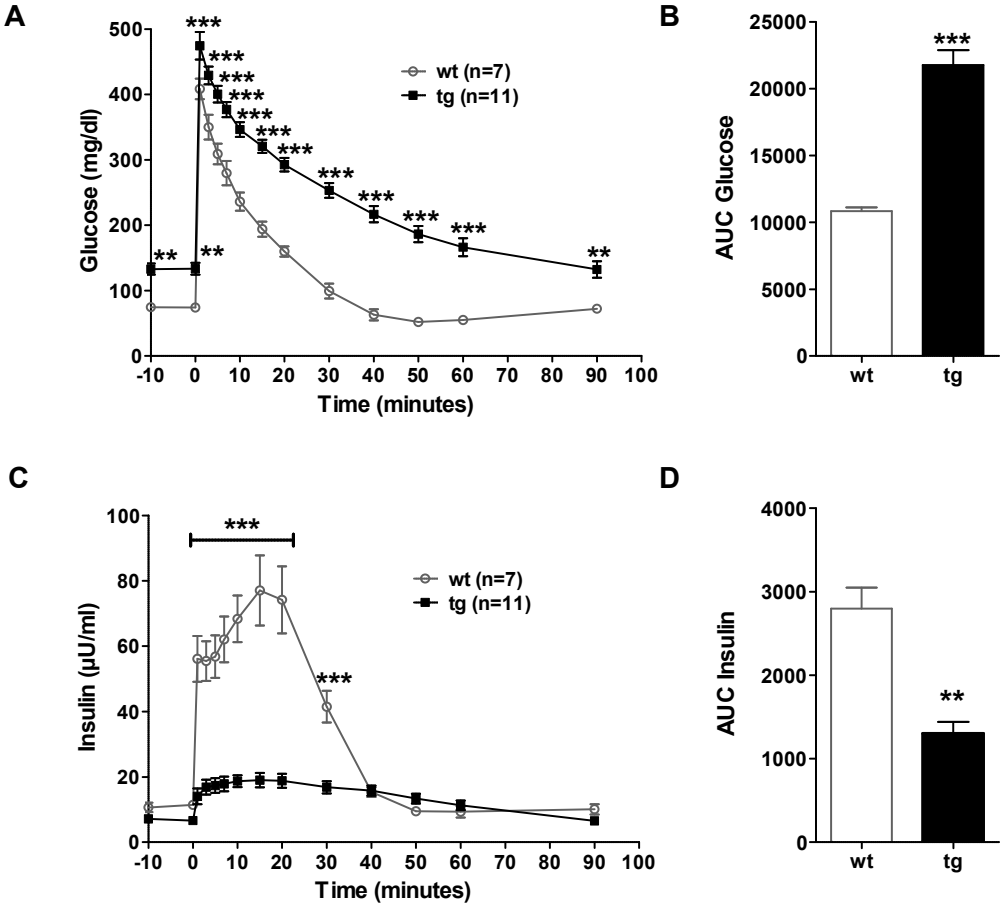

Figure 12: Intravenous glucose tolerance in 7-month-old *INS*^{C93S} transgenic pigs

(**A**) Plasma glucose levels; (**B**) AUC glucose: area under the glucose curve; (**C**) Plasma insulin levels; (**D**) AUC insulin: area under the insulin curve; *INS*^{C93S} transgenic animals (tg) and non-transgenic littermate control animals (wt) of the F1 and F4 generation; data are means ± SEM; (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$.

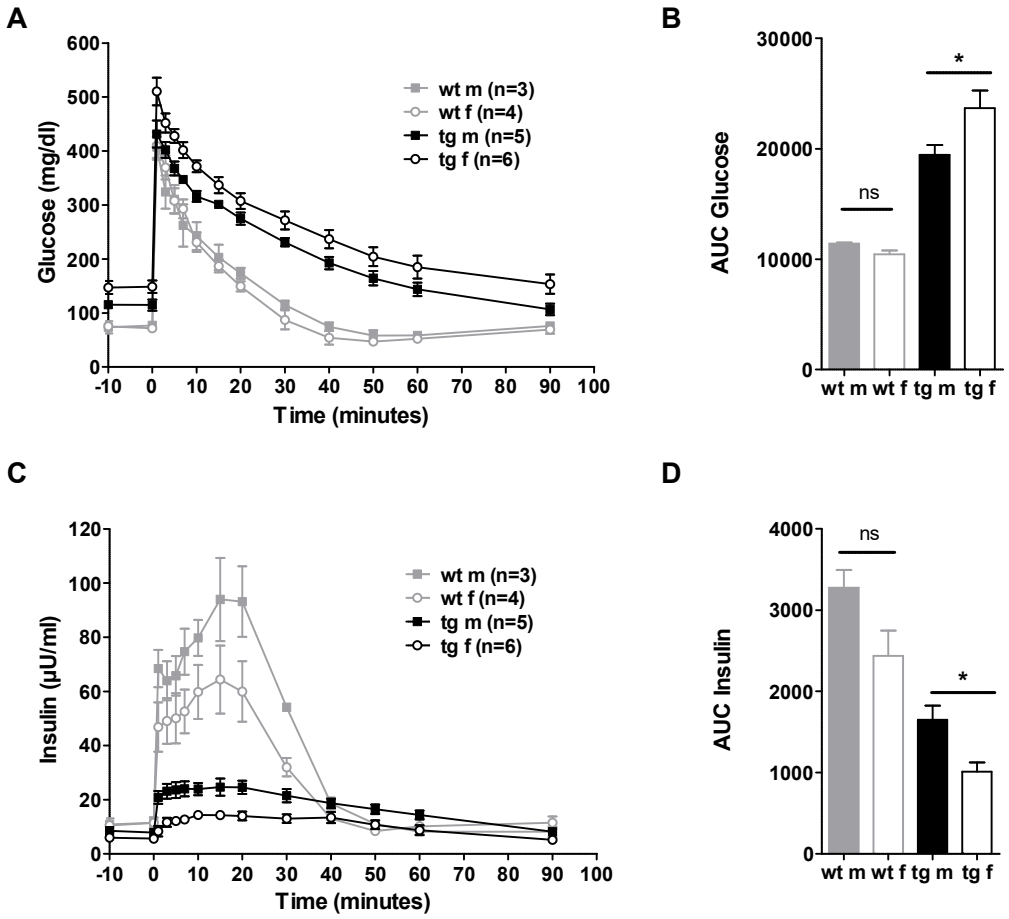

Figure 13: Intravenous glucose tolerance in 7-month-old *INS*^{C93S} transgenic pigs, gender effects

(**A**) Plasma glucose levels; (**B**) AUC glucose: area under the glucose curve; (**C**) Plasma insulin levels; (**D**) AUC insulin: area under the insulin curve; *INS*^{C93S} transgenic animals (tg) and non-transgenic littermate control animals (wt); (m) male; (f) female; data are means ± SEM; (*) $p<0.05$.

Furthermore, the same animal group was subjected to MMGTTs. Oral digestion of dietary constituents present in a standard mixed meal combined with a glucose bolus represents a more physiological test compared with the intravenous glucose challenge. Following oral glucose uptake, glucose-induced insulin secretion is potentiated by incretin hormones produced in enteroendocrine cells and also other factors like gastric emptying play a role for glucose clearance and are not considered following an intravenous glucose bolus. MMGTTs were performed in 4-month-old transgenic pigs (n=11) and seven wt littermate controls as well as in 7-month-old transgenic pigs (n=9) and six littermate controls. FPG levels tended to be reduced at 4 months of age and

were significantly reduced at seven months of age in *INS*^{C93S} transgenic pigs as compared to controls ($FPG_{4months}$= 144.0 ± 8.085 vs. 88.1 ± 2.870, p= 0.058) and ($FPG_{7months}$= 140.7 ± 14.066 vs. 81.9 ± 1.286, p=0.036) (Figure 14 A and Figure 15 A). Like in IVGTTs, fasting plasma insulin levels were unaltered in both age groups (Insulin $_{4\ months}$ = 9.234 ± 1.159 vs. 9.471 ± 1.396, p= 0.9676) and (Insulin $_{7\ months}$ = 7.574 ± 0.818 vs. 9.811 ± 1.642, p= 0.7986) (Figure 14 C and Figure 15 C). After an oral glucose challenge (2 g/kg body weight) mixed with commercial pig food adjusted for body weight, blood glucose concentrations started to increase at time point 15 minutes relative to the oral glucose plus food load in both groups. In both age groups, glucose tolerance was reduced in *INS*^{C93S} tg animals as compared to wt controls (AUC glucose $_{4\ months}$ = 29051 ± 2834 vs. 13209 ± 497, p<0.001) and (AUC glucose $_{7\ months}$ = 26489 ± 2764 vs. 12891 ± 582, p<0.001) (Figure 14 A, B and Figure 15 A, B). In line with increased glucose levels, insulin secretion was reduced in *INS*^{C93S} transgenic animals. Although at four months of age, total AUC insulin was not different from wt animals (AUC insulin $_{4\ months}$ =3801 ± 403 vs. 4121 ± 341, p=0.651), *INS*^{C93S} transgenic pigs showed a significant delay in peak insulin secretion. While wt animals reached maximum plasma insulin levels at 30 min, *INS*^{C93S} transgenic pigs reached their maximum insulin levels only at 90 min, and the insulin peak level of tg animals never reached that of non-transgenic controls (AUC $_{4\ months}$ 0-30min = 1202.7 ± 135.882 vs. 643.0 ± 71.417, p=0.007) (Figure 14 C, D). However, at seven months of age, insulin secretion was significantly reduced in *INS*^{C93S} transgenic pigs (AUC $_{7\ months}$ = 2901 ± 324 vs. 5627 ± 863, p<0.05) (Figure 15 C, D). Compared with insulin secretion four months of age, *INS*^{C93S} transgenic pigs secreted 24 % less insulin at seven months of age (AUC $_{4\ months}$ = 3801.4 ± 403.241 vs. AUC $_{7\ months}$ = 2901 ±324, p=0.197) (Figure 15 E).

In addition, two patterns of insulin curves in wt animals were observed during the MMGTT: a monophasic curve at four months and a biphasic curve at seven months of age. In the monophasic curve, insulin concentrations peaked at 30 minutes (50.8 ± 4.269 µU/mL) after the glucose intake and started to constantly decrease thereafter (Figure 14 B). In the biphasic curve two insulin peaks were observed. A first insulin peak (56.0 ± 13.689 µU/mL) at 30 minutes followed by a decrease in insulin concentration up to 60 minutes and a second insulin peak (70.2 ± 17.128 µU/mL) that occurred at 90 minutes followed by a constant decrease in insulin concentration until the end of the MMGTT, i.e. at 120 minutes (Figure 15 B). These fluctuations were

observed in parallel to the respective glucose curves. *INS*^{C93S} transgenic animals only revealed monophasic curves. In summary, *INS*^{C93S} transgenic pigs showed impaired oral glucose tolerance at four and seven months of age. Different from IVGTT, insulin secretion was delayed but not reduced at four months and reduced at seven months of age.

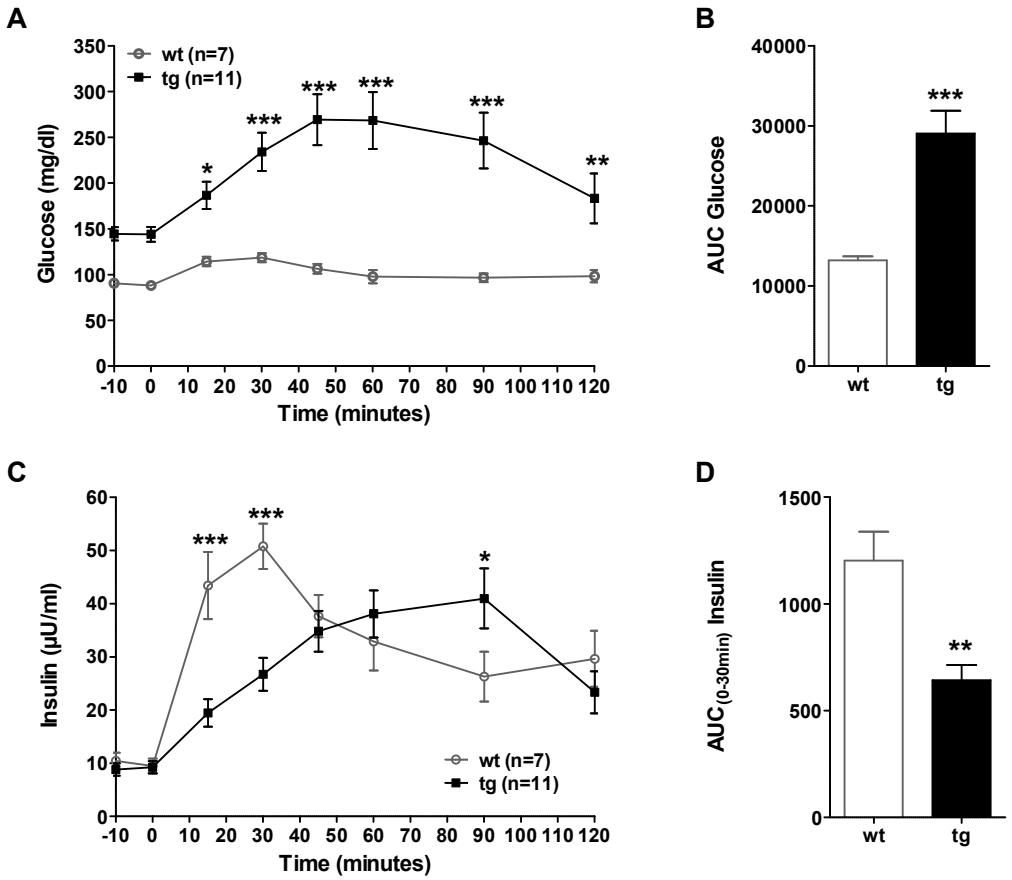

Figure 14: Oral glucose tolerance in 4-month-old *INS*^{C93S} transgenic pigs

(**A**) Plasma glucose levels; (**B**) AUC glucose: area under the glucose curve; (**C**) Plasma insulin levels; (**D**) AUC insulin: area under the insulin curve; *INS*^{C93S} transgenic pigs (tg) and non-transgenic littermate control animals (wt) from F1 and F4 generation; data are means ± SEM; (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$.

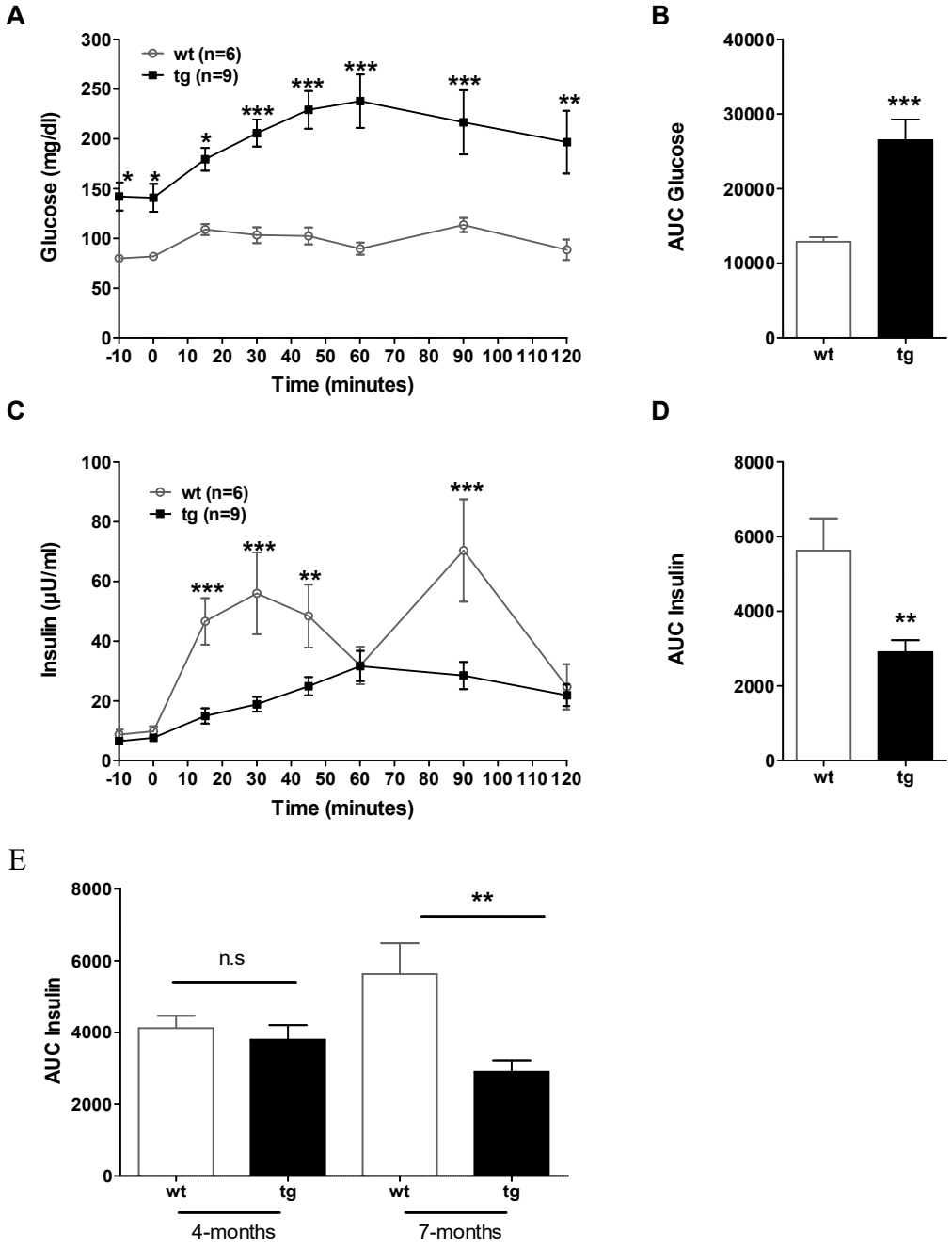

Figure 15: Oral glucose tolerance in 7-month-old *INS*^{C93S} transgenic pigs.

(**A**) Plasma glucose levels; (**B**) AUC glucose: area under the glucose curve; (**C**) Plasma insulin levels; (**D**) AUC insulin: area under the insulin curve; (**E**) AUC insulin at four and seven months; *INS*^{C93S} transgenic pigs (tg) and non-transgenic littermate control animals (wt); data are means ± SEM; (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$.

F1-offspring from founder boar 9748 revealed a similar phenotype, i.e. reduced glucose tolerance and insulin secretion in an IVGTT and OGTT at four and seven months of age. However, the effect of *INS*^{C93S} expression on FBG levels was less

pronounced. Therefore, all further investigations were performed in offspring from founder 9776.

2.4. Low-grade reduction of the total β-cell volume in *INS*^{C93S} pigs

Next, total β-cell volume was investigated. In total, four *INS*^{C93S} transgenic and three age-matched wildtypes were analyzed. At the time of necropsy animals were 389 ± 19.5 days of age. No significant difference in pancreas weight between *INS*^{C93S} transgenic animals and wt controls could be detected at the time of necropsy (217.9 ± 15.0 vs. 191.8 ± 26.6, p=0.289), respectively. Quantitative stereological analysis of the pancreas revealed a reduced, although not significant, total β-cell volume in *INS*^{C93S} transgenic pigs compared to wt littermates. Volume density of β-cells in the pancreas and total β-cell volume was reduced by 35 % ($Vv_{(\beta\text{-cell/Pan})}$ = 0.875 ± 0.07, n=4 vs 1.34 ± 0.29, n=3, p=0.114) and 22 %, ($V_{(\beta\text{-cell,Pan})}$ = 1779.34 ± 198.16, n=4 vs. 2295.70 ± 355.89, n=3, p= 0.114), respectively Figure 16 A and B).

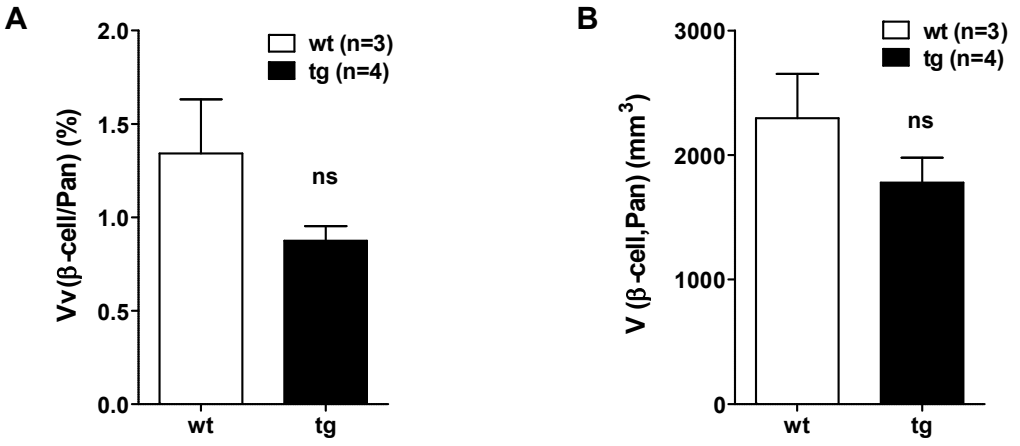

Figure 16: Quantitative stereological analysis of the pancreas

(**A**) Volume density of β-cells in the pancreas ($Vv_{(\beta\text{-cell/Pan})}$) and (**B**) total β-cell volume ($V_{(\beta\text{-cell, Pan})}$); total β-cell volume is decreased by 22 % in tg pigs compared to non-transgenic littermates (p=0.114). Data are means ± SEM; ns: p>0.05.

3. Phenotype of *INS*^{C93S} tg sows during pregnancy

Next, glucose and insulin metabolism in the context of pregnancy were investigated. Physiological metabolic changes during pregnancy, were characterized in wt-pregnant (wt-p) sows compared with wt non-pregnant (wt-np) controls, hence, serving as a control group for the effects of pregnancy in sows. Moreover, metabolic changes in prediabetic *INS*^{C93S} transgenic pregnant sows (*INS*^{C93S}-p) were investigated.

3.1. Fasting glucose levels in *INS*^{C93S} transgenic sows during pregnancy

Eighteen hours fasting glucose levels were monitored twice weekly in *INS*^{C93S}-p (n=3), wt-p (n=4), *INS*^{C93S}-np (n=3) and wt-np (n=4) during gestational week three to sixteen. Pregnant and non-pregnant *INS*^{C93S} transgenic sows exhibited similar significantly elevated fasting glucose levels (*INS*^{C93S}-p: 88.952 ± 2.122 and *INS*^{C93S}-np: 90.306 ± 2.888) compared to wt-p (53.330 ± 0.858 and wt-np 50.330 ± 1.119) sows ($p < 0.0001$) (Figure 17 A, B). No further increase in fasting glucose levels was observed during pregnancy in *INS*^{C93S}-p sows compared to *INS*^{C93S}-np sows (Figure 17 A, B). This was also the case in wt-p and wt-np sows. These results indicate that pregnancy did not affect fasting glucose levels of wt and *INS*^{C93S} transgenic sows.

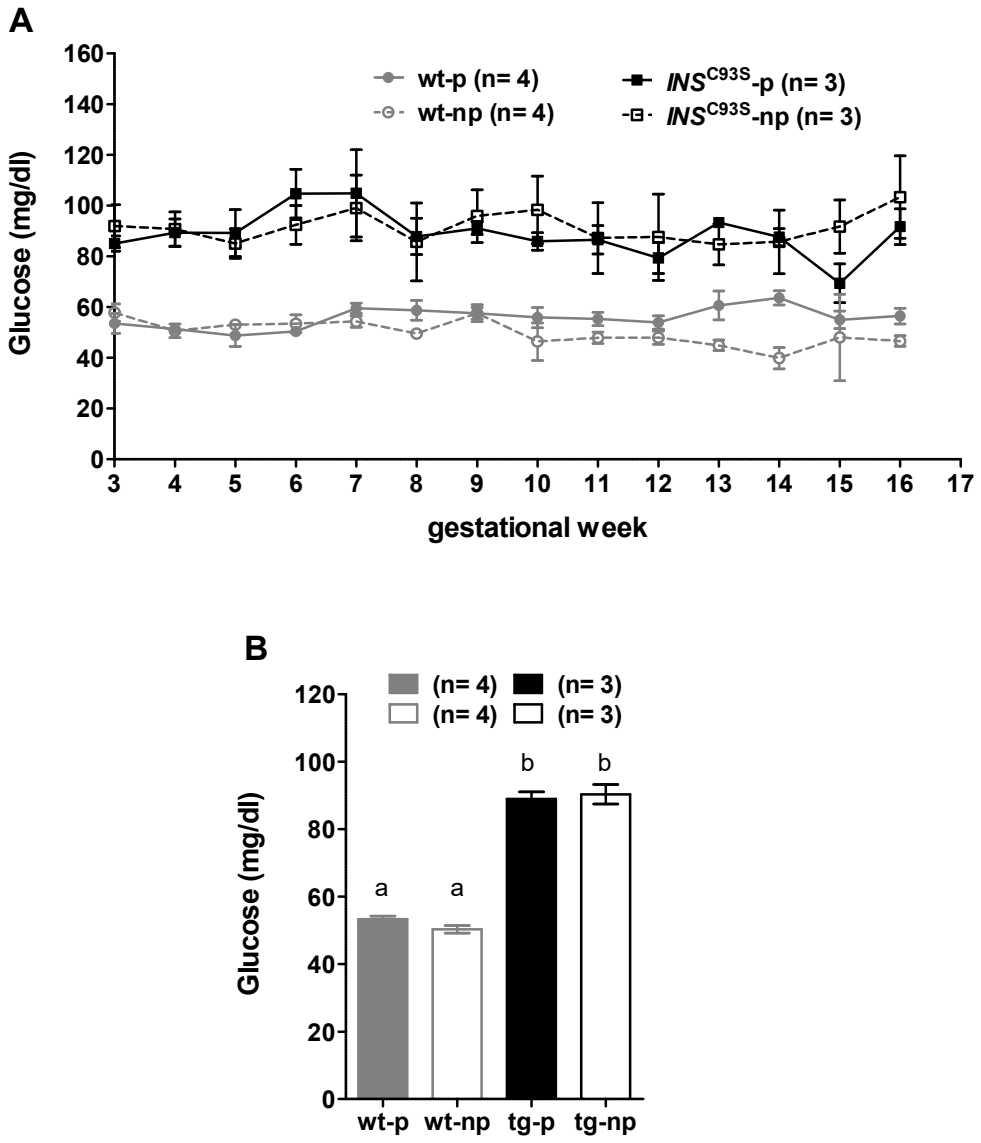

Figure 17: Fasting glucose levels in *INS*^{C93S} transgenic sows during pregnancy

(A) Fasting glucose concentrations from gestational week three to 16. **(B)** Mean glucose levels from gestational week three to 16 of pregnant (*INS*^{C93S}-p) and non-pregnant (*INS*^{C93S}-np) *INS*^{C93S} transgenic sows as well as pregnant (wt-p) and non-pregnant (wt-np) wildtype sows. Data are means ± SEM. a, b: different letters depict significant differences.

3.2. Insulin sensitivity is reduced to the same extent in pregnant *INS*^{C93S} transgenic and wt sows

HIC were performed to investigate changes in insulin sensitivity during pregnancy (gestational week 80 ± 2.30). Initially, insulin sensitivity of wt-p (n=4) and wt-np (n=3) was investigated. In a second step, insulin sensitivity in the pregnant state of wt-p (n=4) and *INS*^{C93S}-p (n=3) was evaluated. Steady state conditions were successfully achieved at 130 min in the two groups. This was shown by constant glucose infusion rates during 130 and 180 minutes. Additionally, plasma insulin (wt-p 0.374 ± 0.057 ng/mL vs. wt-np 0.321 ± 0.047 ng/mL, p=0.488 and tg-p 0.383 ± 0.060ng/mL vs. wt-p 0.450 ± 0.065 ng/mL, p=0.456) and glucose (wt-p 67.2 ± 1.880 mg/dl vs. wt-np 67.1 ± 1.965 mg/dl, p= 0.968 and tg-p 70.3 ± 4.066 mg/dl vs. wt-p 70.9 ± 1.656 mg/dl, p= 0.893) levels were maintained during that period in all four groups (Figure 18 A and Figure 19 A). GIR was reduced by 35 % in wt-p sows compared with wt-np (9.020 ± 0.267 vs. 14.064 ± 0.930, p = 0.028) (Figure 18 B and C), whereas GIR in *INS*^{C93S}-p sows was reduced to the same extend as in wt-p sows (6.335 ± 0.456 vs 6.834 ± 0.317, p=0.633) (Figure 19 B and C). Importantly, the coefficient of variance (CV) of the GIR during the steady state was ≤5 % in both groups (wt-p 4.53 % vs. wt-np 5.13 % tg-p 4.62 % vs. wt-p 3.0 %). Taken together, these results demonstrate that pregnancy reduced insulin sensitivity in pigs, however, insulin sensitivity in pregnant *INS*^{C93S} transgenic sows was not further impaired compared to wt-p sows.

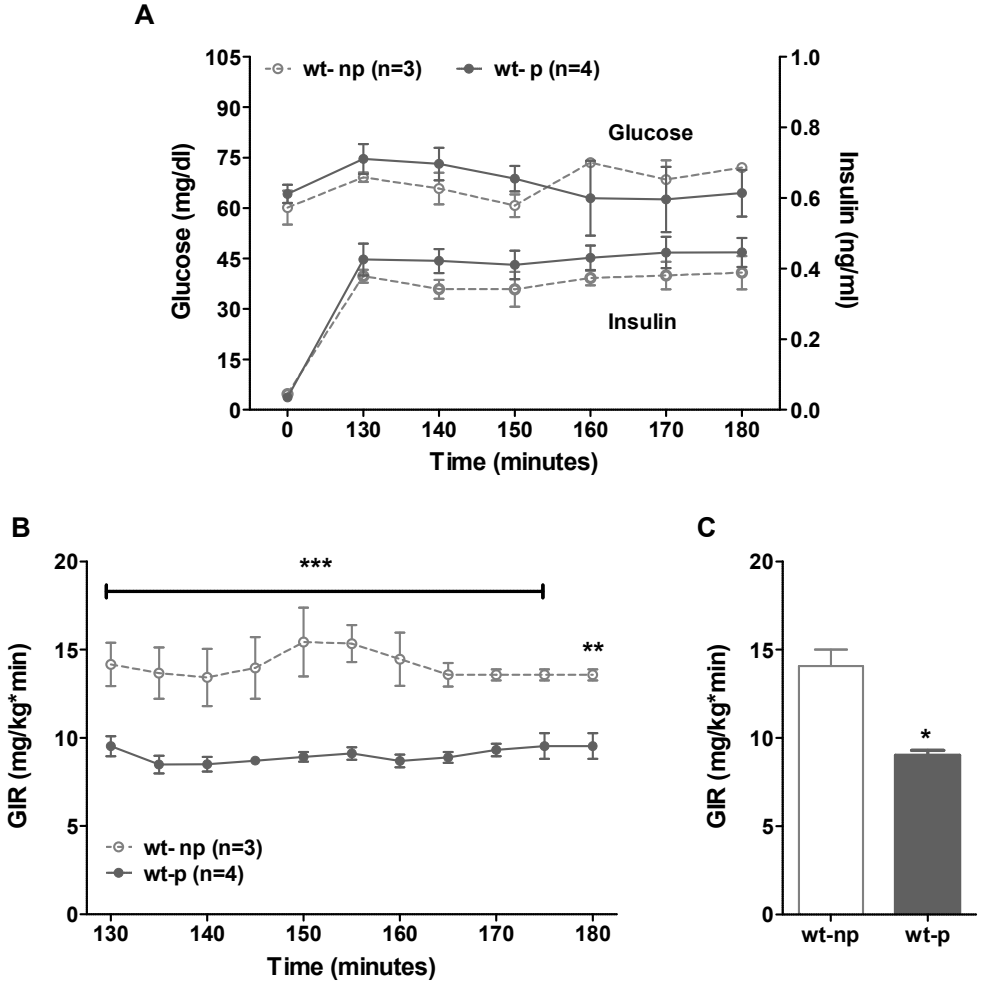

Figure 18: Hyperinsulinemic-euglycemic clamps in wt sows during pregnancy

(A) Plasma glucose and insulin concentrations during steady-state of the HIC. **(B)** Glucose infusion rate (GIR) during steady-state. **(C)** Average GIR during steady state. Pregnant wildtype sows (wt-p); non-pregnant wildtype sows (wt-np); Data are expressed as means ± SEM. (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

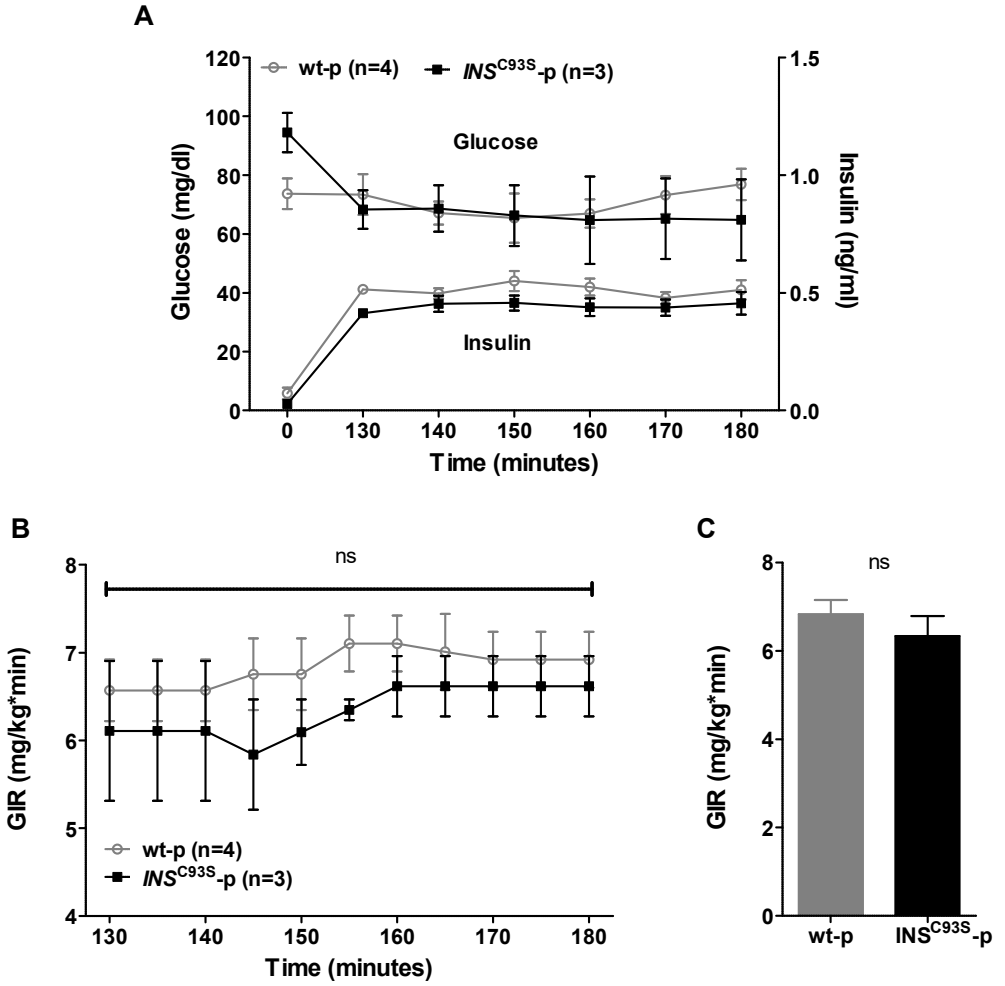

Figure 19: Hyperinsulinemic-euglycemic clamps in tg sows during pregnancy

(A): Plasma glucose and insulin concentrations during steady-state of the HIC. **(B)** Glucose infusion rate (GIR) during steady-state. **(C)** Average GIR during steady state. Pregnant *INS*^{C93S} transgenic sows (*INS*^{C93S}-p); pregnant non-transgenic controls (wt-p); Data are expressed as means ± SEM. (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

3.3. ß-cell function is significantly impaired in *INS*^{C93S}-p sows

HGC tests were performed to assess β-cell function of *INS*^{C93S}-p and wt-p sows (gestational week 86 ± 2.82). Animals were clamped at a hyperglycemic level of 300 mg/dl (*INS*^{C93S}-p 326.7 mg/dl ± 4.97 vs. wt-p 326.6 mg/dl ± 10.03, p= 0.993) by a variable infusion of a 40% glucose solution during the time course of the clamp (Figure 20 A). First phase insulin secretion (0-10 min) did not significantly differ between *INS*^{C93S}-p and wt-p sows (0.114 ng/mL ± 0.006 vs 0.264 ± 0.073, p= 0.189) (Figure 20 B). However, second phase insulin response (10-90 min) was significantly impaired in *INS*^{C93S}-p compared with wt-p sows as indicated by significantly lower plasma insulin concentrations in all time points (Figure 20 B). Overall total insulin response was 71 % reduced in *INS*^{C93S}-p sows compared to wt-p sows (AUC= 19.9 ± 5.49 vs 79.7 ± 8.81, p= 0.0045) (Figure 20 C). Correspondingly, the reduced insulin secretion, GIR was lower in *INS*^{C93S}-p compared with wt-p sows, and significantly different at time points 20, 25, 60 and 70 min (Figure 20 D). However, total GIR did not reach significance between *INS*^{C93S}-p and wt-p sows (9.041 ± 0.324 vs. 12.694 ± 1.368, p=0.077) (Figure 20 D and E). Both *INS*^{C93S}-p and wt-p responded with increased insulin secretion to the arginine bolus at 50 min (Figure 20 B). Despite the reduced insulin secretion in *INS*^{C93S}-p, the increment in insulin response (50 to 60 min) following the arginine bolus was not different compared to wt-p (0.141 ± 0.03 vs 0.214 ± 0.026, p=0.229). Together these results indicate that *INS*^{C93S}-p sows have reduced insulin secretion, reflecting impaired β-cell function.

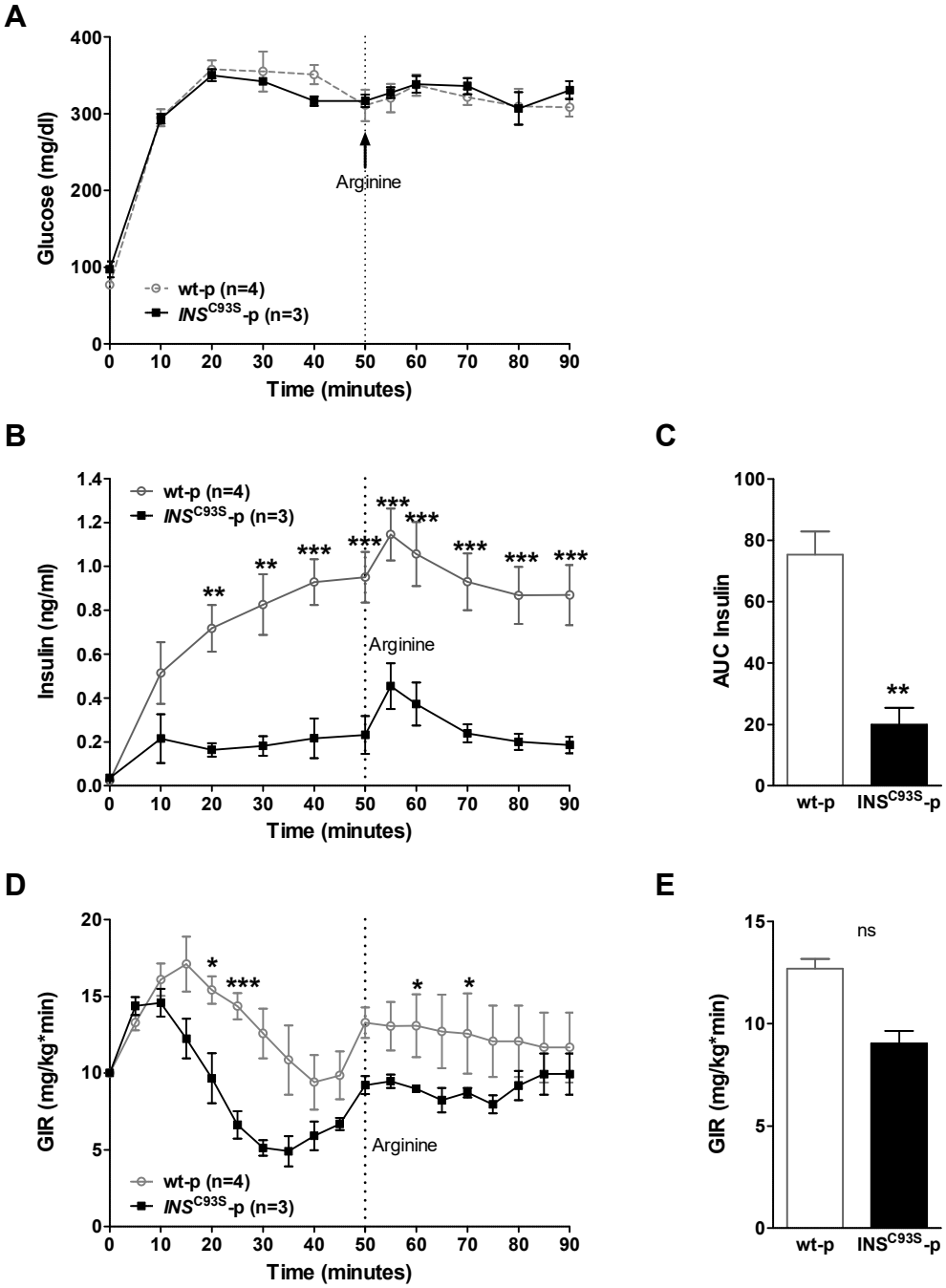

Figure 20: Hyperglycemic clamps during pregnancy

(A) Plasma glucose concentration; (**B**) Plasma insulin levels; (**C**) Area under the insulin curve (AUC insulin); (**D**) Glucose infusion rate (GIR); (**E**) Mean glucose infusion rate; pregnant *INS*^{C93S} transgenic sows (*INS*^{C93S}-p); pregnant non-transgenic littermate controls (wt-p); - non-pregnant controls (wt-np); Date are expressed as means ± SEM; (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$.

3.4. Low-grade impairment of glucose tolerance in wt-p and pronounced impairment in *INS*^{C93S}-p sows

MMGTs were performed in nine wt-p, three *INS*^{C93S}-p and three age-matched wt-np sows. This allowed characterization of glucose metabolism and insulin response in wild-type pigs during pregnancy, as well as to investigate how *INS*^{C93S} transgenic sows respond to extra metabolic demands during the pregnant state as compared to wt-p sows. Wt-np and wt-p pigs showed unaltered fasting glucose levels (65.0 ± 0.996 vs. 67.0 ± 4.272 mg/dl, p=0.863), whereas *INS*^{C93S}-p sows exhibited significantly elevated, borderline hyperglycemic values (104.8 ± 1.530, p<0.01) (Figure 21 A). Although *INS*^{C93S}-p sows exhibited elevated fasting glucose, fasting insulin levels did not differ from wt-p control sows (5.45 ± 3.17 vs. 7.33 ± 1.77, p= 0.911) (Figure 21 B). Upon oral glucose challenge (2 g/kg body weight), glucose levels rose in all three groups and reached maximum levels at 30 min for both wt-np and wt-p sows, respectively (93.7 ± 2.554 and 114.1 ± 5.027 mg/dl, p=0.077). *INS*^{C93S}-p sows showed rising glucose levels until 60 min (209.7 ± 1.888 mg/dl). From 30 min after oral glucose load, wt-p sows had significantly elevated glucose levels until the end of the MMGTT as compared to wt-np sows, but glucose levels were significantly lower than in *INS*^{C93S}-p sows for all time points (Figure 21 A). This resulted in a significantly increased AUC glucose in wt-p as compared to wt-np (AUC= 3866 ± 642.8 vs. 1254.9 ± 183.1, p<0.01) (Figure 21 B) but significantly reduced AUC glucose compared with *INS*^{C93S}-p sows (3866 ± 642.8 vs. 8754.7 ± 989.6, p<0.05) (Figure C). Insulin response was enhanced in wt-p and impaired in *INS*^{C93S}-p sows compared to wt-np. Wt-p sows which showed significantly higher glucose levels than wt-np sows secreted significantly more insulin from time point 30 min compared to wt-np sows (Figure 21 D). Despite not significant, AUC insulin of wt-p tended to be higher than AUC insulin of wt-np (7984 ± 881.1 vs. 4726.7 ± 682.0, p=0.0636) (Figure 21 E). In parallel, tg-p sows which showed impaired glucose tolerance revealed reduced and delayed insulin secretion as compared to wt-p sows (Figure 21 B). This led to a significant reduction in AUC insulin between tg-p and wt-p sows (4607.7 ± 119.2 vs. 7984 ± 881.1, p<00.1) (Figure 21 F).

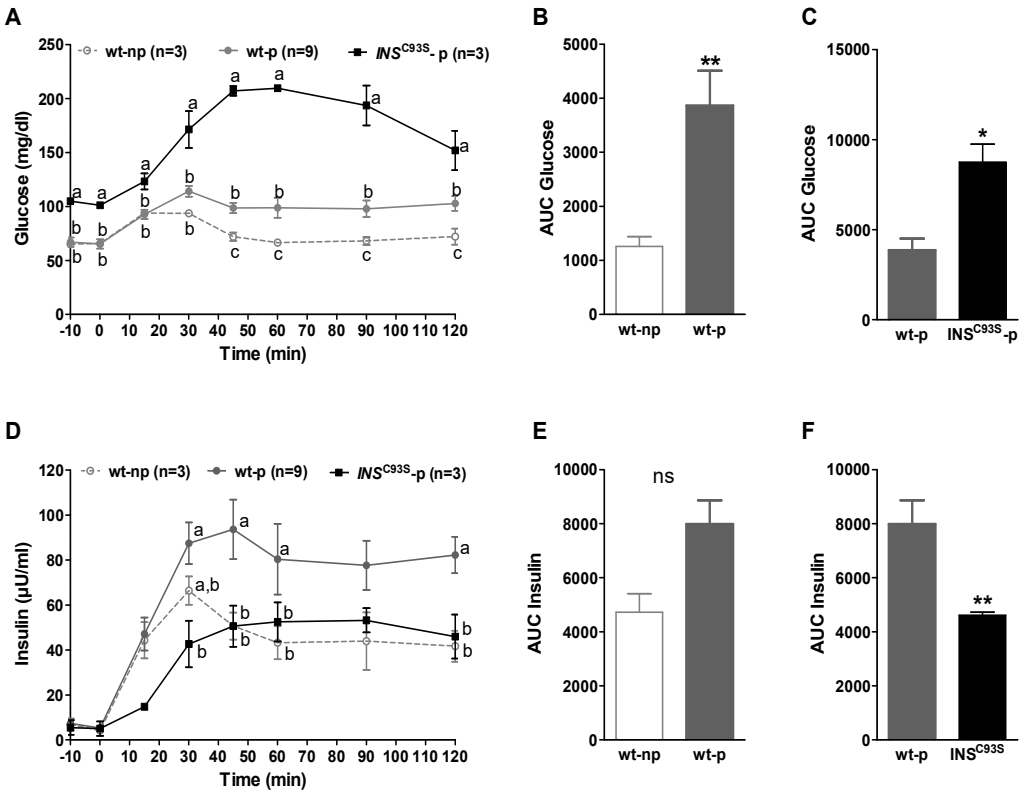

Figure 21: MMGTT during pregnancy.

(A) Plasma glucose levels; (**B, C**) AUC glucose: area under the glucose curve; **(D)** Plasma insulin levels; (**E, F**) AUC insulin: area under the insulin curve; pregnant *INS*^{C93S} transgenic sows (*INS*^{C93S}-p), pregnant non-transgenic control animals (wt-p) and non-pregnant, non-transgenic control animals (wt-np); Data are means ± SEM; a, b, c: different letters depict significant differences; (*) $p<0.05$; (**) $p<0.01$.

4. Effects of mild maternal hyperglycemia on offspring

4.1. Wt piglets born to mild diabetic *INS*^{C93S} transgenic sows revealed elevated FPG and a tendency of reduced glucose tolerance despite increased insulin secretion at birth

Effects of chronic exposure to mild hyperglycemia *in utero* were investigated in newborn piglets. Differences in glucose metabolism and insulin response were investigated during OGTTs at birth in 18 wt piglets born to wt sows and 12 wt piglets born to *INS*^{C93S} transgenic sows. At birth wt/tg piglets showed elevated glucose levels compared to wt/wt piglets (83.4 ± 9.353 vs. 54.133 ± 4.810, p= 0.0553) (Figure 22 A). Results from fasting insulin revealed that 58 % of wt/tg piglets showed insulin concentrations above the detection limit of the ELISA (≤ 1.611 µU/mL) as compared to 11 % of wt/wt, suggesting that wt/tg piglets have higher insulin levels as compared to wt/wt piglets (Figure 22 B). Following oral glucose administration (2 g/kg body weight), wt/tg exhibited significantly elevated glucose levels from 30 min until the end of the OGTT (Figure 23 A). When corrected to baseline values, AUC glucose was not significant although, it tended to be higher in wt/tg compared to AUC glucose of wt/wt piglets, (14278.3 ± 1520.7 vs. 11438.6 ± 783.7, p=0.0790) (Figure 23 B). Insulin response to glucose load was also different in wt/tg piglets which exhibited elevated insulin levels especially at the end (120 min.) of the OGTT (27.8 ± 6.834 vs. 14.2 ± 1.573, p<0.001) (Figure 23 C). Total AUC insulin of wt/tg piglets (corrected to baseline levels) tended to be higher than total AUC insulin from wt/wt (2108.2 ± 380.967 vs. 1417.3 ± 234.390, p= 0.0864) (Figure 23 D). Together these results suggest that chronic exposure to maternal mild hyperglycemia has effects on early glucose response and insulin secretion in wt/tg piglets.

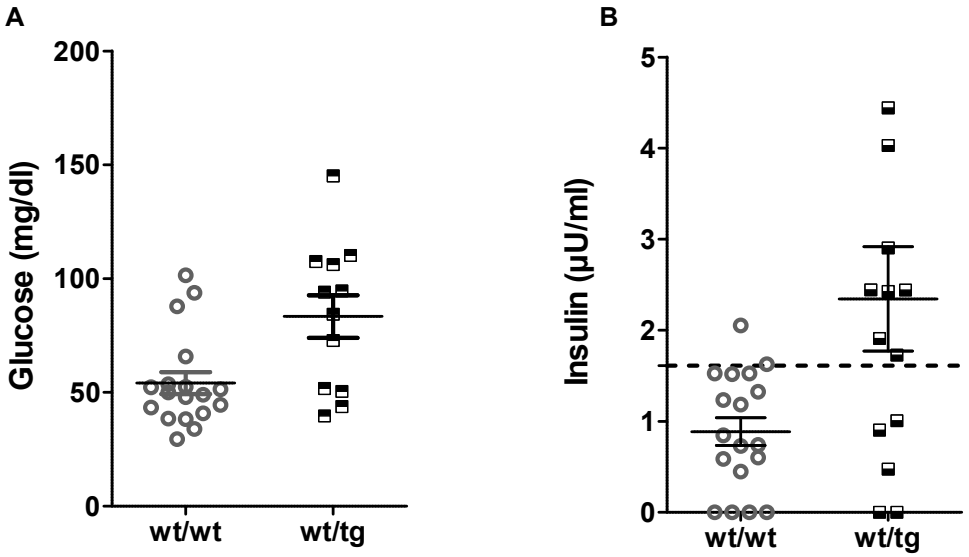

Figure 22: Fasting glucose and insulin levels at birth in offspring of *INS*^{C93S} tg and wt control sows

(**A**) Plasma glucose levels and (**B**) Plasma insulin levels in 18 wt/wt piglets and 12 wt/tg piglets); wt piglets born to wt sows (wt/w); wt piglets born to *INS*^{C93S} transgenic sows (wt/tg); dotted line depicts the detection limit of the insulin assay (1.611 µU/mL); (**) $p<0.01$.

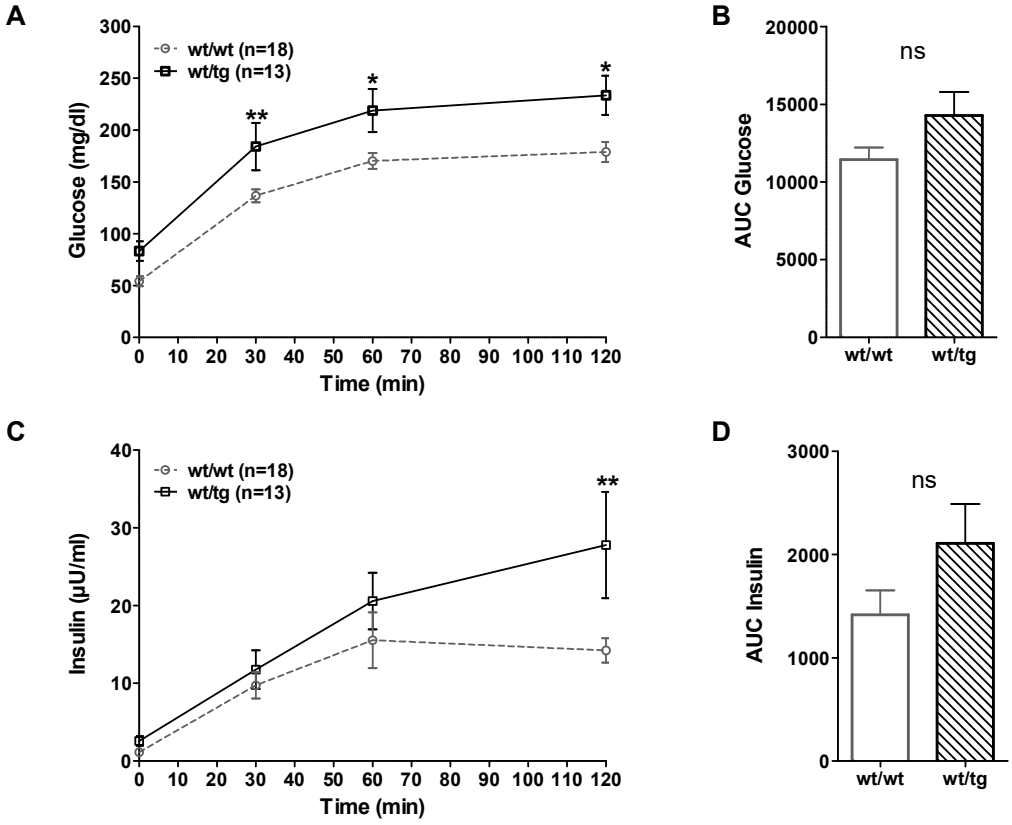

Figure 23: Oral glucose tolerance tests in newborn piglets.

(**A**) Plasma glucose levels; (**B**) AUC glucose: area under the glucose curve; (**C**) Plasma insulin levels; (**D**) AUC insulin: area under the insulin curve; wt piglets born to wt sows (wt/wt); wt piglets born to *INS*^{C93S} tg sows (wt/tg); Data are means ± SEM; (*) $p<0.05$; (**) $p<0.01$.

4.2. Prenatal exposure to mild hyperglycemia altered lipid metabolism and glycolysis-related metabolites in wt offspring.

Plasma samples were collected at 0 and 120 min during OGTTs to determine changes in metabolites involved in carbohydrate and lipid. Six wt/tg and six wt/wt piglets were investigated. At birth (0 min), wt/tg piglets exhibited significantly increased concentrations of total cholesterol (43.4 ± 3.624 vs. 31.7 ± 2.826, p= 0.034) (Figure 24 A), LDL (36.2 ± 2.360 vs. 29.4 ± 1.557, p=0.041) (Figure 24 B) and HDL (19.4 ± 1.777 vs. 14.0 ± 1.557, p=0.025) (Figure 24 C) compared to wt/wt. Triglycerides, NEFA and lipase were not changed (Figure 24 D, E and F). When comparing metabolite changes during OGTT (0 to 120 min), most of the alterations occurred in metabolites directly involved in glucose metabolism. Glucose was significantly

increased at 120 in both groups and was significantly different in wt/tg compared to wt/wt (208.1 ± 20.162 vs. 168.1± 14.231, p=0.0465) (Figure 25 A). Lactate concentrations decreased in both groups after 120 min but with less extended in wt/tg (4.413 ± 0.780 vs. 2.390 ± 0.185, p= 0.149) (Figure 25 B). LDH was significant increased after 120 in wt/tg compared to wt/wt (626.0 ± 79.845 vs. 452.0 ± 39.615, p= 0.028) (Figure 25 and Figure 24 C). These results indicate that wt/tg piglets showed differential lipid profile at birth and key elements of the glycolysis pathway were differently regulated upon 120 min OGTT in wt/tg compared to wt/wt.

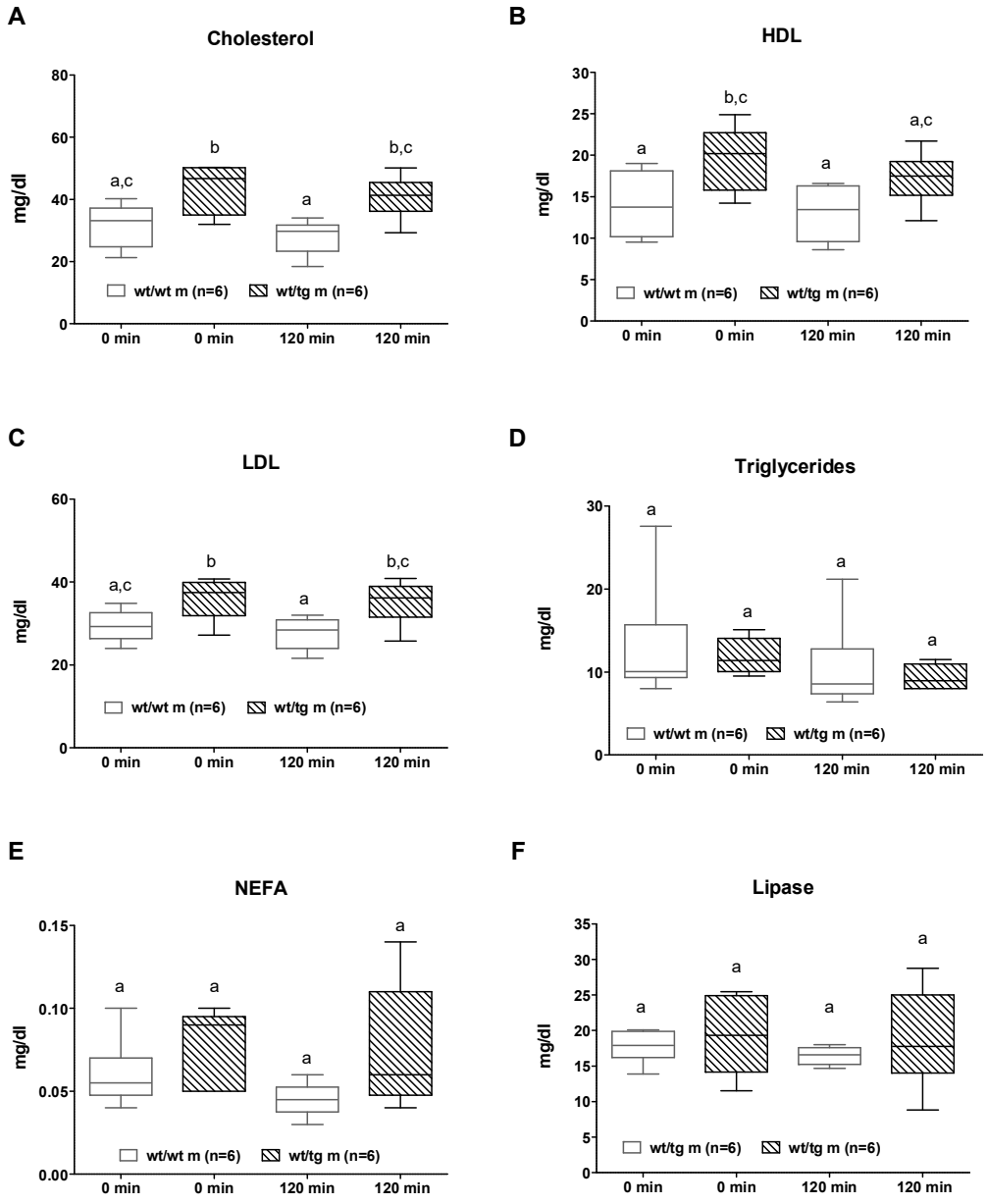

Figure 24: Lipid metabolic parameters in offspring of *INS*^{C93S} tg sows and wt controls

(**A**, **B**, **C**, **D**, **E** and **F**) Differential lipid parameters evaluated in plasma samples collected at 0 min (right after birth) and 120 min (time after oral glucose bolus). Data are means ± SEM, a, b and c: different letters depict significant differences.

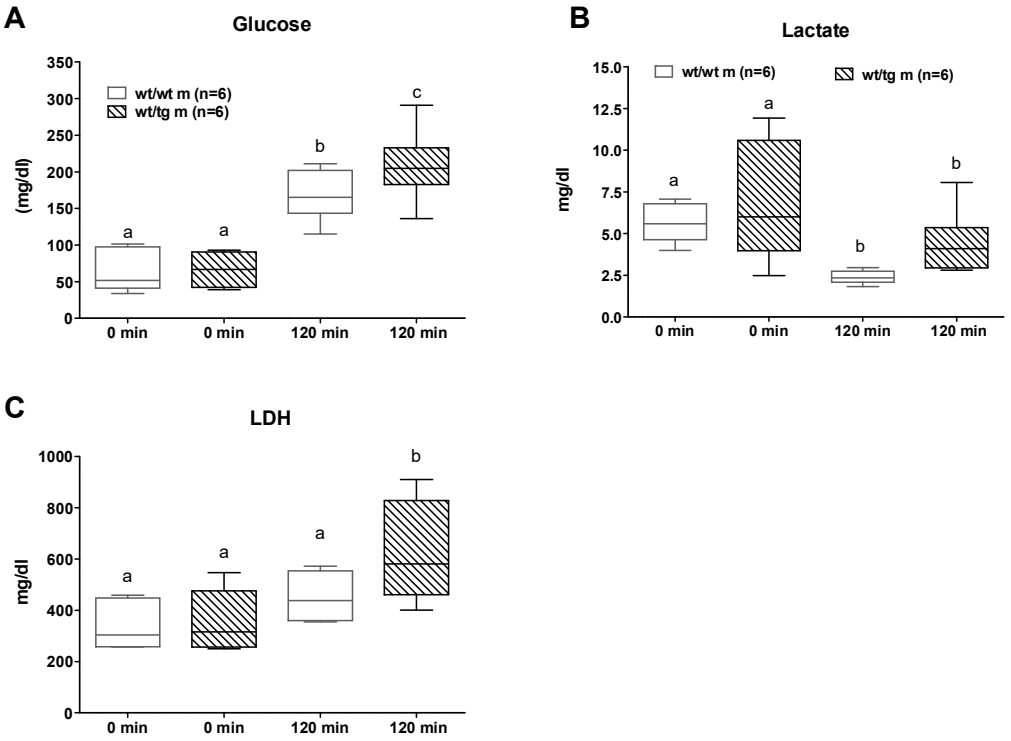

Figure 25: Carbohydrate metabolic parameters in offspring born to *INS*^{C93S} tg sows and born to wt controls

(**A**, **B** and **C**) Carbohydrate parameters of the glycolytic pathway evaluated in plasma samples collected at 0 min (right after birth) and 120 min (time after oral glucose bolus). Data are means ± SEM, a, b and c: different letters depict significant differences.

4.3. Unaltered morphological parameters in wt piglets chronically exposed to mild maternal hyperglycemia

At the end of the study, body and organ weights were investigated in all 14 wt/tg and 11 wt/wt piglets at day one of age. No significant difference in absolute and relative body weights, CRL and organ weights could be detected between the two groups. Although not significant, relative liver and heart weights tended to be higher in wt/tg compared wt/wt animals (Table 9).

Table 9: Body and organ weights in 1-day-old piglets

Parameter	*LSMeans*		*Pvalue*
	wt/tg	wt/wt	
Body weight (day 0) (g)	1358.775	1399.369	0.756
Body weight (day 1) (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

Relative to the body weight (*rel*)

V. DISCUSSION

Maternal hyperglycemia is detrimental for the fetus as it increases the risk to develop metabolic diseases as obesity or diabetes in adulthood. Effects of maternal diabetes and underlying mechanisms are difficult to study in humans and have not been fully clarified by animal models. Transgenic pigs expressing the mutant insulin C93S were characterized before and during pregnancy. This novel animal model enables the investigation of deleterious effects of chronic intrauterine exposure to mild forms of hyperglycemia on the offspring at different developmental stages, e.g. embryo, fetus and offspring.

1. Generation and phenotypic characterization of *INS*^{C93S} transgenic pigs before pregnancy

Five living *INS*^{C93S} transgenic founders resulted from SCNT and embryo transfer experiments (KUROME et al., 2015). The nuclear transfer efficiency was 2.42 %, which is in line with the range of SCNT cloning efficiency (0.5 % to 5 %) in the pig (AIGNER et al., 2010). Different integration patterns of the *INS*^{C93S} transgene among the five *INS*^{C93S} founders were observed in Southern blot analyses (Figure 5 A). This result is expected as these animals resulted from pools of stably transfected cell clones whereby the *INS*^{C93S} construct has randomly integrated into the genome. Gene expression data revealed differences in the expression level of the *INS*^{C93S} transgene between the five transgenic founders (Figure 7). This outcome is also expected and results from effects of random integration. It is well known that expression levels are dependent on the number of integration sites as well as they are influenced by the integration site due to differences in chromatin remodeling of neighboring DNA (WOLF et al., 2000; RECILLAS-TARGA, 2006). We selected founders 9776 and 9748 as they showed the highest expression levels of the *INS*^{C93S} transgene and revealed IGT and reduced insulin secretion during an IVGTT (Figure 9). These results not only proved that the phenotype of *INS*^{C93S} transgenic pigs is specifically triggered by the expression of the mutant insulin C93S rather than triggered by disruption of a functional gene (possibly caused by random integration of the *INS*^{C93S} transgene) as they indicate that the effects of *INS*^{C93S} expression in the development of a mild diabetic phenotype are dose-dependent. Moreover, we demonstrated that *INS*^{C93S} is stable within the genome and is inherited by germline transmission to offspring.

Transgenic F1 offspring of 9776 (Figure 7) and 9748 (data not shown) showed the same *INS*^{C93S} integration pattern and also a similar *INS*^{C93S}/*INS* transcript ratio in the pancreas as their respective founder (Figure 7). These results argue in favor of one single integration site of the *INS*^{C93S} transgene copies.

INS^{C93S} transgenic pigs develop a mild diabetic phenotype which is different from severe diabetes observed in humans carrying heterozygous *INS* mutations and different from the existing *INS* mutant animal models, including *INS*^{C94Y} transgenic pigs (HERBACH et al., 2007; RENNER et al., 2013; LIU et al., 2015). In humans, dominant *INS* mutations cause a heterogeneous repertoire of severe forms of diabetes, including permanent neonatal diabetes, infancy-onset diabetes (i.e. diabetes diagnosed before 12 months of age), type 1b diabetes (i.e. non-autoimmune type 1 diabetes), maturity-onset diabetes of the young (MODY), and early-onset type 2 diabetes (STOY et al., 2007; COLOMBO et al., 2008; EDGHILL et al., 2008; MOLVEN et al., 2008; POLAK et al., 2008). The Munich *Ins2*^{C95S} mouse model (corresponding mutation of the *INS*^{C93S} transgenic pig), develops hyperglycemia and hypoinsulinemia from the fourth week of life (HERBACH et al., 2007). However, in contrast to humans and mouse models with mutant insulin genes, our pig model expresses the mutant *INS*^{C93S} as a transgene, while the endogenous *INS* alleles are intact. Consequently, the phenotype of the founder animals was variable due to random transgene insertion and consequently different expression levels of *INS*^{C93S}.

Previously, transgenic pigs expressing the mutant insulin C94Y were generated, and one line developed permanent neonatal diabetes within the first week of life (RENNER et al., 2013). This *INS* mutation results in a proinsulin with one disrupted interchain disulfide bond (between the A- and B-chain of the insulin) (RENNER et al., 2013). In *INS*^{C93S} transgenic pigs, the cysteine to serine exchange at position 93 leads to a disruption of an intra-A-chain disulfide bond of the proinsulin (Figure 3: Proinsulin amino acid sequence

Proinsulin amino acid sequence composed of the A-chain, B-chain and the connecting peptide (C-peptide). The A- and B-chain of the insulin are connected by two interchain and one intrachain disulphide bonds. The arrow depicts the amino acid exchange cysteine to serine at position 93 (C93S) within the mutant insulin. The differences in the phenotype between these two pig models could be attributed to (i) the expression level of the mutant *INS* (which was 20 % higher in *INS*^{C94Y} transgenic pigs as compared to the *INS*^{C93S} transgenic founder 9776 with the highest expression level of

the mutant insulin C93S (RENNER et al., 2013) and (ii) possibly to differences in intracellular mechanisms to rescue different forms of misfolded insulin (LIU & KAUFMAN, 2003; LIU et al., 2005).

The molecular mechanisms going along with the expression of the mutant insulin C93S were not explored in this study. However, it is well established that disruption of disulfide bonds causes misfolding of the mutant proinsulin (CHANG et al., 2003), blockage of its maturation process, and activation of ER stress response pathways, ultimately impairing β-cell function and resulting in β-cell death (IZUMI et al., 2003; ZUBER et al., 2004; LIU et al., 2005; PARK et al., 2010; RAJAN et al., 2010). Therefore, the translated misfolded proinsulin likely also cause a stress responses in the β-cells of *INS*^{C93S} transgenic pigs (RENNER et al., 2013). Importantly, co-expression of wt and mutant INS proteins *in vitro* interfered with secretion of the wt insulin, and this was caused by intracellular trapping of wt insulin by expression of insulin mutant forms (LIU et al., 2010). Since *INS*^{C93S} transgenic pigs express both the mutant and the endogenous insulin one can speculate that the C93S mutation also interferes with wt insulin maturation and secretion by similar mechanisms.

1.1. *INS*^{C93S} transgenic pigs develop a mild diabetic phenotype

We have performed a detailed characterization of glucose tolerance and insulin secretion in *INS*^{C93S} transgenic pigs of the F1 and F4 generation of founder 9776 by means of IVGTT and MMGTTs at four and seven months of age. First, we showed that, like in humans, glucose and insulin curve responses were influenced by the route of glucose administration (ABDUL-GHANI et al., 2006a). When glucose was administrated orally, glucose and insulin concentrations were gradually increased in wt pigs which reflected the slower rate of glucose absorption as compared with the abrupt rise in plasma glucose and insulin concentration following an intravenous glucose load. Interestingly, we observed that the insulin responsiveness of 4-month-old *INS*^{C93S} transgenic pigs during a MMGTT was better as during an IVGTT. Following an intravenous glucose load insulin secretion was reduced while during a MMGTT insulin response was only delayed but overall insulin secretion unaltered. (Figure 11, Figure 12, Figure 14). It is well known that glucose-induced insulin secretion is potentiated when glucose is given orally compared with intravenous administration. This is known as the incretin effect (ELRICK et al., 1964; MCINTYRE et al., 1964) and is mediated by the two incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) reviewed in

(RENNER et al., 2016b). Both hormones are secreted by enteroendocrine cells in the intestine in response to nutrients (DRUCKER, 2006) and upon binding to their specific receptors in pancreatic β-cells they potentiate insulin secretion accounting for 20 to 60 % of the total insulin response (NAUCK et al., 1986a; NAUCK et al., 1986b). Thus, these results indicate that although pancreatic β-cells have a functional defect due to expression of mutant insulin, they still respond, at least to some extent, to incretins. Secondly, we demonstrated that IGT in *INS*^{C93S} transgenic pigs was aggravated at seven months of age as total insulin secretion was not only significantly reduced following during IVGTT (Figure 13 C and D) but also during MMGTT (Figure 15 C and D). Additionally, first phase (0-10 min) insulin secretion was absent in *INS*^{C93S} transgenic pigs during IVGTT at seven months (Figure 12 C).

Altered kinetics of insulin secretion are also described in human studies of individuals with both IFG and/or IGT (HANEFELD et al., 2003; FESTA et al., 2004; ABDUL-GHANI et al., 2006b; ABDUL-GHANI et al., 2006a; FÆRCH et al., 2009). Isolated IFG in humans is characterized by a decrease in the first phase (first 10 min) insulin response during IVGTT and reduced early-phase (first 30 min) insulin secretion during OGTT while late-phase (60-120 min) insulin response is maintained normal. Individuals with IGT show normal fasting glucose levels but both early- and late-phase insulin secretion are accompanied with insulin secretion defects following OGTT. In this study, *INS*^{C93S} transgenic pigs exhibited elevated FPG levels and IGT with impairment of both insulin secretion phases corresponding to those seen in pre-diabetic humans. In humans, the pathogenesis of IFG and IGT seems to be different and a combination of impaired insulin sensitivity with defects in insulin secretion (CARNEVALE SCHIANCA et al., 2003; NATHAN et al., 2007). Hepatic insulin resistance and insulin secretion defects in individuals with IFG accounts for excessive gluconeogenesis, resulting in elevated FPG and excessive early rise of glucose during first hour of OGTT (BOCK et al., 2007). However, maintenance of normal muscle insulin sensitivity and proper late phase insulin secretion enables plasma glucose levels in IFG to decline in late phase to near base line values at the end of OGTT. Contrary, individuals with IGT reveal muscle and hepatic insulin resistance as well as progressive defects in insulin secretion and thus resulting in elevated glucose levels directly after the glucose load and towards the end of OGTT (NATHAN et al., 2007; AOYAMA-SASABE et al., 2016). At both four and seven months of age, elevated stimulated glucose levels in *INS*^{C93S} transgenic pigs were clearly associated with an

insulin secretion defect caused by the expression of the mutant insulin C93S. These results are consistent with reduced β-cell mass at 14 months of age (Figure 15). Elevated FPG levels in *INS*^{C93S} transgenic pigs at four and seven months of age cannot be solely explained by defects in insulin secretion as fasting insulin levels in *INS*^{C93S} transgenic pigs were only slightly lower than in wt controls (Figure 11 C, Figure 12 C, Figure 14 C and Figure 15 C). Since reduced hepatic insulin sensitivity is often an acquired defect contributing for elevated fasting glucose levels in humans with IFG, hepatic insulin resistance might also play a role in IFG of *INS*^{C93S} transgenic pigs. To address this hypothesis, hyperinsulinemic-euglycemic-clamps with a tracer would be required, and these will be done in future experiments.

Finally, we observed different patterns of insulin secretion curves (monophasic curve and biphasic curve) that match decrease and increase of the respective glucose curves during MMGTTs (Figure 14 C and Figure 15 C). These distinct curve shapes are most likely related to the particular glucose challenge test type used in this study. MMGTTs are frequently used in pigs but differ from the standard OGTT protocols widely used in the clinic to diagnose human patients with IGT and diabetes mellitus (ADA, 2016). Unlike OGTTs in humans, where solely glucose is administrated orally, in MMGTTs, glucose plus a certain amount of pig food are mixed as to facilitate oral glucose consumption in pigs. As a result, glucose absorption may be influenced by the diet. In fact, fiber components are known to affect postprandial absorption of glucose giving rise to lower glucose and insulin concentrations, as well as to interfere with gastric emptying (JENKINS et al., 1978; TORSDOTTIR et al., 1989). In fact, as to counteract these disparities between tests, a recent study in 8-week-old pigs described a redefined OGTT method in pigs that resembles the OGTT in humans (MANELL et al., 2016). Nevertheless, it is important to point out that different glucose and insulin curve shapes have also been identified in humans during OGTTs (TSCHRITTER et al., 2003; NOLFE et al., 2012; HAYASHI et al., 2013). While, different insulin curve shape patterns strongly predict risk of developing diabetes (HAYASHI et al., 2013), others also indicate that individual characteristics such as gender and age are also associated to different oral glucose tolerance curve shapes (TSCHRITTER et al., 2003).

In summary, expression of the mutant *INS*^{C93S} in pigs triggers an impaired insulin response with development of mild hyperglycemia at fasting and reduced glucose tolerance following an oral and intravenous glucose challenge. This mild diabetic

phenotype in *INS*^{C93S} transgenic pigs resembles some pre-diabetic phenotype forms in humans.

1.2. Females are slightly more glucose intolerant than male *INS*^{C93S} transgenic pigs

During IVGTTs performed at seven months of age, female *INS*^{C93S} transgenic pigs showed a more pronounced IGT phenotype as compared to male *INS*^{C93S} transgenic pigs (Figure 13 A, B, C and D). This was accompanied by reduced insulin secretion in females as compared to males. This result is contradictory to the results from the previously described Munich *Ins2*^{C95S} mutant mouse model, in which female mice depict a significant milder disease phenotype compared to males (HERBACH et al., 2007). In the Munich *Ins2*^{C95S} mutant mouse model, this difference could be in part attributed to the protective effect of estrogen leading to a less pronounced phenotype in females (SCHUSTER, 2011). In fact, studies demonstrated that estrogens ameliorate oxidative stress and reduce apoptosis of pancreatic β-cells (LE MAY et al., 2006), stimulate pancreatic β-cell secretion (ROPERO et al., 1999; BALHUIZEN et al., 2010), increase insulin sensitivity (LEE et al., 1999; GONZALEZ et al., 2001) and in ovariectomized *Ins2*^{C95S} mutant females it improved glucose levels although it did not rescue β-cell loss (SCHUSTER, 2011) . Studies in the Göttingen Minipig revealed sex-related differences with females being more prone for the development of the metabolic syndrome and surprisingly revealed that males have higher concentrations of both testosterone but also estradiol (CHRISTOFFERSEN et al., 2007). Therefore, it could also be the case that in domestic pigs, males are more protected by effects of steroid hormones than females. This hypothesis is supported by the fact that this gender difference was observed in 7-month- but not in 4 months-old pigs. Seven months corresponds to the time when pigs reach sexual maturity.

1.3. Postprandial insulin secretion deficit is not sufficient to alter growth rate of *INS*^{C93S} transgenic pigs

Not surprising is the unaltered growth rate of *INS*^{C93S} transgenic pigs compared with wt littermate controls. In humans, *INS* mutant carriers diagnosed with permanent neonatal diabetes are often born small for their gestational age as a consequence of the absence of insulin *in utero*, which acts as a growth factor and therefore promotes fetal growth (CAVE et al., 2000; POLAK & CAVE, 2007). In addition, insulin also impacts growth during postnatal life as shown by reduced growth rates in prepubertal children

with early onset of diabetes mellitus and poor glycemic control (TATTERSALL & PYKE, 1973; EDELSTEN et al., 1981; JACKSON, 1984). Transgenic pigs expressing the mutant insulin C94Y do not show reduced growth at birth but exhibit a progressively reduced growth rate from 2.5 month of age when not rescued with exogenous insulin, resulting in 40% reduced BW at 4.5 months of age (RENNER et al., 2013). In pigs the major postnatal growth rate occurs within the period from birth to six months of age (CORSON et al., 2008a). Contrary to *INS*^{C94Y} transgenic pigs, *INS*^{C93S} transgenic pigs do neither develop severe fasting hyperglycemia at birth, nor during juvenile ages and fasting plasma insulin levels are unaltered compared to controls. The insulin deficit in *INS*^{C93S} transgenic pigs is manifested during postprandial periods when insulin secretion capacity is limited in these animals. Reduction of β-cell mass is also less pronounced in *INS*^{C93S} transgenic pigs which are not dependent on insulin treatment. Thus, the degree of insulin deficiency is less marked in *INS*^{C93S} than in *INS*^{C94Y} transgenic pigs and although insulin secretion is impaired following a glucose challenge, the amount of insulin secreted seems to be enough to sustain normal growth.

1.4. Total β-cell volume is mildly reduced in *INS*^{C93S} transgenic pigs

We reported no differences in absolute and relative pancreas weight and this result is in line with unaltered growth rates in *INS*^{C93S} transgenic pigs. When total β-cell volume was determined at 14 months of age in F1 offspring of founder 9776, a slight reduction (-22 %) of total β-cell volume was observed. Pancreatic β-cells secrete insulin in response to plasma glucose concentrations and thus, maintaining plasma glucose levels within a physiological range (NEWSHOLME et al., 2014). Loss of β-cell mass, is a feature of both type 1 and type 2 diabetes with reports showing 20 % - 65 % β-cell loss in type 2 diabetes (KLOPPEL et al., 1985; BUTLER et al., 2003; RAHIER et al., 2008) and a substantial loss of 99 % in type 1 diabetes (MEIER et al., 2005). Evidence from human studies indicate that depletion of approximately 65 – 80 % of β-cell mass leads to insufficient insulin requirement and development of diabetes (KENDALL et al., 1990; MEIER et al., 2012). In a study involving eight NGT, 10 IGT and 11 type 2 diabetic patients who underwent pancreatic surgery, it was demonstrated that β-cell area was directly correlated with glucose control in these patients, and the β-cell area reduction was 11.4 % in IGT (not significantly different from NGT controls) and 65 % in type 2 diabetic patients (MEIER et al., 2009). However, insulin and C-peptide levels were not significantly decreased in these IGT patients compared with NGT

indicating that despite a small decrease of β-cell mass, β-cell function was still preserved in this particular group. In contrast, *INS*^{C93S} transgenic pigs have an acquired β-cell function defect by the expression of the mutant INS. Hence, *INS*^{C93S} transgenic pigs depict a model in which expression of the mutant INS^{C93S} is the primary defect that alters β-cell function and leads to impairment of insulin secretion especially during stimulatory conditions. Under increased secretory demand, increased ER stress mediated by misfolding of the mutant INS can thus progressively trigger pancreatic β-cell death with loss of pancreatic β-cell mass (IZUMI et al., 2003; ZUBER et al., 2004; LIU et al., 2005; PARK et al., 2010; RAJAN et al., 2010). This hypothesis is also supported by *INS*^{C94Y} transgenic pigs as these animals develop severe hyperglycemia within the first week of age despite unaltered β-cell mass at that stage (RENNER et al., 2013). Another factor contributing to progressive reduction of β-cell mass is exposure to chronic hyperglycemia. *In vitro* studies demonstrated that hyperglycemic conditions induce β-cell stress by increasing cellular oxidative stress with increased production of reactive oxygen and nitrogen species (IHARA et al., 1999; FEDERICI et al., 2001; KAISER et al., 2003; MAEDLER et al., 2008). Although *INS*^{C93S} transgenic pigs do not develop severe hyperglycemia, prolonged postprandial hyperglycemia levels caused by impaired glucose tolerance in *INS*^{C93S} transgenic pigs could perpetuate a vicious cycle of increased secretory demand of β-cells and thus triggering more ER stress responses ultimately leading to some degree of β-cell apoptosis (IZUMI et al., 2003; HERBACH et al., 2007; LIU et al., 2007; LIU et al., 2015).

2. Metabolic changes of *INS*^{C93S} transgenic pigs during pregnancy

Next, we characterized the phenotype of *INS*^{C93S} transgenic pigs during pregnancy. In healthy humans, normoglycemia is maintained during pregnancy despite several pregnancy-intrinsic-physiological changes that result in a “diabetogenic-like” phenotype characterized by (i) elevated postprandial glucose levels, (ii) increased whole body insulin resistance and (iii) increased circulating lipids. The hallmark for the development of diabetes during pregnancy is the inability to secrete enough endogenous insulin to meet the increased demand of insulin during pregnancy. Evidence from previous studies in wt pigs indicated that like in humans, pregnancy in pigs is a “diabetogenic-like” event where development of insulin resistance and

increased lipid mobilization at the end of gestation was observed (GEORGE et al., 1978; SCHAEFER et al., 1991; PÈRE & ETIENNE, 2007). Therefore, we aimed to first characterize pregnancy changes in pigs and our hypothesis was that *INS*^{C93S} transgenic pigs with IGT and mild elevated fasting glycaemia would not be able to cope with intrinsic metabolic stress of pregnancy and therefore develop a progressive diabetic state during pregnancy.

2.1. Pregnancy induced decrease in insulin sensitivity in pigs is not further impaired in *INS*^{C93S}-p sows

In vivo peripheral insulin sensitivity was measured in the third trimester by the gold-standard technique, the hyperinsulinemic-euglycemic clamp (HIC). An insulin infusion rate of 1 mU/kg BW/min that was supposed to fully suppress hepatic insulin production was chosen (DEFRONZO et al., 1978; KOOPMANS et al., 2006). Blood glucose levels were clamped at a concentration of 75 mg/dl by a varying glucose infusion rate resembling a mean normal fasting blood glucose concentration for pigs. Glucose infusion rates were 35 % reduced in wt-p sows compared with wt-np controls (Figure 18 B and C), whereas GIR was not further reduced in *INS*^{C93S}-p sows (Figure 18 F and G). This indicates that, like in humans, insulin sensitivity is physiologically reduced during late pregnancy in wt pigs and that the expression of the mutant insulin C93S did not further impair insulin sensitivity (although a tendency towards reduced insulin sensitivity in tg pigs was observed). The finding of reduced insulin sensitivity during pregnancy is also supported by previous studies in the pig (GEORGE et al., 1978; SCHAEFER et al., 1991; PÈRE et al., 2000; PÈRE & ETIENNE, 2007). The fact that insulin sensitivity is not further impaired in *INS*^{C93S}-p sows is not surprising, since the molecular pathomechanism(s) of expression of the mutant *INS*^{C93S} is reflected in defects in insulin secretion, rather than changes in peripheral insulin sensitivity. Women with GDM have defects in insulin sensitivity which may precede pregnancy (as part of either one or both genetic and environmental contribution) which is further impaired during pregnancy (RYAN et al., 1985; BUCHANAN et al., 1990; CATALANO et al., 1993; HOMKO et al., 2001). Nevertheless, GDM results from an endogenous insulin supply that is insufficient to meet the insulin demand (BUCHANAN & XIANG, 2005; BUCHANAN et al., 2007). An interesting point is why insulin resistance physiologically occurring during pregnancy combined with the intrinsic genetic defect in insulin secretion is not sufficient to trigger distinct hyperglycemia in *INS*^{C93S}-p sows. One possible explanation is the difference in the

degree of physiological insulin resistance during pregnancy. *In vivo* studies measuring insulin sensitivity by means of HIC technique in GDM women indicate that insulin sensitivity is reduced by 50 % to 80 % compared with only 35 % in *INS*^{C93S}-p sows (RYAN et al., 1985; BUCHANAN et al., 1990; CATALANO et al., 1991; CATALANO et al., 1992; CATALANO et al., 1993). Interestingly, GIRs reduced to a similar extent are reported among pregnant women with NGT were reported in our wt-p group. The molecular mechanisms underlying desensitization to insulin during pregnancy in humans are not fully understood. Nevertheless, data from human and murine studies, in healthy pregnancy and GDM, suggest that insulin resistance is multifactorial, involving a combination of increased maternal adiposity (with increased levels of adipokines and cytokines) in addition to desensitization effects of placental associated hormones (BARBOUR et al., 2007). Muscle and adipose tissue biopsy samples obtained from elective caesarian delivery reveal significant changes in IR activity, a decrease in total IRS1 protein concentrations and significantly decreased tyrosine phosphorylation of IR and IRS proteins that are aggravated in GDM (FRIEDMAN et al., 1999; SHAO et al., 2000; CATALANO et al., 2002; BARBOUR et al., 2011). As insulin resistance is physiologically developed in pigs, the same molecular mechanisms can be speculated and they can help for a deeper understanding on the development of insulin resistance during pregnancy. Understanding of these mechanisms can help to develop novel treatment strategies as to reduce insulin resistance in GDM patients.

2.2. Pregnancy is not enough to cause distinct hyperglycemia in *INS*^{C93S} transgenic pigs

Pregnancy did neither alter FPG in wt nor in *INS*^{C93S} transgenic pigs. Unaltered FPG concentrations during gestation in wt pregnant sows are also reported by others and thus our results confirm these previous findings (AHERNE et al., 1969; REYNOLDS et al., 1985; DUEE et al., 1987; SIMOES NUNES et al., 1987; PÈRE et al., 2000; PÈRE & ETIENNE, 2007). In human pregnancy, different studies report a decrease in FPG throughout the course of gestation and, although the molecular mechanisms are not clear, it has been attributed to an increase in distribution volume with the establishment of pregnancy and to the growth of the fetal-placental unit in the last gestational phase (KALHAN et al., 1979; HADDEN & MCLAUGHLIN, 2009; ANGUEIRA et al., 2015). In the post-absorptive phase (fasting phase), the liver is the main contributor to FPG concentrations obtained by hepatic glucose production and

the rate of hepatic glucose production reflects the rate of glucose uptake (DEFRONZO et al., 1989). Metabolite turnover rates using tracer isotope dilution methods provide a better overview on metabolism as compared with point analysis concentrations which give only a static information for a parameter. Using these methods, it was shown that glucose turnover rate (appearance of glucose in the maternal circulation) during the post-absorptive phase was 16 % increased during pregnancy. This observation goes along with decreased glucose concentrations as a result of an increased distribution space (KALHAN et al., 1979). Insulin and glucagon are the two major glucoregulatory hormones that have a pivotal role in regulating hepatic glucose production (CAHILL et al., 1966; FELIG & WAHREN, 1971; UNGER, 1971; ALFORD et al., 1974). During prolonged fasting in humans, the decline in plasma insulin levels is a major hormone trigger to regulate hepatic glucose production and basal levels of glucagon are necessary to maintain hepatic glucose production. During pregnancy in humans, basal insulin levels rise, especially with progression of pregnancy (SPELLACY & GOETZ, 1963; BLEICHER et al., 1964; CATALANO et al., 1991; CATALANO et al., 1993) and reports indicate that glucagon levels do not differ from the non-pregnant state (LEBLANC et al., 1976). During our study, insulin levels were not monitored throughout pregnancy. However, we do have evidence that at the end of gestation basal insulin secretion was not different between the three groups (wt-np, wt-p and tg-p) (Figure 21 D). Therefore, it seems that regulation of basal glucose metabolism during pregnancy in pigs differs from humans. This hypothesis would also explain why FPG in *INS*^{C93S}-p sows were not further impaired during pregnancy and remain unaltered compared with *INS*^{C93S}-np controls. Women with GDM develop hyperglycemia that is a consequence of their inability to compensate for the increased insulin demand due to reduced insulin sensitivity compared to healthy pregnant women (RYAN et al., 1985; CATALANO et al., 1993; HOMKO et al., 2001). We show that insulin resistance is not further impaired in *INS*^{C93S}-p (Figure 18 E and F) and in fact, fasting plasma insulin concentrations during the third trimester indicate no additional insulin demand in the fasting state (Figure 21 D). Therefore, it could be expected that pregnancy-induced metabolic stress per se is not sufficient to provoke a distinct hyperglycemic state or overt diabetes in *INS*^{C93S} transgenic sows. One possible way to achieve this would be the feeding of a high-energy-high-fat-diet. It was previously shown that the fat level in the diet fed to a sow during gestation is directly related to impaired glucose tolerance (VAN DER PEET-SCHWERING et al., 2004). Feeding a

high-fat diet combined to *INS*^{C93S} transgenic pigs during pregnancy could be therefore a visible strategy in future studies to combine genetically and environmental factors to achieve a distinct diabetic state during pregnancy.

2.3. HGCs confirm reduced β-cell function in pregnant *INS*^{C93S} transgenic sows

In vivo β-cell function was evaluated by HGC, in which plasma glucose levels were acutely raised and sustained for 90 min at a hyperglycemic plateau of 300 mg/dL by variable glucose infusion rates. Because the same steady-state of hyperglycemia was achieved in *INS*^{C93S}-p and wt-p sows (Figure 20 A), insulin response under maximal stimulatory conditions could be directly compared in the two groups. HGC confirmed that insulin secretion is severely impaired (- 71 %) in *INS*^{C93S} transgenic compared with wt sows during pregnancy (Figure 20 B and C). This indicates that during pregnancy, under hyperglycemic conditions, *INS*^{C93S}-p transgenic sows fail to compensate with an adequate insulin response but are able to react in a similar manner to an arginine bolus.

2.4. Pregnancy impaired glucose tolerance in wt-p sows and further aggravated glycemic control in *INS*^{C93S}-p sows due to failure to compensate for an increased insulin demand

In addition to HGC, β-cell function and glucose tolerance were evaluated during MMGTT, allowing us to determine glucose and insulin response in a closer natural scenario. We demonstrated that pregnancy induced a slight but significant degree of IGT in wt sows as shown by increased glycemia following meal ingestion that was sustained during one hour upon challenge (Figure 21 A). Wt-p sows secreted more insulin as to maintain normoglycemia despite a reduced insulin sensitivity compared to np controls (Figure 21 D and E). These results indicate that, like in humans, insulin is less effective for controlling circulating glucose in the later pregnancy stages which was also verified by others (SCHAEFER et al., 1991; PERE, 2001; PÈRE & ETIENNE, 2007), and altogether demonstrate that similar to healthy humans, wt-p pigs adapt as to keep sufficient glucose available for the fetuses during times of interrupted feeding (BUTTE, 2000; DI CIANNI et al., 2003). The molecular events underlying β-cell compensatory mechanisms (either by increasing β-cell function or mass) during pregnancy in humans have not been clarified. Rodent islets not only differ in terms of islet architecture compared with human and porcine islets

(CABRERA et al., 2006; KIM et al., 2009; STEINER et al., 2010), they also feature a much higher regeneration capacity (CHICK & LIKE, 1970; BONNER-WEIR et al., 1983; FINEGOOD et al., 1995; MENGE et al., 2008). From the few studies analyzing β-cell mass adaption during pregnancy in humans (VAN ASSCHE et al., 1978; BUTLER et al., 2010) a much smaller increment in β-cell mass is seen as compared with the dramatic β-cell mass expansion in murine pregnancy, suggesting that human β-cells compensate insulin demand by increasing β-cell function of preexisting islets. How pigs regulate β-cell mass during pregnancy is not known. Future studies on this topic are important and may also shed light on human β-cell mass regulation during pregnancy.

Furthermore, we showed that IGT was aggravated in mildly hyperglycemic, pregnant *INS*^{C93S} transgenic sows compared to pregnant controls (Figure 21 A). The postprandial hyperglycemic burden observed in pregnant *INS*^{C93S} transgenic sows is due to inappropriate compensatory insulin secretion capacity that can be explained by the expression of the mutant insulin C93S (Figure 21 D and F). The importance of β-cell function in pregnancy is highlighted in *in vitro* stimulation studies using isolated islets of pregnant rats. It was demonstrated that islets isolated from pregnant rats (GREEN & TAYLOR, 1972) or isolated from rats with prolonged exposure to physiological progesterone and estrogen concentrations mimicking pregnancy conditions (COSTRINI & KALKHOFF, 1971), secreted more insulin to lower glucose levels compared with isolated female islets of non-pregnant controls. Increased insulin content and insulin secretion was also observed in glucose-stimulated pancreas tissue isolated from pregnant rats in comparison to pancreas from non-pregnant controls (MALAISSE et al., 1969). In addition, increased rates of proinsulin biosynthesis followed with increments in intracellular levels of adenylate cyclase and cyclic AMP were found in isolated islets of pregnant rats (BONE & TAYLOR, 1976), supporting evidence of increased needs in insulin production by β-cells during pregnancy. Because *INS*^{C93S} transgenic animals show insulin secretion defects (Figure 21 D and F) and expression of mutant insulin C93S interferes with wt endogenous proinsulin secretion (LIU et al., 2010), these mechanisms may explain the deficit in response towards extra insulin demand during pregnancy in *INS*^{C93S} transgenic sows.

2.5. Wildtype neonates born to pre-diabetic *INS*^{C93S} mothers reveal impaired glucose tolerance despite increased insulin secretion

We demonstrated that wt offspring born to *INS*^{C93S} mothers developed an insulin resistance phenotype at birth with elevated FPG and IGT (Figure 22 A and Figure 23). These results are the first to describe *in vivo* assessment of metabolic changes directly after birth in wt offspring in a large animal model of mild maternal hyperglycemia. The elevated FPG directly after birth in wt/tg might be a consequence of changes occurring during gestation in maternal glucose concentrations *in utero*, which were simultaneously transferred to the fetus. It is well documented that the fetus relies primarily on maternal glucose as energy source and that glucose is transferred to the fetus by a maternal-placental-fetal glucose gradient (KALHAN et al., 1979; KALHAN, 2004). Studies in humans demonstrate a linear relationship between maternal and fetal glucose concentrations, and this relationship is observed during euglycemia as well as hyperglycemia (WHALEY et al., 1966; TOBIN et al., 1969; FOWDEN et al., 1982; SOLTESZ et al., 1985; BOZZETTI et al., 1988; PERE, 1995). Like in humans the fetal pig uses glucose as the main substrate for development and growth (FORD et al., 1984; PERE, 1995) and elevation of maternal glucose correlates with increased fetal glucose levels in the umbilical vein of chronic catheterized fetuses (FOWDEN et al., 1982; PERE, 2001).

Fetal/neonatal hyperinsulinemia is a feature of pregnancies complicated by diabetes, and is correlated with maternal degree of hyperglycemia (GROUP et al., 2008; METZGER et al., 2010). We could not demonstrate neonatal hyperinsulinemia as the large majority of insulin values obtained by RIA assay were below the detection limit of this assay. Nevertheless, we have some indication that wt/tg secreted more insulin at birth (time 0 min) (Figure 22 B) and showed that upon glucose challenge wt/tg piglets adapted with increased insulin secretion as compared to wt/wt offspring (Figure 23 C and D), indicating that fetal pancreatic endocrine alterations must be present as to overcome elevated glucose levels in *INS*^{C93S} mothers. *In vitro* evaluation of neonatal endocrine pancreas from neonates born to mothers with reduced carbohydrate tolerance showed an increased proportion of β-cells (VAN ASSCHE & GEPTS, 1971). Islet hyperplasia and increased pancreatic insulin content is observed in the rat fetal pancreas exposed to a mild-hyperglycemia-induced-model of diabetes (KERVRAN et al., 1978; BIHOREAU et al., 1986a). We observed low insulin concentrations (less than 5 µU/mL) directly at birth in both groups and this is in line

with other reports of insulin measurements in the fetal pig during late gestation (103-107 days) (FOWDEN et al., 1982; MARTIN et al., 1984; PERE, 1995). The low insulin levels are characteristic for pigs in comparison with sheep and cows (HOVE & BLOM, 1973; SIERS & TRENKLE, 1973; BASSETT, 1974) and may explain, in part, the scarce amount of fat in the piglet at birth compared with other neonates (WIDDOWSON, 1950). Human neonates are a remarkable exception in this respect as they show a greater fat mass to BW percentage at birth (12 %) compared with less than 2 % in piglets (LITTEN-BROWN et al., 2010).

We report that wt/tg offspring secreted more insulin than wt/wt offspring although, glucose concentrations remain significantly higher during OGTT indicating IGT due to insulin resistance (Figure 23 A and C). There is a clear evidence that offspring prenatally exposed to an intrauterine hyperglycemic environment are at risk to develop IGT (SILVERMAN et al., 1995; PLAGEMANN et al., 1997) and type 2 diabetes with increasing age (DABELEA et al., 2000; LINDSAY et al., 2000). Whether this is also the case in a pre-diabetic maternal environment and the time window to which these alterations may occur is less explored. Two human studies addressed the presence of insulin resistance at birth as to understand if the same pathophysiological processes preceding onset of metabolic syndrome and diabetes in adults would be present before and at birth. The study from Dyer et al., performed in a Hispanic cohort of neonates (a population group at high risk to develop a metabolic syndrome) showed increased insulin resistance following a shortened-frequently-sampled-intravenous-glucose-tolerance test in large-for-gestational-age term neonates born to mothers with and without gestational diabetes compared with that in poorly grown and normal for gestational age neonates between 24–48 h of birth (DYER et al., 2007). The study from Catalano et al in samples collected from the umbilical vein during delivery revealed increased insulin resistance measured by homeostasis model of insulin resistance in neonates of obese mothers with a positive correlation between fetal adiposity and insulin resistance (CATALANO et al., 2009). Our findings support and extend those suggesting that fetal metabolic programming can be influenced by chronically exposure to milder forms of maternal glycemia with metabolic alterations manifested at birth. We are aware that increased fetal/neonatal body fat mass and fetal overgrowth was not observed in this study. Although not significant, relative liver and heart weights tented to be higher in wt/tg compared with wt/wt (Table 9). It is worth to mention that excessive fetal growth (macrosomia) observed in human neonates

exposed to a diabetic intrauterine environment may be a difficult point to assess in pigs. This is due to intrinsic natural variation in BW at birth within the same litters (FOWDEN et al., 1997). The pig is a polytocous species in which littermates may compete for maternal glucose supply (FOWDEN et al., 1997). Therefore, although fetal glucose levels are determined primarily by the maternal nutritional state, in pigs, the relative placental mass of each fetus and the number of fetuses in the litter may influence fetal glucose consumption and fetal glucose concentrations (WIDDOWSON, 1971; COMLINE et al., 1979; FOWDEN et al., 1997). In addition, differences in placenta morphology among humans and pigs may impact placenta-nutrient-transport-efficiency, and impact fetal growth. Contrary to the high invasive/permeable hemochorial placenta in humans, pigs have a less permeable type of placenta, the epitheliochorial (e.g. placenta transport of NEFAs is limited in the pig whereas it occurs in the human placenta) (LITTEN-BROWN et al., 2010).

In our study, elevated insulin levels in wt/tg piglets following an oral glucose challenge suggest an excessive β-cell response with development of IGT. This is further supported in a prospective study where excess fetal insulin secretion in utero measured in the amniotic fluid strongly predicts IGT in childhood, and although most of the children with IGT were obese, IGT was not associated with macrosomia by multiple logistic analysis (SILVERMAN et al., 1995). In addition, a cross-section study involving pre-puberty children revealed that elevated maternal glucose levels during gestation are specifically associated with poor insulin sensitivity in the children and are positively associated with the offspring's β-cell responsiveness independent of the children´s adiposity grade (BUSH et al., 2011). Interestingly, other studies reported lower insulin secretion among offspring of diabetic pregnancies (including GDM) (GAUTIER et al., 2001; SOBNGWI et al., 2003; SALBE et al., 2007; KELSTRUP et al., 2013). However, in these studies, insulin secretion was analyzed in the offspring at adulthood. Animal studies also support this evidence in which increased insulin secretion at younger ages follows reduced insulin secretion in adult life (BOLOKER et al., 2002; HOLEMANS et al., 2003). Reduced insulin secretion is also observed in adult rats which were exposed to mild-hyperglycemia (110 – 140 mg/dL) during gestation (GAUGUIER et al., 1991). Thus one could speculate that overstimulated β-cell response in early life can precede reduced β-cell function and mediate β-cell decline and full blown diabetes in later life. The molecular mechanisms by which maternal hyperglycemia modulates the offspring´s insulin sensitivity and increased β-

cell response are not fully understood. As previously mentioned, alterations in the morphology of the fetal pancreas with increased β-cell mass and insulin content were observed in animal and human studies (VAN ASSCHE & GEPTS, 1971; KERVRAN et al., 1978; BIHOREAU et al., 1986a). In addition, alterations in skeletal muscle glucose uptake resulting from decreased protein levels of GLUT1 and GLUT4 transporters have been demonstrated in murine offspring exposed to diabetes *in utero* (BOLOKER et al., 2002). Together these data demonstrate that milder forms of hyperglycemia as seen in *INS*^{C93S} tg sows can affect *in utero* programming of metabolic disorders which are already manifest early in the postnatal period. Combined environmental risk factors such as overnutrition in later life, triggering exaggerated β-cell response may contribute to exhaustion and decline of β-cell function. Ultimately, this may explain increased prevalence of metabolic syndrome and type 2 diabetes in the offspring of diabetic mothers.

In addition, we demonstrate that maternal intrauterine exposure to mild hyperglycemia has effects on different clinical-chemical parameters in plasma of the offspring. During fetal development, mobilization of lipids for fat storage varies tremendously among humans and other mammalian species (JONES, 1982). In humans, body fat deposition occurs essentially in the last trimester of intrauterine life and accounts for approximately 16 % of neonatal body mass at birth (mainly in the form of white-adipose tissue) (HERRERA & AMUSQUIVAR, 2000). In the pig white adipose tissue in significant amounts could not be detected macroscopically in 1-day-old piglets by our group and low body fat mass in neonatal piglets is also supported by others (WIDDOWSON, 1950; LITTEN-BROWN et al., 2010). These observations are consistent with the low levels of triglycerides, NEFAs and lipase observed at birth in both wt/tg and wt/wt piglets (Figure 24). We do observe a significant increase in total cholesterol which is consistent with increased levels of LDL and HDL in wt/tg compared with wt/wt piglets (Figure 24). We recognize that there is limited information of comparison of lipid profiles in neonates at term of GDM or diabetic mothers with offspring from healthy pregnancies. Lipeski et al. studied children (7-9 years of age) 20 born to GDM and 22 born to healthy control mothers (matched for family history of diabetes and hypercholesterolemia) and reported no differences in mean of cholesterol, LDL and total HDL among GDM offspring and controls (LIPESKI et al., 1998). Others have determined the prevalence of metabolic markers

for the metabolic syndrome (including elevated FPG, triglycerides, HDL, waist circumference) in children (7-11 years of age) born to GDM and control mothers and indicated that 25 % of GDM offspring had at least one significantly changed metabolic marker with increased triglycerides being most prevalent (KEELY et al., 2008). In children dyslipidemia, obesity, insulin resistance and glucose intolerance are factors related to the metabolic syndrome and increase the risk for the development of diabetes and cardiovascular diseases in adulthood (BURNS et al.; SCHUBERT et al.; FRANKS et al., 2007; JUONALA et al., 2008). Our results suggest that mild maternal hyperglycemia had differential effects in lipid metabolism of wt/tg compared with controls at birth.

Furthermore, we evaluated lactate and LDH metabolism as these substrates are directly related to glucose metabolism i.e. glucose is the major source of lactate and lactate is the major substrate for endogenous glucose production (ADEVA-ANDANY et al., 2014). Patients with diabetes mellitus show severe alterations in intracellular metabolism of glucose in insulin-sensitive tissues including enhanced non-oxidative glycolysis with increased lactate production (THORBURN et al., 1990; DEL PRATO et al., 1993). In healthy subjects most of the glucose disposal during the postprandial period (43.5 %) is metabolized via the oxidative pathway (with pyruvate entering the mitochondria and follow the tricarboxylic cycle), 33 % is used for glycogen synthesis whereas 23.5 % follows the non-oxidative glycolysis pathway with formation of lactate (WOERLE et al., 2003). Although not significantly different, wt/tg piglets showed increased plasma lactate at two hours post oral glucose challenge compared with wt/wt (Figure 25 B). Consistently LDH was significantly higher two hours post oral glucose challenge in wt/tg (Figure 24 C). These results suggest that energy production during postprandial period in wt/tg is being enhanced through glycolysis via the non-oxidative pathway as compared to controls. Determination of levels of pyruvate dehydrogenase (PDH) as well as pyruvate would be necessary to confirm these preliminary observations. Interestingly, expression levels of genes involved in the mitochondrial oxidative pathway were found reduced in the skeletal muscle from patients with diabetes as compared to healthy controls (MOOTHA et al., 2003; PATTI et al., 2003), and reduced activity of PDH complex enzymes, are found reduced *in vitro,* in muscle of diabetic patients (ABBOT et al., 2005). Studies in chronic catheterized sows and fetal piglets indicate that lactate concentrations are increased in the fetal circulation compared to the maternal circulation (PERE, 1995, 2001) which

is also true for humans (GILFILLAN et al., 1985; BELL et al., 1989), sheep (BURD et al., 1975; CHAR & CREASY, 1976), guinea pigs (CARSTENSEN et al., 1982) and rats (SHAMBAUGH et al., 1977), suggesting that lactate is produced from glucose by the placenta. In pregnancies complicated by diabetes, excessive maternal blood glucose could facilitate overproduction of lactate in the placenta. Indeed, in vitro perfusion studies in human placentas from uncomplicated pregnancies perfused with gradually increased glucose concentrations indicate that the amount of lactate produce by the placenta is proportional to glucose concentration (HAUGUEL et al., 1986). Although, this is not confirmed in perfusion studies from placentas from GDM women (OSMOND et al., 2000). In contrast, another study indicates a 23% increase in the lactate concentration in the umbilical vein in GDM pregnancies (TARICCO et al., 2009). We report no differences in basal levels of lactate at birth between wt/tg and wt/wt, suggesting that there were no differences in the placental lactate production among the two groups in this study.

VI. CONCLUDING REMARKS AND PERSPECTIVES

INS^{C93S} transgenic pigs expressing the mutant insulin C93S develop mild diabetes characterized by elevated fasting glucose levels and impaired glucose tolerance. This phenotype is specific to the mutant *INS*^{C93S} primarily causing a β-cell function defect, leading to impairment of insulin secretion, which is mostly evident under glucose-stimulated conditions. Thus, *INS*^{C93S} transgenic pigs represent a model of a subtle degree of diabetes which resembles a pronounced pre-diabetic state in humans. Pregnancy *per se* was not sufficient to further impair glycemic control in *INS*^{C93S} transgenic pigs. However, it could be demonstrated that even milder degrees of hyperglycemia directly impact glucose control at birth in newborn wt piglets. Within this work two important questions were raised and should be addressed in future studies. First, which molecular events contributed to the metabolic alterations at birth in wt/tg piglets? Possible compensatory pancreatic alterations, such as increased β-cell mass or function was present in these animals. Development of insulin resistance was also suggested, and thus it would be important to determine if impairment of insulin signaling is present in peripheral tissues. Organ weight analysis showed that livers of wt/tg piglets tended to be heavier and clinical-chemical parameters also indicate increased liver metabolism. Therefore, it would be important to determine if the observed alterations in plasma metabolites are also associated with liver transcriptome changes of associated pathways. In addition, it would be mandatory to evaluate if *in utero* programming of metabolic disorders also persists during later developmental stages and if additional environmental risk factors, such as feeding a high-fat-high-energy diet to the mothers, can contribute to the development of metabolic complications in this predisposed offspring.

Other study applications that go beyond the maternal diabetes field can be explored in the *INS*^{C93S} transgenic pigs. The pronounced pre-diabetic phenotype of these animals offers an excellent opportunity to identify possible early biomarkers related to β-cell dysfunction and compared to those found in the mild diabetic GIPR^{dn} pig model with impaired incretin hormone function (RENNER et al., 2010). Moreover, high-fat diet manipulation in *INS*^{C93S} transgenic pigs can possibly lead to an aggravated hyperglycemic phenotype and therefore, allowing to follow disease progression stages in a model integrating different comorbidities of diabetes (i.e. excessive weigh gain, dyslipidemia and possibly insulin resistance).

VII. SUMMARY

Impaired glucose control in newborn piglets exposed to mild hyperglycemia *in utero*: study in a novel transgenic pig model for mild maternal diabetes

Two types of maternal diabetes can be distinguished: preconceptional diabetes, i.e. preexisting type 1 or type 2 diabetes in the mother or gestational diabetes (GDM) with its first onset around the 24th week of pregnancy. Maternal diabetes negatively affects fetal development as well as triggers intrauterine programming of diseases in the offspring's later life like obesity, impaired glucose tolerance and type 2 diabetes mellitus. The deleterious effects of maternal hyperglycemia on mother, fetus and early offspring are especially difficult to study in humans. Hence, experimental animal models are essential to better understand the consequences of maternal diabetes. The pig is an excellent animal model as pigs share many similarities with humans, e.g. fetal development is completed intrauterine in humans and pigs while mice are born at a more immature state. Until now, the majority of animal models for maternal diabetes was established in rodents by different approaches as pancreatectomy, chemical or dietary diabetes induction as well as genetic engineering. So far only few models with a milder phenotype similar to the mild hyperglycemic levels in mothers due to a tightly regulated glycemic control exist. Here, we established a novel porcine model of mild maternal diabetes using transgenic pigs expressing the mutant insulin C93S.

INS^{C93S} transgenic pigs were generated by somatic cell nuclear transfer and embryo transfer. Before pregnancy glucose control was investigated by an IVGTT and MMGTT and ß-cell mass was determined by quantitative-stereological analyses. For pregnancy studies, three *INS*^{C93S} transgenic (tg) and nine wildtype (wt) sows were artificially inseminated with semen of the same wt boar while three wt sows served as non-pregnant controls. Fasting blood glucose was monitored weekly throughout pregnancy. Within the third trimester, hyperinsulinemic-euglycemic (HIC) and hyperglycemic clamps (HGC) as well as and mixed-meal glucose tolerance tests (MMGTT) were performed. At birth, wt piglets born to wt sows (wt/wt, n=18) and wt piglets born to tg sows (wt/tg, n=13) underwent an oral glucose tolerance test (OGTT) before first colostrum intake. Piglets were necropsied on day one for tissue collection.

INS^{C93S} transgenic pigs show mild impaired fasting glycaemia (IFG), impaired glucose tolerance (IGT) and insulin secretion as well as a slightly decreased in β-cell mass (-

22 %). As observed in humans, insulin sensitivity was reduced in pregnant wt sows compared to non-pregnant wild-type controls within the third trimester. Insulin sensitivity of pregnant *INS*^{C93S} transgenic sows was reduced to the same extent. β-cell function was severely reduced in *INS*^{C93S} transgenic pregnant sows, displayed by reduced insulin secretion and glucose infusion rate during the HGC. In a MMGTT glucose tolerance was nearly, however not fully sustained in wt pregnant vs. non-pregnant sows by increased insulin secretion, while *INS*^{C93S} transgenic sows did not meet the increased insulin demand. Fasting hyperglycemia in *INS*^{C93S} tg sows did not deteriorate further throughout pregnancy. Wildtype piglets born to wt sows (wt/wt) revealed unaltered birth and organ weights compared to wt piglets born to transgenic sows (wt/tg). However, glucose tolerance of wt/tg piglets was significantly reduced despite increased insulin secretion indicative of an insulin-resistant state. Additionally, wt/tg piglets showed significantly increased cholesterol as well as LDL and HDL levels.

In summary, we established a novel transgenic pig model which reveals mild IFG and IGT representing a pronounced pre-diabetic state. As pigs and humans exhibit an insulin resistant-state during late pregnancy, *INS*^{C93S} transgenic pigs seems to be a valuable model for the evaluation of consequences of mild maternal hyperglycemia on the offspring. Indeed, it was demonstrated in our study that mild maternal hyperglycemia resulted in impaired glucose tolerance despite increased insulin secretion as well as altered lipid metabolism in piglets at birth. This work can be further extended as to understand which molecular events contributed to the metabolic alterations at birth. Additionally, further studies will show if *in utero* programming of metabolic disorders also persists during later developmental stages, and how additional environmental risk-factors, such as feeding a high-fat-high-energy diet to the mothers affects their own as well as their offspring's metabolism.

VIII. ZUSAMMENFASSUNG

Verminderte Glukosetoleranz in neugeborenen Ferkeln infolge einer Exposition von maternaler Hyperglykämie: eine Studie in einem neuen transgenen Schweinemodell für geringgradigen maternalen Diabetes

Bislang sind zwei Formen von maternalem Diabetes bekannt, einmal der präkonzeptionelle Diabetes, d.h. ein bereits vor der Schwangerschaft bestehender Typ 1 oder Typ 2 Diabetes sowie Gestationsdiabetes, welcher zum ersten Mal zumeist um die 24. Gestationswoche auftritt. Maternaler Diabetes kann negative Auswirkungen auf die fötale Entwicklung sowie die Entwicklung von Krankheiten wie Adipositas, reduzierte Glukosetoleranz und Typ 2 Diabetes mellitus bei den Nachkommen begünstigen. Die Untersuchung der Auswirkungen des maternalen Diabetes auf die Mutter sowie frühe Entwicklungsstadien der Nachkommen ist beim Menschen kaum möglich. Aus diesem Grund sind aussagekräftige Tiermodelle von großer Bedeutung. Generell ist das Schwein ein exzellentes Tiermodell, da es sehr viele Ähnlichkeiten mit dem Menschen hat, die auch für die Untersuchung von Konsequenzen des maternalen Diabetes relevant sind. So wird beim Schwein wie auch beim Menschen die fötale Entwicklung intrauterin abgeschlossen, während Mäuse in einem unreiferen Stadium geboren werden. Bis heute wurden die meisten Tiermodelle zu diesem Thema im Nager mittels von Pankreatektomie, Diabetesinduktion durch chemische Substanzen wie Streptozotocin, durch spezielle Diäten oder mittels genetischer Modifikationen etabliert. Nur wenige der bislang etablierten Tiermodelle zeigen einen milden Phänotyp, d.h. nur eine geringgradige Hyperglykämie, die der Situation von in der Regel aufgrund einer engmaschigen Blutzuckerkontrolle gut eingestellten Schwangeren entspricht. Deshalb haben wir in der vorliegenden Studie ein neues Schweinemodell erstellt, welches eine milde Form von maternalem Diabetes zeigt. Transgene Schweine, die das mutierte Insulin C93S exprimieren, wurden mittels somatischen Kerntransfers mit anschließendem Embryotransfer erstellt. Zunächst wurde die Glukosehomöostase mittels eines intravenösen sowie eine „mixed-meal“ Glukosetoleranztests untersucht. Im Anschluss daran wurden weibliche Tiere mit Sperma desselben Ebers besamt und innerhalb des dritten Trimesters hyperinsulinämische-euglykämische Clamps (HIC), hyperglykämische Clamps (HGC) sowie ein „mixed-meal“ Glukosetoleranztest durchgeführt. Unmittelbar nach der Geburt und vor der ersten Kolostrumaufnahme wurden bei Ferkeln von nicht

transgenen Kontrollsauen (wt/wt, n=18) sowie bei nicht-transgenen Ferkeln von *INS*^{C93S} transgenen Sauen orale Glukosetoleranztests durchgeführt. Zur Organprobenentnahme wurden die Ferkel am ersten Lebenstag euthanasiert und seziert.

INS^{C93S} transgene Schweine entwickeln geringgradig erhöhte gefastete Blutglukosespiegel, eine reduzierte Glukosetoleranz und Insulinsekretion sowie eine reduzierte ß-Zellmasse (-22%). Ähnlich wie beim Menschen zeigten Kontrollschweine eine reduzierte Insulinsensitivität im dritten Trimester, die bei *INS*^{C93S} transgenen Schweinen ähnlich ausgeprägt war. Die ß-Zellfunktion während der Trächtigkeit war jedoch hochgradig gestört, was durch eine signifikant reduzierte Insulinsekretion sowie reduzierte Glukoseinfusion im HGC gezeigt werden konnte. Im MMGTT war die Glukosetoleranz bei trächtigen wt Sauen aufgrund einer signifikant erhöhten Insulinsekretion zur Kompensation der reduzierten Insulinsensitivität beinahe vollständig erhalten, während *INS*^{C93S} transgene Sauen den erhöhten Insulinbedarf nicht vollständig kompensieren konnten und deshalb signifikant erhöhte Blutglukosespiegel zeigten. Die bereits bestehende gefastete Hyperglykämie bei *INS*^{C93S} transgene Sauen verschlechterte sich während der Trächtigkeit jedoch nicht. Wt/wt Ferkel hatten ein unverändertes Geburtsgewicht sowie Organgewichte im Vergleich zu wt/tg Ferkeln. Die Glukosetoleranz von wt/tg Ferkeln war jedoch trotz einer signifikant erhöhten Insulinsekretion reduziert, was auf einen Status von Insulinresistenz hinweist. Zusätzlich zeigten wt/tg Ferkel signifikant erhöhte Plasmakonzentrationen von Gesamt-, sowie LDL- und HDL-Cholesterin.

In der vorliegenden Studie wurde ein neues transgenes Schweinemodell, das geringgradig erhöhte, gefastete Blutglukosespiegel sowie eine reduzierte Glukosetoleranz entsprechend einem fortgeschrittenem prä-diabetischem Stadium entwickelt, etabliert und charakterisiert. Da Schweine wie auch Menschen während der späten Trächtigkeit eine Insulinresistenz entwickeln, erscheint das *INS*^{C93S} transgene Schweinemodell als gut geeignetes Modell, um Auswirkungen des maternalen Diabetes auf die Mutter wie auch die Nachkommen untersuchen zu können. Es konnte in unserer Studie gezeigt werden, dass Nachkommen von prädiabetischen Sauen bereits bei Geburt Veränderungen des Glukosemetabolismus wie eine reduzierte Glukosetoleranz sowie Veränderungen im Lipidstoffwechsel zeigen. Zukünftige Studien müssen klären, ob diese Veränderungen fortbestehen, sich verschlimmern und zu einem Diabetes mellitus führen, welche molekularen

Mechanismen zugrunde liegen, und wie zusätzliche Faktoren, wie Adipositas, induziert durch das Füttern einer fettreichen-hochkalorischen Diät, sich auf Mütter und Nachkommen auswirken.

IX. INDEX OF ABBREVIATIONS

ACTB	ß actin
ADA	American Diabetes Association
AUC	area under the curve
BMI	body mass index
BW	body weight
cDNA	complementary deoxyribonucleic acid
CETP	cholesterol ester transfer protein
CRL	crown-rump length
C-terminal	carboxy-terminal
CV	coefficient of variance
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
FFA	free fatty acids
FPG	fasting plasma glucose
GDM	gestational diabetes mellitus
GLUT	glucose transporter
HAPO	hyperglycemia and Adverse Pregnancy Outcomes
HbA_{1c}	glycated hemoglobin
HDL	high-density lipoproteins
HFD	high fat diet
HGC	hyperglycemic clamp
HIC	hyperinsulinemic-euglycemic clamp
hPGH	human placental growth hormone
hPL	human placental lactogen
IADPSG	International Association of the Diabetes Pregnancy Study Groups
IDF	International Diabetes Federation
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
INS	insulin
IR	insulin receptor tyrosine kinase
IRS	insulin-receptor substrate
IVGTT	intravenous glucose tolerance test
LDL	low density lipoproteins
MMGTT	mixed-meal glucose tolerance tests
MODY	maturity-onset diabetes of the young
NEFA	Non esterified fatty acids
N-terminal	amino-terminal
OGTT	oral glucose tolerance test

PCDM	preconceptional diabetes mellitus
PCR	polymerase chain reaction
PG	postprandial glucose
PI3K	phosphatidylinositol 3-kinase
PKB/AKT	protein kinase B
PKC	atypical protein kinase C
PRL	prolactin
RIA	radioimmunoassay
SCNT	somatic cell nuclear transfer
SEM	standard error of means
TNF-α	tumor necrosis factor alpha
UV	ultraviolet
VLDL	very low density lipoproteins

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