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Effects of the long-acting glucagon-like peptide-1 receptor agonist liraglutide in adolescent pigs with impaired glucose-dependent insulinotropic polypeptide receptor function

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TABLE OF CONTENTS

I. INTRODUCTION

Diabetes mellitus is a major public health problem that shows an alarming steady increase in prevalence numbers (Scully 2012; Shaw et al. 2010). Over 90% of all diabetic patients suffer from type 2 diabetes, a progressive metabolic disorder with dangerous and fatal impact on the organism (ADA 2013a). Therefore, new treatment strategies are urgently needed that address the core problem responsible for its clinical manifestation and progressive course: The failure and decline of pancreatic beta-cells (Prentki et al. 2006). Glucagon-like peptide-1 receptor (GLP-1R) agonists as representatives for incretin-based therapies are a promising approach for diabetes treatment. It has been shown for this class of drugs that they seem to have beneficial effects on pancreatic beta-cells: *In vitro* they showed the potential to stimulate beta-cell proliferation and decrease apoptosis rate and *in vivo* they were able to increase the beta-cell volume in rodent animal models (Bregenholt et al. 2005; Friedrichsen et al. 2006; Miao et al. 2013; Rolin et al. 2002; Sturis et al. 2003; Tews et al. 2009; Tourrel et al. 2002; Tourrel et al. 2001; Xu et al. 1999). However, the beta-cells of rodents show a much higher capacity for regeneration and proliferation compared to the human beta-cells and it is therefore questionable if the liraglutide mediated increases of the beta-cell volume in rodents are representative for the human organism (Butler et al. 2007; Menge et al. 2008). Data are still completely lacking about the *in vivo* effect of GLP-1R agonists on beta-cells in humans because there are no appropriate non-invasive imaging techniques for the quantification and evaluation of human total beta-cell volume (Malaisse 2005). Furthermore, the effect of GLP-1R agonists on adolescent organisms has not been appropriately investigated yet and these drugs are only approved for the use in adult type 2 diabetic patients (Amylin 2012, 2011; EMEA 2009b; Novo Nordisk 2010). However, appropriate pharmacotherapy for younger type 2 diabetic patients gets more and more important given the facts that prevalence numbers of adolescent people suffering from this disease are also steadily increasing (Bloomgarden 2004; Flint et al. 2011; George et al. 2013). A large animal model like the pig can help to close the gap between rodent models and human patients because of its strong physiological and pathophysiological similarities to human beings (Aigner et al. 2010; Swindle et al. 2012). Thus, the effect of the GLP-1R agonist liraglutide on

glycemic control, body weight, food intake and especially the total beta-cell volume was evaluated in adolescent transgenic pigs that express a dominant-negative glucose-dependent insulinotropic polypeptide receptor ($GIPR^{dn}$) in the pancreatic beta-cells. This transgenic pig model seemed to be particularly suitable for this study because it shows key findings of a prediabetic state including impaired function of the incretin hormone glucose-dependent insulinotropic polypeptide (GIP) as well as a progressive decline of beta-cells (Renner et al. 2010).

II. REVIEW OF THE LITERATURE

1. Type 2 diabetes mellitus

1.1. Prevalence of diabetes mellitus

According to the American Diabetes Association (ADA) diabetes mellitus is defined as a "group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both" (ADA 2013a). Due to its frequent and progressive occurrence this disease has become a major public health problem, also indicated by alarming steady increases in prevalence (Scully 2012): While in 2010 it was estimated that about 6.4% of adult people worldwide were suffering from diabetes, already 7.7% will suffer from this disease in 2030. In numbers, this means an increase from 285 to 439 million diabetic patients, excluding undiagnosed cases (Shaw et al. 2010). The highest prevalence can be found in upper-middle as well as middle-income countries like China or India with over 10% of diabetic people, while prevalence numbers are lower in high-income countries like Japan or most of the European countries (Scully 2012; Shaw et al. 2010). Diabetes mellitus is not only a priority health problem, it is also a huge burden regarding consequential costs: In 2010, about 12% of the total global health expenditure (approximately 376 billion US dollars) were invested in diabetes mellitus, an amount of money that will not be sufficient in coming years regarding the rapid increase of diabetes prevalence (Scully 2012; Zhang et al. 2010). With a proportion of 90-95% type 2 diabetes is the most common form of diabetes mellitus worldwide. As it was formerly known as "adult-onset diabetes" it has to be mentioned that also the prevalence numbers of children and adolescent people suffering from type 2 diabetes are steadily increasing within the last years (ADA 2013a; Bloomgarden 2004). Taken together this underlines the urgent need of a better comprehension of diabetes pathogenesis and following consequences to slow down the explosive expansion of this disease by development of prevention methods and appropriate treatment strategies (Zimmet et al. 2001).

1.2. Pathophysiology and consequences of type 2 diabetes

The development of type 2 diabetes mellitus is a complex and often longsome process as it can take years before a clinically overt diabetes characterized by chronic hyperglycemia takes shape (Campbell 2009b). Although the pathophysiologic aspects during this progressive development process are not completely understood yet, two major pathogenetic principles are included: Insulin resistance and beta-cell failure (Prentki et al. 2006). Insulin resistance mainly describes the inability of insulin to appropriately stimulate glucose uptake in peripheral tissues, especially in muscle tissue, and additionally to suppress glucose output and production from the liver, all in all going along with the development of a prediabetic state (DeFronzo 2004). However, in an early state of insulin resistance normoglycemia and normal glucose tolerance are conserved because the pancreatic beta-cells show enhanced function and compensate for the metabolic disorder by increasing insulin secretion and also total beta-cell volume (Prentki et al. 2006). Provided that beta-cells are completely functional, this compensation can be effective for the whole human lifetime (Campbell 2009a). The principle reason for the manifestation of a clinically overt type 2 diabetes mellitus is the dysfunction of susceptible beta-cells characterized by decreased insulin secretory capacity, impaired glucose tolerance and increased beta-cell apoptosis rate followed by progressive loss of functional beta-cell volume (Butler et al. 2003; Campbell 2009a; Prentki et al. 2006). Beta-cell failure and decrease of beta-cell volume are even worsening during the course of the disease due to several reasons: As beta-cells are quite sensitive to elevated glucose levels, the chronic hyperglycemia shows destructive effects because it causes decreased insulin secretion as well as multiple cellular stress response processes and supports increased beta-cell apoptosis. Additionally, elevated levels of free fatty acids as well as the accumulation of cytotoxic islet amyloid polypeptide (IAPP) in the beta-cells can further trigger beta-cell destruction (Campbell 2009a; Wajchenberg 2007). Plenty of risk factors like obesity, hypertension, lack of physical activity, smoking or excessive consummation of alcohol increase the danger of developing type 2 diabetes and are unfortunately more and more becoming part of modern lifestyle (Olokoba et al. 2012). Development of overweight or obesity, particularly intra-abdominal obesity, is a major cause for the development of insulin resistance and thereby one of the most important risk factors that is tightly connected to type 2 diabetes (Wajchenberg 2007). Based on

a long-term observation Ford et al. even suggested that every kilogram body weight gain accounts for a 4.5% higher risk of developing diabetes (Ford et al. 1997). This is especially alarming knowing that the prevalence of obesity is also steadily increasing (Campbell 2009b). Additionally, genetic predispositions play an important role as risk factors for type 2 diabetes (ADA 2013a; Olokoba et al. 2012). The disease occurs more often in aging people, females that showed gestational diabetes before or people suffering from depression. Race and ethnicity seem to influence the susceptibility to develop type 2 diabetes, as for example African Americans show a higher risk for this disease than white people (Deshpande et al. 2008). The long-term impact of a chronic hyperglycemia on the organism is dangerous: It promotes pathologic changes in small blood vessels leading to nephropathy right up to end-stage renal failure, retinopathy leading to blindness, impotence or peripheral neuropathy going along with foot ulcers right up to extremity amputation. Macroangiopathies also reflect a big problem because they can promote cardiovascular diseases like stroke, heart attack, congestive heart failure or coronary heart disease that account for about 65% of all cases of death in diabetic patients (ADA 2013a; Campbell 2009b; Deshpande et al. 2008).

1.3. Diagnosis criteria for type 2 diabetes in humans

A committee of the ADA developed a categorization system for the clinical diagnosis of diabetes that is steadily updated. It is based on fasting plasma glucose (FPG) as well as postprandial glucose (PG) levels two hours after a standardized oral glucose tolerance test in humans that should indicate the state of glucose tolerance. Glycated hemoglobin (HbA_{1c}), a monitoring parameter of long-term blood glucose, can also be used as diagnostic criteria (ADA 2013a; Little et al. 2013). The disease diabetes mellitus can be diagnosed in 4 cases: HbA_{1c} shows values over 6.5% or FPG is greater than 126 mg/dl or PG reaches levels higher than 200 mg/dl or a patient shows values over 200 mg/dl in a random blood sample and additionally classical clinical symptoms of elevated blood glucose. Furthermore, FPG between 100-125 mg/dl, PG between 140-199 mg/dl or HbA_{1c} between 5.7–6.4% can be interpreted as a prediabetic state in which a human shows higher risk of developing diabetes mellitus (ADA 2013a).

1.4. Therapeutic approaches for type 2 diabetes

Regarding the complex pathogenesis of type 2 diabetes mellitus and the manifold consequences on the organism, the successful therapy of this disease can be challenging. Main goals of therapy are to fight hyperglycemia and get glucose homeostasis under control to prevent further complication, particularly damages of blood vessels (Campbell 2009b; Patel et al. 2008). But even more important should be the effort to protect and restore beta-cells, as their progressive dysfunction and loss is responsible for the development and worsening of the course of diabetes mellitus (Campbell 2009a). Additionally, it is necessary to work on strategies that lead to a complete prevention of a clinical manifestation of the disease during a prediabetic state (Chaturvedi 2007). Although therapy goals have to be defined individually for every patient, the general state of glycemic control as recommended by the ADA should include HbA_{1c} levels <7%, preprandial glucose values between 70 and 130 mg/dl and postprandial glucose values lower than 180 mg/dl (ADA 2013b). Classical diabetes treatment starts with the attempt to improve the metabolic situation by changes of lifestyle including controlled diet and promotion of physical activity with the main goal to achieve a reduction of body mass index (ADA 2013b; Klein et al. 2004). If this approach does not show the desired success pharmacological agents are used, mainly starting within the group of oral antidiabetic drugs. Metformin, a biguanide that acts blood glucose lowering because it primarily decreases hepatic glucose production and increases glucose uptake in peripheral tissues, is recommended as initial pharmacotherapy for type 2 diabetic patients and is thereby commonly used. It does not affect body weight or even slightly decreases it and gastrointestinal disorders are the mostly seen side effects (ADA 2013b; Campbell 2009b; Nathan et al. 2009; Olokoba et al. 2012). If glycemic control does not improve appropriately after 3-6 months, combination therapy with other pharmacological agents can be introduced. Besides biguanides the following groups of oral antidiabetic drugs are available: Sulfonylureas like glimepiride, glyburide or glypizide and glinides like repaglinide directly influence the beta-cells to produce more insulin and thereby lower blood glucose, but mainly sulfonylureas are therefore also connected with the risk of hypoglycemia and additionally cause weight gain (Nathan et al. 2009; Olokoba et al. 2012). Thiazolidinediones like pioglitazone or rosiglitazone enhance the sensitivity of the organism (mainly skeletal muscles and the liver) to insulin. Unfortunately this

group of drugs also shows adverse effects like an increased risk for congestive heart failure, peripheral edema and can cause weight gain (Nathan et al. 2009; Olokoba et al. 2012; Singh et al. 2007). Reduction of glucose uptake within the intestine by inhibition of α-glucosidase and thereby improvement of postprandial blood glucose is caused by drugs like acarbose and voglibose. However, the application of α-glucosidase inhibitors is not widespread in diabetes treatment because of their frequent gastrointestinal side effects (Campbell 2009b; Olokoba et al. 2012). Another group of orally active substances that cause a reduced reabsorption of glucose in the kidney by inhibiting the responsible sodium glucose cotransporter-2 (SGLT-2) with following glucosuria but also lower blood glucose levels is currently under development (Ferrannini et al. 2012). Subcutaneous injection of exogenic insulin is one of the most effective methods to lower blood glucose levels and is applied for a long time in diabetes treatment. However, insulin therapy needs an appropriate individual management to prevent the risk of hypoglycemic episodes and additionally weight gain has to be considered as side effect (Campbell 2009b; Nathan et al. 2009). A new therapeutic approach on the market is represented by drugs that are based on the incretin hormone system, namely dipeptidyl peptidase-4 (DPP-4) inhibitors and GLP-1R agonists. Beside of a glucose-dependent stimulation of insulin secretion without the risk of severe hypoglycemia these agents show weight reducing or weight neutral effects (Russell 2012). They are the first group of drugs that seem to address the fundamental cause of type 2 diabetes mellitus as it was shown in rodent models that they have the potential to preserve beta-cell function and volume (Mu et al. 2006; Rolin et al. 2002; Shimizu et al. 2012; Sturis et al. 2003; Tourrel et al. 2002; Tourrel et al. 2001; Xu et al. 1999).

2. The incretin hormone system

2.1. Discovery of the incretin hormones and the incretin effect

During studies with dogs Bayliss and Starling discovered in 1902 a substance that is produced by intestinal epithelial cells independent from innervation and that this substance they called “secretin” is able to activate pancreatic juice secretion (Bayliss et al. 1902). From this time on the prospect that the intestine can give some sort of signal to the endocrine pancreas in response to nutrient ingestion and affect the disposal of carbohydrates was born. La Barre introduced in 1932 for the

first time the term “incretin”, describing an extract from the upper gut mucosa that is able to provoke reduced blood glucose levels in dogs, but does not induce exocrine pancreatic secretion (La Barre 1932). Rapid progress concerning incretin hormone research was made after the development of the radioimmunoassay (RIA) for insulin in 1960 by Yalow and Berson (Yalow et al. 1960) when insulin plasma concentrations could be determined reliably: It was shown in humans that a glucose load given orally provokes a greater and more sustained increase in plasma insulin than the same amount of glucose given intravenously, indicating an additional stimulus for insulin release (Elrick et al. 1964; McIntyre et al. 1964). This phenomenon was later called the incretin effect and the search for responsible hormones to clarify the underlying mechanisms was intensified. The sought-after incretin hormones should fulfill the following classical properties: Secreted from enteroendocrine cells in response to nutrient ingestion, especially carbohydrates, they should be able to stimulate insulin release at states of elevated glucose levels (Creutzfeldt 2005). The first hormone that fulfilled these requirements was discovered by Brown and colleagues in extracts of porcine small intestine and originally named “gastric inhibitory peptide” for its capability to inhibit gastric acid secretion in dogs (Brown et al. 1975). However, further research showed that this hormone at physiological levels also triggers insulin release glucose dependently in animals as well as in humans and thereby reveals classical insulinotropic properties (Dupre et al. 1973; Pederson et al. 1975). To grant this function priority over the inhibition of gastric acid secretion that was only seen after the administration of pharmacological doses, GIP was renamed into “glucose-dependent insulinotropic polypeptide” while keeping the same abbreviation. As the incretin effect after immunoneutralization of endogenous GIP with the help of antisera was blunted, but not completely inhibited, it was suspected that another substance might contribute to the incretin effect (Ebert et al. 1982). While sequencing mammalian proglucagon genes two fragments with similar sequence to glucagon were found: glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2). As they were synthesized and tested for their biological function GLP-1 revealed, contrary to GLP-2, potent glucose-dependent insulinotropic activity and was therefore characterized as the second incretin hormone (Bell et al. 1983; Schmidt et al. 1985). Both incretin hormones work in an additive manner, while the contribution of GIP to the incretin effect quantitatively seems to be stronger than that of GLP-1 (Nauck et al. 1993a).

Together, they seem to be responsible for up to 60% of total postprandial insulin secretion in normal healthy subjects after administration of an oral glucose load (Nauck et al. 1986).

2.2. Glucagon-like peptide-1 (GLP-1)

2.2.1. Biosynthesis, secretion and degradation of GLP-1

The sequence of GLP-1 is encoded in the proglucagon gene that is mainly expressed in alpha-cells within the pancreas, L-cells of the intestinal mucosa and additionally in neurons of specialized regions of the brainstem and hypothalamus (Baggio et al. 2007). The posttranslational processing of the proglucagon precursor is cell-type specific: In pancreatic alpha-cells glucagon, glicentin-related polypeptide (GRPP) and major proglucagon peptide (MPGF) are the mainly delivered products, whereas intestinal L-cells and neurons in the brain mostly liberate GLP-1, GLP-2, glicentin and oxyntomodulin (Larsen et al. 1997; Mojsov et al. 1986). Although pigs show higher density of GLP-1 immunoreactive L-cells along the intestine compared to humans, cell distribution seems to be similar between the two species throughout small and large intestine with highest amounts of L-cells in the distal jejunum, ileum and colon (Eissele et al. 1992). The main stimulators of GLP-1 secretion are nutrients like fat and carbohydrates (Brubaker 2006) and the following biphasic secretion pattern results in a rapidly starting increase in GLP-1 plasma concentration within 5 to 15 minutes followed by a second secretion interval after 30 to 60 minutes (Herrmann et al. 1995). As the early GLP-1 secretion pattern can hardly be explained by direct contact of nutrients with L-cells at rather distal parts of the intestine it is likely that mechanisms of GLP-1 secretion are complex and also involve other stimulators like neural and endocrine factors (Brubaker 1991; Roberge et al. 1993; Rocca et al. 1999). GLP-1 appears in the organism in two biologically active and functionally equal forms: The mainly circulating GLP-1 (7-36)-amide and the minor GLP-1 (7-37), that is extended by a glycine residue (Orskov et al. 1994; Orskov et al. 1993). These bioactive forms show a very short half-life of less than 2 minutes due to partial proteolytic cleavage by the enzyme DPP-4 and thereby conversion to the inactive forms GLP-1 (9-36)-amide or GLP-1 (9-37) (Deacon et al. 1995a; Deacon et al. 1995b; Mentlein et al. 1993). As DPP-4 is *inter alia* membrane-bound to endothelial cells of capillaries regulating blood outflow of the

intestinal mucosa, it is in close vicinity to GLP-1 secreting L-cells. Thereby the degree of degradation is extensive, as 50% of secreted bioactive GLP-1 is already converted to inactive forms by DPP-4 before even entering the systemic blood circulation (Hansen et al. 1999).

2.2.2. GLP-1 receptor (GLP-1R) and signal transduction

Like the receptor for glucagon and GIP, the GLP-1R belongs to the class 2 of heterotrimeric guanine nucleotide-binding protein (G-protein) coupled receptors containing a N-terminal extracellular domain for ligand binding, seven membrane-spanning domains linked by intra and extracellular loops and a C-terminal cytoplasmic domain for further intracellular signaling (Mayo et al. 2003). However, binding of the ligand GLP-1 to its receptor is highly specific as GIP cannot bind to the GLP-1R and glucagon only with 100- to 1000-fold less affinity (Doyle et al. 2007; Fehmann et al. 1994; Thorens 1992). Expression of the GLP-1 receptor is confirmed for a lot of tissues including alpha-, beta- and delta-cells in the pancreas as well as brain, heart, lung, kidney, stomach, intestine, pituitary, skin, thyroid C-cells and specialized regions of the peripheral and central nervous system like the vagus nerve, the hypothalamus or the hippocampus (Baggio et al. 2007; Kim et al. 2008). Binding of GLP-1 to the N-terminal extracellular domain leads to a conformational change of the receptor with further activation and dissociation of the alpha subunit of the G-protein that is located at the third intracellular loop. This causes multiple and complex effects on further messenger systems (Hallbrink et al. 2001). The enzyme adenylate cyclase (AC) gets activated going along with enhanced production of cyclic adenosine monophosphate (cAMP) (Drucker et al. 1987). Increased cAMP concentrations lead to the activation of two main important downstream effectors: Protein kinase A (PKA) and guanine nucleotide exchange factor 2 (Epac2) that subsequently activate further messenger systems (Holz 2004; Kang et al. 2001; Wheeler et al. 1993). Additionally, GLP-1 binding to its receptor can lead to the activation of phosphatidylinositol-3-kinase (PI3K) as well as mitogen-activated protein kinase (MAPK) and their downstream messenger systems (Buteau et al. 1999; Montrose-Rafizadeh et al. 1999).

2.2.3. Effects of GLP-1 on the endocrine pancreas

The binding of GLP-1 to the GLP-1R located on the cell membrane of pancreatic beta-cells leads to multiple cellular actions that mediate its strong glucose-dependent insulinotropic effect, mainly caused by increased cAMP levels in the cell with further activation of PKA and Epac2 (Holst et al. 2004). The most important cellular events are the closure of ATP-dependent potassium channels, leading to depolarization of the cell membrane and opening of voltage-dependent calcium channels with further influx of calcium. Cytosolic calcium concentration gets additionally increased by mobilization of calcium from intracellular stores, which altogether triggers fusion of insulin containing secretory granules and exocytosis of insulin (Holst 2007; Holst et al. 2004). An important factor in this cascade which may cause about 70% of the stimulatory action of GLP-1 on insulin secretion is the ability of cAMP to increase the number of insulin containing granules that are rapidly ready for release independently of PKA and Epac2 (Gromada et al. 1998). The effect of GLP-1 induced promotion of insulin secretion absolutely requires a certain threshold of glucose levels in the beta-cell (Holst et al. 2004; Nauck et al. 1993c; Qualmann et al. 1995). Activation of the GLP-1R also results in an up-regulation of insulin biosynthesis and gene transcription by cAMP/PKA-dependent but also -independent messenger pathways and thereby secures intracellular insulin reserves (Drucker et al. 1987; Fehmann et al. 1992). Furthermore it was shown that GLP-1 has beneficial trophic effects on the beta-cells as it is able to inhibit apoptosis rate in isolated human islets as well as in the beta-cells of rodent models (Farilla et al. 2003; Farilla et al. 2002; Li et al. 2003) and stimulates beta-cell proliferation in rodents (Farilla et al. 2002; Perfetti et al. 2000; Stoffers et al. 2000). GLP-1 signaling is able to provide the differentiation of progenitor cells in pancreatic duct epithelium to insulin-producing beta-cells (Zhou et al. 1999). These findings were particularly interesting regarding the prospect that GLP-1 could be a new treatment option for type 2 diabetic patients that show decreased beta-cell volume going along with increased apoptosis rate, but they still have to be confirmed in humans *in vivo* (Butler et al. 2003). The underlying molecular mechanisms of the protective function of GLP-1 on the beta-cells are complex and not completely understood. Messenger pathways including cAMP/PKA, PI3K and MAPK systems *inter alia* result in an up-regulation of insulin receptor 2 substrate (*Irs2*) gene expression and increased transcription of the pancreatic and duodenal homeobox gene 1

(*Pdx1*) (Baggio et al. 2007). Beside its impact on the beta-cells, GLP-1 is able to inhibit glucagon secretion in humans, perhaps supported by its ability to stimulate somatostatin secretion which was shown in pig and rat pancreata as well as in isolated islets (Creutzfeldt et al. 1996; Fehmann et al. 1995; Orskov et al. 1988; Schmid et al. 1990). The stimulatory action on somatostatin release seems to be independent of glucose levels and may be caused by direct contact of GLP-1 to a GLP-1R on pancreatic delta-cells (Fehmann et al. 1995; Fehmann et al. 1991).

2.2.4. Extrapancreatic effects of GLP-1

GLP-1 shows additional functions in other tissues beside its effect on the pancreas as also indicated by the versatile distribution of GLP-1Rs within the organism (Baggio et al. 2007; Kim et al. 2008). First of all, GLP-1 seems to be involved in the regulation of appetite, as short-term as well as long-term administration of GLP-1 forces up the feeling of satiety resulting in a distinct and sustained reduced food intake with the consequence of lower body weight gain (Flint et al. 1998; Gutzwiller et al. 1999; Zander et al. 2002). Moreover, GLP-1 reduces gastric secretion and most notably delays dose-dependently gastric emptying, which consequently leads to decelerated appearance of glucose from the meal in the bloodstream (Nauck et al. 1997; Wettergren et al. 1993; Wishart et al. 1998). Thereby GLP-1 shows a relevant second blood glucose-lowering effect that is independent from an increase in insulin secretion and is even going along with decreased postprandial insulin levels (Meier et al. 2003; Nauck et al. 1997). An important factor gaining more and more interest in recent research is the effect of GLP-1 on the cardiovascular system. Although in rodent models GLP-1 seems to increase blood pressure as well as heart rate (Barragan et al. 1994; Yamamoto et al. 2002), these findings are not in agreement with the neutral effects on these parameters seen in humans (Toft-Nielsen et al. 1999). In dogs with dilated cardiomyopathy continuous infusion with recombinant GLP-1 over 48 hours improved left ventricular and systemic hemodynamics and furthermore it was able to decrease myocardial infarction size in rat models of ischemia/reperfusion, indicating a cardioprotective function of GLP-1 (Bose et al. 2005; Nikolaidis et al. 2004). In humans it was shown that GLP-1 has beneficial effects on endothelial function in type 2 diabetic patients suffering from coronary artery disease and enhances myocardial function during chronic heart failure, making GLP-1 and GLP-1R agonists a promising approach for the application in the treatment of

cardiovascular diseases (Nystrom et al. 2004; Thrainsdottir et al. 2004). The central nervous system seems to play a major role mediating functions of GLP-1 as GLP-1 and GLP-1Rs are expressed in the brainstem, the hypothalamus, the area postrema or the nodose ganglion of abdominal vagal afferent nerve fibers which all are specialized regions implicated in the regulation of appetite and feeding behavior, gastric motility and cardiovascular function (Baggio et al. 2007). Thereby it is likely that these effects caused by GLP-1 are partially due to direct interactions with GLP-1Rs, for example on stomach and heart, but mainly influenced by central neuronal regulation (Kim et al. 2008). But GLP-1 also shows further effects on the organism: Because of its neuroprotective effects studies are ongoing in rodent models to prove the use of GLP-1 and GLP-1R agonists in the therapy of neurodegenerative diseases like Alzheimer´s disease (Holscher 2010). The glucose-lowering effect of GLP-1 is additionally supported by an enhanced glucose uptake and storage in fat and muscle tissue as well as decreased glucose production but enhanced glycogen storage in the liver (Baggio et al. 2007). GLP-1 seems to influence the hypothalamic-pituitary axis because it is able to stimulate secretion of thyroid-stimulating hormone, luteininizing hormone, corticosterone, adrenocorticotropin and vasopressin in rodent models or rodent cell lines (Kim et al. 2008). However, GLP-1R deficient mice (*GLP1r*$^{-/-}$) show no considerable disorders regarding function of the hypothalamic-pituitary axis (MacLusky et al. 2000). In thyroid C-cells, GLP-1 increases release of calcitonin which could be correlated with an indirect function on bone, because calcitonin is an inhibitor of the activity of osteoclasts and it was shown that *GLP1r*$^{-/-}$ mice show great bone fragility and decrease of bone density (Kim et al. 2008; Lamari et al. 1996).

2.3. Glucose-dependent insulinotropic polypeptide (GIP)

2.3.1. Biosynthesis, secretion and degradation of GIP

The second incretin hormone GIP is a 42-amino acid peptide that is synthesized in K-cells within the intestinal mucosa that can mainly be found along the duodenum and proximal jejunum (Buchan et al. 1978; Moody et al. 1984). Release of GIP from these cells is mostly stimulated by nutrient absorption in a species-specific manner: While fat uptake is the greatest inducer of GIP secretion in humans, carbohydrates are the main stimulus in rodents and pigs (Baggio et al. 2007). The

way of degradation for GIP is similar as for GLP-1 because also in this case the enzyme DPP-4 splits the molecule at the N-terminus, resulting in a conversion to the biological inactive form GIP (3-42) and thereby a short half-life of 5-7 minutes in humans (Deacon et al. 2000).

2.3.2. GIP receptor (GIPR) and signal transduction

As the GLP-1R, the GIPR belongs to the heterotrimeric G-protein coupled receptors (Mayo et al. 2003). While the N-terminal extracellular domain is responsible for ligand binding and the first transmembrane domain for receptor activation, the third intracellular loop of the receptor is particularly important for the interaction with the G-protein and thereby for the further signal transduction (Brubaker 1991; Cypess et al. 1999; Gelling et al. 1997; Harmar 2001; Salapatek et al. 1999). With following activation of the enzyme AC to produce cAMP and further messenger systems including PKA, MAPK and PI3K the molecular signal mechanisms of GLP-1R and GIPR clearly coincide, which is also reflected in a lot of similar biological functions (Baggio et al. 2007). The GIPR shows a widespread distribution within the organism including expression in pancreatic alpha- and beta-cells, intestine, heart, lung, kidney, adrenal cortex, pituitary, bone, adipose tissue and specialized areas within the brain (Kim et al. 2008).

2.3.3. Biological functions of GIP

Regarding the beta-cells, GIP generally shows the same effects as described for GLP-1 mainly via similar molecular mechanisms: A glucose-dependent boost of insulin secretion, up-regulation of insulin biosynthesis and gene transcription as well as pro-proliferative and anti-apoptotic effects on the beta-cells mainly shown *in vitro* (Dupre et al. 1973; Fehmann et al. 1995; Kim et al. 2005; Trumper et al. 2002; Trumper et al. 2001). With this knowledge it is understandable that GIP and GLP-1 work in an additive manner mediating the incretin effect (Nauck et al. 1993a). In contrast to GLP-1, GIP is only a poor stimulator of somatostatin release and does not influence glucagon secretion except simultaneous glucose levels of 5 mmol/l or lower are present, then it even stimulates glucagon secretion (Fehmann et al. 1995). The ability to inhibit gastric acid secretion was the first described function of GIP and the decisive factor for its former name “gastric inhibitory peptide” (Brown et al. 1975). Stimulation of progenitor cell proliferation in the brain and of fatty acid synthesis in adipocytes as well as an

anabolic effect on bone can additionally be counted to the functions of GIP (Baggio et al. 2007).

3. Liraglutide as an incretin-based therapeutic agent

3.1. Fundamental concept of incretin-based therapeutics

Type 2 diabetic patients show a different metabolic reaction pattern in response to nutrient uptake compared to healthy human subjects: During food or nutrient ingestion their enteroendocrine L-cells and K-cells also secrete the two incretin hormones, but while the function of GLP-1 is preserved GIP shows a clearly diminished insulinotropic action, leading to a distinct reduced incretin effect (Nauck et al. 1986; Nauck et al. 1993b). As postprandial secretion levels of GIP are similar in comparison to healthy subjects (Toft-Nielsen et al. 2001; Vilsboll et al. 2001) the underlying mechanism for the body´s inability to respond to GIP is not completely clarified yet. Reasons could be sequence variants in the coding region of the GIPR gene leading to altered structure and/or expression of the receptor (Almind et al. 1998; Kubota et al. 1996; Lynn et al. 2001; Saxena et al. 2010). The lack of responsiveness of GIP in type 2 diabetic patients leads to disturbed insulin response and higher blood glucose levels. These findings led to the idea to compensate the insufficient GIP function by potentiating the preserved insulinotropic function of GLP-1 and thereby improve glucose homeostasis. GLP-1 shows desirable properties for the clinical treatment of type 2 diabetes: An existing hyperglycemia can be lowered to physiological levels by enhancing of insulin secretion and, as insulin secretion only gets stimulated during hyperglycemic stages, the danger of hypoglycemia is, in comparison to classical insulin treatment, very low (Drucker 2003; Holst 1999). Another useful function of GLP-1 for patients suffering from type 2 diabetes and often showing excessive overweight is that it acts as a mediator of satiety and delays gastric emptying (Alvarez et al. 2005; Nauck et al. 1997). Two big groups of incretin-based drugs are available. As native GLP-1 administration is not practical because of the very short half-life (< 2 min) (Deacon et al. 1995a) the GLP-1 sequence was either changed to ensure a longer half-life while directly stimulating the GLP-1R (GLP-1R agonists) or molecules were developed that slow down GLP-1 degradation by inhibiting the enzyme DPP-4 and thereby increase endogenous GLP-1 concentration (DPP-4 inhibitors).

3.1.1. DPP-4 inhibitors

Endogenous as well as exogenous administered GLP-1 gets rapidly and extensively degraded to biological inactive GLP-1 (9-36)-amide or GLP-1 (9-37) by partial proteolytic cleavage of the enzyme DPP-4 (Deacon et al. 1995a; Deacon et al. 1995b; Mentlein et al. 1993). From the 1990s up to the present, major advances have been made in developing small molecules that block the active center of this enzyme and thereby save GLP-1 from degradation. Studies with different rodent models showed that molecules like Ile-thiazolidide or valine-pyrrolidide are capable to augment insulin response and improve glucose tolerance (Ahren et al. 2000; Pauly et al. 1999; Pederson et al. 1998). It was also shown in anesthetized pigs that one single administration of DPP-4 inhibitor valine-pyrrolidide is able to completely block the conversion from biological active GLP-1 into its degradation product (Deacon et al. 1998a). Nowadays, advanced DPP-4 inhibitors have already shown their potential to improve glycemic control while having neutral effects on body weight in type 2 diabetic patients treated by monotherapy or add-on therapy (Ahren 2009; Idris et al. 2007; Karagiannis et al. 2012; Richter et al. 2008). Generally these drugs seem to be well tolerated (Duez et al. 2012) and show potential for preserving beta-cell function and volume in rodent models (Jelsing et al. 2012b; Mu et al. 2006; Shimizu et al. 2012). Already approved DPP-4 inhibitors are sitagliptin (Januvia®), vildgliptin (Galvus®), saxagliptin (Onglyza®) and linagliptin (Trajenta®). It has to be considered that unit cost is increased in comparison to older drugs like sulfonylureas while having similar effects on glucose levels and additionally long-term safety is not completely assured yet (Karagiannis et al. 2012). Beside its enzymatic action DPP-4 is a membrane-associated molecule on the surface of T-cells (named CD26) and plays a role in transduction of activation signaling dependent on the interaction with other membrane-associated antigens (Fleischer 1994; Torimoto et al. 1991). It seems that the enzymatic activity of DPP-4 is not obligatory for the activation of T-cells so that blockage of the active catalytic center should not compromise its immune functions (von Bonin et al. 1998). As GLP-1 is not the only substrate for DPP-4 but also a number of other regulatory peptides including members of the pancreatic polypeptide and glucagon family as well as some interleukins and analgesic brain peptides, it is important to further determine in what way blockage of DPP-4 activity influences metabolism of these peptides (Mentlein 1999). Interestingly, DPP-4 deficient

Fischer rats are viable and show normal phenotype, also regarding glucose tolerance (Pederson et al. 1996).

3.1.2. GLP-1R agonists

The aim during development of GLP-1R agonists was to create and discover molecules with elongated duration of action while full biological GLP-1 actions are conserved. A lot of studies characterized structural determinants of GLP-1 action: It was shown that analogues carrying a substitution at position 8 of GLP-1 (7-37) are resistant to DPP-4 degradation, show longer half-life *in vivo* but still are biologically active (Deacon et al. 1998b). This is comprehensible with the knowledge that DPP-4 is a specialized exopeptidase that removes dipeptides from bioactive peptides when amino acids proline or alanine are the penultimate N-terminal residues (Mentlein 1999). Native GLP-1 (7-37) contains an alanine as penultimate N-terminal residue and is thereby an excellent substrate for the DPP-4 (Kieffer et al. 1999). In contrast to the native GLP-1 structure N- and C-terminally truncated peptides show less or no biological action (Gefel et al. 1990; Mojsov 1992; Suzuki et al. 1989), while C-terminally extended peptides show nearly identical efficacy compared to GLP-1 (Goke et al. 1993; Nathan et al. 1992). Particularly the side chain of position 7 (histidine), but also of position 10, 12, 13, 15, 28, 29 seem to be important for receptor interaction, as substitution leads to significant loss in receptor affinity (Adelhorst et al. 1994; Hareter et al. 1997). The attachment of fatty acids enforces the ability to bind serum albumin and thereby slows down renal elimination of GLP-1 (Knudsen et al. 2000). Nowadays several GLP-1 agonists are available for subcutaneous injection and play an important role in modern diabetes treatment. Exenatide was the first GLP-1R agonist on the market, gaining U.S. Food and Drug Administration (FDA) approval in 2005 under the trade name Byetta® (Amylin 2011). It is a synthetic form of exendin-4, a naturally existing GLP-1 mimetic that was originally isolated from the salivary gland of the lizard *Heloderma suspectum* and shares 53% sequence homology to native GLP-1 (Eng et al. 1992). With a half-life of 2.4 hours it shows higher DPP-4 resistance but still strong affinity to the GLP-1R (Bray 2006; Goke et al. 1993). Byetta® is indicated as an adjunct to diet or exercise in adult patients with type 2 diabetes mellitus or in combination with other oral antidiabetic drugs and has to be administered subcutaneously twice daily (Amylin 2011). During research and clinical trials Byetta® showed

biological effects of native GLP-1 desired for diabetes treatment: It has beneficial effects on glucose homeostasis by enhancing insulin secretion in a glucose dependent manner, reducing postprandial hyperglycemia and suppressing glucagon secretion in humans (Bunck et al. 2009; Buse et al. 2004; Cervera et al. 2008; DeFronzo et al. 2005; Fehse et al. 2005; Kolterman et al. 2003; Moretto et al. 2008) as well as in rodent and primate models of diabetes (Greig et al. 1999; Parkes et al. 2001; Young et al. 1999). Gastric emptying is slowed down dose-dependently and satiety is increased by the use of Byetta®, leading to weight loss (Buse et al. 2004; Linnebjerg et al. 2008; Meier et al. 2003; Moretto et al. 2008; Toft-Nielsen et al. 1999; Vilsboll et al. 2012; Young et al. 1999). Beneficial effects on blood pressure and lipid profiles have also been reported (Klonoff et al. 2008). A great point of interest is the fact that exenatide seems to be able to increase beta-cell volume and function, thereby leading to a cessation or even a reversing of disease progression. These effects on pancreatic beta-cells were not shown in humans so far, but there are several reports using cell lines or rodent models: exendin-4 showed 10-fold higher potency to stimulate the conversion of AR42J cells that were derived from a chemically induced pancreatic tumor from negative for islet hormones to insulin, pancreatic polypeptide and glucagon producing cells (Zhou et al. 1999). Treatment with exendin-4 led to an increase of the beta-cell volume going along with amplified beta-cell proliferation and/or decreased beta-cell apoptosis rate in several diabetic rodent models, e.g. partially pancreatectomized rats, streptozotocin-treated Wistar rats and C57BL/6 mice, intrauterine growth-retarded neonatal Sprague-Dawley rats, Goto-Kakizaki rats, diabetic *db/db* mice or *fa/fa* Zucker rats (Gedulin et al. 2005; Li et al. 2003; Park et al. 2008; Stoffers et al. 2003; Tourrel et al. 2002; Tourrel et al. 2001; Wang et al. 2002; Xu et al. 1999). Although it is a priority goal of diabetes research there is still a lack of approaches for reliably non-invasive visualization of beta-cells that could prove this theory in humans (Malaisse 2005). To further increase patient compliance a new formulation of exenatide called Bydureon® was approved in 2012 (Amylin 2012). By encapsulation of exenatide into small spherical particles the drug is released over an extended period and only has to be injected once weekly subcutaneously while having similar effects as Byetta® (Aroda et al. 2011; DeYoung et al. 2011). Liraglutide under the trade name Victoza® is the third GLP-1R agonist formulation on the market with approval of the European Medicine Agency (EMEA) in 2009 (EMEA 2009a). As it is the drug used in the

present study pharmacokinetics and pharmacodynamic effects should be clarified in more detail (see II 3.2, II 3.3, II 3.4). With agents like albiglutide and lixisenatide that are currently undergoing phase 3 trials there is an ongoing development of new GLP-1R agonists (Khan et al. 2012). In conclusion GLP-1R agonists show a bigger extent of reduction in glycated hemoglobin and weight as well as greater treatment satisfaction and efficiency compared to DPP-4 inhibitors and will sure gain an even more important role in the treatment of type 2 diabetes mellitus (Russell 2012).

3.2. Pharmacokinetics of liraglutide

The success and effectiveness of exenatide amplified the efforts to develop GLP-1R agonists with prolonged half-life suitable for a less frequent subcutaneous administration. During these studies liraglutide with the former name NN2211 was discovered, an incretin mimetic that shows 97% homology to native GLP-1. The peptide portion of liraglutide was produced recombinantly in *Saccharomyces cerevisiae* and shows only one difference to the structure of human GLP-1(7-37): Lysine at position 34 is replaced by arginine. Additionally, a C16 palmitoyl fatty acid chain is chemically attached to lysine at position 26 via glutamic acid spacer, allowing intensified binding to serum albumin as well as self-association into heptamers at the injection site and thereby resulting in delayed absorption from the subcutis (Drucker et al. 2010; Knudsen et al. 2000; Novo Nordisk 2010; Steensgard DB 2008). Because of these modifications liraglutide can be cleaved by DPP-4 in the same position as GLP-1, but at a much slower rate (Malm-Erjefalt et al. 2010). It still retains high affinity to the GLP-1 receptor while showing a plasma half-life of about 11-15 hours after subcutaneous administration in humans and is thereby suitable for a once daily administration (EMEA 2009a; Knudsen et al. 2000; Novo Nordisk 2010). While pigs show similar terminal half-life of about 14 hours it seems to be shorter in mice, rats, rabbits and monkeys (4-8 hours) (EMEA 2009a). After subcutaneous injection liraglutide is slowly absorbed in the human organism with maximum plasma concentrations obtained 9-14 hours after dosing (Agerso et al. 2002; Elbrond et al. 2002). Maximum peak liraglutide concentration after subcutaneous injection of 0.6 mg in human is approximately 9.4 nmol/l and increases proportionally with dose in the therapeutic range from 0.6 mg up to 1.8 mg (Agerso et al. 2002; EMEA 2009b). Although a slight tendency towards accumulation was noticed in mice, rats and monkeys,

accumulation ratio was low (< 2%) and comparable to the results seen in humans. Overall bioavailability accounts approximately 53% for monkeys and 55% for humans while pigs show higher bioavailability of 76% (EMEA 2009a). Despite slight variances concerning relative bioavailability it was shown in humans that the pharmacokinetic properties of a 0.6 mg liraglutide dose did not differ when administered to different injection sites like thigh, upper arm or abdomen (Kapitza et al. 2011b). Additionally, liraglutide seems to be able to cross the blood-brain barrier as well as the placental barrier (EMEA 2009a; Hunter et al. 2012). The volume of distribution after subcutaneous dosing (0.6 mg) is approximately 13 liter with a mean apparent clearance of 1.2 liter/hour. During the initial 24 hours after administration this GLP-1 agonist seems to be mainly distributed in the plasma compartment in its intact form and extensively (> 98%) bound to proteins (Malm-Erjefalt et al. 2010; Novo Nordisk 2010). As no intact liraglutide can be detected in urine or feces it seems likely that it is completely catabolized within the body into amino acids and fatty acid fragments that either get recycled or eliminated. Two minor metabolites were determined, but they don´t seem to have relevant activities (Malm-Erjefalt et al. 2010). Plasma exposure of liraglutide was not increased in patients suffering from renal dysfunction or hepatic impairment, leading to the conclusion that the kidney or the liver do not play a single major role during elimination (Flint et al. 2010; Jacobsen et al. 2009). Pharmacokinetics seems not to be influenced by age, gender, race or ethnicity and is similar between healthy men and patients with type 2 diabetes (Damholt et al. 2006; Novo Nordisk 2010). As liraglutide delays gastric emptying it is important to evaluate the influence of this GLP-1 agonist on absorption rate and drug-drug interaction of simultaneously given oral drugs. Liraglutide seems not to interfere with function of cytochrome P450 enzymes and shows no clinically significant interactions with the co-administered drugs acetaminophen, atorvastatin, griseofulvin, digoxin, lisinopril or an oral contraceptive formulation (EMEA 2009a; Jacobsen et al. 2011; Kapitza et al. 2011a; Malm-Erjefalt M 2008; Novo Nordisk 2010).

3.3. Pharmacodynamic effects of liraglutide in animal models

3.3.1. Pharmacodynamic effects of liraglutide in animal models of diabetes

3.3.1.1. Liraglutide administration in rodent models of diabetes

A consistent key finding during investigations of liraglutide administration in hyperglycemic rodent models of diabetes was the potent, dose-dependent and long-lasting blood glucose lowering effect during basal glucose profiling as well as after glucose challenge (Brand et al. 2009; Larsen et al. 2008; Rolin et al. 2002; Shimoda et al. 2011; Sturis et al. 2003; Vrang et al. 2012). No effect on basal blood glucose was seen in normoglycemic rodent models, indicating that this effect is glucose-dependent (Bock et al. 2003b; Shimoda et al. 2011). A part of the glucose-lowering effect seems to be due to a reduction of food intake that mostly could be observed acutely after liraglutide administration (Rolin et al. 2002) and was even persistent during the whole liraglutide treatment period in some studies (Larsen et al. 2008; Sturis et al. 2003). As a consequence body weight reductions were monitored in liraglutide-treated animals and analysis of body composition in obese rats revealed that the weight loss was mainly due to a decrease of fat mass (Raun et al. 2007a). On the one hand, the decrease of food intake and body weight is provoked by a delayed gastric emptying caused by liraglutide, but on the other hand regulation of the central nervous system seems to be involved, particularly regarding long-term body weight reduction (Jelsing et al. 2012a). A reducing effect on food intake and body weight could also be shown in non-diabetic normal and obese rats (Bock et al. 2003b; Larsen et al. 2001b). Native GLP-1 is known to increase insulin secretion in a glucose-dependent manner and thereby it *inter alia* mediates its anti-hyperglycemic effect (Holst et al. 2004). An increase of insulin secretion during basal insulin profiling or after glucose challenging caused by liraglutide administration could also be shown in diabetic ZDF rats (Brand et al. 2009; Sturis et al. 2003) and diabetic *ob/ob* mice (Rolin et al. 2002). In diabetic *db/db* mice fasting insulin was increased after 2 days of liraglutide treatment, but decreased after 2 weeks of liraglutide administration. In normoglycemic *m/m* mice no difference was seen regarding fasting insulin levels (Shimoda et al. 2011). In UCD-T2DM (University of California, Davis, type 2 diabetes mellitus) rats, a model of polygenic obesity that shows a late onset of diabetes, liraglutide

treatment caused a delayed development of diabetes and this was going along with significantly reduced fasting plasma insulin and glucose, indicating an improvement in insulin sensitivity (Cummings et al. 2010). In obese but non-diabetic Sprague Dawley rats no significant differences in insulin levels were seen during an oral glucose tolerance test after 12 weeks of liraglutide treatment in comparison to placebo treatment (Raun et al. 2007a). As native GLP-1 is known to have trophic effects on beta-cells (Farilla et al. 2003; Farilla et al. 2002) most studies using rodent models also determined the effect of the GLP-1R agonist liraglutide on the beta-cell volume, beta-cell proliferation and apoptosis showing variable results: Liraglutide treatment (200 µg/kg twice daily) for 15 days could cause a beta-cell volume increase of about 35% as well as a distinct increase in beta-cell proliferation in diabetic *db/db* mice in comparison to placebo-treated animals, but same treatment interval (100 µg/kg twice daily) provoked only a tendency of increased beta-cell proliferation and beta-cell volume without significant differences in diabetic *ob/ob* mice (Rolin et al. 2002). Also in a study of Shimoda et al. two weeks of liraglutide administration (200 µg/kg twice daily) provoked a rise of beta-cell volume of approximately 36% in the diabetic *db/db* mouse model, accompanied by greater beta-cell proliferation rate, suppressed beta-cell apoptosis rate and decreased oxidative stress rate. In this study liraglutide additionally caused a 30% increase of beta-cell volume and increased beta-cell proliferation in a normoglycemic *m/m* mouse model (Shimoda et al. 2011). Sturis et al. investigated the effects of liraglutide in the male ZDF rat model. A treatment period of 6 weeks (30 µg or 150 µg/kg twice daily) in ZDF rats that in this time interval developed hyperglycemia provoked an increase of beta-cell volume in comparison to placebo treatment. Liraglutide administration over a shorter term of 2 weeks caused significantly lower beta-cell volume and proliferation in ZDF rats that did not develop hyperglycemia during this treatment interval compared to placebo treatment. The authors concluded that a certain level of glucose could be necessary for an effect of liraglutide on beta-cell dynamics (Sturis et al. 2003). However, no differences in beta-cell volume were seen in older severely diabetic male ZDF rats after 6 weeks of liraglutide treatment (200 µg/kg twice daily) or 30 days of liraglutide treatment (15 µg or 50 µg/kg twice daily) in comparison to placebo treatment (Brand et al. 2009; Larsen et al. 2008). On the other hand, Vrang et al. could show that 13 weeks of liraglutide administration (1 mg/kg/day) leads to increased beta-cell volume in female, but

not in male diabetic ZDF rats (Vrang et al. 2012). Non-diabetic Sprague Dawley rats showed greater beta-cell volume after one week of liraglutide administration (200 µg/kg twice daily) compared to placebo, but this effect was vanished after 6 weeks as no more differences in beta-cell volume were visible (Bock et al. 2003b). 12 weeks of liraglutide treatment (200 µg/kg twice daily) in candy-fed non-diabetic obese Sprague Dawley rats caused weight loss and reduced beta-cell volume compared to the obesity-associated increase of beta-cells in placebo-treated rats (Raun et al. 2007a).

3.3.1.2. Liraglutide administration in porcine models of diabetes

Although the bigger part of animal studies was carried out with rodent models there are still a few reports of anti-hyperglycemic and body weight reducing effects of liraglutide in the Göttingen Minipig model (Raun et al. 2007b; Ribel et al. 2002): During a study of Ribel et al. streptozotocin was used to induce either diabetes or impaired glucose tolerance in male Göttingen Minipigs (Ribel et al. 2002). During hyperglycemic clamp experiments these pigs acutely pre-treated with liraglutide required a higher glucose infusion rate to hold blood glucose on a constant level and still they even showed a weak tendency of lower plasma glucose levels, while insulin plasma levels were distinctly and glucose-dependently increased in comparison to placebo pre-treatment. During a chronic treatment study with daily subcutaneous liraglutide administration of 3.3 µg/kg for 4 weeks liraglutide-treated pigs showed significantly improved oral glucose tolerance during oral glucose tolerance tests while insulin responses did not change in comparison to placebo-treated animals. Additionally, it was shown that liraglutide administration significantly reduced gastric emptying in this pig model and improved insulin sensitivity as indicated by the parameter glucose to insulin ratio (Ribel et al. 2002). In a study of Raun et al. *ad libitum* fed adult female Göttingen Minipigs were used that did not show diabetes or impaired glucose tolerance, but developed insulin resistance while showing intense obesity (Raun et al. 2007b). After a 3-week acclimatization period where subcutaneous liraglutide dosage was titrated for each pig, animals were treated with 7 µg/kg for another 4 weeks. Liraglutide caused a strong and sustained suppression of food intake that accounted over 60% in comparison to untreated obese minipigs, going along with reductions in body weight of about 4-5% (Raun et al. 2007b). An *in vitro* study showed that liraglutide administration improves survival of isolated porcine islets

going along with reduction of beta-cell apoptosis, improved glucose responsiveness and cellular viability seen at 24 h of culture (Emamaullee et al. 2009). In pancreaticoduodenectomized Yucutan miniature pigs that received an infusion of a marginal mass of pancreatic islets into the portal circulation, a 6-week administration of liraglutide (20 µg/kg maintenance dose) could enhance metabolic function by increasing serum insulin during glucose tolerance testing. However, the quantitative proportion of beta-cells in the transplanted islet did not differ between liraglutide- and placebo-treated animals (Emamaullee et al. 2009).

3.4. Pharmacodynamic effects of liraglutide in human clinical trials

Liraglutide under the trade name Victoza® is an isotonic colorless solution that has to be injected subcutaneously once daily. In Europe, it is not approved as monotherapy but in combination with metformin and/or a sulphonylurea as well as metformin and a thiazolidinedione with the main goal to improve glycemic control (EMEA 2009a). It is recommended in humans to start with the dosage 0.6 mg once daily and rise to 1.2 mg not before one week later. For some patients it can be beneficial to further increase the dosage to a maximum of 1.8 mg once daily to achieve better therapy results (Novo Nordisk 2009). Before the EMEA and FDA granted marketing authorization for Victoza® in 2009, comprehensive clinical study programs were performed to evaluate the potential, clinical efficacy and safety of this drug as monotherapy or in combination with other commonly used therapeutics for diabetes treatment as reviewed in Ryan et al. (2011). The first clinical trial study was published by Matsbach et al. in 2004 and already showed that 12 weeks of liraglutide treatment in type 2 diabetic patients at dosages up from 0.6 mg can significantly decrease HbA_{1c} in comparison to placebo (Madsbad et al. 2004). During further phase 1 and 2 development trials, when liraglutide was mainly administered short-term or within a maximum duration of 14 weeks, the glucose-lowering effect was also seen, going along with a glucose-dependent increased insulin secretory response, weight loss and decrease of systolic blood pressure in diabetic patients (Schmidt 2010). Altogether these studies and findings showed the promising abilities of liraglutide in the treatment of type 2 diabetes and reinforced the efforts of more detailed and longer-term research. Therefore, the largest clinical trials were performed during the phase 3 clinical trial program and included six extensive studies that together form the Liraglutide Effect and Action in Diabetes (LEAD) program. All LEAD

studies were carried out for 26 weeks, except LEAD-3 that lasted even longer for 52 weeks, and liraglutide initial dosages of 0.6 mg mostly increasing to 1.2 mg or 1.8 mg were used (Buse et al. 2009; Garber et al. 2009; Marre et al. 2009; Nauck et al. 2009; Russell-Jones et al. 2009; Zinman et al. 2009). A large number of overall about 4500 type 2 diabetic patients was included into the LEAD program at different therapeutic stages of the disease: Some of them showed early phase diabetes and thereby had not yet been treated or only for a short time with one oral antidiabetic drug, others were used to be treated with a combination of two oral antidiabetic drugs that still failed to reach the therapeutic goal of maintained glucose control. In the six LEAD trials, liraglutide efficacy and safety as monotherapy, dual-drug therapy or triple-drug therapy was evaluated mostly compared to other standard diabetic treatments like glimepiride, rosiglitazone or exenatide as active comparators (Nauck 2012).

3.4.1. Efficacy of liraglutide on glycemic control during LEAD trials

The improvement of glycemic control by liraglutide treatment was proven in detail during LEAD trials and demonstrated with the influence on parameters including HbA_{1c}, FPG and PG: HbA_{1c} after liraglutide treatment was generally reduced in comparison to mean baseline HbA_{1c} prior to liraglutide administration, both when liraglutide was administered alone and in combination with one or two oral antidiabetic drugs (Blonde et al. 2009; Buse et al. 2009). When used as monotherapy (LEAD-3), 1.2 mg liraglutide could cause HbA_{1c} reduction of 0.84% from the baseline prior to the treatment while 1.8 mg even mediated a decrease of 1.14%, and thereby greater reduction than seen for the sulfonylurea glimepiride (Garber et al. 2009). Liraglutide in combination with other oral antidiabetic drugs additionally provoked greater HbA_{1c} reductions compared to exenatide twice daily (Buse et al. 2009), thiazolidinedione rosiglitazone (Marre et al. 2009) and insulin glargine (Russell-Jones et al. 2009) that also were administered in combination with the same oral antidiabetic drugs. Throughout all LEAD studies liraglutide monotherapy as well as combination therapy with oral antidiabetic drugs provoked a reduction of FPG and PG in comparison to baseline values at the beginning of the trials and placebo treatment (Blonde et al. 2009; Buse et al. 2009). The extent of these reductions caused by 1.2 mg or 1.8 mg liraglutide combination treatment was similar to active comparators like insulin glargine or glimepiride (Nauck et al. 2009; Russell-Jones et al. 2009) or even greater

compared to rosiglitazone (Marre et al. 2009). Liraglutide therapy decreased FPG significantly more than exenatide twice daily when both agents were combined with other pharmacological agents. However, PG reductions were similar after lunch but showed greater extent in response to exenatide combined treatment after breakfast and dinner (Buse et al. 2009). When given as monotherapy, liraglutide mediated greater reductions in FPG and PG than glimepiride monotherapy (Garber et al. 2009).

3.4.2. Efficacy of liraglutide on beta-cell function during LEAD trials

Beta-cell function during LEAD studies was evaluated by calculating parameters using fasting insulin and glucose levels, like the homeostasis model assessment of insulin resistance (HOMA-IR) and of beta-cell function (HOMA-β) or by determination of the proinsulin : insulin ratio (Blonde et al. 2009). HOMA-β was improved between 20% and 44% by liraglutide treatment in all six LEAD studies compared to baseline at the beginning of the trial. Therefore the increase in HOMA-β was greater compared to the one caused by rosiglitazone (LEAD-1) or exenatide (LEAD-6) treatment with concomitant background therapies respectively (Garber 2011). HOMA-IR was not continuously determined in all LEAD studies, but Garber et al. assessed that liraglutide monotherapy mediated significantly decreased HOMA-IR values in comparison to glimepiride monotherapy (Garber et al. 2009). Furthermore, the proinsulin : insulin ration was found to be lower compared to baseline at the beginning of the trial during all LEAD studies (Matthews 2008). Taken together, these calculations propose beneficial effects on beta-cell function and improvement of insulin sensitivity by liraglutide treatment (Garber 2011).

3.4.3. Efficacy of liraglutide on body weight and blood pressure during LEAD trials

Regarding body weight, liraglutide administration with or without concomitant drugs caused weight loss in comparison to baseline values or placebo during LEAD studies 2-6. During 52 weeks of liraglutide monotherapy (LEAD-3), diabetic patients showed a mean body weight loss of 2.5 kg when being treated with 1.8 mg liraglutide and 2.1 kg when being treated with 1.2 mg liraglutide. This body weight reduction mainly occurred within the first 16 weeks of treatment and was sustained thenceforward (Blonde et al. 2009; Garber et al. 2009).

Liraglutide administration caused similar weight loss as exenatide twice daily when both drugs were combined with oral antidiabetic agents (Buse et al. 2009). When liraglutide was combined with metformin (LEAD-2) or with metformin and rosiglitazone (LEAD-4), the weight lowering effect was found to be dose-dependent (Nauck et al. 2009; Zinman et al. 2009). LEAD-1 was the only study that reported weight-neutral effects for liraglutide therapy combined with the sulfonylurea glimepiride (Marre et al. 2009). Generally, the extent of weight loss seems to be greater in diabetic patients that additionally show a high initial body weight and mainly includes the loss of visceral adipose tissue (Blonde et al. 2009). During all LEAD studies liraglutide treatment alone as well as dual or triple treatment was associated with decreased systolic blood pressure (SBP) (Blonde et al. 2009; Buse et al. 2009). When given as monotherapy for a period of 52 weeks, 1.2 mg liraglutide caused SBP reductions of 2.1 mm Hg and 1.8 mg liraglutide even 3.6 mm Hg in comparison to baseline prior to the treatment. Regarding diastolic blood pressure there was also a tendency observed for reductions after liraglutide treatment but this effect was not significant (Garber et al. 2009).

3.4.4. Safety and tolerability of liraglutide during LEAD trials

Liraglutide was generally well tolerated during all trials with low rates of hypoglycemia (Blonde et al. 2009). During monotherapy, 12% of patients treated with 1.2 mg liraglutide and 8% of patients treated with 1.8 mg liraglutide showed minor hypoglycemic events defined as plasma glucose <3.1 mmol/l (Garber et al. 2009). However, rate of hypoglycemia seemed to increase when liraglutide treatment was coupled with a sulfonylurea like glimepiride. Mild to moderate gastrointestinal symptoms like a different range of nausea, vomiting and diarrhea were the main detected adverse effects (Ryan et al. 2011). Generation of antibodies in response to liraglutide treatment was observed in about 8.6% of patients included in the LEAD studies. However, appearance of antibodies seemed not to change pharmacodynamic actions of liraglutide (Perry 2011). With the knowledge that GLP-1 agonists are able to stimulate calcitonin release in rodent thyroid C-cells and increase the occurrence of C-cell hyperplasia and C-cell tumor formation in rats it was additionally important to accurately evaluate the impact of liraglutide on C-cells (Bjerre Knudsen et al. 2010). Analysis of clinical data from the six LEAD studies and three additional clinical studies up to

a treatment period of about two years revealed no evidence for a connection of liraglutide to pathological changes of thyroid C-cells (Chiu et al. 2012). Although some studies reported the occurrence of pancreatitis combined with liraglutide treatment, the incidence rate during LEAD studies was low compared to the general susceptibility of type 2 diabetic patients to develop pancreatitis described in previous studies (Noel et al. 2009; Ryan et al. 2011).

4. The GIPR^{dn} transgenic pig model

4.1. The pig as a large animal model for diabetes research

In regard to the increasing prevalence of type 2 diabetes mellitus appropriate animal models play an irreplaceable role to clarify its pathogenesis as well as to test the efficacy and safety of new drugs for the development of novel therapeutic concepts. According to the Federal Ministry for Food, Agriculture and Consumer Protection, rodents (mainly mice and rats) have been the most commonly used mammalian laboratory animals in Germany with a percentage of about 95% in 2011, followed by rabbits (3%) and pigs (0.6%) (BMELV 2013). Reasons for the predominant use of rodent animal models are the availability of strains with well-defined genetic background adapted to a lot of important fields of medical research, early sexual maturity and short reproductive cycle, high cost effectiveness and the possibility of good experimental standardization (Bogue 2003; Clee et al. 2007). However, the translation of scientific results gained from rodents in basic research to human clinical application is often difficult. In this context the use of the pig as a large animal model can help to close the gap between rodent and man by sharing a lot of anatomical, physiological and pathophysiological similarities with humans, especially with regard to diabetes research (Aigner et al. 2010; Douglas 1972; Larsen et al. 2004; Lunney 2007; Matsunari et al. 2009; Swindle et al. 2012). Porcine and human sequences of GLP-1 are highly conserved (Kieffer et al. 1999) and GIP amino acid sequence only distinguishes at residue 18 (human: histidine, porcine: arginine) and residue 34 (human: asparagine, porcine: serine) (Moody et al. 1984). Structurally, porcine insulin differs from human insulin by one single amino acid at the C-terminal alanine, position 30 of the B-chain, and seems to have similar therapeutic efficacy in clinical use (Brogden et al. 1987; Heinemann et al. 1993; Richter et al. 2005). Despite some differences regarding pancreatic duct system both pig and human

pancreas show comparable size, anatomical orientation and localization as well as similar pancreatic blood supply (Ferrer et al. 2008; Murakami et al. 1997; Swindle 1998; Swindle et al. 2012; Truty et al. 2008). In both species pancreatic endocrine cells are mainly located in the islets of Langerhans with some single cells or small cell clusters arranged in the exocrine pancreatic tissue (Jay et al. 1999; Wieczorek et al. 1998). Islet structure of pig and human shows differences regarding proportion of each type of endocrine cells. Although insulin-producing beta-cells are the main fraction of endocrine cells in both species, the amount of beta-cells in porcine islets (< 80%, depending on age) is higher than in human islets in situ (< 50%). Accordingly human islets show a bigger percentage of non-beta-cells (mostly alpha-cells) (Cabrera et al. 2006; Dufrane et al. 2012). While pigs at the age of 5 weeks show diffuse islet structure containing beta-cells with small diameter, the development of larger cell clusters is visible as the age of the pigs increases. That is the reason why islet structure of older pigs is comparable to islet structure of adult humans (Jay et al. 1999). The number of islets in the whole pancreas seems to differ greatly between different pig breeds (Ulrichs 1995). As in humans, IAPP is expressed mainly in the beta-cells but has a changed sequence in the amyloidogenic domain (Betsholtz et al. 1989; Lukinius et al. 1996). Thereby dangerous accumulation of cytotoxic amyloid plaque accompanied by a progressive increase of beta-cell apoptosis rate does not occur in pigs (Clark et al. 1988; Potter et al. 2010). Physiological blood glucose levels are comparable (human: 70-100 mg/dl, porcine: 70-115 mg/dl), with the exception of the minipig that shows lower values (Classen 2004; Kixmöller 2004; Kraft 2005; Larsen et al. 2004; Larsen et al. 2001a; Plonait 1988; Waldmann 2001). For interpretation of glucose tolerance it is important to consider that pigs show less increase in plasma glucose during an oral glucose tolerance test and dispose an intravenous glucose load more efficiently than humans (Anderson 1973; Ferrannini et al. 1985; Hanawalt et al. 1947; Larsen et al. 2002a; Larsen et al. 2002b). The capacity for insulin secretion after stimulation with glucose *in vivo* is extensive and peripheral insulin concentrations show very rapid dynamics (Kjems et al. 2001; Larsen et al. 2003). Both species show a slight deterioration in glucose tolerance with age, going along with higher glucose and insulin plasma levels (Broughton et al. 1991; Larsen et al. 2001a; Rosenthal et al. 1982). The morphology and the physiology of the porcine gastrointestinal system resemble those of humans with both species being omnivorous and thereby showing similar ingesta transit times, ion transport,

motility and digestive effectiveness (Miller et al. 1987; Swindle 1998). Pigs are a desirable model for testing new treatment strategies given subcutaneously. Composition, permeability, metabolic properties and sparse hair coat of porcine skin are comparable to man and enable similar percutaneous absorption rate, kinetics and dynamics of chemical compounds after injection (Benech-Kieffer et al. 2000; Bode et al. 2010; Sullivan et al. 2001; Swindle et al. 2012).

4.2. Generation and characterization of GIPR^{dn} transgenic pigs

To clarify the role of a reduced function of GIP on metabolism and its role in the pathogenesis of type 2 diabetes genetically modified pigs were developed that express a GIPR^{dn} in the pancreatic beta-cells (Renner et al. 2010). The GIPR^{dn} differs from the endogenous GIPR by an eight amino acid deletion (amino acid position 319-326, nucleotide position 955-978) and two point mutations (amino acid position 340, nucleotide position 1018-1020) that further lead to an amino acid exchange from alanine to glutamate in the sequence of the third intracellular loop of the receptor. As it is known that this loop is especially important for signal transduction (Cypess et al. 1999; Harmar 2001; Salapatek et al. 1999) GIP can bind to the GIPR^{dn} with almost equal affinity as it has to the endogenous GIPR, but the binding does not provoke any further biological functions (Herbach et al. 2005). As a result of the competition between GIPR^{dn} and endogenous GIPR for their ligand GIP a reduction of the insulinotropic action of GIP can be seen, but not a complete loss. Thereby the metabolic situation observed in type 2 diabetic patients is well imitated. GIPR^{dn} transgenic pigs were created by a highly efficient gene transfer technology based on lentiviral vectors that in this case consisted of the complementary DNA of the human GIPR^{dn} under the control of the rat insulin 2 (*Ins 2*) gene promoter (RIP2) (Hofmann et al. 2003; Renner et al. 2010). Vectors were injected into the perivitelline space of pig zygotes and subsequently embryos were laparoscopically transferred to cycle synchronized recipient gilts. After birth pigs were genotyped using polymerase chain reaction (PCR) and Southern blot. Expression of GIPR^{dn} mRNA was successfully proven in isolated pancreatic islets using real-time PCR. Transgenic offspring was established by mating male founder boars to wild-type sows. During the characterization of this pig model it was shown that GIPR^{dn} transgenic pigs develop normally and do not show a clinically overt diabetes mellitus, at least throughout an evaluation period of 24 months, indicated by normal blood glucose and fructosamine levels (Renner et al.

2010). In contrast, the also well characterized GIPR^{dn} transgenic mouse model shows a severe diabetic phenotype just before weaning going along with glucosuria, elevated serum glucose levels, reduced insulin levels and increased glucagon concentration (Herbach et al. 2005). However, different physiological tests and quantitative-stereological analyses in the GIPR^{dn} transgenic pig model showed distinct modifications in comparison to non-transgenic control groups: Stimulation tests with GIP and exendin-4, a potent GLP-1R agonist, showed that the insulinotropic effect of intravenously administered GIP was blunted, whereas GLP-1R agonist exendin-4 elicited significantly higher serum insulin levels in GIPR^{dn} transgenic pigs than in wild-type controls. This proves that GLP-1R function is, in comparison to GIPR function, undisturbed and even compensatory hyperactive in this animal model (Renner et al. 2010). Immunohistochemical staining of GIPR and GLP-1R showed that there is no distinct difference in expression levels and spatial distribution of the receptors between transgenic and wild-type control pigs. Glucose tolerance tests provided more information regarding glucose homeostasis and insulin secretion. During an oral glucose tolerance test GIPR^{dn} transgenic pigs at the age of 11 weeks showed clearly higher blood glucose level as well as delayed insulin secretion while total insulin release was unchanged. At 5 months of age oral glucose tolerance was already disturbed due to a significant decreased insulin secretion (Figure 2). Intravenous glucose tolerance tests showed no abnormalities in 11-week-old transgenic pigs, but an ongoing deterioration regarding intravenous glucose tolerance and insulin secretion fully developed at the age of 11 months (Figure 1) (Renner et al. 2010).

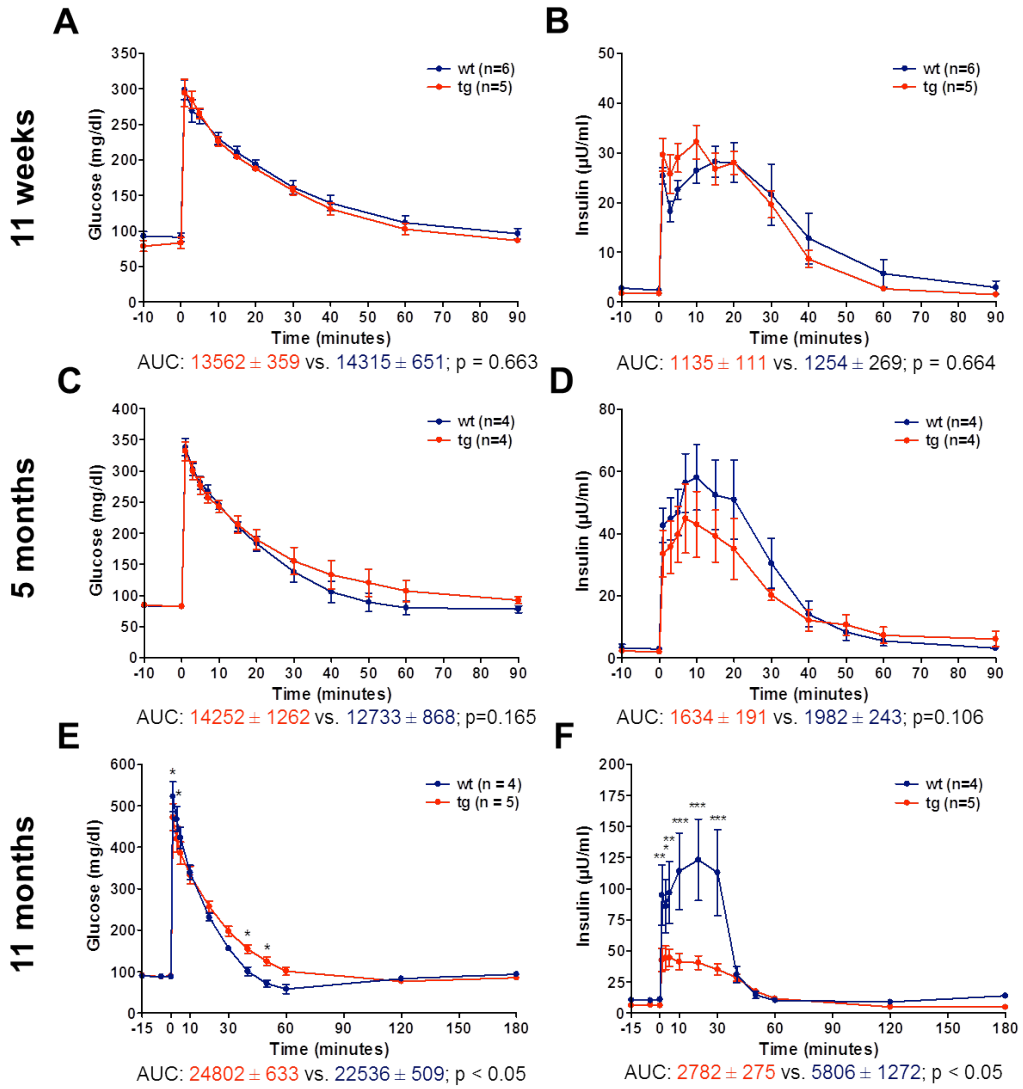

Figure 1: Intravenous glucose tolerance observed during the characterization of the GIPR^{dn} transgenic pig model

Unchanged intravenous glucose tolerance **(A)** and insulin secretion **(B)** in GIPR^{dn} transgenic pigs (tg) at the age of 11 weeks; tendency of higher blood glucose **(C)** and reduced insulin secretion **(D)** at the age of 5 months; significantly disturbed intravenous glucose tolerance **(E)** and reduced insulin secretion **(F)** at the age of 11 months compared to wild-type controls (wt); 0 minutes = point of glucose administration; n: number of animals investigated. Data are means ± SEM; *: $p<0.05$ vs. control, **: $p<0.01$ vs. control, ***: $p<0.001$ vs. control. (Published in Renner et al., 2010, Copyright 2010 American Diabetes Association, From Diabetes®, Vol. 59, 2010, 1228-1238, reprinted with permission from *The American Diabetes Association*)

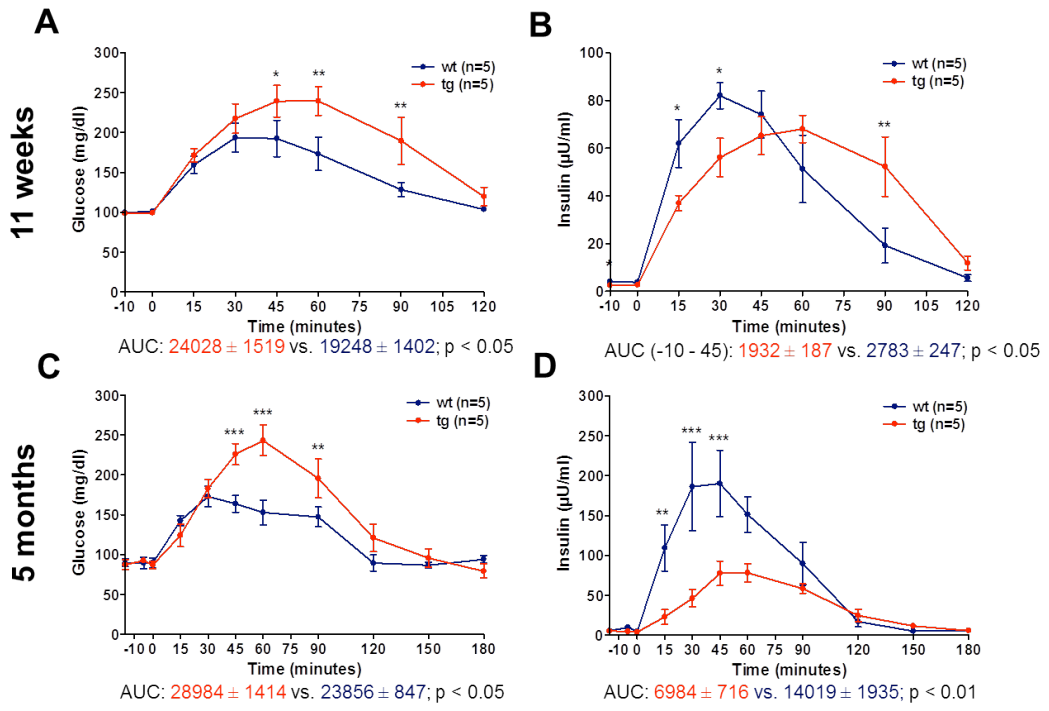

Figure 2: Oral glucose tolerance observed during the characterization of the GIPR^{dn} transgenic pig model

Disturbed oral glucose tolerance in GIPR^{dn} transgenic pigs (tg) at the age of 11 weeks **(A)** and 5 months **(C)** going along with delayed insulin secretion **(B)** and reduced insulin secretion **(D)** compared to wild-type controls (wt); 0 minutes = point of glucose administration; n = number of animals investigated. Data are means ± SEM; *: p<0.05 vs. control, **: p<0.01 vs. controls, ***: p<0.001 vs. controls. (Published in Renner et al., 2010, Copyright 2010 American Diabetes Association, From Diabetes®, Vol. 59, 2010, 1228-1238, reprinted with permission from *The American Diabetes Association)*

These findings suggested the idea that an expression of the GIPR^{dn} could cause a general disturbance of insulin secretion and/or changes in structure and integrity of pancreatic islets of Langerhans with increasing age. This suspicion was confirmed by the result of quantitative-stereological analyses of the pancreata. The total beta-cell volume was significantly decreased in 5-month-old GIPR^{dn} transgenic pigs (35% reduction) and even more in 1 to 1.4-year-old animals (60% reduction) (Figure 3). Expression of the GIPR^{dn} also led to a significant reduction

of beta-cell proliferation rate in 11-week-old pigs that was no longer visible in older transgenic pigs, and a tendency of higher beta-cell apoptosis rate in 1 to 1.4-year-old transgenic animals. These results show for the first time in a large animal model that GIP plays an important role in the physiological development and destiny of pancreatic beta-cells.

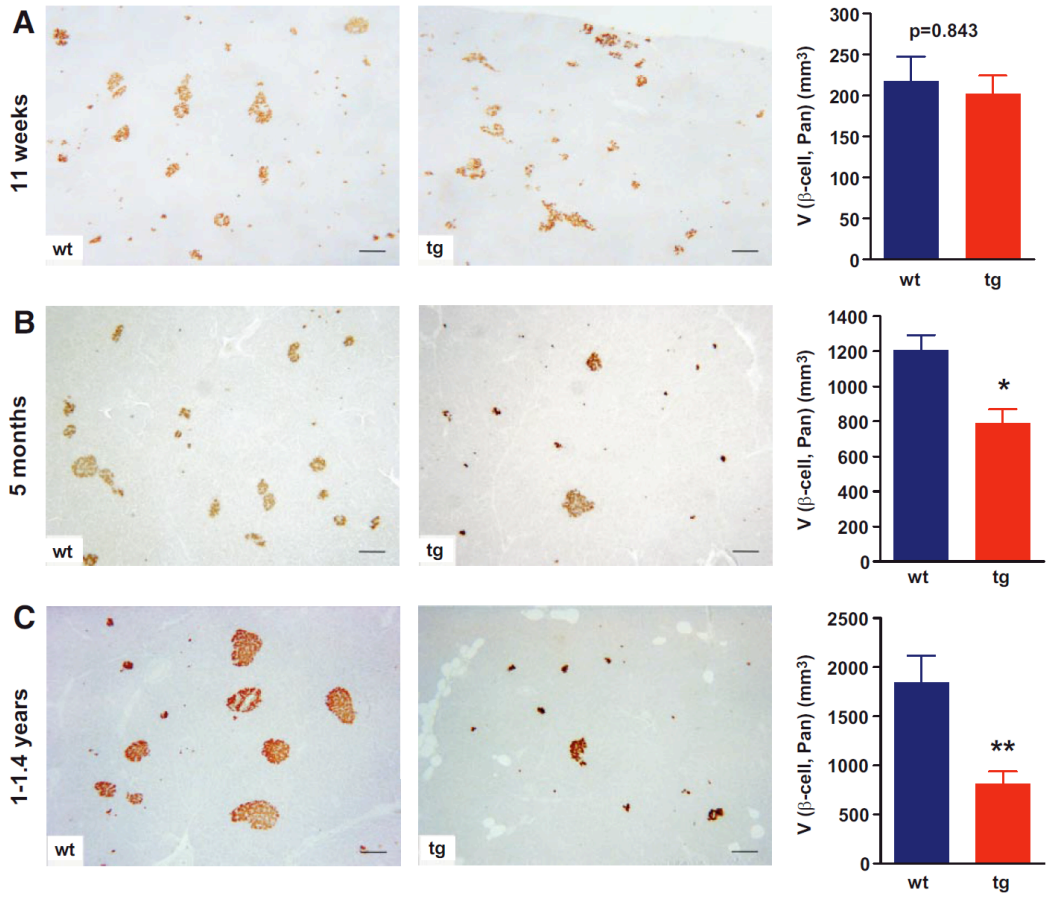

Figure 3: Beta-cell volume of GIPR^{dn} transgenic pigs at different age

(A) Unchanged beta-cell volume in 11-week-old GIPR^{dn} transgenic pigs (tg), but (B, C) reduced beta-cell volume in 5-month-old and 1-1.4-year-old GIPR^{dn} transgenic pigs compared to non-transgenic control wild-type pigs (wt). Data are means ± SEM; n = number of animals investigated; *: $p<0.05$ vs. control, **: $p<0.01$vs. control. *(*Published in Renner et al., 2010, Copyright 2010 American Diabetes Association, From Diabetes®, Vol. 59, 2010, 1228-1238, reprinted with permission from *The American Diabetes Association*)

Taken together, GIPR^{dn} transgenic pigs show reduced insulinotropic action of GIP, decreased glucose tolerance and insulin secretion as well as a progressive age-related reduction of beta-cell volume. Thereby this interesting pig model reflects important aspects of prediabetes seen in type 2 diabetic patients and offers manifold options for translational diabetes research like for example the evaluation of new treatment strategies for type 2 diabetes (Renner et al. 2010). Thus, the GIPR^{dn} transgenic pig model was used in the present study for the evaluation of the effects of the GLP-1R agonist liraglutide on glycemic control, growth, food intake and especially on the total beta-cell volume.

III. RESEARCH DESIGN

The present study was designed to evaluate the effects of the GLP-1R agonist liraglutide on glucose control, food intake, growth and especially on the total beta-cell volume of GIPR^{dn} transgenic pigs. Overall twenty-nine GIPR^{dn} transgenic pigs were randomly chosen by lot to be either treated with liraglutide or 0.9% sodium chloride as placebo, injected subcutaneously for 90 days once daily. During this treatment period the animals had access to an *ad libitum* chow. Liraglutide dosages in the range of 0.6 mg to 1.8 mg were used based on the recommended human dosages and pig body weight. Food intake, weight gain as well as the health status were monitored on a regular basis and levels of somatostatin and components of the insulin-like growth factor (IGF) system were determined prior to and after the treatment period. Additionally, metabolic tests including intravenous and oral glucose tolerance tests were carried out prior to the therapy and at the end to evaluate changes in glucose control. After the final post-treatment metabolic tests GIPR^{dn} transgenic pigs were euthanized, pancreata were harvested, systematically sampled and quantitative-stereological analyses were carried out for the determination of the total beta-cell volume. Two different age groups of GIPR^{dn} transgenic pigs were investigated in this study:

Prophylactic group: This group (n=18) consisted of GIPR^{dn} transgenic pigs that were either treated with liraglutide (n=9; 5 female, 4 male) or placebo (n=9; 5 female, 4 male) from 2 months until 5 months of age. It is known from previous studies that GIPR^{dn} transgenic pigs around the age of 2 months show undisturbed intravenous, but disturbed oral glucose tolerance. Although beta-cell volume is unaltered at an age of 2 months, it will be reduced about 35% at the time these pigs are 5 months of age, going along with deterioration in oral glucose tolerance and delayed insulin secretion during intravenous glucose tolerance test (Renner et al. 2010). The aim of this study part was to evaluate in what way a therapy of GIPR^{dn} transgenic pigs with liraglutide influences metabolic status, glucose homeostasis and especially if it is able to cause a retardation of the forthcoming reduction of the total beta-cell volume or if it can even prophylactically prevent it in comparison to placebo-treated GIPR^{dn} transgenic pigs.

Therapeutic group: GIPR^{dn} transgenic pigs in this group (n=11) were treated with liraglutide (n=5, 3 female, 2 male) or placebo (n=6, 3 female, 3 male) from 5 months until 8 months of age. At 5 months of age GIPR^{dn} transgenic pigs normally show a clear reduction of their total beta-cell volume of about 35% that even deteriorates with age to a reduction of 60% at the age of about one year, associated with disturbed oral and intravenous glucose tolerance (Renner et al. 2010). Beside effects on metabolic status and glucose homeostasis, this study part should show if a therapy of GIPR^{dn} transgenic pigs with liraglutide can prevent or slow down the progressive reduction of beta-cell volume or even restores beta-cells in comparison to placebo-treated GIPR^{dn} transgenic pigs.

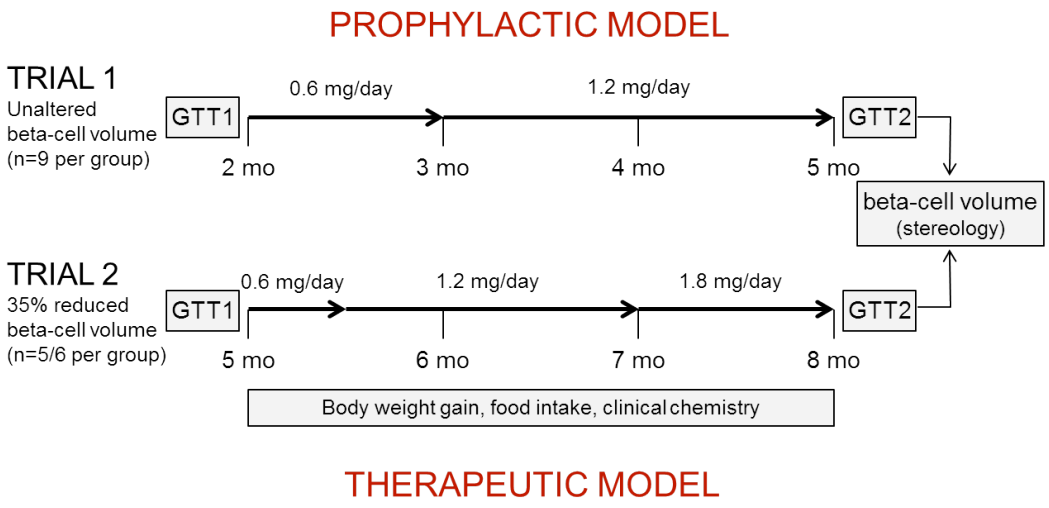

Figure 4: Study outline of liraglutide treatment trial

Overview of general research design with two determined age groups (trial 1: prophylactic group, trial 2: therapeutic group) and liraglutide dosage regimen; n = number of animals investigated; mo = months of age; GTT = glucose tolerance test; mg = milligram.

IV. ANIMALS, MATERIALS AND METHODS

1. Animals

Animals included in this study were hemizygous male and female transgenic pigs expressing a dominant-negative GIP receptor. During the whole treatment period pigs were housed in planar single pens covered with straw and had *ad libitum* access to water and a commercial diet (Table 1). Careful training before treatment and experimental tests ensured the work with conscious animals. All animal experiments were approved by the responsible animal welfare authority (Regierung von Oberbayern, Munich; AZ-55.2-1-54-2532-43-11).

Table 1: Diet composition
Diets were produced by Zimmerer-Werk, Landshut, Germany

	Ferkelstarter UNI (piglets up to 25 kg)	**Zuchtschwein Getreidemischung UNI** (growing and adult pigs)
MJ ME/kg	13.1	10.9
Crude protein %	17.5	13.6
Crude fat %	2.9	3.2
Crude ash %	5.3	5.9
Crude fiber %	3.5	7.9
Calcium %	0.7	0.9
Phosphorus %	0.6	0.6
Sodium %	0.2	0.2
Magnesium %	0.2	0.3

ME: metabolizable energy

2. Materials

2.1. Apparatuses

Accu-jet® pro pipette controller	Brand, Wertheim
Agarose gel electrophoresis chamber	OWL Inc., USA
Analytic balance	Sartorius, Göttingen
Analytic balance MS 100	Schippers GmbH, Kerken

Animal balance Fstar 125	Meier-Brakenberg GmbH, Exertal
AU 400 autoanalyzer	Olympus, Hamburg
Benchtop 96 tube working rack	Stratagene, USA
BX41 light microscope	Olympus, Hamburg
Celltek® blood cell counter	Bayer Diagnostics, Fernwalde
DP72 video camera	Olympus, Hamburg
Gel documentation system	Bio Rad, Munich
HM 315 microtome	Microm, Walldorf
Incubator 37°C	Wagner + Munz, Munich
Incubator 60°C	Memmert, Schwabach
Inhalation anaesthesia device K1	Koch KG, Bad Ems
Hitachi 911® autoanalyzer	Boehringer, Ingelheim
LB 2111 γ-counter	Berthold, Bad Wildbad
Object micrometer	Zeiss, Oberkochen
Mastercycler® gradient	Eppendorf, Hamburg
Multipipette® plus	Eppendorf, Hamburg
MS1 minishaker vortexer	IKA®-Werke GmbH, Staufen
Pipettes (1000 µl, 200 µl, 100 µl, 10 µl, 2 µl)	Gilson Inc., USA
Power Pac 300 gel electrophoresis unit	Bio Rad, Munich
Shandon Citadel tissue processor 1000	Thermo Fisher Scientific, Schwerte
Sony video graphic printer UP-895CE	Sony, USA
TBS 88 paraffine embedding system	Medite, Burgdorf
Tru Trak® 3900P pulse oximeter	Datex-Ohmeda, Finland

Centrifuges:

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

2.2. Consumables

Adhesive tape	Tesa SE, Hamburg
Adhesive tissue tape	Henry Schein® Vet GmbH, Hamburg
Aluminium spray	CP – Pharma, Burgdorf
BD Micro-Fine Ultra™ pen needles (0.33 x 12.7 mm; 29 G)	Becton Dickinson GmbH, Heidelberg
Cavafix® Certo® central venous catheter	B. Braun, Melsungen
Combitips® plus (1 ml, 10 ml)	Eppendorf, Hamburg
Cover slips (24x40 mm)	VWR International GmbH, Darmstadt
Disposable shaver	Wilkinson GmbH, Solingen
Disposable syringes (2, 5, 10, 20 ml)	Henry Schein® Vet GmbH, Hamburg
Disposable tubes for γ-counter	Sarstedt, Nümbrecht
Falcon® Centrifuge tubes (15 ml)	Becton Dickinson, Heidelberg
Gazin® gauze swab	Lohmann + Rauscher, Neuwied
Glass cuvettes with rack insert	VWR GmbH, Darmstadt
Hypodermic needles (18 G, 20 G)	Henry Schein® Vet GmbH, Hamburg

Monovette® blood collection system (Serum, EDTA)	Sarstedt, Nümbrecht
Omnican® 40 disposable insulin syringe	B. Braun, Melsungen
OP-Cover (60 x 90 cm)	A. Albrecht, Aulendorf
Parafilm® M	American Can Company, USA
PCR reaction tubes (0.2 ml)	Braun, Wertheim
Perfusor® cable (50 cm)	B. Braun, Melsungen
Pipette tips	Eppendorf, Hamburg
Pipette tips with filter	Axygen Inc., USA
Safe-Lock reaction tubes (1.5 ml, 2 ml)	Eppendorf, Hamburg
SafeGrip® latex gloves	SLG, Munich
Scalpel blade sterile No.36	Medicon eG, Tuttlingen
Sempermed® supreme latex OP gloves	Sempermed, USA
Skin adhesive spray	A. Albrecht, Aulendorf
3-way-stopcock Variostop®	Clinico GmbH, Bad Hersfeld
Star Frost® microscope slides	Engelbrecht, Edermünde
Surgicryl® suture material (USP 2-0)	SMI AG, Belgium
Test tube peg wrack	Polylab, India
TSK Supra sterile cannula (1.2 x 100 mm)	TSK, Japan
Uni-Link embedding cassettes	Engelbrecht, Edermünde
Vasofix® indwelling cannula (20 G, 22 G)	B. Braun, Melsungen
Vasofix® mandrin (20 G, 22 G)	B. Braun, Melsungen

2.3. Chemicals

Comment: Chemicals were used in p.a. quality unless marked otherwise

Acetic acid	Merck, Darmstadt

Agarose Universal	Bio Sell, Nürnberg
Aprotinin (3.0 PEU/mg)	Roth, Karlsruhe
Bromophenolblue	Roth, Karlsruhe
DAB -3,3´diaminobenzidine tetrahydrochloride	KemEnTec, Denmark
Diprotin A	Sigma, Taufkirchen
1.4-Dithiothreitol (DTT)	Biomol GmbH, Hamburg
EDTA (Ethylenediaminetetraacetic acid)	Roth, Karlsruhe
Ethanol	Roth, Karlsruhe
Ethidiumbromide (1 mg/ml)	Merck KGaA, Darmstadt
4% Formaldehyde	SAV LP, Flintsbach
Glucose 50% solution	B. Braun, Melsungen
Glycerol	Roth, Karlsruhe
Hydrogen peroxide (30%)	NeoLab, Heidelberg
Magnesium chloride	Merck, Darmstadt
Magnesium chloride (25 mM)	Qiagen GmbH, Hilden
Mayer´s Hemalum solution	Applichem GmbH, Darmstadt
Rotipuran® benzyl alcohol	Roth, Karlsruhe
Sodium chloride solution (0.9%)	B. Braun, Melsungen
Sodium hydroxide (2 N)	Roth, Karlsruhe
Tris-(hydroxymethyl)-aminomethane (TRIS)	Roth, Karlsruhe
Xylene	Applichem GmbH, Darmstadt

2.4. Antibodies, drugs, enzymes, oligonucleotides

2.4.1. Antibodies

Primary antibody:

Polyclonal guinea pig anti-porcine insulin	Dako Cytomation, Hamburg

Secondary antibodies:

AP-conjugated goat anti-guinea pig IgG	Southern Biotech, USA
HRP-conjugated rabbit anti-guinea pig IgG	Dako Cytomation, Hamburg

2.4.2. Drugs

Azaperon (Stresnil®)	Jansen Pharmaceutica, Belgium
Cefquinom (Cobactan® 2.5%)	Intervet, Unterschleißheim
Embutramid, Mebezonium, Tetracain (T61®)	Intervet, Unterschleißheim
Heparin-Sodium (25,000 IU/5 ml)	B. Braun, Melsungen
Isobar® Isoflurane	Intervet, Unterschleißheim
Ketamine hydrochloride (Ursotamin®)	Serumwerke Bernburg Bernburg
Liraglutide (Victoza®) (6 mg/ml injection solution in pre-filled pen)	Novo Nordisk, Denmark
Metamizol-Sodium (Vetalgin®)	Intervet, Unterschleißheim
Meloxicam (Metacam®)	Boehringer Ingelheim, Ingelheim
Xylazine (Xylazin 2%)	WDT, Garbsen

2.4.3. Enzymes

Taq DNA Polymerase (5 U/ml)	Agrobiogen, Hilgertshausen

2.4.4. Oligonucleotides

RIP2 (sense):	5′-TAGTCGACCCCCAACCACTCCAAGTGGAG-3′
RIP2 (antisense):	5′-TAGGATCCCTCGAGTCTAGAGTTAGGGCTG-3′
ACTB (sense):	5′-TGGACTTCGAGCAGAGATGG-3′
ACTB (antisense):	5′-CACCGTGTTGGCGTAGAGG-3′

2.5. Buffers, media and solutions

Water deionized in a Millipore machine (Easypure® II, pure Aqua, Schnaitsee) was used as solvent and termed aqua bidest. unless indicated otherwise. All buffers, media or solutions were stored at room temperature unless otherwise noted.

2.5.1. Aprotinin dilution for liraglutide profiling

100 mg Aprotinin (3 PEU/mg)

Add 19.82 ml sodium chloride solution (0.9%)

Add 180 µl benzylalcohol

Vortexed, aliquoted, stored at -80°C

2.5.2. Buffers, media and solutions for PCR and agarose gels

2.5.2.1. dNTP-mix

2 mM dATP, dCTP, dGTP, dTTP

mixed in aqua bidest. and stored at -20°C in aliquots

2.5.2.2. Loading dye for DNA (6x)

10% glycerol in aqua bidest.

1 spatula tip of bromophenolblue

Add 0.5 M NaOH until color turns blue

Aliquoted, stored at -20°C

2.5.2.3. TAE buffer (50x)

242 g Tris

100 ml 0.5 M EDTA (ph 8.0)

57 ml AcOH

Add 1000 ml aqua bidest.

Buffer was diluted to single concentration before use.

2.5.3. Buffers, media and solutions for immunohistochemistry

2.5.3.1. DAB solution

1 DAB tablet was dissolved in 10 ml aqua bidest. for 45 minutes under light protection, filtered, aliquoted and stored at -20°C.

2.5.3.2. TBS buffer for immunohistochemistry (10x)

90 g NaCl

60.5 g Tris

Ad 1000 ml aqua bidest.

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

2.5.3.3. 100 mM Tris HCl (pH 8.5)

12.114 g Tris

Ad 1000 ml aqua bidest.

Adjusted to pH 8.5, autoclaved

2.6. Kits

Nexttec™ Genomic DNA Isolation Kit	Nexttec GmbH, Leverkusen
OCTEIA™ IGF-I ELISA Kit	Immunodiagnostic Systems (IDS) Inc., USA
Porcine Insulin RIA Kit	Millipore, USA
Somatostatin (Human, Rat, Mouse, Porcine) RIA kit	Phoenix Pharmaceuticals Inc., USA
Vector® Red Substrate Kit (AP)	Biozol, Eching

2.7. Other reagents

dNTPs (dATP, dCTP, dGTP, dATP)	MBI Fermentas, St. Leon Roth
Braunol® solution	B. Braun, Melsungen
Gene Ruler™ (1 kb DNA ladder)	MBI Fermentas, St. Leon Roth

Goat serum	MP Biomedicals, France
Histokitt	Glaswarenfabrik Hecht, Sondheim / Röhn
Kodan® Tinktur Forte	Schülke + Mayr GmbH, Norderstedt
10 x PCR buffer	Qiagen GmbH, Hilden
Porcine serum	MP Biomedicals, France
Puc Mix Marker 8	MBI Fermentas, St. Leon Roth
Rabbit serum	MP Biomedicals, France
Q-Solution	Qiagen GmbH, Hilden

2.8. Software

Endnote® version X6	Reuters, USA
Graph Pad Prism® version 5.02	GraphPad Software Inc., USA
Olympus Visiomorph™ image analysis	Visiopharm, Denmark
SAS version 8.2	SAS Institute Inc., USA
SPSS version 21.0	IBM, USA

3. Methods

3.1. Genotyping polymerase chain reaction (PCR)

3.1.1. DNA isolation

Ear punches were obtained from 2-day-old piglets and stored at -20°C until further processing. For isolation of genomic DNA a Nexttec™ Genomic DNA Isolation Kit (Nexttec GmbH, Leverkusen) was used according to the manufacturer´s instruction. In brief, ear punches (ca. 5 mm in diameter) were minced and transferred to 1.5 ml reaction tubes containing the following lysis buffer:

Lysis buffer: 265 µl G1

10 µl G2

25 µl G3

3 µl DTT

Samples were incubated over night at 60°C. The next day 120 µl lysate was transferred to equilibrated Nexttec™ clean columns, incubated for three minutes at room temperature and centrifuged at 700 x g for one minute. The eluate containing the purified DNA was either immediately used for further genotyping PCR or stored at 4°C until further processing.

3.1.2. PCR conditions

The following transgene-specific primers were used to identify the GIPR^{dn} transgene:

RIP2 (sense): 5′-TAGTCGACCCCCAACCACTCCAAGTGGAG-3′

RIP2 (antisense): 5′-TAGGATCCCTCGAGTCTAGAGTTAGGGCTG-3′

To proof DNA integrity PCR was also carried out using beta-actin (*ACTB*)-specific primers as listed below. *ACTB* is a house-keeping gene coding for a component of the cytoskeleton in eukaryotic cells and is thereby ubiquitously expressed.

ACTB (sense): 5′-TGGACTTCGAGCAGAGATGG-3′

ACTB (antisense): 5′-CACCGTGTTGGCGTAGAGG-3′

PCR components were prepared on ice in 0.2 ml reaction tubes. Ingredients of master mix and PCR conditions are listed below:

Table 2: Reaction batch for RIP2-hGIPR^{dn} PCR

RIP2-hGIPR^{dn}	
Aqua bidest.	15.5 µl
10 x PCR buffer (Qiagen)	2.5 µl
$MgCl_2$ (15 mM)	2.5 µl
dNTPs (2 mM)	2.5 µl
Primer sense (10 µM)	0.4 µl
Primer antisense (10 µM)	0.4 µl
Taq DNA Polymerase (5 U/µl)	0.2 µl
DNA Template	1 µl
Total volume	25 µl

Table 3: Reaction conditions RIP2-hGIPR^{dn} PCR

RIP2-hGIPR^{dn} PCR			
Denaturation	95°C	4 min	
Denaturation	95°C	30 sec	35 x
Annealing	62°C	30 sec	
Elongation	72°C	45 sec	
Termination	4°C	15 min	

Table 4: Reaction batch for *ACTB* PCR

ACTB	
Aqua bidest.	8.75 µl
Q-solution (Qiagen)	4 µl
10 x PCR buffer (Qiagen)	2 µl
$MgCl_2$ (25 mM)	1.25 µl
dNTPs (2 mM)	2 µl
Primer sense (10 µM)	0.4 µl
Primer antisense (10 µM)	0.4 µl
Taq DNA Polymerase (5 U/µl)	0.2 µl
DNA Template	1 µl
Total volume	20 µl

Table 5: Reaction conditions *ACTB* PCR

ACTB PCR			
Denaturation	95°C	4 min	
Denaturation	95°C	30 sec	35 x
Annealing	58°C	30 sec	
Elongation	72°C	45 sec	
Termination	4°C	15 min	

Genomic DNA of an already genotyped piglet was used as positive control and DNA of a wild-type pig served as negative control. Additionally, aqua bidest. was utilized as non-template control.

3.1.3. Agarose gel electrophoresis

During agarose gel electrophoresis amplified DNA strands were separated according to their length. A 2% agarose gel was produced by heating a mixture of Universal Agarose and 1x TAE buffer in the microwave until solution was clear. After cooling to 55°C ethidiumbromide (0.5 µg/ml) was added because it intercalates in DNA and thereby allows visualization under UV-light. Gel solidified in a gel electrophoresis chamber that was additionally filled with 1 x TAE buffer. Amplified DNA samples from PCR were mixed with 2.5 µl DNA loading dye (10x) and pipetted into gel slots. A Gene Ruler^{TM} 1 kb DNA molecular weight standard as well as a puc Mix Marker 8 were used to determine fragment size. By connecting chamber to an electric circuit (130 volt) for the time of approximately 1 hour DNA fragments were separated and visualized under UV-light.

3.2. Clinical and metabolic analyses during the liraglutide/placebo treatment period

3.2.1. Accomplishment of liraglutide/placebo administration

Treatment period started at 2 months (67 days) of age in the prophylactic group and at 5 months (147 days) of age in the therapeutic group. Either liraglutide or placebo was administered subcutaneously to GIPR^{dn} transgenic pigs once daily between 8 and 9 a.m. for a period of 90 days. Afterwards therapy was carried on for one week during the time of the post-treatment glucose tolerance tests and liraglutide profiling until necropsy. For liraglutide administration a prefilled pen (Victoza®, 6 mg/ml) was used. An equal volume of sodium chloride solution (0.9%) was filled in disposable insulin syringes and was subcutaneously injected as placebo. Victoza® pens and prepared placebo syringes were stored at 4°C until use. The lateral neck region behind the ear was chosen as injection site. Based on the recommended human dosage and pig body weight the following dose regimen was used (Table 6):

Table 6: Liraglutide/Placebo dose regimen

Prophylactic group (n=18)	**Liraglutide (n=9)**	**Placebo (n=9)**
Day of therapy 1-30	0.6 mg (100 µl)	100 µl
Day of therapy 31-90	1.2 mg (200 µl)	200 µl
Therapeutic group (n=11)	**Liraglutide (n=5)**	**Placebo (n=6)**
Day of therapy 1-16	0.6 mg (100 µl)	100 µl
Day of therapy 17-60	1.2 mg (200 µl)	200 µl
Day of therapy 61-90	1.8 mg (300 µl)	300 µl

3.2.2. Monitoring of body weight, food intake as well as clinical-chemical parameters

Body weight of all GIPR^{dn} transgenic pigs included in the study was measured to the nearest 0.5 kg once weekly using a standard large animal scale. Weighing was started during the pre-treatment period at an age of 32 days in the prophylactic group and at an age of 50 days in the therapeutic group and continued during the whole treatment period. To ensure an *ad libitum* food intake feeding devices were used that were continuously backfilled when necessary. During the treatment period food intake was determined every third day in the prophylactic group and once a week in the therapeutic group. Spilled food was collected daily, weighed and subtracted from food intake. Total body weight gain during the 90-day therapeutic period was divided by entire food intake to receive an index named feeding efficiency. Every pig included in the study was monitored daily by general examination for side effects like obvious signs of nausea, vomiting, diarrhea or obstipation. Additionally, blood samples were taken prior to the treatment period and subsequently once a month for evaluation of clinical-chemical parameters. Therefore pigs were fixed with a restraining device after an 18-hour overnight fasting period and blood was taken by puncture of the right *Vena jugularis externa*. Blood samples (serum, EDTA-plasma) were immediately stored on ice and centrifuged (1,500 x *g*, 15 min, 4°C). Supernatant was carefully separated and aliquoted. Serum parameters (glucose, bilirubin, urea, creatinine, total protein, albumin, sodium, potassium, chloride, calcium, phosphorus, iron, magnesium, aspartate aminotransferase (AST), γ-glutamyltransferase (γGT), alkaline phosphatase (AP)) were determined using Autoanalyzer Hitachi 911® and adapted reagents from Roche Diagnostics GmbH, Unterhaching. Leucograms were compiled from EDTA-plasma samples using the fully automated blood cell counter Celltek®.

3.2.3. Determination of growth-regulating polypeptides

3.2.3.1. Determination of plasma somatostatin levels by radioimmunoassay

Somatostatin levels were determined in EDTA-plasma samples of liraglutide- (n=4) and placebo-treated (n=4) $GIPR^{dn}$ transgenic pigs from the prophylactic and the therapeutic group respectively, taken before the start and at the end of the treatment period, using a commercial somatostatin RIA kit (Phoenix Pharmaceuticals, Burlingame, USA). This assay is based on the competition between a radioactive-labeled somatostatin peptide and the not-labeled somatostatin peptide in the plasma sample for binding sites at a limited quantity of antibodies in each reaction mixture. As the concentration of somatostatin in the unknown sample increases, the amount of radioactive ^{125}I-peptide able to bind to the antibody is decreased and *vice versa*. This was measured by separating antibody-bound from free radioactive tracer and counting the antibody-bound fraction in a γ-counter. Samples were measured in duplicates and only accepted with a coefficient of variance (CV) less than 10 %, otherwise measurement was repeated. Chemicals and buffers were diluted with aqua bidest. according to the manufacturer´s instructions. Using the provided somatostatin standard (12.8 µg/ml) eight serial dilutions were prepared for later set up of a standard curve. Tubes were labeled and filled according to the following assay flow chart:

Table 7: Overview of somatostatin RIA assay set-up
TC: total count tubes, NSB: non-specific binding tubes, Bo: reference/total binding tubes, AB: assay buffer, QC: quality control, SAB: somatostatin antibody

Tube	AB	standard / QC / sample	SAB
1, 2 (TC)	----	----	----
3, 4 (NSB)	200 µl	----	----
5, 6 (Bo)	100 µl	----	100 µl
7, 8	----	100 µl of 10 pg/ml	100 µl
9, 10	----	100 µl of 20 pg/ml	100 µl
11, 12	----	100 µl of 40 pg/ml	100 µl
13, 14	----	100 µl of 80 pg/ml	100 µl
15, 16	----	100 µl of 160 pg/ml	100 µl
17, 18	----	100 µl of 320 pg/ml	100 µl
19, 20	----	100 µl of 640 pg/ml	100 µl
21, 22	----	100 µl of 1280 pg/ml	100 µl
23, 24	----	100 µl of QC	100 µl
25-n	----	100 µl of unknown sample	100 µl

After vortexing covered tubes were incubated for 20 hours at 4°C. Afterwards 50 µl of ^{125}I-tracer peptide were added to each sample, tubes were again vortexed, covered and incubated for 20 hours at 4°C. On day three, 100 µl of Goat Anti-Rabbit IgG serum and 100 µl of Normal Rabbit Serum were added to each tube except total count tubes and after vortexing the mixture was incubated for 90 minutes at room temperature. Subsequently 500 µl assay buffer were added, samples were centrifuged (1,900 x g, 30 min, 4°C), supernatant was carefully decanted and tubes stayed inverted for 45 seconds with the exception of the total count tubes. Remaining pellets were immediately measured for 1 minute in the γ-counter. Prior to actual sample measurement linearity of concentrations was proven by sample dilution experiments.

3.2.3.2. Determination of serum insulin-like growth factor binding protein-2 and -3 (IGFBP-2 and -3) levels by Western ligand blot analysis

Serum levels of IGFBP-2 and -3 were analyzed by quantitative Western ligand blotting as described before (Metzger et al. 2011) in cooperation with Dr. Andreas Hoeflich at the Leibniz Institute for Farm Animal Biology (FBN) in Dummerstorf, Germany. In brief, serum samples were diluted 1:3 with phosphate buffer (pH 7.4) and 1:2 with sample buffer containing 2% sodium dodecyl sulfate (SDS). Proteins were heat denatured for 5 minutes at 95°C and separated by SDS polyacrylamide gel electrophoresis. For quantitation diluted human recombinant IGFBPs were used as standards. After protein transfer to a polyvinyl fluoride membrane (Millipore, Schwalbach), blots were developed using a commercial Western ligand blotting kit containing biotinylated IGF-II and streptavidin-conjugated peroxidase (IBT, Binzwangen). IGFBPs were detected by the ECL Advance Western Blotting Detection kit (GE Healthcare, Freiburg) and a Kodak image station (Kodak, Berlin). Quantification of original IGFBP-2 and -3 levels was performed using the ImageQuant software package (Molecular Dynamics, USA).

3.2.4. Determination of serum insulin-like growth factor-I (IGF-I) levels by enzyme linked immuno sorbent assay (ELISA)

Serum IGF-I levels were kindly measured in cooperation with Dr. Maximilian Bielohuby at the Endocrine Research Unit, Medizinische Klinik und Poliklinik IV, LMU, Munich. A commercially available OCTEIA^{TM} Rat/Mouse IGF-I ELISA kit (Immunodiagnostic Systems (IDS) Inc., Boldon, UK) was used that has

been shown to also recognize porcine IGF-I (Tatara et al. 2007). As per manufacturer´s instruction, serum samples were pretreated with a release reagent and sample diluent to avoid interference from IGF-binding proteins. Accordingly, samples as well as kit controls were pipetted into slots of a microtiter plate coated with anti-IGF-I antibodies. Anti-rat IGF-I biotin conjugate was also given to slots and plate was allowed to incubate for 2 hours under constant shaking. After a washing step, Streptavidin-horseradish peroxidase conjugate was added and the plate was washed again after an incubation time of 30 minutes. Subsequently, tetramethylbenzidine was added as chromogen substrate. After 20 minutes incubation time the reaction was stopped by adding acidic stop solution. The IGF-I concentration of the unknown samples was calculated by plotting the mean absorbance, measured at a wave length of 450 nm (reference wave length 620 nm), of the unknown samples against the absorbance of a known calibration curve. The calibrators used in this assay were derived from human material and showed concentrations of 10, 22, 86, 261 and 892 ng/ml.

3.3. Metabolic tests

Metabolic tests included an intravenous glucose tolerance test (IVGTT) as well as an oral glucose tolerance test (OGTT). In the prophylactic as well as the therapeutic group these tests were performed prior to the liraglutide/placebo treatment and were repeated at the end of the treatment period. Every test was performed in freely moving animals in single pens after an 18-hour overnight fasting period.

3.3.1. Surgical implantation of central venous catheters

To ensure a stress-free frequent blood sampling in unrestrained animals during the glucose tolerance tests two central venous catheters were surgically inserted into the external jugular vein under general anesthesia. After a premedication of azaperone (0.5 ml per 10 kg body weight (BW) intra muscular (i.m.)) and ketamine hydrochloride (2 ml per 10 kg BW i.m.) anesthesia was initiated by inhalation of 5% isoflurane. After reaching the status of surgical tolerance anesthesia was maintained by an inhalation of 1% isoflurane. Metamizol (1 ml per 10 kg BW) was administered through an indwelling venous catheter in the ear vein and meloxicam (0.2 ml per 10 kg BW) was given i.m. to assure peri- surgical analgesia. During surgery nasal septum as well as dew claws were regularly

stimulated to check the depth of anesthesia. Oxygen saturation and pulse rate were continuously monitored using a pulse oximeter. Animals were fixed in back position, hair was shaved around the neck and the surgical field was extensively cleaned and disinfected. A skin incision of about five centimeters length was made in the *sulcus jugularis* followed by careful preparation through subcutaneous tissue and cutaneous muscle. The external jugular vein was exposed, surrounding tissue was removed and two fixation sutures were set proximally and distally around the vein. After venotomy two central venous catheters were inserted 10 to 15 cm into the vein relative to pig size to reach the desired position near the heart base. A proximal ligature inhibited blood reflux and a distal ligature fixed catheters and saved them from slipping out of position. Subsequently, the operation wound was sutured in two layers. For further external fixation of the catheters they were attached with a single suture to the skin and covered with gauze swab and adhesive tape up to the ridge. Exterior catheter ends were connected to 3-way stopcocks and coiled in a pouch out of gauze to provide easy access for blood sampling (Figure 5). After surgery, cefquinom 2.5% was administered i.m. once daily for three days (2 ml per 25 kg BW) to prevent infections. Metamizol (1 ml per 10 kg BW) was administered intravenously to assure post-surgical analgesia. Both catheters were flushed once daily with 50 IU heparin/ml 0.9% isotonic sodium chloride solution. At the time of the metabolic tests all animals showed normal behavior and food intake, indicating full recovery from surgical procedure.

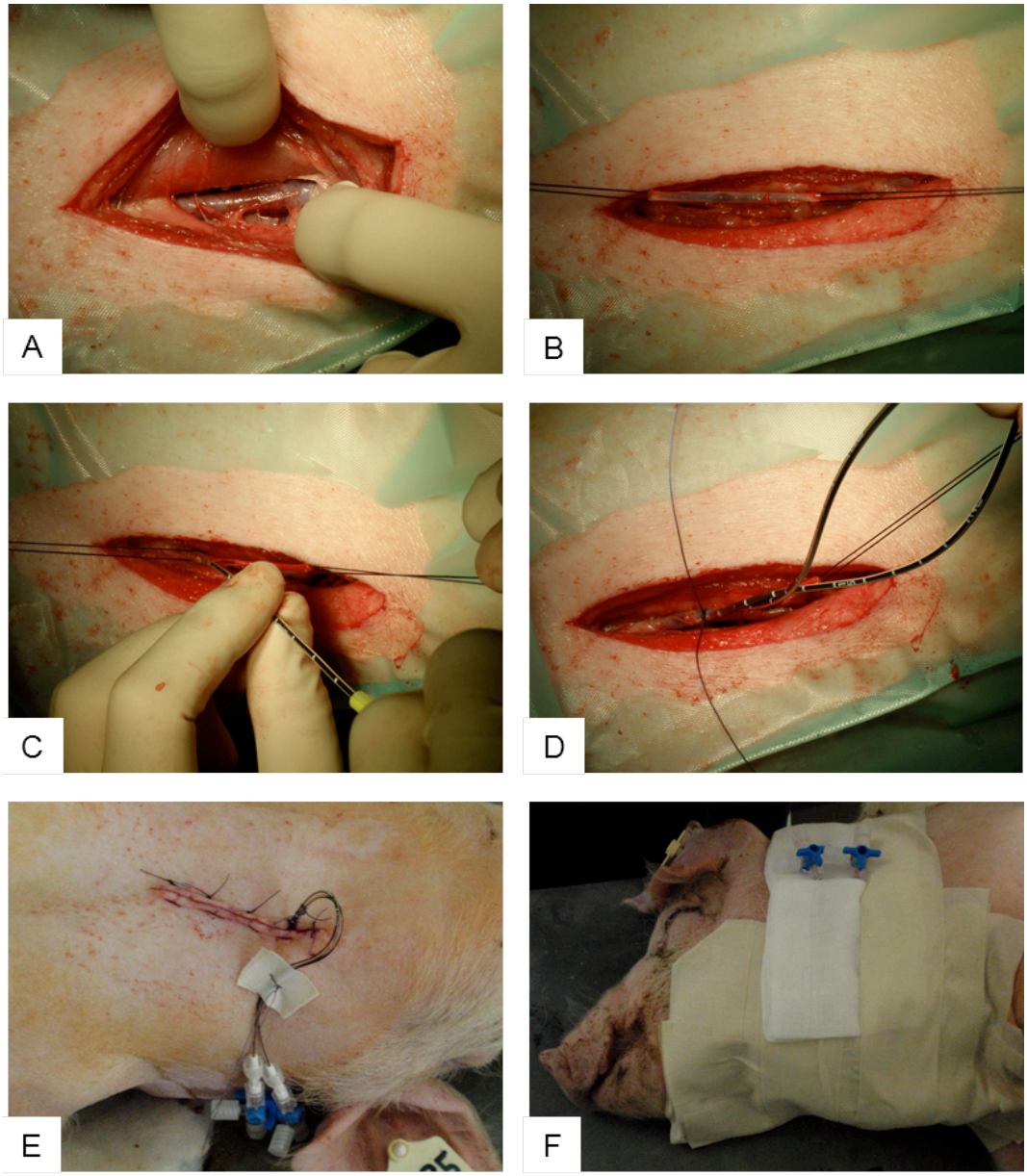

Figure 5: Surgical implantation of central venous catheters
(A) Exposure of *Vena jugularis externa,*
(B) Tensioning of the vein using two holding sutures
(C) Placement of the first central venous catheter after venotomy
(D) Placement of the second central venous catheter and fastening of the distal holding suture
(E) Skin suture and external catheter fixation
(F) Formed gauze pouch for easy access covered with adhesive tape

(Pictures were kindly provided by Dr. Christiane Fehlings and Dr. Simone Renner, Institute for Molecular Animal Breeding and Biotechnology, Oberschleißheim, Germany)

3.3.2. Intravenous glucose tolerance test (IVGTT)

After an 18-hour fasting period overnight a bolus injection of concentrated 50% glucose solution (0.5 g per kg BW) was administered as quickly as possible through one marked central venous catheter. The catheter was then cleared from residual glucose by flushing it with 20 ml of 0.9% isotonic NaCl solution. Blood samples were taken through the second catheter at -10, 0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50, 60 and 90 minutes relative to the glucose load. Before obtaining each sample a small amount of blood was withdrawn through the catheter and discarded. After every blood collection the catheter was flushed with 3-4 ml of 0.9% isotonic sodium chloride solution. Blood was drawn in EDTA monovettes and at once put on ice. After centrifugation (1,500 x *g*, 15 min, 4°C) and separation plasma was stored at -80°C until further processing. Plasma glucose levels were measured using an AU400 autoanalyzer (Olympus) and plasma insulin levels were determined in duplicate by a porcine insulin RIA kit (Millipore) as described in IV 3.3.4.

3.3.3. Oral glucose tolerance test (OGTT)

For OGTT pigs were offered a mixed meal consisting of 2 g glucose per kg BW (given as 50% glucose solution) mingled with commercial pig fodder (Deuka porfina U, for composition see Table 8) after an 18-hour fasting period.

Table 8: Diet composition of Deuka porfina U

Deuka porfina U	
MJ ME/kg	12.6
Crude protein %	16.5
Crude fat %	0.9
Crude ash %	5.5
Crude fiber %	6.0
Calcium %	0.85
Phosphorus %	0.55
Sodium %	0.2

The amount of chow used for the mixed meal was adapted to the amount of glucose solution dependent on body weight of the pigs. For the prophylactic group (treated from 2–5 months) 50 g pig fodder was used in the OGTT prior to the treatment and 200 g in the OGTT after the treatment. In the therapeutic group (treated from 5–8 months) 150 g chow was used in the pre-treatment OGTT and

300 g in the post-treatment OGTT. The mixed meal was given at time point 0 and was eaten from a bowl under supervision. Pigs had to eat the whole meal within 15 minutes; otherwise the test was stopped and repeated one day later. Blood samples were taken at -10, 0, 15, 30, 45, 60, 90 and 120 minutes relative to the beginning of glucose feeding and further processed as explained in IV 3.3.2.

3.3.4. Measurement of plasma insulin levels by radioimmunoassay

Plasma insulin levels of samples taken during glucose tolerance tests were measured in duplicates using a porcine insulin RIA kit (Millipore) according to the manufacturer´s instructions. Like the RIA for somatostatin, this is a competitive radioimmunoassay and is based on the same principle (see IV 3.2.3.1). With the help of six standard reaction mixtures it was possible to set up a standard curve with increasing concentrations of unlabeled antigen and from this curve the amount of antigen in the unknown samples could be calculated. Values were only accepted when the CV of duplicate measurements was less than 10%; otherwise the measurement was repeated.

3.3.4.1. Standard preparation

After 1.0 ml of assay buffer was added to six labeled tubes serial dilutions were prepared using the provided 200 µU/ml standard according to the following schedule:

Table 9: Overview of standard preparation for insulin RIA

Tube	Standard Concentration	Volume of Standard to add
1	100 µU/ml	1.0 ml of 200 µU/ml
2	50 µU/ml	1.0 ml of 100 µU/ml
3	25 µU/ml	1.0 ml of 50 µU/ml
4	12.5 µU/ml	1.0 ml of 25 µU/ml
5	6.25 µU/ml	1.0 ml of 12.5 µU/ml
6	3.125 µU/ml	1.0 ml of 6.25 µU/ml

Standards were stored at -20°C.

3.3.4.2. Assay set-up

Samples were thawed, vortexed and centrifuged at full speed for 10 minutes at 4°C. Disposable tubes for γ-counter were labeled and filled according to the following assay flow chart:

Table 10: Overview of insulin RIA assay set-up
AB: assay buffer, QC: quality control, IT: insulin tracer, AB: insulin antibody, TC: total count tubes, NSB: non-specific binding tubes, Bo: reference / total binding tubes

Tube	AB	Standard/QC/sample	^{125} I-IT	AB
1, 2 (TC)	----	----	50 µl	----
3, 4 (NSB)	150 µl	----	50 µl	----
5, 6 (Bo)	100 µl	----	50 µl	----
7, 8	50 µl	50 µl of 3.125 µU/ml	50 µl	50 µl
9, 10	50 µl	50 µl of 6.25 µU/ml	50 µl	50 µl
11, 12	50 µl	50 µl of 12.5 µU/ml	50 µl	50 µl
13, 14	50 µl	50 µl of 25 µU/ml	50 µl	50 µl
15, 16	50 µl	50 µl of 50 µU/ml	50 µl	50 µl
17, 18	50 µl	50 µl of 100 µU/ml	50 µl	50 µl
19, 20	50 µl	50 µl of 200 µU/ml	50 µl	50 µl
21, 22	50 µl	50 µl of QC 1	50 µl	50 µl
23, 24	50 µl	50 µl of QC 2	50 µl	50 µl
25-n	50 µl	50 µl of unknown sample	50 µl	50 µl

Tubes were vortexed, covered with parafilm and incubated for 22 hours at 4°C. On day two 500 µl of cold precipitating reagent were added to every tube except total count tubes. Mixture was vortexed, incubated for 20 minutes at 4°C and centrifuged (2,700 x g, 30 min, 4°C). All tubes except total count tubes were at once inversed for 45 seconds to decant supernatant and excess liquid was blotted from lip of the tubes. The remaining pellet was counted in a γ-counter for one minute.

3.3.5. Calculation of insulin sensitivity indices

For assessment of insulin sensitivity in GIPR^{dn} transgenic pigs prior to and after liraglutide/placebo therapy different insulin sensitivity/resistance indices were calculated.

3.3.5.1. Homeostasis model assessment (HOMA)

The index homeostasis model assessment (HOMA) was calculated according to Matthews et al. using fasting plasma glucose and insulin values to determine the degree of insulin resistance (HOMA-IR) and beta-cell function (HOMA-β) (Matthews et al. 1985). The following formula was used:

HOMA-IR = (I_0 x G_0) / 22.5

HOMA-β = 20 * I_0 / (G_0 – 3.5)

I_0 = Fasting plasma insulin (µU/ml)

G_0 = Fasting plasma glucose (mmol/l)

3.3.5.2. Quantitative insulin sensitivity check index (QUICKI)

This insulin sensitivity index proposed by Katz et al. was also calculated using fasting plasma glucose/insulin values (Katz et al. 2000).

QUICKI = 1 / (log I_0 + log G_0)

I_0 = Fasting plasma insulin (µU/ml)

G_0 = Fasting plasma glucose (mg/dl)

3.3.5.3. Matsuda Index (ISI $_{(Matsuda)}$)

For this index not only fasting plasma glucose/insulin values but also mean values during OGTT (time points 0, 15, 30, 45, 60, 90, 120 minutes relative to glucose mixed meal) were used.(Matsuda et al. 1999).

$ISI_{(Matsuda)}$ = 10000 / √(G_0 * I_0 * G_{mean} * I_{mean})

I_0 = Fasting plasma insulin (µU/l)

G_0 = Fasting plasma glucose (mg/dl)

I_{mean} = mean plasma insulin concentration during OGTT (µU/l)

G_{mean} = mean plasma glucose concentration during OGTT (mg/dl)

3.4. Liraglutide profiling

At the end of the 90-day treatment period plasma liraglutide levels were determined over a period of 16 hours. Therefore, all liraglutide-treated $GIPR^{dn}$ transgenic pigs from the prophylactic and the therapeutic group were investigated. During profiling pigs had access to an *ad libitum* chow and were not fasted. For blood collection central intravenous catheters were used that pigs still had from the post-treatment glucose tolerance tests (see IV 3.3.1). Before obtaining each sample a small amount of blood was withdrawn through the catheter and discarded. After every blood collection catheter was flushed with 3-4 ml 0.9%

isotonic sodium chloride solution. Animals of the prophylactic group received 1.2 mg liraglutide, while pigs of the therapeutic group received 1.8 mg liraglutide. Victoza® was injected as usual into the lateral side of the neck at 8 a.m. One blood sample was taken prior to injection (t=0 minutes), thereby representing a value where the last liraglutide injection the day before was 24 hours ago. Subsequently blood samples were taken at 4, 8, 12 and 16 hours relative to the Victoza® injection. EDTA monovettes were prepared with DPP-4 inhibitor diprotin A (3 mM, 50 µl/ml blood sample) and protease inhibitor aprotinin (0.6 TIU/ml blood sample). After centrifugation (1,500 x *g*, 15 min, 4°C) plasma was separated, aliquoted and stored at -80°C until further processing. Plasma liraglutide levels were measured by Novo Nordisk A/S using an in-house luminescence oxygen channeling immunoassay validated for pig plasma.

3.5. Morphological evaluation of GIPR^{dn} transgenic pigs at the end of the treatment period

3.5.1. Necropsy

Anesthesia was induced by intramuscular injection of ketamine hydrochloride (2 ml per 10 kg BW) and azaperone (0.5 ml per 10 kg BW) followed by insertion of an indwelling cannula into the ear vein. The animals were euthanized by intravenous injection of T61 (1 ml per 10 kg BW). During general pathologic examination pancreas, lung, heart, kidney, liver, spleen and stomach were weighed for further calculation of absolute and relative organ weights.

3.5.2. Pancreas preparation and sampling

The whole pancreas was taken out of the abdominal cavity as quick as possible after euthanasia of the animals. Fat tissue, connective tissue and vessels were removed and the organ was weighed. The pancreas was cut through between the *lobus pancreatis sinister* and the connective lobe to the *lobus pancreatis dexter* (Figure 6) and brought into straight position. For subsampling the length of the organ was determined and tissue slides of 0.5 cm thickness were cut out every 2.5 cm over the whole pancreas and prefixed in 4% neutral buffered formalin for 8 hours. Subsequently slides were turned to their left side and covered by a 1 cm^2 point-counting grip. Every point that encountered pancreatic tissue was marked, counted and the sum of hitting points was divided by the number 20 and called quotient Y. A random number X between one and quotient Y was chosen and

samples of about 1 cm^2 were taken out of the tissue slides at position X, X+Y, X+2*Y, X+3*Y until X+20*Y. Excised samples were placed in an embedding cassette right cut surface downwards, fixed in 4% neutral buffered formalin overnight and further routinely processed for paraffin embedding. Sections of about 4 µm thickness were cut from every paraffin block using a HM 315 microtome, put in aqua bidest., mounted on 3-aminopropyltriethoxy-silane-treated microscope glass slides for immunohistochemistry and incubated at 37°C overnight.

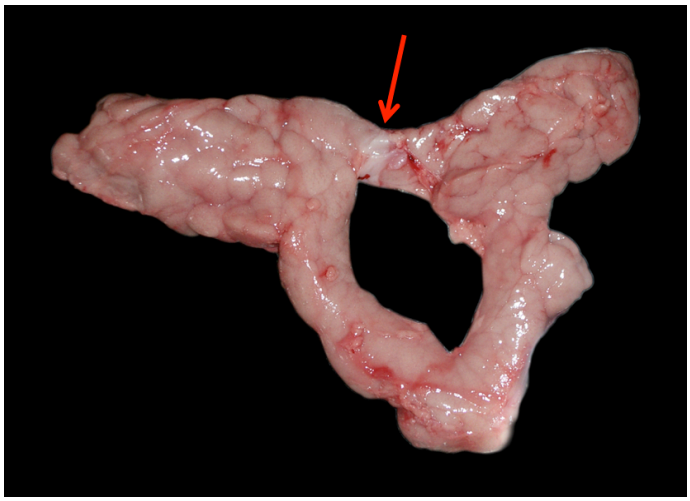

Figure 6: Pancreas preparation for quantitative-stereological analyses
Red arrow indicates the separation site between *lobus pancreatis sinister* and the connective lobe to the *lobus pancreatis dexter*.

3.5.3. Immunohistochemical staining for insulin

The indirect alkaline phosphatase (AP) method was used for the detection of insulin containing cells in paraffin sections of all pigs included in this study for further quantitative-stereological analyses. Pancreatic tissue sections on microscope slides (see IV 3.5.2) were deparaffinized in xylene for 15 minutes and rehydrated in descending alcohol concentrations (99%, 96%, 70% alcohol, aqua bidest.). Then sections were further processed as indicated in Table 11.

Table 11: Flow chart of immunohistochemistry for evaluation of the beta-cell volume

	Chemical	Incubation
STEP 1	Hydrogen peroxide solution 1%	15 minutes
STEP 2	TBS buffer	10 minutes
STEP 3	Goat serum diluted 1:10 in TBS buffer	30 minutes
STEP 4	Primary antibody diluted 1:1000 in TBS buffer (Polyclonal guinea pig anti-porcine insulin)	60 minutes
STEP 5	TBS buffer	10 minutes
STEP 6	Secondary antibody diluted 1:100 in TBS buffer (AP-conjugated goat anti-guinea pig IgG) + 5% porcine serum	60 minutes
STEP 7	TBS buffer	10 minutes
STEP 8	Vector® Red chromogen diluted in 100 mM Tris HCl (pH 8.5)	20 minutes
STEP 9	Floating distilled water	5 minutes
STEP 10	Mayer´s haemalum solution	10 seconds
STEP 11	Floating distilled water	5 minutes

Afterwards, slides were dehydrated in ascending alcohol concentrations (aqua bidest., 70%, 96%, 99% alcohol), cleared in xylene and mounted under cover slips using quick-hardening mounting medium histokitt. This method was used for all pancreatic tissue sections used for quantitative-stereological analyses. For nuclear profile counting sections were processed similarly using a slightly different flow chart as follows:

Table 12: Flow chart of immunohistochemistry for nuclear profile count

	Chemical	Incubation
STEP 1	Hydrogen peroxide solution 1%	15 minutes
STEP 2	TBS buffer	10 minutes
STEP 3	Rabbit serum diluted 1:10 in TBS buffer	30 minutes
STEP 4	Primary antibody diluted 1:1000 in TBS buffer (Polyclonal guinea pig anti-porcine insulin)	60 minutes
STEP 5	TBS buffer	10 minutes
STEP 6	Secondary antibody diluted 1:50 in TBS buffer (HRP-conjugated rabbit anti-guinea pig IgG) + 5% porcine serum	60 minutes
STEP 7	TBS buffer	10 minutes
STEP 8	DAB chromogen activated with 1 µl 30% hydrogen peroxide solution per 1 ml	3 minutes
STEP 9	Floating distilled water	5 minutes
STEP 10	Mayer´s haemalum solution	4 minutes
STEP 11	Floating distilled water	5 minutes

3.5.4. Quantitative-stereological analyses of pancreata

During pancreas sampling 20 tissue specimens were obtained from each animal included in the study and immunostained for insulin using Vector® Red as chromogen (see IV 3.5.3). Quantitative-stereological analyses were carried out using the computer-assisted Olympus Visiomorph™ image analysis system coupled to a light microscope and a color video camera. In 50% of every tissue slide area of insulin-positive cells as well as of total pancreatic area was determined in 400 x final magnification on a color monitor displaying images of stained samples with the help of the semiautomatic stereological software Visiomorph™. This software can differentiate between colors and thereby measured areas of red stained insulin-positive beta cells and additionally of the blue casted surrounding pancreatic tissue. Isolated beta-cells were not manually counted; they were also recognized by the system by definition of insulin-positive red stained cells covering an area smaller than 250 μm^2. Total beta-cell areas and total pancreatic section areas on every slide of one pig where summed up and named $A_{(isoβ\text{-}cell,50\%)}$, $A_{(β\text{-}cell,50\%)}$ and $A_{(Pan,50\%)}$, whereas total area of isolated beta-cells was also included into $A_{(β\text{-}cell,50\%)}$, and area of $A_{(β\text{-}cell,50\%)}$ was also included into $A_{(Pan,50\%)}$. To avoid impreciseness due to tissue shrink during histological processing, total pancreas volume ($V_{(Pan)}$) before paraffin embedding was calculated by the quotient of pancreas weight at necropsy ($W_{(Pan)}$) and the specific weight of the pig pancreas (sp. $W_{(Pan)}$, 1.07 g/cm^3). This specific weight was determined by the submersion method in which a displacement of isotonic hydroxide solution caused by the pancreas volume was investigated by weighing (Scherle 1970). $V_{(Pan)}$ as well as pancreatic volume density of beta-cells ($Vv_{(β\text{-}cells/Pan)}$) and isolated beta-cells ($Vv_{(isoβ\text{-}cell/Pan)}$) as well as pancreatic total beta-cell volume ($V_{(β\text{-}cell,Pan)}$) and isolated beta-cell volume ($V_{(isoβ\text{-}cell,Pan)}$) were computed according to the following formulae:

$$\mathbf{V_{(Pan)} = W_{(Pan)} / sp.\ W_{(Pan)}}$$

$$\mathbf{Vv_{(β\text{-}cell/Pan)} = A_{(β\text{-}cell,50\%)} / A_{(Pan,50\%)}}$$

$$\mathbf{V_{(β\text{-}cell,Pan)} = Vv_{(β\text{-}cells/Pan)} * V_{(Pan)}}$$

$$\mathbf{Vv_{(isoβ\text{-}cell/Pan)} = A_{(isoβ\text{-}cell,50\%)} / A_{(Pan,50\%)}}$$

$$\mathbf{V_{(isoβ\text{-}cell,Pan)} = Vv_{(isoβ\text{-}cells/Pan)} * V_{(Pan)}}$$

Total beta-cell volume referred to body weight ($V_{(\beta\text{-cell,Pan})}$ / BW) was calculated by dividing the total beta-cell volume ($V_{(\beta\text{-cell,Pan})}$) by the individual body weight of the pigs at the day of necropsy. Images were taken from representative tissue slides of each animal group and treatment type under equal conditions. For illustration of dimensions an object micrometer was photographed in the same magnification as slides to design accurate scale bars.

3.5.5. Counting of nuclear profiles

Within the prophylactic group, 4 liraglutide-treated and 4 placebo-treated animals were randomly chosen for nuclear profile counting within a defined insulin-positive stained area. From each animal 4 tissue slides also used for the evaluation of beta-cell volume were randomly selected, stained for insulin using DAB as chromogen and additionally with haemalum for a strong nuclei staining (see 3.5.3) and randomly sampled until 25 islets per slide were detected. Thus, in total 100 islets per animal were evaluated. Per islet the insulin-positive stained area was measured at 400 x final magnification using the semiautomatic stereological software Visiomorph^{TM} and within this area nuclear profiles were manually counted. The nuclear profile count was expressed as the number of nuclear profiles per insulin-positive stained area of 10^5 μm^2.

3.6. Statistics

All data are presented as means ± standard error of means (SEM). The results of oral and intravenous glucose tolerance tests were statistically evaluated by analysis of variance (Linear Mixed Models; SAS 8.2) taking the fixed effects of Group (liraglutide/placebo-treated), Time (relative to glucose administration), interaction of Group*Time as well as random effect of animal into account. The same analysis of variance was used for the evaluation of body weight and food intake taking the fixed effects of Group (liraglutide/placebo-treated), duration of treatment/Age and the interaction Group*duration of treatment/Age 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 (liraglutide/placebo-treated), Age (before/during different time points of the treatment period) and the interaction Group*Age into account. Absolute and relative organ weights were statistically evaluated by analysis of variance (General Linear Model, SPSS 21.0) taking the fixed effect of Group

(liraglutide/placebo-treated) into account. Statistical significance between liraglutide- and placebo-treated GIPR^{dn} transgenic pigs regarding quantitative-stereological analyses, nuclear profile counting as well as ratios of somatostatin, IGF-I, IGFBP-2 and -3 were evaluated by Mann-Whitney-U test in combination with an exact test procedure (SPSS 21.0). Areas under the curve for insulin and glucose were created using Graph Pad Prism® software (version 5.02) and statistical significance between liraglutide- and placebo-treated GIPR^{dn} transgenic pigs was also evaluated by Mann-Whitney-U test in combination with an exact test procedure (SPSS 21.0). P values less than 0.05 were considered to be significant.

V. RESULTS

1. Genotyping of GIPR^{dn} transgenic pigs by PCR

Pigs used in this study were hemizygous F4-generation GIPR^{dn} transgenic pigs. They were bred by naturally mating one GIPR^{dn} transgenic boar to overall 8 non-transgenic sows. Altogether, 82 piglets were born of which 43 were identified as transgenic by evaluation of isolated DNA from ear punches using PCR. PCR of DNA samples from GIPR^{dn} transgenic pigs resulted in a 720-bp RIP2-specific band, while there was no band visible in DNA of non-transgenic littermates. To avoid false-negative results DNA integrity was proven by simultaneous PCR using *ACTB*-specific primers, resulting in a band of 331 bp if the DNA sample was intact (Figure 7). Among the GIPR^{dn} transgenic animals, 18 pigs were included in the prophylactic group and 11 pigs in the therapeutic group.

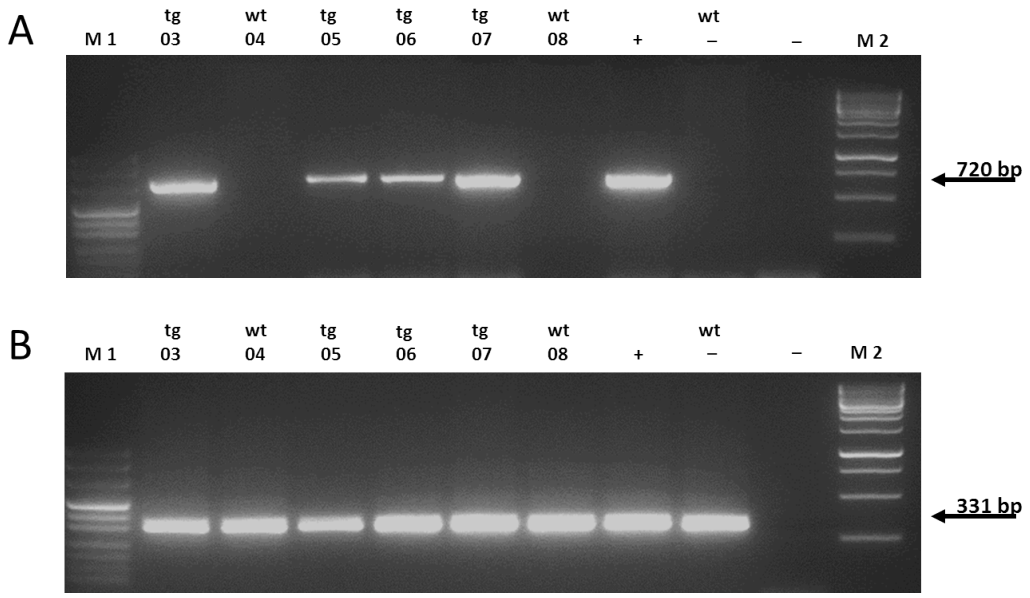

Figure 7: Genotyping of GIPR^{dn} transgenic pigs and non-transgenic littermates by PCR analysis

(A) Specific PCR analysis for GIPR^{dn} transgene using RIP2 primers; **(B)** Control of DNA integrity using *ACTB* primers; **(A/B)** tg: GIPR^{dn} transgenic pig; wt: non-transgenic littermate; +: genomic DNA of already genotyped GIPR^{dn} transgenic pig as positive control; wt -: genomic DNA of non-transgenic pig as negative control; -: aqua bidest. as non-template control; M1: puc Mix Marker 8; M2: Gene ruler^{TM} 1 kb DNA ladder.

2. Plasma liraglutide levels in GIPR^{dn} transgenic pigs

To determine the amount of subcutaneously injected liraglutide that is actually arriving in the blood, liraglutide plasma levels were evaluated by Novo Nordisk A/S, Denmark at the end of the treatment period, using an in-house luminescence oxygen channeling immunoassay. All GIPR^{dn} transgenic pigs from both groups that were treated with liraglutide were included (prophylactic group: n=9, therapeutic group: n=5). While the course of liraglutide levels in the therapeutic group was similar, 3 out of 9 pigs included in the prophylactic group showed distinctly lower liraglutide levels than the remaining six pigs. This was not correlated with the body weight of the animals. Right before administration of 1.2 mg liraglutide at time point 0 minutes the prophylactic group showed mean steady state concentration of 24,522.2 ± 1,332.3 pmol/l (mean ± SEM) while pigs of the therapeutic group that got the last subcutaneous injection of 1.8 mg liraglutide 24 hours ago exhibited mean concentrations of 29,820 ± 2,069.88 pmol/l. Within the 5 defined time-points where blood samples were taken the highest plasma liraglutide levels were determined in both groups 8 hours after dosing with the therapeutic group showing 52,260 ± 2,163.24 pmol/l and the prophylactic group showing 43,233.3 ± 2,959.35 pmol/l. During the whole profile both groups showed relatively constant plasma liraglutide levels with the therapeutic group revealing between 11-18% higher concentrations compared to the prophylactic group at all measurement time-points (Figure 8).

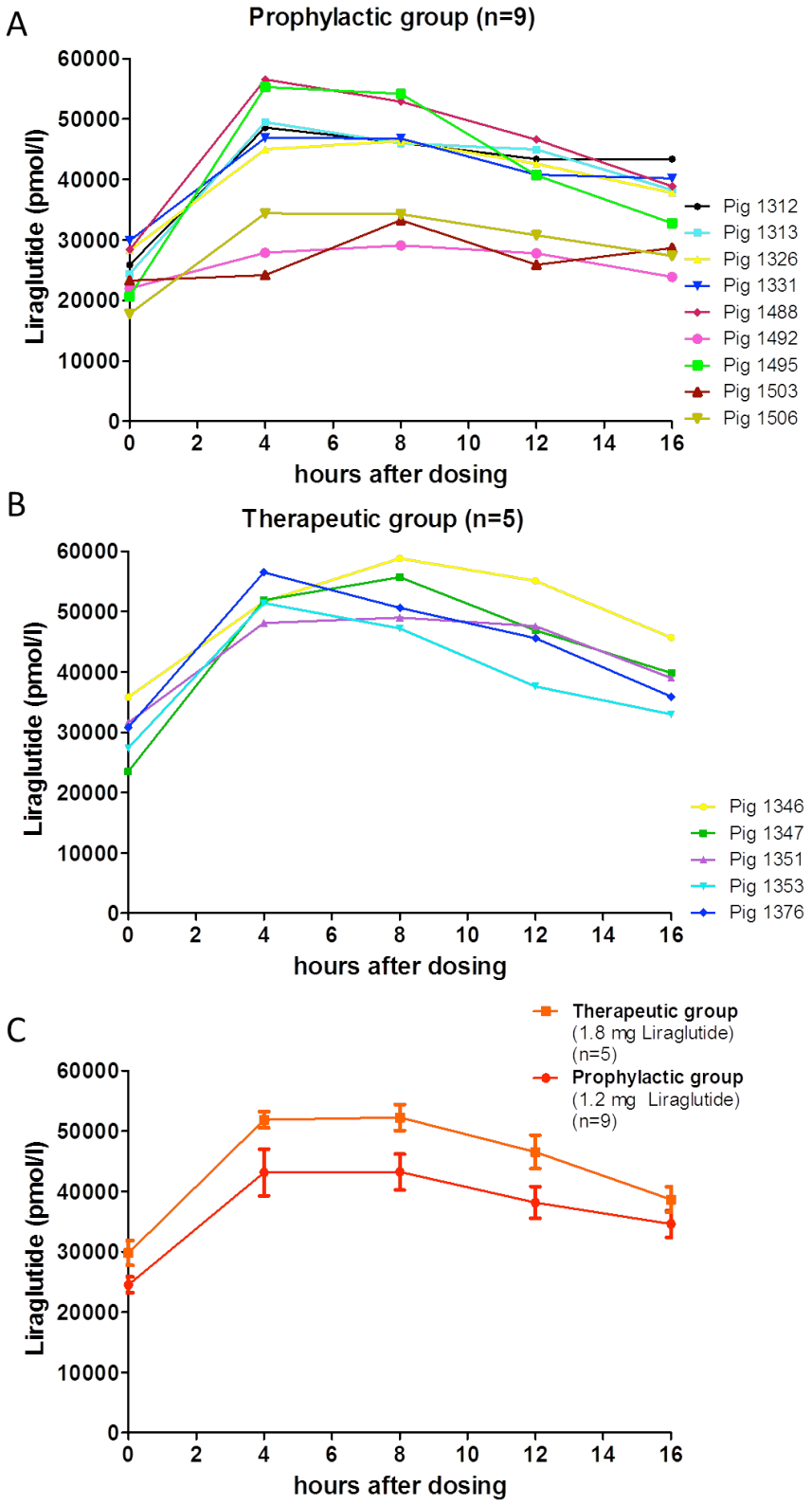

Figure 8: Individual and mean liraglutide plasma levels in GIPR^{dn} transgenic pigs

Individual curves of each liraglutide-treated GIPR^{dn} transgenic pig included in the prophylactic **(A)** or therapeutic **(B)** group and mean plasma liraglutide concentrations **(C)** in the prophylactic (red) and therapeutic (orange) group; 0 hours = point of liraglutide administration; n = number of animals investigated. Data are means ± SEM.

3. Monitoring of clinical, metabolic and clinical-chemical parameters during liraglutide therapy

3.1. Clinical-chemical parameters and unchanged health status in liraglutide-treated GIPR^{dn} transgenic pigs

During the treatment period GIPR^{dn} transgenic pigs of the prophylactic and the therapeutic group were regularly examined. At all times animals showed normal behavior and undisturbed general condition, even when being treated with the maximum dosage of liraglutide recommended for the treatment of human patients (1.8 mg per day). Adverse effects including gastrointestinal symptoms like vomiting, obstipation, diarrhea or noticeable signs of nausea were not obvious. To further check health status blood samples were taken from all animals after an 18-hour fasting period before the beginning of the treatment (prophylactic group: 8 weeks of age, therapeutic group: 20 weeks of age) and subsequently every four weeks until the end of the treatment period. Significant differences between the different time points of blood sampling were frequently seen, independent of the treatment type of the pigs, indicating changes of these parameters with age and growth. Most clinical-chemical parameters including leucocytes, bilirubin, urea, sodium, potassium, chloride, magnesium, AST and µGT did not significantly differ between liraglutide- and placebo-treated GIPR^{dn} transgenic pigs (Table 13+14). This is also true for serum blood glucose that stayed in normoglycemic ranges (70-115 mg/dl) (Kraft 2005; Plonait 1988; Waldmann 2001) during the whole treatment period in liraglutide-treated as well as in placebo-treated GIPR^{dn} transgenic pigs. However, total protein, albumin as well as phosphorus were significantly lower in liraglutide-treated compared to placebo-treated GIPR^{dn} transgenic pigs in both groups. Furthermore, liraglutide-treated animals of the therapeutic group showed significant lower levels of creatinine, calcium and iron compared to placebo-treated pigs. Within the prophylactic group a significant increase of AP was detected in liraglutide-treated GIPR^{dn} transgenic pigs compared to placebo-treated ones.

Table 13: Clinical-chemical parameters in GIPR^{dn} transgenic pigs of the prophylactic group

Clinical-chemical parameters in liraglutide- **(L, n=9)** and placebo-treated **(P, n=9)** GIPR^{dn} transgenic pigs of the prophylactic group prior to (8 weeks of age) and during the treatment period.

Parameter	Group	Age												Analysis of variance		
		8 weeks			12 weeks			16 weeks			20 weeks			Group	Age	G × A
Leucocytes	L	14.0	±	0.7	13.3	±	0.8	18.1	±	1.0	16.6	±	0.8	n.s.	***	n.s.
[x 10^9/l]	P	15.0	±	1.1	12.4	±	1.4	16.6	±	1.2	16.1	±	1.1			
Glucose	L	89.9	±	2.8	95.7	±	4.1	82.9	±	1.4	81.1	±	1.9	n.s.	*	n.s.
[mg/dl]	P	89.7	±	2.1	86.5	±	6.2	80.9	±	3.3	88.3	±	4.0			
Bilirubin	L	2.2	±	0.4	2.4	±	0.5	1.9	±	0.2	2.0	±	0.3	n.s.	*	n.s.
[µmol/l]	P	2.3	±	0.3	4.1	±	0.9	2.2	±	0.4	1.8	±	0.3			
Urea	L	2.9	±	0.3	3.7	±	0.4	3.1	±	0.5	3.4	±	0.3	n.s.	n.s.	n.s.
[mmol/l]	P	2.9	±	0.2	3.7	±	0.3	2.9	±	0.3	2.6	±	0.3			
Creatinine	L	44.0	±	1.6	58.4	±	5.9	101.3	±	3.6	100.1	±	2.3	n.s.	***	n.s.
[µmol/l]	P	49.3	±	2.4	61.2	±	3.9	96.8	±	4.3	105.6	±	5.3			
Total protein	L	46.2	±	1.1	46.9	±	0.8	53.6	±	1.2	51.2	±	1.0	***	***	n.s.
[g/l]	P	46.1	±	0.7	51.5	±	1.3	58.5	±	1.4	55.9	±	1.2			
Albumin	L	25.8	±	1.3	29.3	±	1.1	37.4	±	1.2	26.6	±	1.4	**	***	n.s.
[g/l]	P	24.3	±	1.0	33.0	±	1.6	41.9	±	1.3	31.7	±	1.5			
Sodium	L	137.9	±	0.6	140.1	±	0.9	141.3	±	0.8	140.8	±	0.6	n.s.	***	n.s.
[mmol/l]	P	138.0	±	0.7	141.8	±	0.7	143.2	±	0.9	140.8	±	0.5			
Potassium	L	4.1	±	0.1	4.8	±	0.2	4.9	±	0.2	4.7	±	0.4	n.s.	*	n.s.
[mmol/l]	P	4.4	±	0.2	4.7	±	0.1	4.8	±	0.1	4.1	±	0.1			
Chloride	L	98.2	±	0.8	97.6	±	0.8	99.0	±	0.7	97.9	±	1.0	n.s.	n.s.	n.s.
[mmol/l]	P	97.8	±	0.6	98.0	±	0.4	98.8	±	0.4	98.2	±	0.6			
Calcium	L	2.3	±	0.04	2.5	±	0.04	2.5	±	0.1	2.1	±	0.1	n.s.	***	n.s.
[mmol/l]	P	2.3	±	0.05	2.5	±	0.03	2.5	±	0.03	2.3	±	0.03			
Phosphorus	L	3.2	±	0.1	3.0	±	0.1	2.4	±	0.1	2.6	±	0.01	**	***	*
[mmol/l]	P	3.1	±	0.1	3.4	±	0.1	3.0	±	0.1	2.7	±	0.1			
Iron	L	17.2	±	1.8	26.2	±	1.8	25.2	±	2.5	20.0	±	1.2	n.s.	***	n.s.
[µmol/l]	P	18.9	±	1.5	27.2	±	3.5	24.3	±	0.9	23.0	±	1.6			
Magnesium	L	1.1	±	0.3	0.8	±	0.01	0.9	±	0.01	0.7	±	0.1	n.s.	n.s.	n.s.
[mmol/l]	P	0.8	±	0.03	0.9	±	0.02	0.8	±	0.03	0.7	±	0.02			
AST	L	30.6	±	4.1	32.5	±	5.3	22.1	±	2.1	27.0	±	2.3	n.s.	n.s.	n.s.
[U/l]	P	29.5	±	3.2	33.6	±	2.2	29.5	±	1.7	33.9	±	5.1			
γGT	L	34.5	±	3.7	40.9	±	4.2	35.6	±	3.2	37.2	±	3.8	n.s.	n.s.	n.s.
[U/l]	P	32.8	±	2.4	37.4	±	4.8	36.6	±	4.1	35.2	±	4.8			
AP	L	190.1	±	9.2	185.6	±	8.9	130.3	±	7.3	109.1	±	5.8	**	***	n.s.
[U/l]	P	157.9	±	12.4	167.5	±	7.7	119.1	±	4.7	98.4	±	3.5			

Table 14: Clinical-chemical parameters in GIPR^{dn} transgenic pigs of the therapeutic group

Clinical-chemical parameters in liraglutide- **(L, n=5)** and placebo-treated **(P, n=6)** GIPR^{dn} transgenic pigs of the therapeutic group prior to (20 weeks of age) and during the treatment period.

Parameter	Group	Age												Analysis of variance		
		20 weeks			24 weeks			28 weeks			32 weeks			Group	Age	G × A
Leucocytes	L	19.1	±	1.3	16.9	±	0.6	16.3	±	1.0	17.4	±	1.4	n.s.	n.s.	n.s.
[x 10^9/l]	P	19.4	±	1.8	15.8	±	2.2	14.3	±	1.2	17.1	±	1.7			
Glucose	L	84.0	±	2.8	88.6	±	3.5	76.4	±	2.0	80.4	±	0.7	n.s.	*	n.s.
[mg/dl]	P	85.0	±	2.2	80.2	±	2.8	76.0	±	4.4	87.7	±	2.5			
Bilirubin	L	1.8	±	0.3	2.1	±	0.3	3.5	±	0.7	2.0	±	0.3	n.s.	n.s.	n.s.
[µmol/l]	P	1.8	±	0.3	2.8	±	0.6	3.8	±	1.9	1.6	±	0.4			
Urea	L	2.1	±	0.5	3.0	±	0.3	3.1	±	0.2	3.0	±	0.3	n.s.	n.s.	n.s.
[mmol/l]	P	2.8	±	0.5	3.3	±	0.3	3.5	±	0.3	2.3	±	0.3			
Creatinine	L	75.1	±	5.6	88.0	±	4.9	111.0	±	6.2	106.9	±	5.1	*	***	n.s.
[µmol/l]	P	84.2	±	5.7	94.1	±	6.5	114.5	±	3.1	137.8	±	16.6			
Total protein	L	57.5	±	1.7	53.5	±	0.9	56.4	±	1.0	55.5	±	0.6	***	n.s.	n.s.
[g/l]	P	59.9	±	2.4	59.2	±	3.5	62.3	±	2.1	64.3	±	1.6			
Albumin	L	25.6	±	1.7	25.2	±	0.7	26.0	±	0.9	27.9	±	1.3	***	n.s.	n.s.
[g/l]	P	31.4	±	2.2	34.2	±	4.5	38.7	±	3.0	41.2	±	2.4			
Sodium	L	138.2	±	0.4	138.8	±	1.2	139.8	±	2.7	139.8	±	0.6	n.s.	n.s.	n.s.
[mmol/l]	P	139.8	±	0.7	139.0	±	1.0	141.8	±	0.7	141.0	±	0.7			
Potassium	L	4.0	±	0.1	4.9	±	0.1	4.5	±	0.3	4.1	±	0.1	n.s.	***	n.s.
[mmol/l]	P	4.1	±	0.1	4.3	±	0.1	4.4	±	0.1	4.0	±	0.2			
Chloride	L	94.6	±	0.7	96.6	±	1.2	97.0	±	1.8	99.8	±	0.7	n.s.	*	n.s.
[mmol/l]	P	96.8	±	1.2	99.2	±	1.2	98.3	±	0.9	99.0	±	0.4			
Calcium	L	2.5	±	0.03	2.4	±	0.03	2.1	±	0.03	2.2	±	0.04	***	***	*
[mmol/l]	P	2.5	±	0.02	2.4	±	0.04	2.4	±	0.1	2.3	±	0.04			
Phosphorus	L	2.8	±	0.1	3.0	±	0.1	3.0	±	0.1	2.2	±	0.1	**	***	n.s.
[mmol/l]	P	2.8	±	0.1	3.2	±	0.1	3.1	±	0.1	2.5	±	0.1			
Iron	L	19.0	±	1.1	20.6	±	1.8	18.0	±	0.6	18.3	±	1.4	**	n.s.	n.s.
[µmol/l]	P	18.7	±	2.5	24.6	±	3.6	28.2	±	2.6	24.4	±	2.8			
Magnesium	L	0.8	±	0.02	0.8	±	0.01	0.8	±	0.03	0.7	±	0.02	n.s.	***	n.s.
[mmol/l]	P	0.8	±	0.02	0.8	±	0.01	0.8	±	0.02	0.7	±	0.01			
AST	L	43.2	±	16.0	20.4	±	3.8	17.7	±	4.4	28.5	±	5.1	n.s.	n.s.	n.s.
[U/l]	P	31.4	±	5.2	29.3	±	4.9	27.2	±	2.8	43.7	±	11.1			
γGT	L	44.1	±	4.5	39.1	±	1.2	49.1	±	9.0	39.8	±	2.1	n.s.	n.s.	n.s.
[U/l]	P	42.5	±	6.4	29.2	±	2.5	47.0	±	10.0	30.2	±	3.2			
AP	L	108.0	±	14.7	107.6	±	10.1	102.4	±	10.9	75.2	±	8.1	n.s.	**	n.s.
[U/l]	P	97.7	±	11.3	104.8	±	8.8	99.2	±	8.8	60.1	±	5.3			

3.2. Reduced food intake and feeding efficiency in liraglutide-treated GIPR^{dn} transgenic pigs

Food intake was determined every third day (prophylactic group) or every week (therapeutic group) during the whole treatment period. In this time animals had constantly access to an *ad libitum* chow. Generally, all pigs of the prophylactic group that were treated from two until five months of age (n=18) showed food intake that was steadily increasing with age, while older pigs included in the therapeutic group treated from five to eight months of age (n=11) revealed a more constant food intake. For both groups, the effect of liraglutide treatment was consistent: Straight from the beginning of the treatment period liraglutide-treated animals showed significant decreases in food intake in comparison to placebo-treated pigs, with differences of 20-35% determined in the prophylactic group and 40-50% in the therapeutic group (Figure 9). This distinction was assessed steadily over the whole treatment period and did not change by an increase of the liraglutide dosage. Additionally, feeding efficiency (body weight gain per food intake) was significantly reduced in liraglutide-treated pigs both in the prophylactic group (0.27 ± 0.01 vs. 0.31 ± 0.01, $p<0.05$) and the therapeutic group (0.16 ± 0.01 vs. 0.24 ± 0.01, $p<0.01$) (Figure 9).

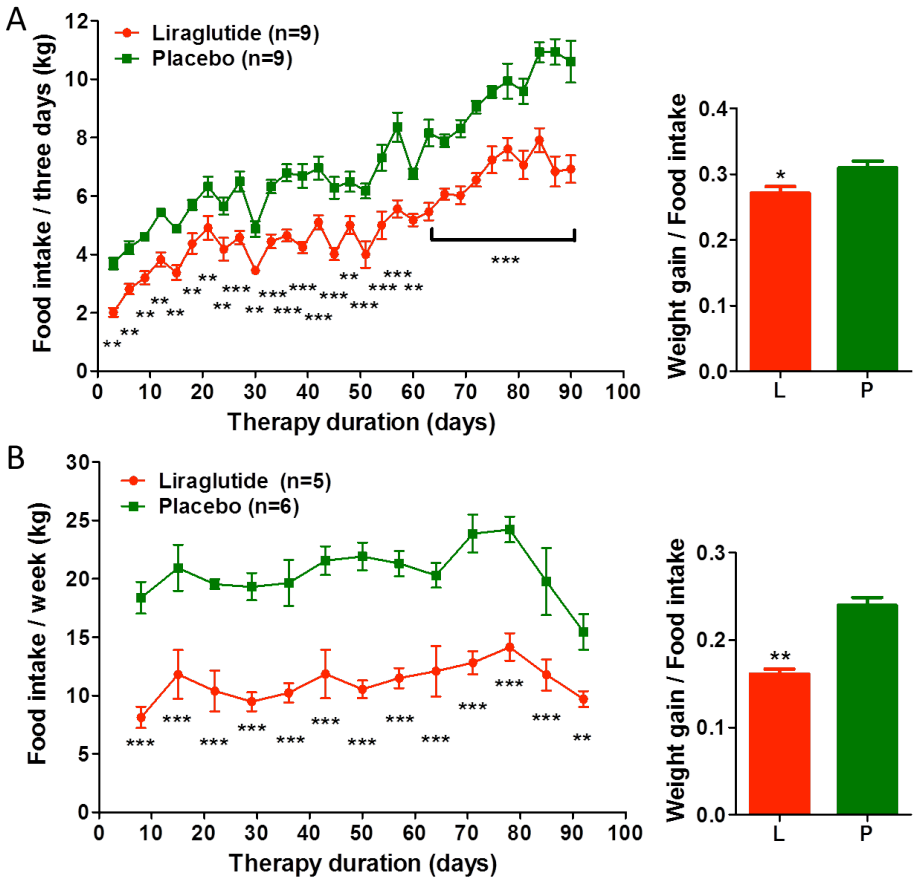

Figure 9: Reduced food intake and feeding efficiency in liraglutide-treated GIPR^{dn} transgenic pigs

Mean food intake (left) and feeding efficiency (right) in the prophylactic **(A)** and the therapeutic group **(B)** during the treatment period in liraglutide- (L) and placebo-treated (P) GIPR^{dn} transgenic pigs; n = number of animals investigated. Data are means ± SEM; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

3.3. Reduced body weight gain in liraglutide-treated GIPR^{dn} transgenic pigs

Regular weekly body weight control was already started during the pre-treatment period at the age of 32 days (prophylactic group, n=18) or 50 days (therapeutic group, n=11) and continued over the whole treatment period until necropsy. Effects of liraglutide treatment on body weight were in accordance with the observed effects on food intake and feeding efficiency. GIPR^{dn} transgenic animals of the prophylactic group gained weight consistently during pre-treatment time. However, a rapid change was observed at the start of therapy: Liraglutide-treated pigs distinctly gained less weight compared to placebo-treated animals. This finding was progressive throughout the whole treatment period and finally

resulted in a body weight difference of 31% (63.7 ± 2.4 kg vs. 91.6 ± 3.7 kg) compared to placebo-treated pigs at the day of necropsy (Figure 10 A). Animals of the therapeutic group were chosen randomly by lot and raised equally, yet they showed – by chance – already slight, but significant differences in body weight before the start of the treatment. But also in this group liraglutide treatment impaired body weight gain, reflected in a final body weight difference of 41% (79.9 ± 4.3 kg vs. 134.2 ± 5.9 kg) in liraglutide-treated pigs in comparison to the placebo-treated pigs (Figure 10 B). Differences in body weight were already clearly visible by looking at the physical appearance of the pigs, whereby liraglutide-treated animals showed distinct smaller shape and size in comparison to placebo-treated pigs.

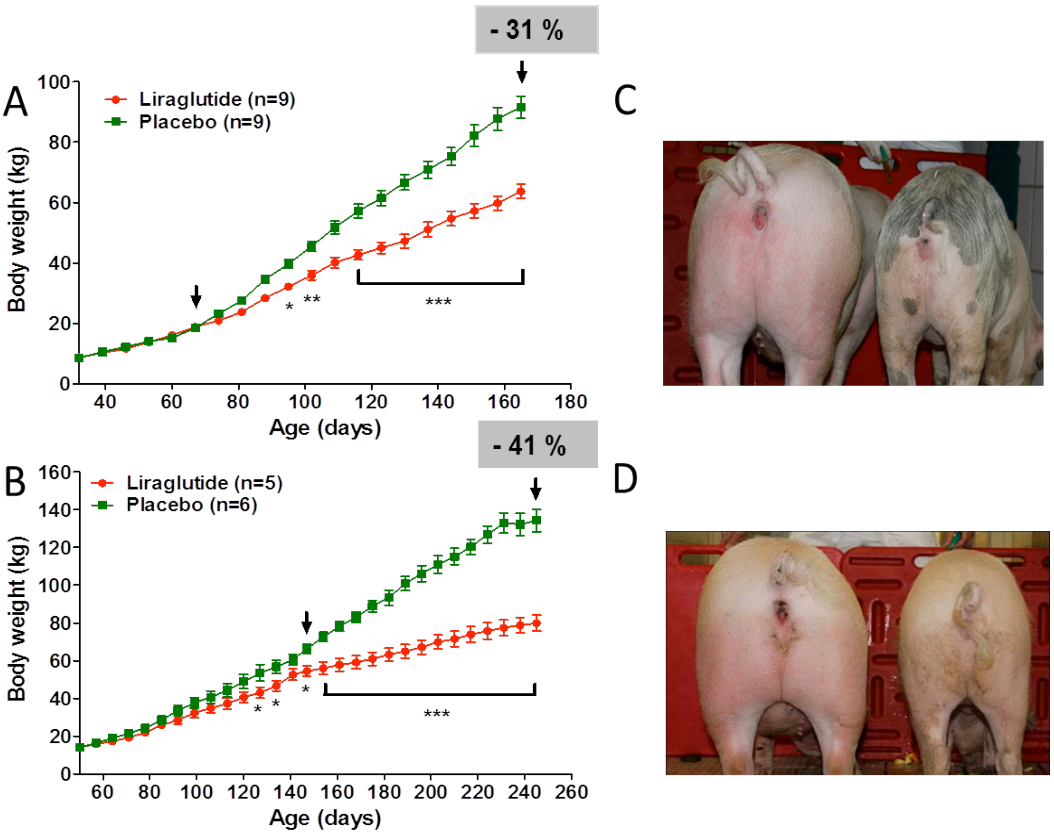

Figure 10: Reduced body weight gain of liraglutide-treated GIPR^{dn} transgenic pigs

Mean body weight gain of the prophylactic **(A)** and the therapeutic **(B)** group during pre-treatment and treatment period; n = number of animals investigated; black arrows indicate the start and the end of the treatment period; data are means ± SEM; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; representative physical appearance of liraglutide-treated pig (right side) and placebo-treated pig (left side) in the last third of therapy in the prophylactic **(C)** and therapeutic **(D)** group.

3.4. Influence of liraglutide treatment on levels of growth-regulating polypeptides

Due to the distinctly reduced body weight gain of liraglutide-treated GIPR^{dn} transgenic pigs in both the prophylactic and the therapeutic trial, it was interesting to evaluate if blood levels of polypeptides involved in growth regulation were altered. Therefore, levels of somatostatin, insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein (IGFBP) -2 and -3 were determined in blood samples of 18-hours fasted animals of the prophylactic and the therapeutic group right before the start and at the end of the treatment period respectively. Due to a large variation between individual animals, the peptide concentrations after the treatment period were divided by the concentrations before the treatment period and this ratio representative for the degree of concentration changes was compared between liraglutide- and placebo-treated GIPR^{dn} transgenic pigs.

3.4.1. Changes in somatostatin levels in liraglutide- and placebo-treated GIPR^{dn} transgenic pigs

Somatostatin levels were determined in plasma samples in randomly chosen liraglutide- (n=4) and placebo-treated pigs (n=4) of the prophylactic group as well as the therapeutic group using RIA. The ratio of the mean somatostatin concentration after the treatment to the concentration prior to the treatment showed no significant differences in pigs of the prophylactic group, but was significantly elevated ($p<0.05$) in liraglutide-treated compared to placebo-treated GIPR^{dn} transgenic pigs of the therapeutic group (Figure 11).

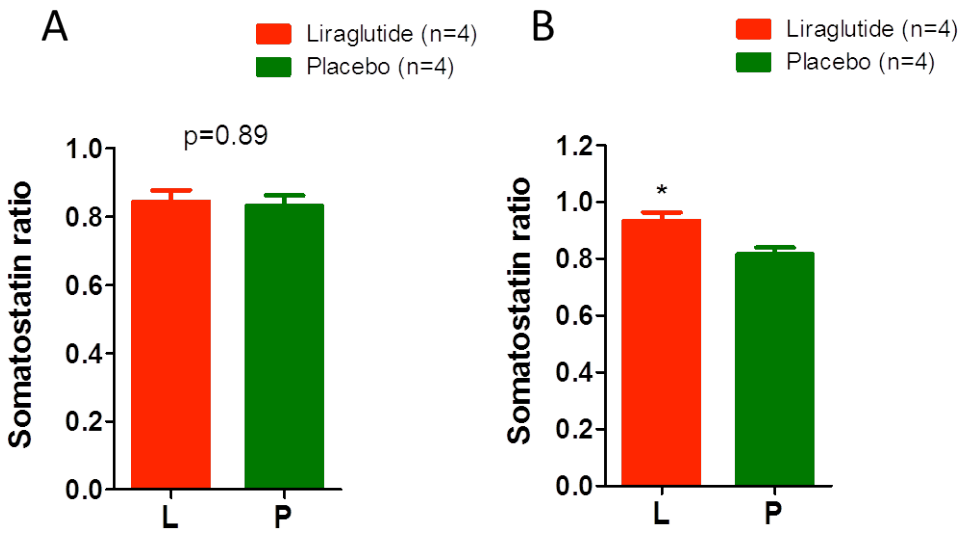

Figure 11: Somatostatin ratio in GIPR^{dn} transgenic pigs

Mean post-treatment/pre-treatment somatostatin ratio in liraglutide- (L) and placebo-treated (P) pigs of the prophylactic **(A)** and the therapeutic **(B)** group; n = number of animals investigated. Data are means ± SEM, *: $p<0.05$.

3.4.2. Changes in IGF-I, IGFBP-2 and-3 levels in liraglutide- and placebo-treated GIPR^{dn} transgenic pigs

IGF-I levels were measured in serum samples using an ELISA kit, while IGFBP-2 and -3 levels were determined also in serum samples by quantitative Western ligand blot analysis. Samples of all animals of the therapeutic group were included (n=11) while 7 liraglutide-treated and 7 placebo-treated pigs were randomly chosen within the prophylactic group. The post-treatment/pre-treatment ratios for IGF-I did not differ between liraglutide- and placebo-treated GIPR^{dn} transgenic pigs in both groups. Additionally, no major effect of liraglutide treatment on IGFBP-2 and -3 was detected as there were also no significant differences seen for IGFBP-2 and -3 post-treatment/pre-treatment ratios compared between liraglutide- and placebo-treated GIPR^{dn} transgenic pigs (Figure 12).

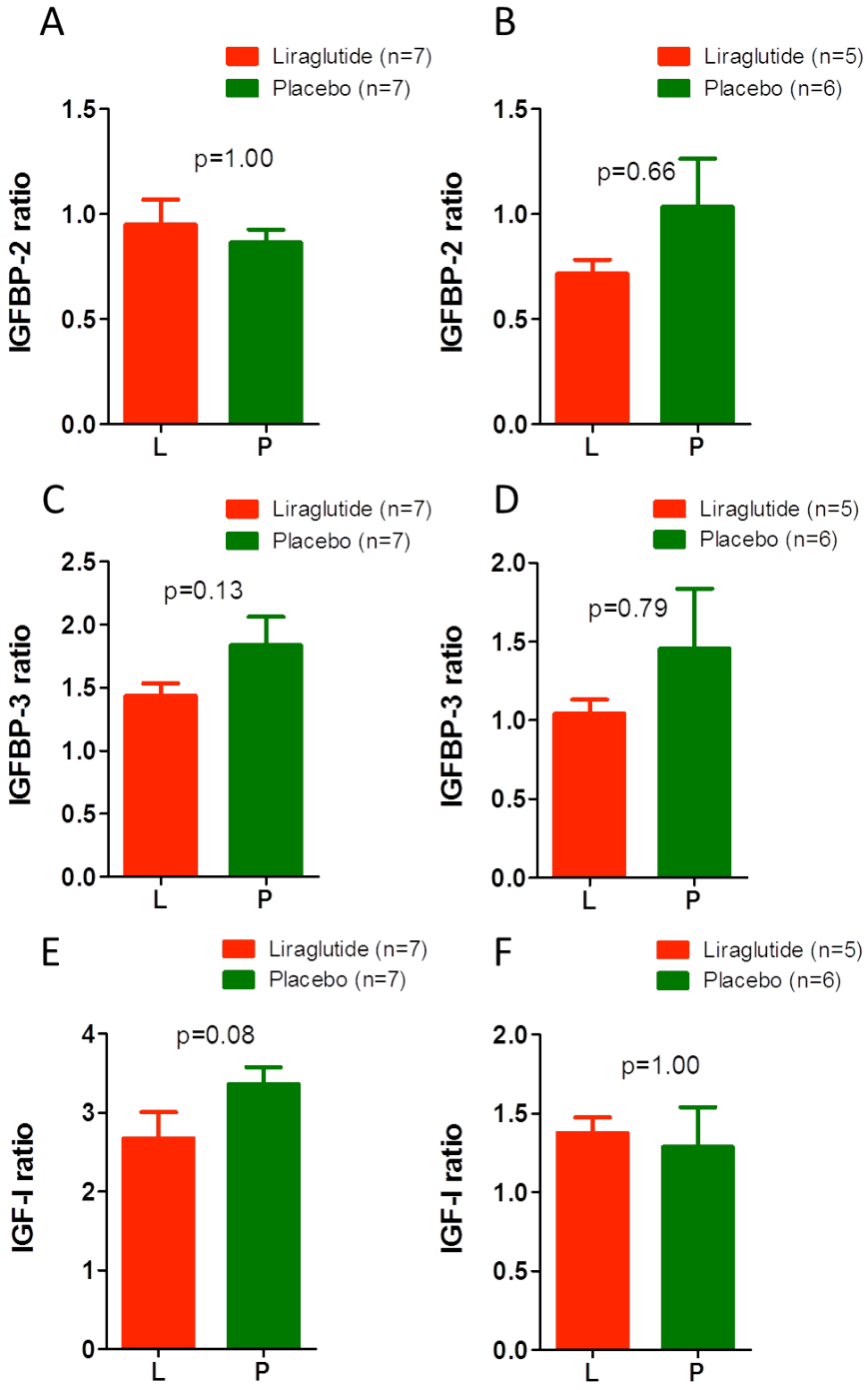

Figure 12: Unchanged IGF-I, IGFBP-2 and -3 ratios in liraglutide-treated GIPR^{dn} transgenic pigs

Mean post-treatment/pre-treatment ratio of IGFBP-2 **(A, B)**, IGFBP-3 **(C, D)** and IGF-I **(E, F)** in liraglutide- (L) and placebo-treated (P) GIPR^{dn} transgenic pigs of the prophylactic group **(A, C, E)** and the therapeutic group **(B, D, F)**; n = number of animal investigated. Data are means ± SEM.

4. Effect of liraglutide treatment on glucose control

Intravenous and oral glucose tolerance tests were performed in all animals included in the study prior to liraglutide/placebo treatment and additionally at the end of the treatment period. Liraglutide/placebo treatment was carried on during the post-treatment glucose tolerance tests. Thus, 3 hours before the start of the post-treatment glucose tolerance tests pigs received their usual dosage of liraglutide (prophylactic group: 1.2 mg, therapeutic group: 1.8 mg) or placebo. Intravenous and oral glucose tolerance tests were performed on consecutive days while pigs showed the following ages and body weights: Pigs of the prophylactic group (n=18) underwent the first glucose tolerance test at 60 ± 1 days of age prior to the start of therapy at 67 days of age, while showing similar body weights of 15 ± 1.0 kg (placebo-treated subgroup, n=9) and 16.1 ± 0.73 kg (liraglutide-treated subgroup, n=9). The post-treatment glucose tolerance tests were conducted at 158 ± 1 days of age and body weight at this time point already showed significant differences with mean values of 87.7 ± 3.84 kg (placebo-treated subgroup) and 59.78 ± 2.32 kg (liraglutide-treated subgroup). The first glucose tolerance test in pigs of the therapeutic group (n=11) was performed at an age of 135 ± 5 days. At this time point animals of the later liraglutide-treated subgroup (n=5) showed mean body weight of 50.7 ± 4.10 kg while the later placebo-treated subgroup (n=6) had mean body weight of 58.6 ± 2.71 kg. Therapy was started in all animals at an age of 147 days and accordingly post-treatment glucose tolerance tests were conducted at an age of 238 ± 2 days. At this age liraglutide-treated pigs showed clearly less body weight compared to placebo-treated animals (78.8 ± 4.10 kg (liraglutide) vs. 132.3 ± 5.94 kg (placebo)).

4.1. Improved oral glucose tolerance and decreased insulin secretion in liraglutide-treated GIPR^{dn} transgenic pigs

4.1.1. Prophylactic group: Results of oral glucose tolerance tests

There was no significant difference seen during the pre-treatment OGTT between GIPR^{dn} transgenic pigs concerning elevation and decline of blood glucose levels after oral stimulation and AUC for glucose was similar (18,375 ± 881 (liraglutide-treated subgroup) vs. 17,839 ± 682 (placebo-treated subgroup), p=0.80) (Figure 13 A). However, treatment with liraglutide led to significant changes in blood glucose levels during the post-treatment OGTT: Liraglutide-treated pigs

showed slower and distinctly less glucose elevation in comparison to placebo-treated pigs. AUC for glucose was reduced by 22.5% (14,018 ± 447 vs. 18,094 ± 659, p<0.001), indicating an improvement in oral glucose tolerance by liraglutide treatment (Figure 13 B).

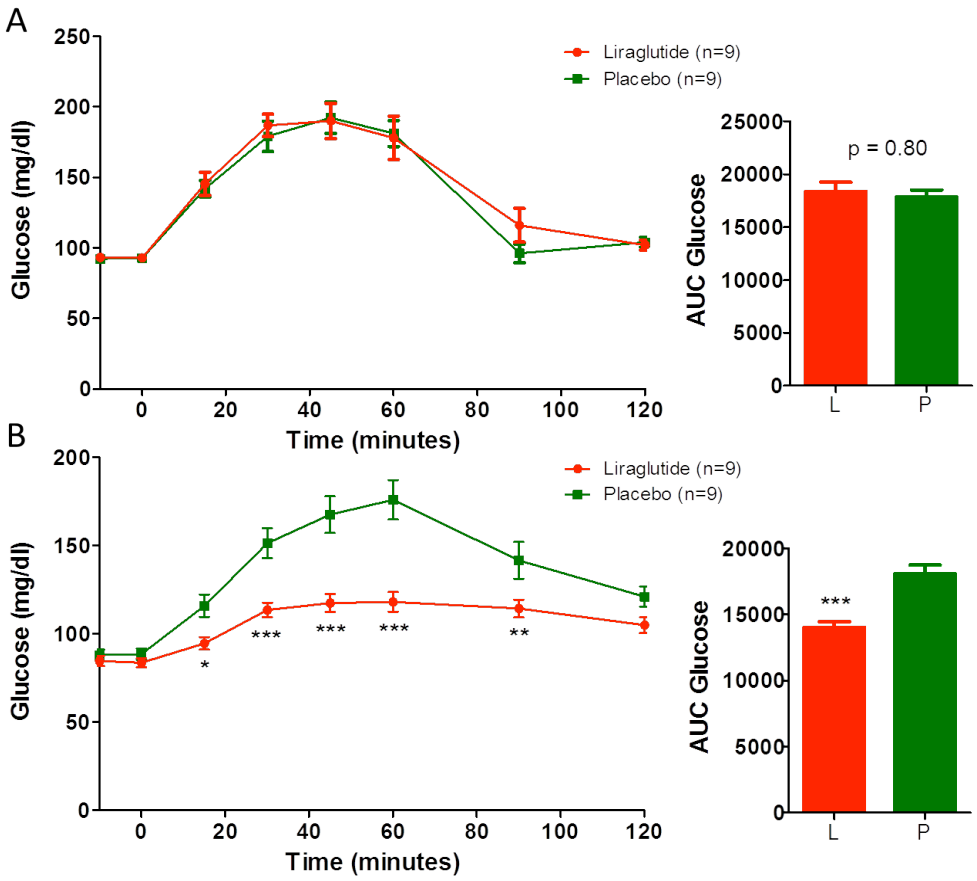

Figure 13: Improved oral glucose tolerance in liraglutide-treated GIPR^{dn} transgenic pigs of the prophylactic group

Similar glucose levels during pre-treatment OGTT **(A)**, but significantly reduced glucose levels and AUC glucose during post-treatment OGTT **(B)** of liraglutide-treated (L) compared to placebo-treated (P) GIPR^{dn} transgenic pig, 0 minutes = point of glucose administration; n = number of animals investigated; AUC glucose = area under the glucose curve. Data are means ± SEM, *: p<0.05, **: p<0.01, ***: p<0.001.

Although plasma insulin levels in animals of the liraglutide-treated subgroup showed a significant increase (p<0.05) 30 minutes after glucose administration during the pre-treatment OGTT, the course of the insulin curve seemed similar and AUC for insulin was not significantly different (3,296 ± 261 (liraglutide-

treated subgroup) vs. 2,956 ± 187 (placebo-treated subgroup), p=0.30) (Figure 14 A). Though, insulin levels of liraglutide-treated animals in response to oral stimulation after the treatment period were significantly decreased in comparison to placebo-treated pigs. Additionally, AUC for insulin in liraglutide-treated animals was reduced by 36% (2,865 ± 283 vs. 4,474 ± 619, p<0.05) (Figure 14 B).

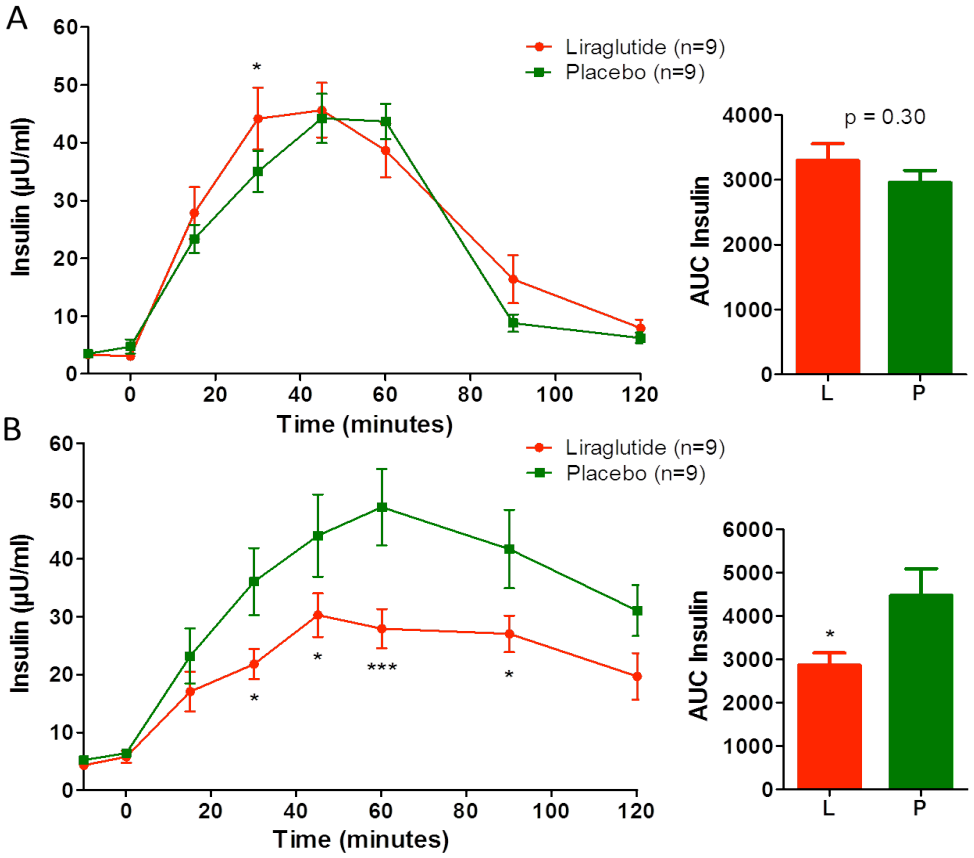

Figure 14: Reduced insulin secretion in liraglutide-treated GIPR^{dn} transgenic pigs of the prophylactic group

Similar insulin level during pre-treatment OGTT **(A)** but significantly reduced insulin levels and AUC insulin during post-treatment OGTT **(B)** in liraglutide-treated (L) compared to placebo-treated (P) GIPR^{dn} transgenic pigs, 0 minutes = point of glucose administration; n = number of animals investigated; AUC insulin = area under the insulin curve. Data are means ± SEM, *: p< 0.05, ***: p<0.001.

4.1.2. Therapeutic group: Results of oral glucose tolerance tests

Generally, results seen during OGTTs in the therapeutic group resembled those determined in the prophylactic group regarding changes in blood glucose and insulin levels: There was no significant difference seen in elevation or decline of

blood glucose levels after oral stimulation during pre-treatment OGTT going along with similar AUC for glucose (16,223 ± 1,612 (liraglutide-treated subgroup) vs. 15,258 ± 647 (placebo-treated subgroup), p=0.93) (Figure 15 A). However, liraglutide treatment led to significantly lower blood glucose elevations only reaching maximum concentrations of 124 mg/dl and AUC for glucose was reduced by 28% (14,561 ± 942 vs. 20,025 ± 1,707, p<0.05). In contrast, placebo-treated pigs showed a peak glucose concentration of 194 mg/dl during the post-treatment OGTT (Figure 15 B). Thus, oral glucose tolerance in GIPR^{dn} transgenic pigs included in the therapeutic group was improved by liraglutide treatment.

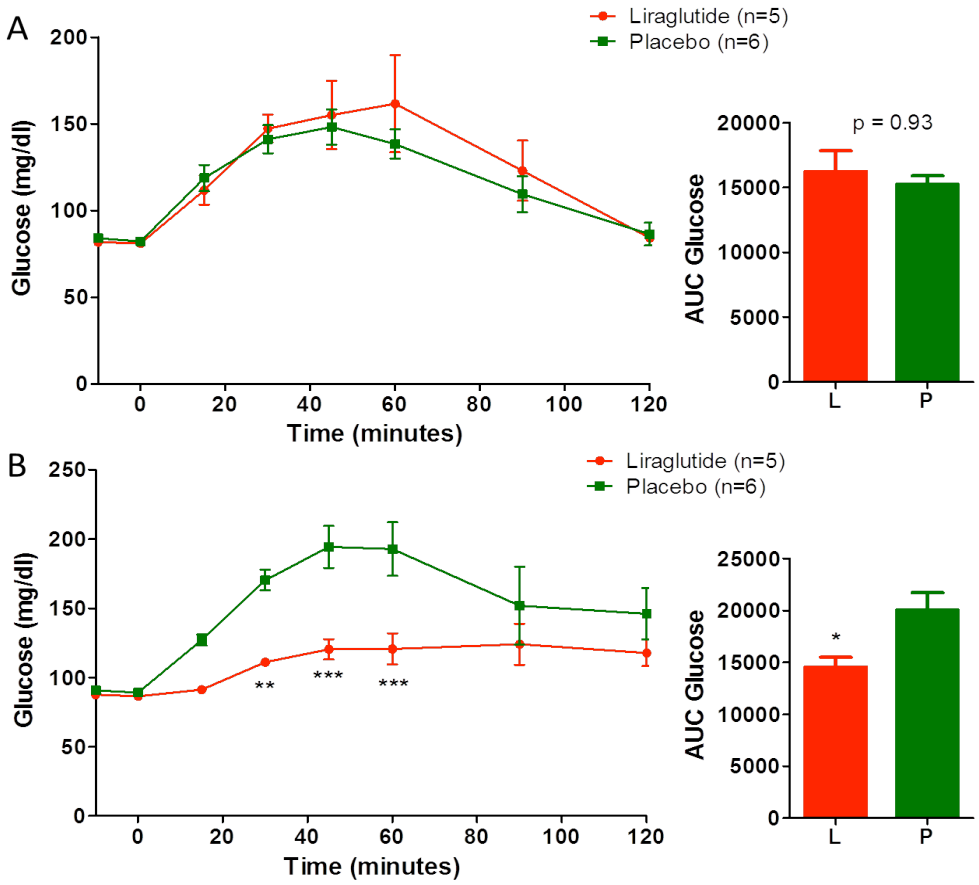

Figure 15: Improved oral glucose tolerance in liraglutide-treated GIPR^{dn} transgenic pigs of the therapeutic group

Unchanged glucose levels during pre-treatment OGTT **(A)**, but significantly reduced glucose levels and AUC glucose during post-treatment OGTT **(B)** of liraglutide-treated (L) compared to placebo-treated (P) GIPR^{dn} transgenic pigs, 0 minutes = point of glucose administration; n = number of animals investigated; AUC glucose = area under the glucose curve. Data are means ± SEM, *: p<0.05, **: p<0.01, ***: p<0.001.

Although insulin secretion in animals of the liraglutide-treated subgroup during the pre-treatment OGTT was lower compared to the placebo-treated subgroup, significance was only reached at time point 45 minutes after glucose administration ($p<0.05$). Likewise the AUC for insulin showed no significant difference (3,268 ± 360 (liraglutide-treated subgroup) vs. 4,671 ± 869 (placebo-treated subgroup), $p=0.25$) (Figure 16 A). However, results of the post-treatment OGTT were different: Insulin levels of placebo-treated pigs showed a distinct increase and a peak insulin concentration of 111.3 µU/ml was reached 60 minutes after glucose administration, thereby being approximately twice as high as maximum concentrations reached during pre-treatment OGTT. In contrast, liraglutide-treated animals only showed a slight rise in insulin levels with low peak concentrations of 37 µU/ml. The difference of insulin secretion between liraglutide- and placebo-treated pigs was significant throughout the whole post-treatment OGTT. Accordingly, AUC for insulin in liraglutide-treated animals was reduced by 71% in comparison to placebo-treated pigs (2,685 ± 103 vs. 9,138 ± 1,400, $p<0.01$) (Figure 16 B).

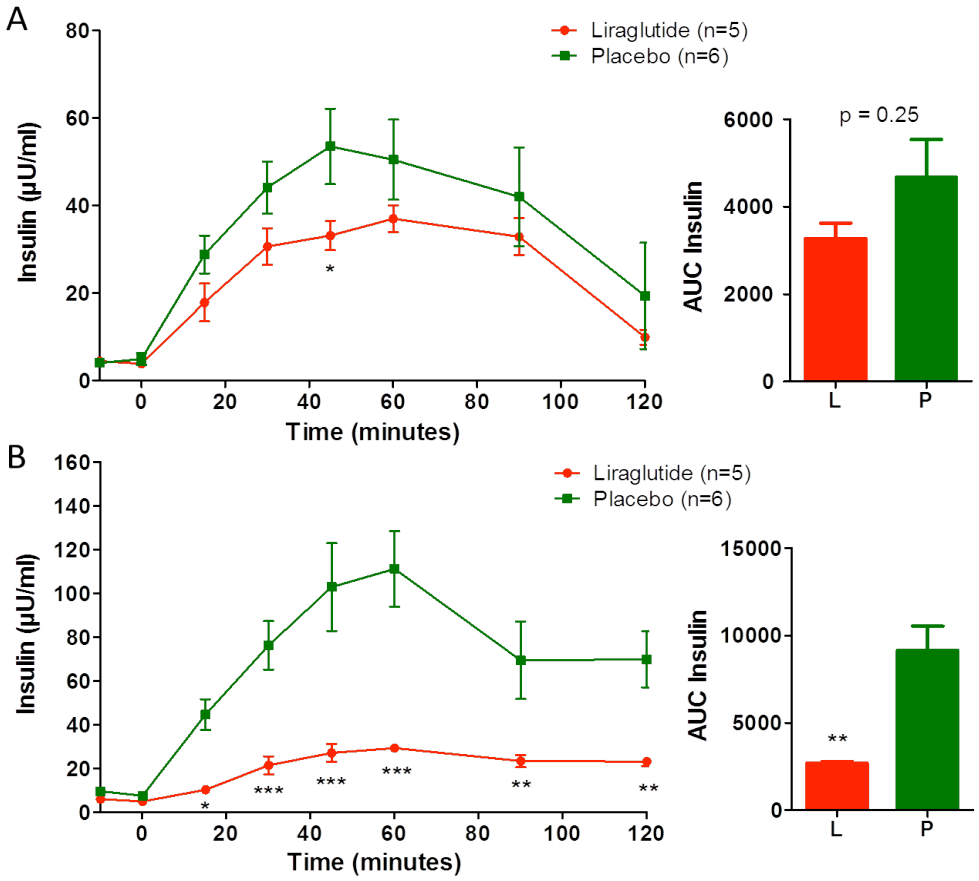

Figure 16: Reduced insulin secretion in liraglutide-treated GIPR^{dn} transgenic pigs of the therapeutic group

Similar insulin level during pre-treatment OGTT **(A)** but significantly reduced insulin levels and AUC insulin during post-treatment OGTT **(B)** in liraglutide-treated (L) compared to placebo-treated (P) GIPR^{dn} transgenic pigs, 0 minutes = point of glucose administration; n = number of animals investigated; AUC insulin = area under the insulin curve. Data are means ± SEM, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

4.2. Improved insulin sensitivity in liraglutide-treated GIPR^{dn} transgenic pigs

Extensive clinical trials with type 2 diabetic patients proposed the ability of liraglutide to improve insulin sensitivity and beta-cell function, indicated by the calculation of different parameters (Blonde et al. 2009; Buse et al. 2009). For evaluation of changes in insulin sensitivity of GIPR^{dn} transgenic pigs prior to and after the treatment with the GLP-1R agonist liraglutide indices were calculated that show good correlation to the euglycemic hyperinsulinemic clamp, which is widely accepted being the gold standard for the validation of insulin sensitivity (Katz et al. 2000; Matsuda et al. 1999; Matthews et al. 1985; Radikova 2003). Basal plasma insulin and glucose values of animals after an 18-hour fasting period were used for calculation of HOMA-IR, HOMA-β and QUICKI. As these indices mainly reflect hepatic insulin sensitivity and basal hepatic glucose production, additionally ISI $_{(Matsuda)}$) was calculated. ISI $_{(Matsuda)}$ includes glucose and insulin levels in the fasting state as well as during OGTT. Thus, not only hepatic but also insulin sensitivity of peripheral tissues can be assessed (Radikova 2003). Results of insulin sensitivity evaluation were similar for the prophylactic group as well as for the therapeutic group: HOMA-IR showed similar values before the start of therapy in all GIPR^{dn} transgenic pigs (prophylactic group: 0.72 ± 0.09 vs. 0.85 ± 0.12, p=0.43, therapeutic group: 0.77 ± 0.07 vs. 0.83 ± 0.16, p=0.58). After the treatment period HOMA-IR of liraglutide-treated pigs had not changed or just slightly increased whereas placebo-treated pigs showed a distinct rise in HOMA-IR, leading to significant differences between HOMA-IR of liraglutide-treated compared to placebo-treated pigs after the therapy (prophylactic group: 0.89 ± 0.10 vs. 1.26 ± 0.11, $p<0.05$; therapeutic group: 0.87 ± 0.12 vs. 1.87 ± 0.21, $p<0.05$). This indicates decreased insulin sensitivity in placebo-treated pigs. Although a trend of lower levels of HOMA-β as an index for beta-cell function was detected in liraglutide-treated pigs after therapy, no significance was reached in comparison to the placebo-treated pigs (prophylactic group: 83.98 ± 7.88 vs. 100.95 ± 13.42, p=0.34; therapeutic group: 67.53 ± 11.21 vs. 112.72 ± 18.14, p=0.18). QUICKI showed similar values prior to the start of the therapy (prophylactic group: 1.17 ± 0.11 vs. 1.03 ± 0.10, p=0.44, therapeutic group: 0.90 ± 0.06 vs. 0.98 ± 0.11, p=0.54). However, values of QUICKI were significantly higher in liraglutide-treated pigs after the therapy in comparison to placebo-treated animals (prophylactic group: 0.87 ± 0.05 vs. 0.69 ± 0.03, $p<0.05$;

therapeutic group: 0.89 ± 0.09 vs. 0.58 ± 0.04, $p<0.05$), indicating an improved insulin sensitivity in liraglutide-treated pigs. The greatest difference between liraglutide- and placebo-treated pigs was seen when glucose and insulin values of OGTTs were additionally included for the calculation of $ISI_{(Matsuda)}$. Before the start of the therapy this index did not differ between the two groups (prophylactic group: 10.24 ± 0.90 vs. 10.00 ± 0.83, $p=0.87$; therapeutic group: 10.78 ± 0.84 vs. 9.82 ± 1.34, $p=0.93$). After the treatment, $ISI_{(Matsuda)}$ of liraglutide-treated animals had hardly changed, while placebo-treated ones showed a distinct decrease of insulin sensitivity in comparison to the pre-treatment values. Thereby, treatment with liraglutide led to a significant increase in $ISI_{(Matsuda)}$ in comparison to placebo treatment (prophylactic group: 12.10 ± 1.28 vs. 7.24 ± 0.72, $p<0.01$; therapeutic group: 11.68 ± 0.74 vs. 3.95 ± 0.57, $p<0.01$). Altogether, calculation of different insulin sensitivity indices showed a significant improvement of insulin sensitivity by a 90-day liraglutide therapy in comparison to placebo treatment, but no significant differences for the index HOMA-β (Figure 17).

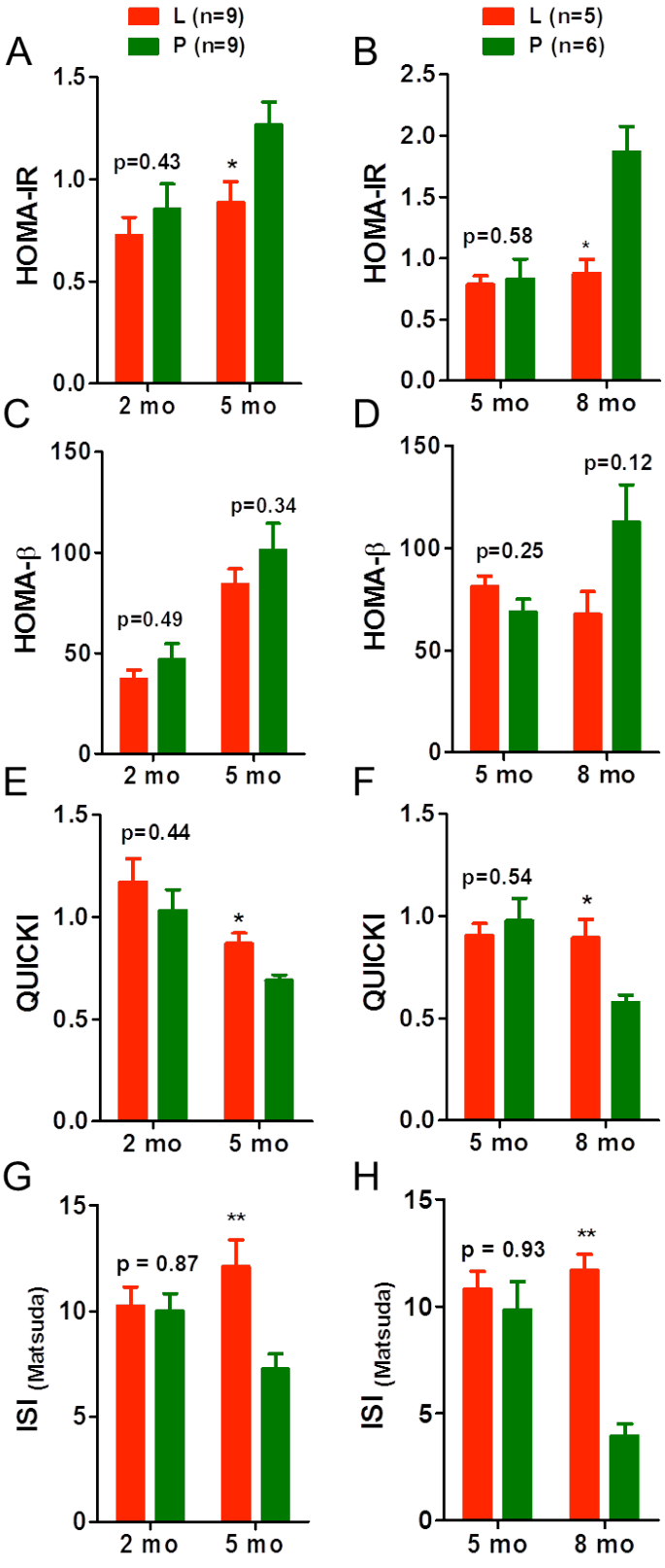

Figure 17: Improved insulin sensitivity in liraglutide-treated GIPR^{dn} transgenic pigs

Different insulin indices representing prophylactic group **(A, C, E, G)** and therapeutic group **(B, D, F, H)** before and at the end of the treatment period respectively; n = number of animals investigated; mo = months of age; L = liraglutide-treated animals (red), P = placebo-treated animals (green). Data are means ± SEM; *: $p<0.05$, **: $p<0.01$.

4.3. Improved intravenous glucose tolerance in liraglutide-treated GIPR^{dn} transgenic pigs

4.3.1. Prophylactic group: Results of intravenous glucose tolerance tests

GIPR^{dn} transgenic pigs of the liraglutide-treated subgroup (n=9) showed higher blood glucose concentrations one minute after glucose administration in comparison to the placebo-treated subgroup (n=9) during pre-treatment IVGTT ($p<0.01$). However, further course of the glucose curves was similar and AUC for glucose showed no significant differences (12,821 ± 458 (liraglutide-treated subgroup) vs. 13,015 ± 592 (placebo-treated subgroup), $p=0.86$) (Figure 18 A). In contrast to these findings, the post-treatment IVGTT revealed a significant improvement in intravenous glucose tolerance by liraglutide treatment in comparison to placebo treatment: AUC for glucose in liraglutide-treated animals was reduced by 15% (12,259 ± 446 vs. 14,445 ± 386, $p<0.01$) compared to placebo-treated ones and although the initial rise in blood glucose levels as well as maximum glucose concentrations were similar in all pigs included in this group, liraglutide-treated pigs showed significantly faster decline of glucose in the bloodstream (Figure 18 B).

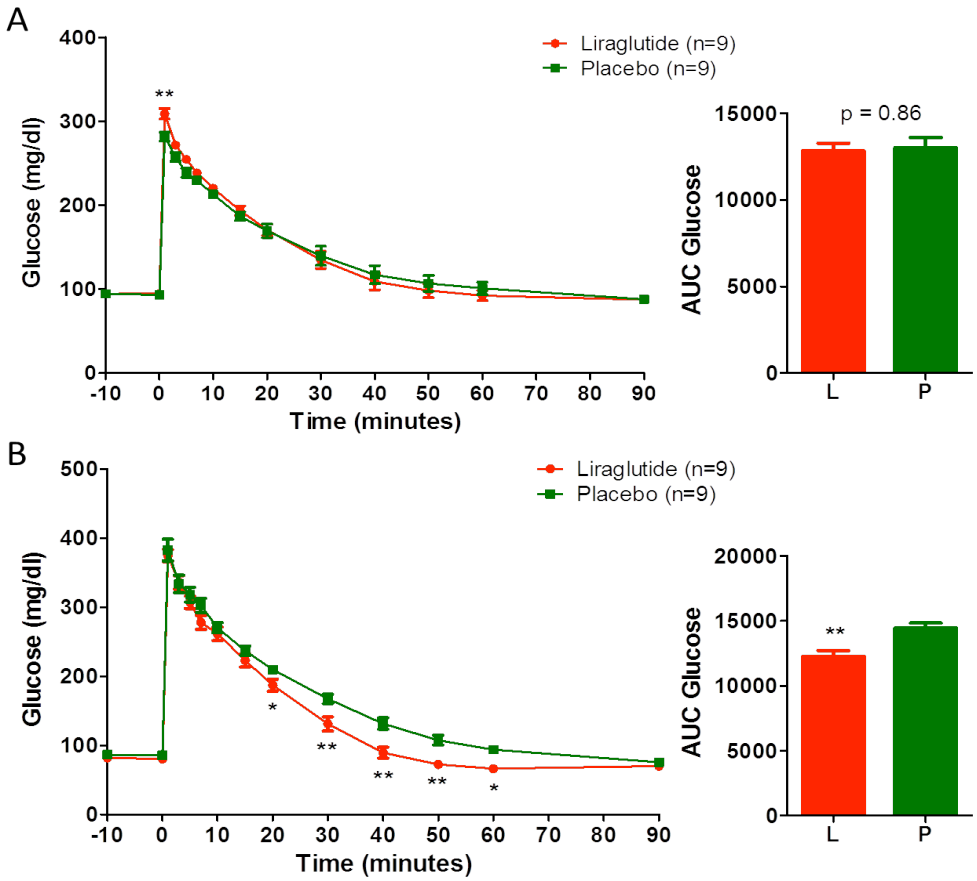

Figure 18: Improved intravenous glucose tolerance in liraglutide-treated GIPR^{dn} transgenic pigs of the prophylactic group

Similar course of glucose curves during pre-treatment IVGTT **(A)**, but significantly faster decline of blood glucose and reduced AUC for glucose during post-treatment IVGTT **(B)** of liraglutide-treated (L) compared to placebo-treated (P) GIPR^{dn} transgenic pigs; 0 minutes = point of glucose administration; n = number of animals investigated; AUC = area under the glucose curve. Data are means ± SEM; *: $p<0.05$, **: $p<0.01$.

Before the start of the treatment period, animals of the liraglutide treatment group revealed significantly higher insulin concentrations 15 minutes after intravenous glucose bolus ($p<0.01$), while no significant differences were seen at all other time points in comparison to the placebo treatment group. AUC for insulin revealed a tendency to be higher in the liraglutide-treated animals (862 ± 75 (liraglutide-treated subgroup) vs. 719 ± 69 (placebo-treated subgroup), $p = 0.07$) (Figure 19 A). Insulin secretion during post-treatment IVGTT showed a higher increase in liraglutide-treated animals reaching significance 20 minutes after

glucose administration ($p<0.05$) and also slightly faster decline, but AUC for insulin was not different in comparison to placebo-treated pigs (1,322 ± 123 (liraglutide-treated subgroup) vs. 1,387 ± 101 (placebo-treated subgroup), p = 0.74) (Figure 19 B).

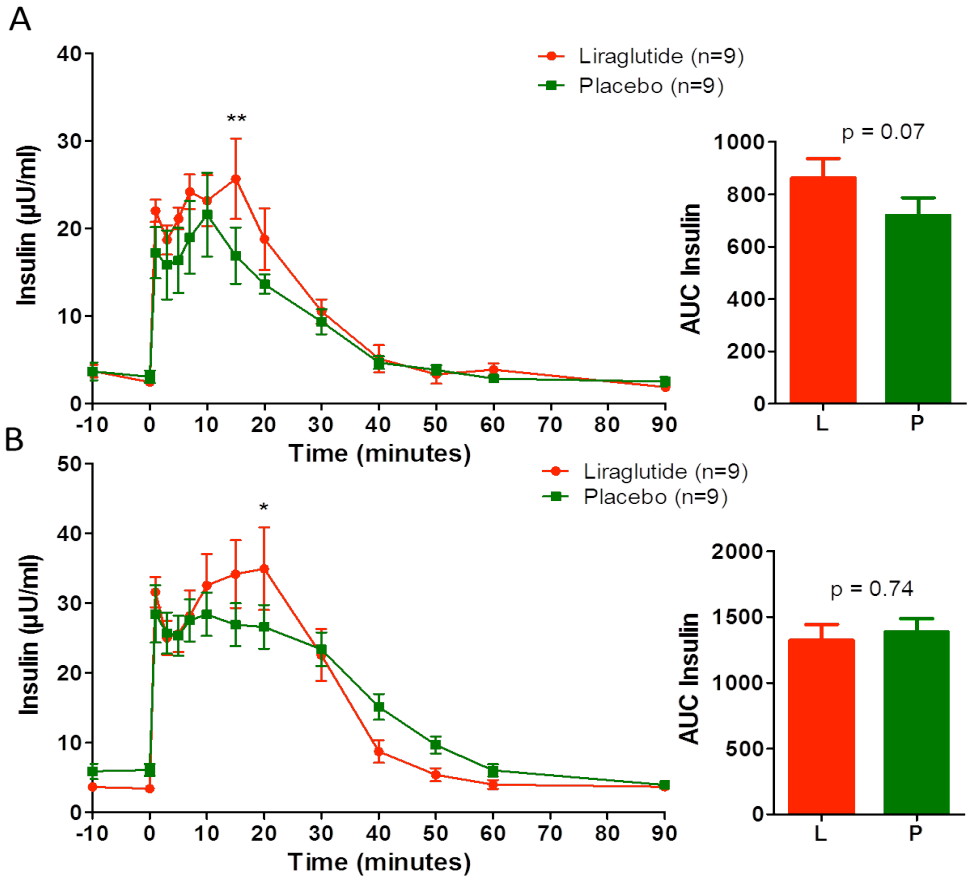

Figure 19: Insulin secretion and AUC for insulin during IVGTT in the prophylactic group

Insulin secretion in liraglutide-treated (L) compared to placebo-treated (P) $GIPR^{dn}$ transgenic pigs during pre-treatment IVGTT **(A)** and post-treatment IVGTT **(B)**; 0 minutes = point of glucose administration; n = number of animals investigated; AUC = area under the insulin curve. Data are means ± SEM; *: $p<0.05$, **: $p<0.01$.

4.3.2. Therapeutic group: Results of intravenous glucose tolerance tests

Results of the intravenous glucose tolerance tests observed in the therapeutic group regarding blood glucose generally reflect the findings determined in the prophylactic group: Prior to the treatment period blood glucose concentration one minute after glucose administration was significantly higher in the liraglutide-

treated subgroup (n=5) compared to the placebo-treated subgroup (n=6) ($p<0.05$), but AUCs for glucose were similar in both subgroups (13,142 ± 918 (liraglutide-treated subgroup) vs. 13,557 ± 359 (placebo-treated subgroup), $p=0.80$) (Figure 20 A). The post-treatment IVGTT also mirrored results seen in the prophylactic group, but to a greater extent: AUC for glucose was reduced by 23% (14,900 ± 614 vs. 19,148 ± 914, $p<0.01$) and glucose levels were generally lower in liraglutide-treated pigs over the whole period of time (90 minutes) following intravenous glucose load compared to placebo-treated pigs (Figure 20 B).

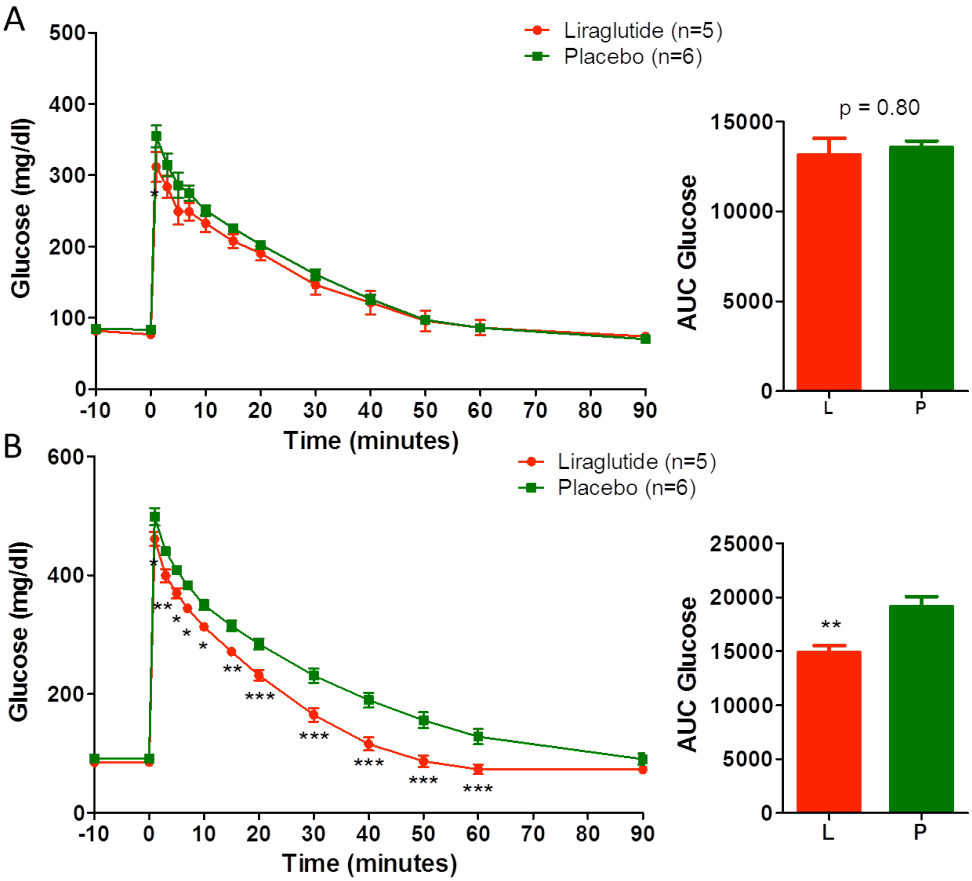

Figure 20: Improved intravenous glucose tolerance in liraglutide-treated GIPR^{dn} transgenic pigs of the therapeutic group

Similar course of glucose curves during pre-treatment IVGTT **(A),** but significantly lower rise and faster decline of blood glucose concentration as well as reduced AUC for glucose during post-treatment IVGTT **(B)** in liraglutide-treated (L) compared to placebo-treated (P) GIPR^{dn} transgenic pigs; 0 minutes = point of glucose administration; n = number of animals investigated; AUC = area under the glucose curve. Data are means ± SEM; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.

Liraglutide-treated pigs of the therapeutic group showed lower insulin secretion both during the pre-treatment and the post-treatment IVGTT. Prior to the start of the therapy, at a time point where pigs were still untreated but yet showed differences in body weight of 13.5%, pigs of the liraglutide-treated subgroup showed significantly lower insulin secretion 7 to 20 minutes after intravenous glucose administration and also lower but not significantly different AUC for insulin compared to the placebo-treated subgroup (1,022 ± 178 (liraglutide) vs. 1,474 ± 243 (placebo), p=0.34) (Figure 21 A). During post-treatment IVGTT insulin secretion in liraglutide-treated pigs was even more decreased, reaching significance from 1 to 50 minutes after glucose bolus, and this was accompanied by significantly lower AUC for insulin in comparison to the placebo-treated animals (1,605 ± 146 vs. 2,991 ± 355, p<0.01) (Figure 21 B).

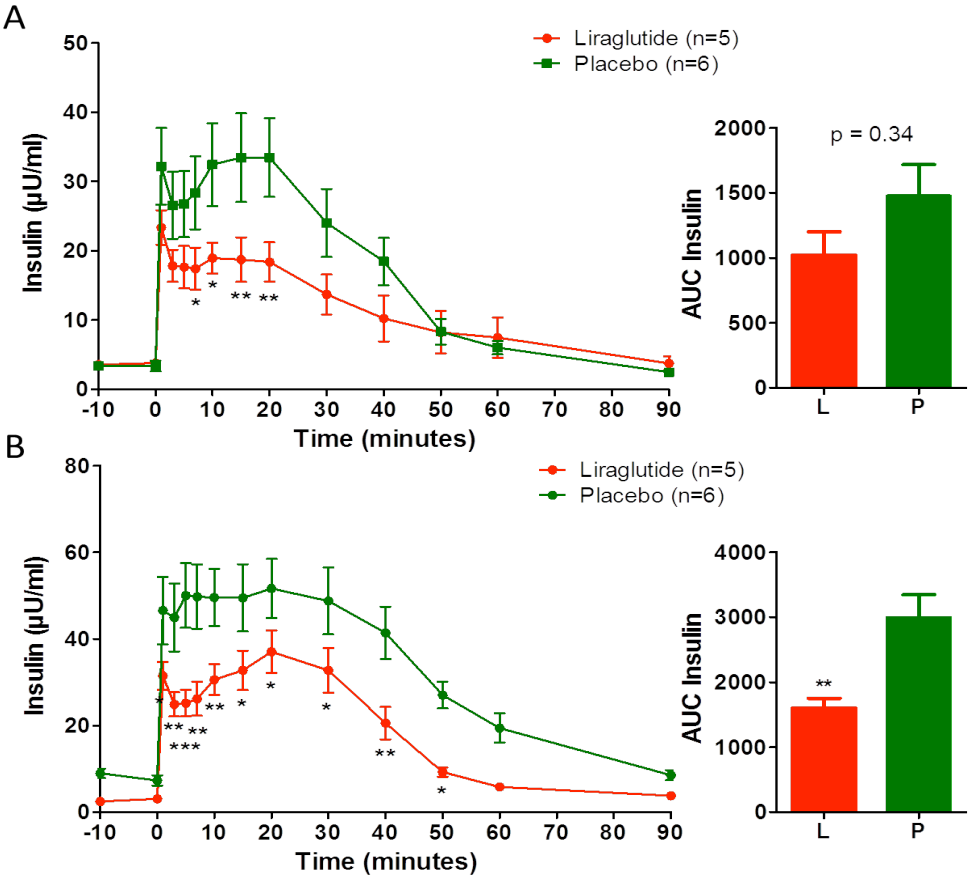

Figure 21: Insulin secretion and AUC for insulin during IVGTT in the therapeutic group

Insulin secretion in liraglutide-treated (L) and placebo-treated GIPR^{dn} transgenic pigs (P) during pre-treatment IVGTT **(A)** and post-treatment IVGTT **(B)**; 0 minutes = point of glucose administration; n = number of animals investigated; AUC = area under the insulin curve. Data are means ± SEM; *: p<0.05, **: p<0.01, ***: p<0.001

5. Effects of liraglutide treatment on the beta-cell volume of GIPR^{dn} transgenic pigs

5.1. Pancreas histology of liraglutide- and placebo-treated GIPR^{dn} transgenic pigs

Pancreatic tissue slides immunohistochemically stained for insulin did not show histological differences, neither between liraglutide- and placebo-treated animals, nor between the prophylactic and the therapeutic group. Insulin staining pattern and intensity were very similar in all animals included in the study and there was no visible difference in size or number of pancreatic islets. Pancreas morphology appeared to be conserved with normal structure of exocrine and endocrine pancreatic tissue (Figure 22).

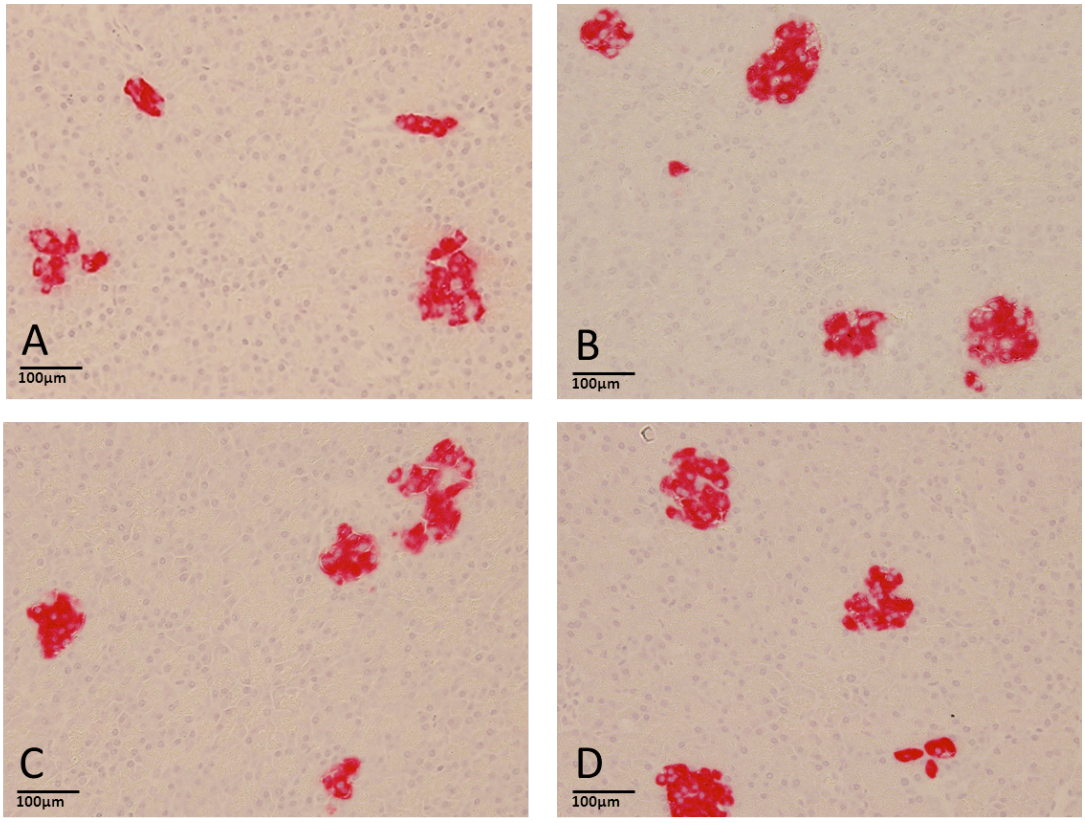

Figure 22: Unaltered insulin staining pattern and islet size in liraglutide-treated GIPR^{dn} transgenic pigs

Representative histological pancreas sections immunohistochemically stained for insulin of liraglutide-treated pigs of the prophylactic **(A)** and the therapeutic group **(C)** in comparison to placebo-treated pigs of the prophylactic **(B)** and the therapeutic group **(D)**, scale bar represents a length of 100 µm.

5.2. Effect of liraglutide treatment on the total beta-cell volume

For evaluation of the effect of liraglutide treatment on the total beta-cell volume quantitative-stereological analyses were performed in specimens obtained by random systematic sampling of pancreata from all $GIPR^{dn}$ transgenic pigs included in the study. The volume density of beta-cells ($Vv_{(\beta\text{-cell/Pan})}$) as well as isolated beta-cells in the pancreas ($Vv_{(iso\beta\text{-cell/Pan})}$) was determined and the total volume of beta-cells ($V_{(\beta\text{-cell, Pan})}$) as well as isolated beta-cells ($V_{(iso\beta\text{-cell,Pan})}$) was calculated. Isolated beta-cells were not determined manually, but recognized by the software Visiomorph™ as red stained insulin-positive cells covering an area smaller than 250 μm^2. Thereby it is possible that not only single cells were counted, but also small cell clusters of approximately up to five insulin-positive stained cells. As liraglutide- and placebo-treated pigs of both groups showed distinct differences in total body weight at the time of the morphological evaluation, total beta-cell volume was also related to body weight (($V_{(\beta\text{-cell, Pan})}$) / BW).

5.2.1. Evaluation of the beta-cell volume in the prophylactic group

Neither volume density of beta-cells in the pancreas ($Vv_{(\beta\text{-cell/Pan})}$, p=0.11) nor the total beta-cell volume ($V_{(\beta\text{-cell, Pan})}$, p=0.07) showed significant differences between liraglutide- (n=9) and placebo-treated pigs (n=9) in the prophylactic group at an age of 5 months. However, these parameters revealed a tendency to be decreased in liraglutide-treated pigs with a 14% lower volume density ($Vv_{(\beta\text{-cell/Pan})}$) and 22% lower total beta-cell volume ($V_{(\beta\text{-cell, Pan})}$) compared to placebo-treated pigs. Also, no differences were observed regarding volume density ($Vv_{(iso\beta\text{-cell/Pan})}$, p=0.55) and total volume of isolated beta-cells in the pancreas ($V_{(iso\beta\text{-cell,Pan})}$, p=0.61). The absolute weight of pancreata revealed no significant differences between liraglutide- and placebo-treated pigs (p=0.26). However, at the time of necropsy liraglutide-treated pigs showed a body weight reduction of 31% compared to the placebo-treated ones. Therefore, the total beta-cell volume related to body weight (($V_{(\beta\text{-cell, Pan})}$) / BW) was additionally calculated. Individual body weights of the pigs at the day of necropsy were used for the calculation. The parameter $V_{(\beta\text{-cell, Pan})}$ / BW was not different between liraglutide- and placebo-treated pigs (p=0.34) (Figure 23).

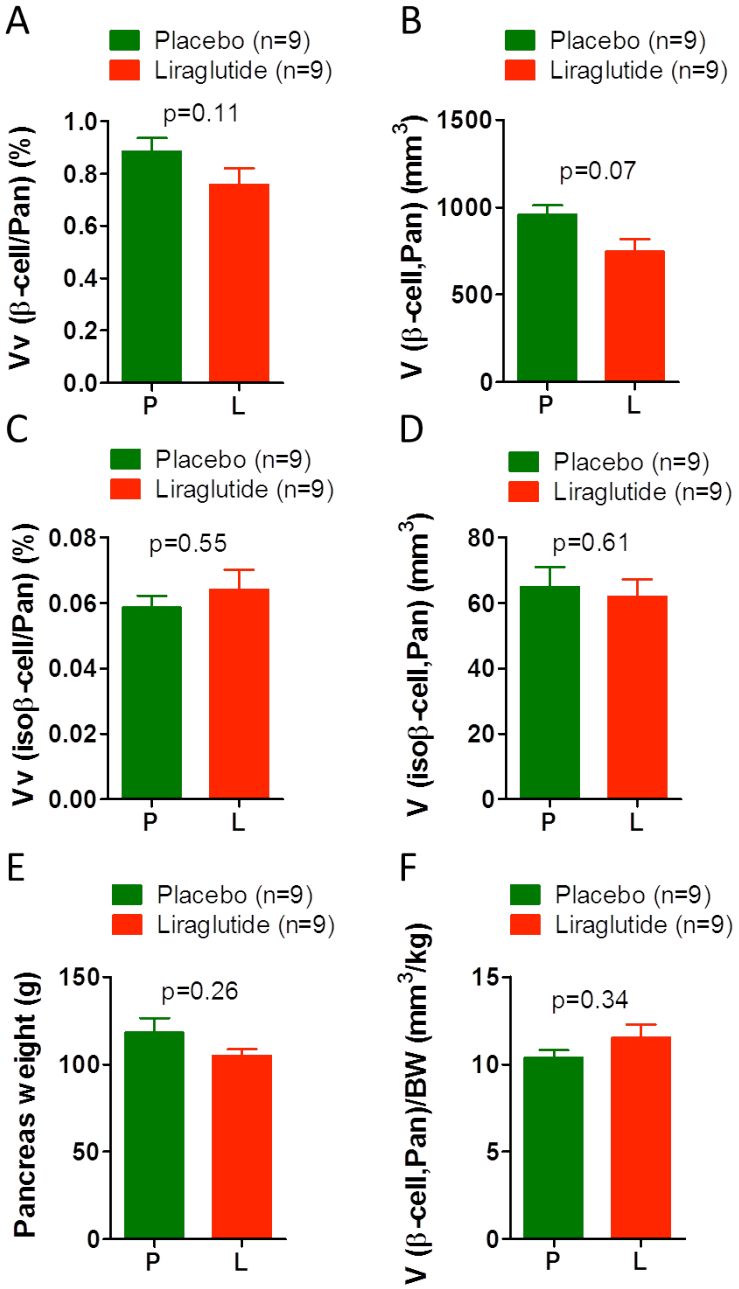

Figure 23: Quantitative-stereological analyses of pancreata from GIPR^{dn} transgenic pigs of the prophylactic group

(A/B) Volume density (Vv $_{(\beta\text{-cell/Pan})}$) and total volume (V $_{(\beta\text{-cell, Pan})}$) of beta-cells in the pancreas; **(C/D)** volume density (Vv $_{(\text{iso}\beta\text{-cell/Pan})}$) and total volume (V $_{(\text{iso}\beta\text{-cell, Pan})}$) of isolated beta-cells in the pancreas; **(E)** mean absolute pancreas weight; **(F)** total beta-cell volume related to body weight (V $_{(\beta\text{-cell, Pan})}$ / BW); L = liraglutide-treated animals, P = placebo-treated animals, n = number of animals investigated. Data are means ± SEM.

5.2.2. Evaluation of the beta-cell volume in the therapeutic group

The trend for lower volume density and total volume of beta-cells in the pancreas of liraglutide-treated pigs observed in the prophylactic group at five months of age did reach significance in the therapeutic group at eight months of age: Eight-month-old liraglutide-treated animals (n=5) showed a 32% ($p<0.01$) reduction in $Vv_{(\beta\text{-cell/Pan})}$ and a 44% ($p<0.01$) reduction of $V_{(\beta\text{-cell, Pan})}$ in comparison to placebo-treated pigs (n=6). In accordance with the findings in the prophylactic group there was no significant difference of $Vv_{(iso\beta\text{-cell/Pan})}$ ($p=0.44$) and $V_{(iso\beta\text{-cell,Pan})}$ ($p=0.67$) between liraglutide- and placebo-treated pigs. At the day of necropsy liraglutide-treated pigs in the therapeutic group also showed distinct less body weight (-41%) than placebo-treated animals. Therefore also in this group the total beta cell volume was related to body weight ($V_{(\beta\text{-cell, Pan})}$ / BW), which resulted in the loss of significant differences between liraglutide- and placebo-treated animals and similar values for $V_{(\beta\text{-cell, Pan})}$ / BW ($p=0.67$) (Figure 24).

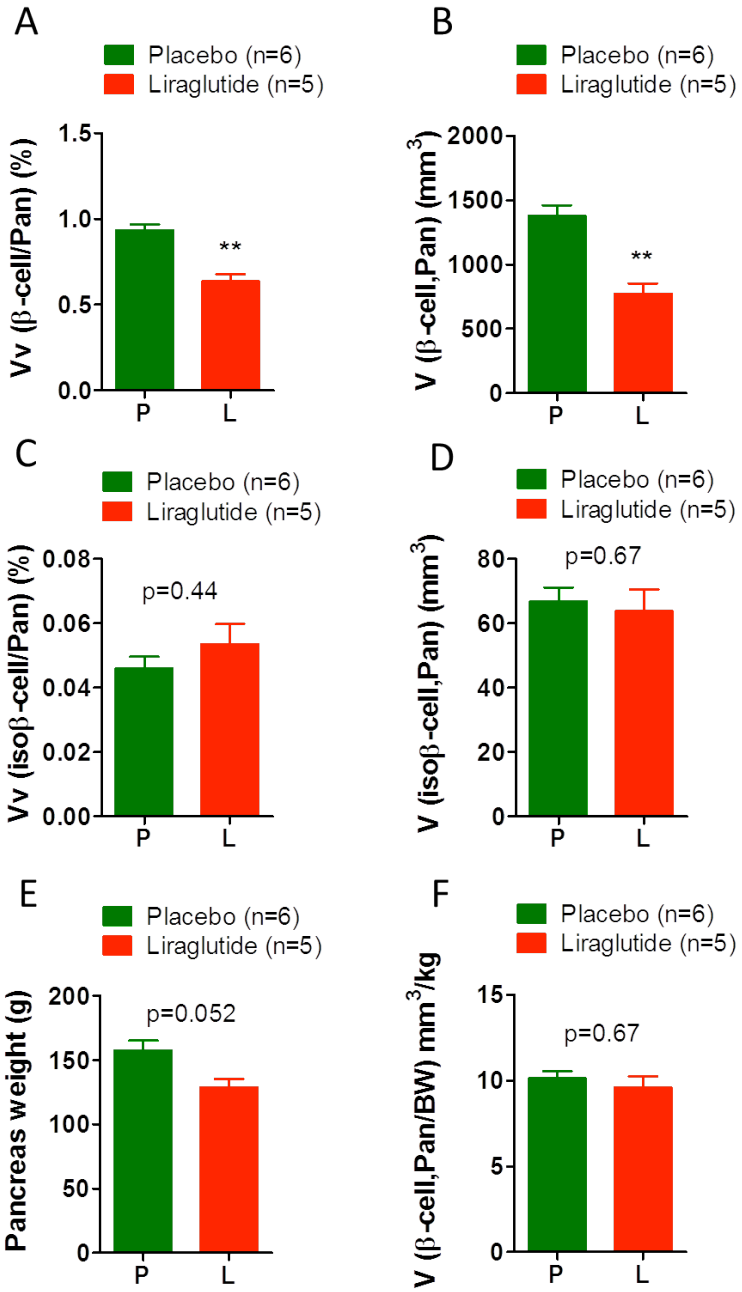

Figure 24: Quantitative-stereological analyses of pancreata from GIPR^{dn} transgenic pigs of the therapeutic group

(A/B) Volume density (Vv $_{(\beta\text{-cell/Pan})}$) and total volume (V $_{(\beta\text{-cell, Pan})}$) of beta cells in the pancreas; **(C/D)** volume density (Vv $_{(iso\beta\text{-cell/Pan})}$) and total volume (V $_{(iso\beta\text{-cell, Pan})}$) of isolated beta cells in the pancreas; **(E)** mean absolute pancreas weight; **(F)** total beta-cell volume related to body weight (V $_{(\beta\text{-cell, Pan})}$ / BW); L = liraglutide-treated animals, P = placebo-treated animals; n = number of animals investigated. Data are means ± SEM; **: $p<0.01$.

5.3. Unchanged nuclear profile count in liraglutide- and placebo-treated GIPR^{dn} transgenic pigs

Due to the knowledge that liraglutide-treated pigs of the prophylactic group showed a strong tendency of decreased total beta-cell volume ($V_{(\beta\text{-cell, Pan})}$) that even got significant in the therapeutic group, nuclear profile counting was carried out to get an idea whether augmented cell division (hyperplasia) or rather enlargement of the cells (hypertrophy) can be detected in placebo-treated animals. Therefore, tissue slides of randomly chosen liraglutide- (n=4) and placebo-treated animals (n=4) of the prophylactic group were immunohistochemically stained for insulin using DAB as chromogen combined with strong nuclear staining using haemalum and subsequently beta-cell nuclei were manually counted within insulin-positive stained area of 100 islets per animal. Nuclear profile counting revealed no significant differences between liraglutide- and placebo-treated animals (p=0.34) (Figure 25).

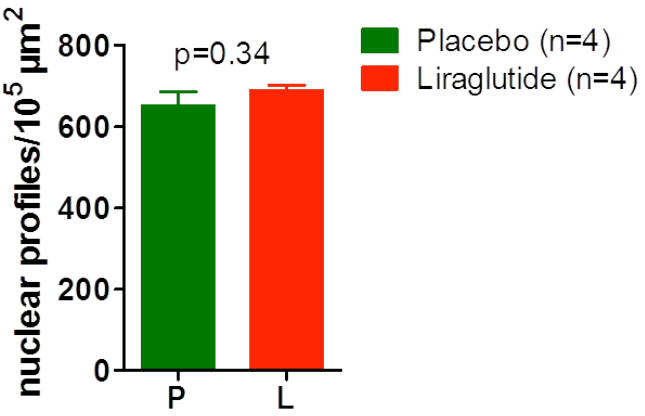

Figure 25: Unchanged nuclear profile count in GIPR^{dn} transgenic pigs of the prophylactic group

Nuclear profile count of liraglutide-treated (L) in comparison to placebo-treated (P) GIPR^{dn} transgenic pigs, n = number of animals investigated, Data are means ± SEM

5.4. Influence of liraglutide treatment on gastric emptying in GIPR^{dn} transgenic pigs

Prior to necropsy, five liraglutide-treated and four placebo-treated pigs of the prophylactic as well as the therapeutic group were fasted for 6 hours, then 1 kg of food was offered for one hour, followed by another 12-hour fasting period until pigs were euthanized. Two hours before necropsy, daily liraglutide and placebo treatment was accomplished, i.e. 1.2 mg (prophylactic group) and 1.8 mg liraglutide (therapeutic group) or the corresponding volume of placebo was subcutaneously injected. After euthanasia, the stomach was opened and content was examined. Differences between the two treatment types were clearly visible: While stomach of placebo-treated pigs was empty or only filled with liquid, stomach content of liraglutide-treated pigs still contained large amounts of remaining food as well as small amounts of liquid.

5.5. Organ weights of liraglutide- and placebo-treated GIPR^{dn} transgenic pigs

With the knowledge that liraglutide-treated pigs of both groups showed distinctly less body weight compared to placebo-treated animals at the end of treatment period, organs were weighed during necropsy for the calculation of absolute and relative organ weights. In the prophylactic group significantly lower absolute organ weights in liraglutide-treated pigs (n=9) could be detected for the kidneys ($p<0.001$), heart ($p<0.001$), spleen ($p<0.01$) and liver ($p<0.01$) in comparison to placebo-treated animals (n=9). Pancreas, stomach and lung also showed this tendency but without reaching significance. When related to body weight, liraglutide-treated pigs in this group showed significantly higher relative organ weights for pancreas ($p<0.001$), stomach ($p<0.05$) and spleen ($p<0.05$), while the remaining organs showed no significant differences. In the therapeutic group liraglutide-treated pigs (n=5) also showed lower absolute organ weights compared to placebo-treated animals (n=6) getting significant for the kidneys ($p<0.01$), heart ($p<0.01$), stomach ($p<0.05$) and liver ($p<0.05$). Regarding relative organ weights liraglutide-treated pigs showed a tendency of higher values. However, no significant differences could be detected compared to placebo-treated pigs (Figure 26).

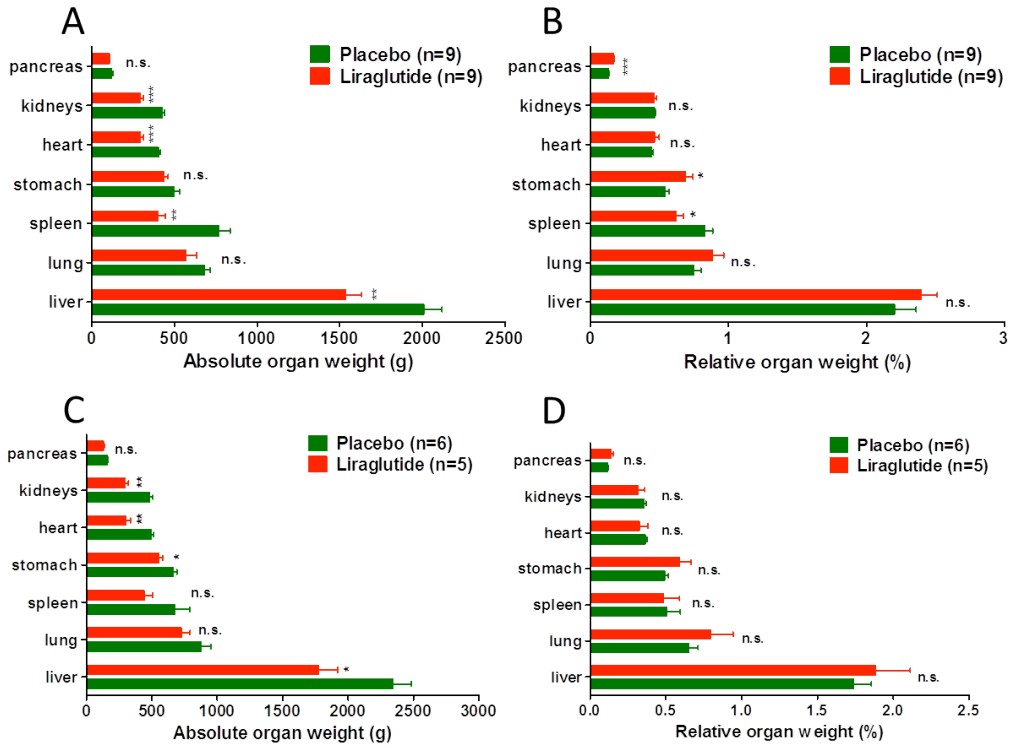

Figure 26: Organ weights of liraglutide- and placebo-treated GIPR^{dn} transgenic pigs

Absolute **(A,C)** and relative **(B,D)** organ weights determined in liraglutide- and placebo-treated GIPR^{dn} transgenic pigs of the prophylactic group **(A,B)** and the therapeutic group **(C,D)**; n = number of animals investigated; n.s. = not significant. Data are means ± SEM; *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

VI. DISCUSSION

The present study evaluated the effect of a 90-day treatment with the long-acting GLP-1R agonist liraglutide on glycemic control, body weight, food intake and the total beta-cell volume in adolescent $GIPR^{dn}$ transgenic pigs. This pig model shows key characteristics of a prediabetic state including an impaired function of the incretin hormone GIP, reduced glucose tolerance and insulin secretion as well as a progressive decline of the total beta-cell volume (Renner et al. 2010). Two different age groups were investigated during this study: The prophylactic group (n=18) was treated with liraglutide/placebo from 2 months (unaltered beta-cell volume) to 5 months of age to investigate if liraglutide would be able to cause a cessation of the beta-cell volume reduction or if it can even prophylactically prevent it. A second group (therapeutic group, n=11) was treated with liraglutide/placebo from 5 months (35% reduced beta-cell volume) to 8 months of age to see if liraglutide treatment can slow down the progressive reduction of the beta-cell volume or if it is even able to restore it.

1. Plasma liraglutide levels in $GIPR^{dn}$ transgenic pigs

Among the approved GLP-1R agonists on the market liraglutide under the brand name Victoza® was the drug of choice for this study because it seems to have the highest pharmacodynamic potency: Compared to exenatide twice daily (Byetta®) the pharmacokinetic profile of liraglutide makes it suitable for once daily administration and it maintains stable steady state concentrations over a time range of 24 hours leading to long-term blood glucose lowering effects with low rates of hypoglycemia in diabetic subjects (Blonde et al. 2009; Knudsen et al. 2000; Rosenstock 2009). In direct comparison to the maximum recommended dosage of exenatide (10 µg twice daily) the maximum recommended dosage of liraglutide (1.8 mg once daily) was able to decrease HbA_{1c} more effective in type 2 diabetic subjects over a treatment period of 26 weeks. Additionally, liraglutide caused less hypoglycemia as well as less persistent nausea and increased HOMA-β to a higher extent than exenatide, indicating greater improvement in beta-cell function (Buse et al. 2009). During an extension of this study the treatment of some patients was switched from exenatide to liraglutide, resulting in further improvement of glucose control (Buse et al. 2010). In a head-to-head trial over a

26 weeks treatment period liraglutide (1.8 mg once daily) also caused a greater decrease in HbA_{1c} compared to the long-acting exenatide formulation Bydureon® (2 mg once weekly) that was recently approved (Buse et al. 2013). These findings further demonstrate the great potential of liraglutide and underline the reasons for its use in the present study. To determine effective plasma liraglutide levels in the liraglutide-treated $GIPR^{dn}$ transgenic pigs included in this study a profile was performed at the end of the 90-day treatment period. Within the five time-points where blood samples were taken (0, 4, 8, 12, 16 hours relative to liraglutide administration) the animals showed relatively constant course of liraglutide plasma level. The highest mean liraglutide plasma levels were 52.3 nmol/l after dosing of 1.8 mg liraglutide (therapeutic group) and 43.2 nmol/l after dosing of 1.2 mg liraglutide (prophylactic group). Although blood sampling during the profile was insufficiently frequent to determine a real peak concentration, the highest liraglutide plasma concentrations were measured in the blood sample 8 hours after dosing. This is similar to the findings in human clinical trials, where liraglutide concentrations peaked within 9-14 hours after dosing (Agerso et al. 2002; Elbrond et al. 2002). In humans it was reported that an injection of 0.6 mg liraglutide causes peak concentrations of about 9.4 nmol/l that seem to increase proportionally with dose in the therapeutic range of 0.6 mg to 1.8 mg liraglutide (Agerso et al. 2002; EMEA 2009b). Although Rosenstock et al. measured maximum concentrations of only 17 nmol/l after subcutaneous administration of 1.8 mg liraglutide in humans, the European Medicine Agency assessed maximum concentrations of 44.7 nmol/l (EMEA 2009a; Rosenstock et al. 2009). Average steady state concentrations over 24 hours after 1.8 mg liraglutide administration were reported to be approximately 34 nmol/l (EMEA 2009b; Novo Nordisk 2010). Compared with the data from humans, pigs included in this study showed higher effective plasma levels of liraglutide after administration of similar dosages. This may partly be explained by the higher overall bioavailability of 76% in pigs compared to the human (55%). The upper tolerance limit of liraglutide dosage is not known for pigs, but during chronic repeat-dose toxicity studies in rats or monkeys liraglutide was still well tolerated when reaching 8- or 70-fold higher plasma levels compared to the clinical exposure (EMEA 2009a). In previous studies with porcine models lower dosages of liraglutide (3.3 µg/kg and 7 µg/kg) were used compared to the present study (Raun et al. 2007b; Ribel et al. 2002). One study reported that Yucutan miniature pigs seem to be particularly

sensitive to liraglutide (Emamaullee et al. 2009). However, the higher liraglutide dosage that is also recommended for humans and the following greater liraglutide plasma levels did not seem to influence the general health status of the pigs included in this study as they showed normal behavior and undisturbed condition throughout the whole treatment period. Furthermore, no adverse effects like vomiting, obstipation and diarrhea were obvious. No signs of nausea were observed, although it has to be mentioned that the assessment of malaise in a pig model is difficult and may be overlooked. Most serum parameters (except total protein, albumin, phosphorus, calcium, AP; see below) were not significantly different between liraglutide- and placebo-treated GIPR^{dn} transgenic pigs and additionally stayed within reference ranges (Kixmöller 2004; Kraft 2005).

2. Dramatic food intake and body weight gain reducing effects due to liraglutide treatment

2.1. Reduced food intake and feeding efficiency in liraglutide-treated GIPR^{dn} transgenic pigs

Liraglutide treatment had a strong and sustained effect on *ad libitum* feeding behavior: Straight from the beginning of the therapy food intake was significantly decreased in liraglutide-treated GIPR^{dn} transgenic pigs with reductions of 20-35% in the prophylactic group and even 40-50% in the therapeutic group compared to placebo-treated GIPR^{dn} transgenic animals. This chronic reduction of nutrient intake may also serve as an explanation for the significant reduction of total serum protein and serum albumin observed in liraglutide-treated GIPR^{dn} transgenic pigs compared to placebo-treated ones. A food intake reducing effect during liraglutide treatment was also shown in studies with normal, obese and diabetic rodents (Larsen et al. 2001b; Larsen et al. 2008; Raun et al. 2007a; Sturis et al. 2003) as well as in hyperphagic adult Göttingen minipigs. These minipigs even showed a sustained decrease in food intake of approximately 60% although they were treated with a lower dosage of liraglutide (7 µg/kg) compared to this study (Raun et al. 2007b). Furthermore clinical studies with type 2 diabetic human patients also showed that liraglutide treatment provoked improved eating behavior going along with less energy intake with one study reporting an *ad libitum* energy intake reduction between 9% to 15% after liraglutide treatment (Fujishima et al. 2012; Horowitz et al. 2012; Horowitz 2008). One of the underlying mechanisms for this

effect on feeding behavior is certainly the ability of liraglutide as a GLP-1R agonist to delay gastric emptying and thereby increase the feeling of fullness and satiety (Horowitz et al. 2012; Ribel et al. 2002). A recent study showed that the effect of delayed gastric emptying was clearly reduced in rats after 14 days of liraglutide treatment compared to treatment day 1, maybe due to tachyphylaxis or GLP-1R desensitization, suggesting that reduced gastric emptying only mediates acute effects on food intake and body weight while other factors mainly seem to contribute to further chronic effects (Jelsing et al. 2012a). In the present study it was observed at the end of the 90-day treatment period that the stomach of liraglutide-treated GIPR^{dn} transgenic pigs still contained large amounts of remaining food after a fasting period of 12 hours. In comparison, the stomach of placebo-treated GIPR^{dn} transgenic pigs was empty or only fluid filled. Although these observations were not quantified, they clearly argue for a delayed gastric emptying in liraglutide-treated GIPR^{dn} transgenic pigs. A similar finding was also reported in a study with Göttingen minipigs after 4 weeks of liraglutide treatment (Ribel et al. 2002). However, it is still likely that further factors like stimulation of GLP-1R within energy-intake regulating regions of the central nervous system (e.g. in the brainstem or hypothalamus) and the occurrence of nausea as adverse effect also contribute to the food intake reducing effects of liraglutide (Flint et al. 1998; Jelsing et al. 2012a; Meece 2009). Liraglutide-treated GIPR^{dn} transgenic pigs also showed significant decreases in feeding efficiency by 12%-32% compared to placebo-treated GIPR^{dn} transgenic pigs, indicating a lower body weight gain per food intake. This could be indicative for an increase in energy expenditure by liraglutide treatment which was also seen in candy-fed obese rats and human type 2 diabetic subjects, although it did not reach significance in these two studies (Horowitz et al. 2012; Raun et al. 2007a). An increase of energy expenditure in liraglutide-treated rats was also suggested as it was observed that liraglutide-treated animals developed similar body weights compared to a restricted-fed control group with significant lower food intake (Cummings et al. 2010). However, no effects on 24-hour energy expenditure where detected in a study with type 2 diabetic patients but it has to be mentioned that only the lowest recommended dosage of liraglutide (0.6 mg) was used for treatment in this case (Harder et al. 2004).

2.2. Reduced body weight gain in liraglutide-treated GIPR^{dn} transgenic pigs

Liraglutide-treated GIPR^{dn} transgenic pigs gained significantly less weight compared to placebo-treated ones during the whole 90-day treatment period. This finding was progressive and resulted in a body weight decrease by 31% in the prophylactic group and even 41% in the therapeutic group compared to placebo-treated pigs at the end of liraglutide therapy. Body composition of the pigs was not determined in the present study so that no statement can be made about what tissues were reduced the most. Absolute organ weights of liraglutide-treated GIPR^{dn} transgenic pigs were decreased compared to their placebo-treated counterparts, while relative organ weights mainly did not show significant differences. This fact suggests that liraglutide had a generalized inhibitory effect on whole body and organ growth. Furthermore, liraglutide-treated GIPR^{dn} transgenic pigs of both groups showed significantly reduced serum levels of phosphorus going along with significantly reduced calcium levels in the therapeutic group and increased levels of AP in the prophylactic group. Calcium and phosphorus are major constituents of bone and it is known that an insufficient mineralization of the organic bone matrix, as for example seen during osteomalacia/rachitis, can go along with increased activity of AP in the blood (Kixmöller 2004; Kraft 2005; Plonait 1980). These findings could suggest a liraglutide associated disturbance of bone metabolism, but it has to be mentioned that AP is an enzyme that is influenced by age, nutrition as well as other organ systems like the liver (Kraft 2005; Plonait 1980). Furthermore, the parameters calcium, phosphorus and AP were significantly different between liraglutide- and placebo-treated GIPR^{dn} transgenic pigs, but they were within reference ranges for pigs (Kixmöller 2004; Kraft 2005). Nevertheless future studies on the effects of liraglutide in adolescents should include a detailed analysis of bone growth and structural characteristics. Body weight lowering effects caused by liraglutide treatment are a consistent finding both during studies in animal models as well as in clinical human trials with type 2 diabetic subjects like the extensive LEAD studies 2-6 (Blonde et al. 2009; Larsen et al. 2001b; Larsen et al. 2008; Raun et al. 2007a; Raun et al. 2007b). An exception was the study LEAD-1 where liraglutide in combination with glimepiride showed weight neutral effects (Marre et al. 2009). A big part of this body weight reducing effect is definitely mediated by the lower food intake caused by liraglutide treatment (see VI 2.1). However, studies

with rats showed that chronic liraglutide treatment over 12 weeks only caused short-term food intake reducing effects, while the body weight reducing effect was nevertheless sustained, indicating that other mechanisms might also be responsible for the weight loss (Guo et al. 2013). Although the reduction of food intake in the present study was sustained, further reasons for the differences in body weight also have to be considered regarding that body weight was so dramatically diverging by 31% (prophylactic group) and 41% (therapeutic group) in liraglutide-treated GIPR^{dn} transgenic pigs compared to placebo treatment. In comparison, liraglutide monotherapy with similar dosages as used in this study (1.2 mg or 1.8 mg) over 52 weeks in type 2 diabetic subjects caused mean weight loss of 2.1 kg to 2.5 kg compared to the beginning of treatment from a baseline that was about 92 kg. Therefore, this means a body weight reduction of only about 2.2% to 2.5% (Garber et al. 2009). Compared with this data the inhibition of body weight gain in liraglutide-treated GIPR^{dn} transgenic pigs included in this study was much higher. This may at least in part be related to the fact that treated patients were adult, while the treated GIPR^{dn} transgenic pigs in the present study were adolescent. A number of hypotheses about the potentially involved mechanisms are discussed below.

2.2.1. Reduced secretion of the anabolic hormone insulin

Liraglutide treatment caused a significant reduction of insulin secretion during a mixed meal oral glucose tolerance test in GIPR^{dn} transgenic pigs compared to placebo treatment. AUC insulin was reduced by 36% in liraglutide-treated GIPR^{dn} transgenic pigs in the prophylactic group (5 months of age) and by 70% in the therapeutic group (8 months of age) as compared to age-matched placebo-treated GIPR^{dn} transgenic pigs. As a mixed meal oral glucose tolerance test mimics normal food intake very well, it can be assumed that liraglutide-treated GIPR^{dn} transgenic pigs in the present study generally show much lower insulin secretion without real peaks during their normal feeding behavior when compared to placebo-treated animals. Insulin is known to be a hormone with strong anabolic and growth-promoting properties as it can *inter alia* directly stimulate protein synthesis as well as reduce proteolysis in different tissues including skeletal muscle cells and indirectly influences the IGF-I hormone system (Demling 2005; Fujita et al. 2006; Hill et al. 1985; Straus 1984). The impact of a lack of insulin can be seen in the growth retardation of children in which diabetes was diagnosed

around puberty and insulin therapy is insufficient (Edelsten et al. 1981; Salardi et al. 1987; Tattersall et al. 1973). GIPR^{dn} transgenic pigs used in this study also were adolescent and in a period of rapid growth. Therefore, it could be assumed that the reduced insulin secretion followed by lower anabolic function of this hormone in the liraglutide-treated GIPR^{dn} transgenic pigs may additionally have mediated the massive body weight gain reducing effects. This would be in accordance with the findings in a previously described transgenic pig model of permanent neonatal diabetes that displayed significantly reduced fasting insulin levels at the age of 4.5 months and showed a distinct concurrent body weight reduction of about 40% in comparison to non-transgenic control littermates (Renner et al. 2012).

2.2.2. No effect of liraglutide on the insulin-like growth factor system

To evaluate the effect of liraglutide treatment on other growth-regulating peptides, serum levels of IGF-I, IGFBP-2 and -3 were measured before and after liraglutide/placebo treatment, in a randomly chosen subset of GIPR^{dn} transgenic pigs respectively. IGF-I is a growth-promoting hormone that is released particularly by the liver mainly due to stimulation by growth hormone (GH), but also insulin can stimulate IGF-I secretion (Holt et al. 2008; Straus 1984). In the circulation IGF-I is mainly bound to high-affinity IGFBPs that regulate its bioavailability. IGFBP-2 is known to rather inhibit IGF-I action and thereby acts growth-inhibiting (Hoeflich et al. 2001; Hoeflich et al. 1999; Jones et al. 1995). IGFBP-3 is the binding protein where most of IGF-I in the circulation is bound to in a ternary complex with an acid-labile subunit (ALS) (Holman et al. 1996; Jones et al. 1995). Liraglutide treatment did not seem to influence the serum levels of these peptides, as the ratio of IGF-I, IGFBP-2 and -3 serum levels after vs. before therapy, indicating the degree of alteration during the treatment period, was not significantly different between liraglutide- and placebo-treated GIPR^{dn} transgenic pigs of the prophylactic and the therapeutic trial. Thus, it seems unlikely that an involvement of these components of the insulin-like growth factor system contributed to the marked decrease in weight gain observed in adolescent liraglutide-treated GIPR^{dn} transgenic pigs.

2.2.3. Somatostatin levels in GIPR^{dn} transgenic pigs

Somatostatin is a peptide hormone that is also involved in the growth-regulating

system: It is *inter alia* secreted from delta-cells within the endocrine pancreas as well as the hypothalamus and functions as an inhibitor of the release of GH, thereby showing growth-inhibiting function (Barnett 2003; Hauge-Evans et al. 2009; Kumar et al. 2010). Somatostatin is additionally able to inhibit insulin as well as glucagon secretion (Hauge-Evans et al. 2009). It was shown in rat and porcine isolated perfused pancreata as well as in cell lines that native GLP-1 has the ability to stimulate somatostatin release (Brubaker et al. 1997; Fehmann et al. 1995; Fehmann et al. 1991; Orskov et al. 1988; Schmid et al. 1990), and similar findings were also reported for the GLP-1R agonist exendin-4 (Egido et al. 2004; Silvestre et al. 2003). In the present study the ratio of somatostatin plasma levels after vs. before therapy did not differ between liraglutide- and placebo-treated $GIPR^{dn}$ transgenic pigs of the prophylactic group, while liraglutide-treated $GIPR^{dn}$ transgenic pigs of the therapeutic group showed a higher post-treatment/pre-treatment ratio. However, the level of significance was low ($p=0.034$) and only a subset of 4 liraglutide- and 4 placebo-treated $GIPR^{dn}$ transgenic pigs was investigated. When the mean concentrations before therapy as well as after therapy were directly compared between liraglutide- and placebo-treated $GIPR^{dn}$ transgenic pigs without calculating the ratio, no significant differences were observed. Thus, a stimulation of somatostatin secretion by liraglutide treatment with a following growth-inhibiting effect seems unlikely.

2.2.4. Liraglutide dosage recommended for humans in adolescent $GIPR^{dn}$ transgenic pigs

In this study the recommended human dosages of Victoza® from 0.6 mg up to 1.8 mg per day (corresponding to 19-33 µg/kg in the prophylactic group and 10-25 µg/kg in the therapeutic group) were used for the treatment of $GIPR^{dn}$ transgenic pigs. Previous studies investigating the effect of liraglutide treatment in minipig models used lower dosages of liraglutide (3.3 µg/kg, 7 µg/kg, 20 µg/kg) (Emamaullee et al. 2009; Raun et al. 2007b; Ribel et al. 2002). Although $GIPR^{dn}$ transgenic pigs showed undisturbed condition and behavior during liraglutide treatment it cannot be excluded that the higher dosage of Victoza® may have contributed to the massive body weight gain reducing effect. Another difference compared to previous studies including rodent models, porcine models and also the clinical human trials is the use of adolescent pigs in a phase of rapid growth and weight gain. Thus, it is possible that liraglutide treatment (particularly when

the recommended human dosage is used) has a greater influence on growth and body weight gain in the adolescent organisms compared to adult subjects. This could also serve as an explanation for a study in which it was shown that liraglutide treatment (7 µg/kg) caused an even stronger reducing effect on food intake compared to this study, but still only body weight reductions of 4-5% in 18-month-old adult minipigs (Raun et al. 2007b). Another study also reported less body weight loss of about 20% in liraglutide-treated adult Yucutan miniature pigs during a treatment period of 6 weeks with up to 20 µg liraglutide/kg body weight for 60 days (Emamaullee et al. 2009). At the moment, Victoza® is only tested and approved for the use in adult type 2 diabetic subjects while appropriate data are lacking about its effect in adolescents suffering from type 2 diabetes (EMEA 2009b; Flint et al. 2011; Novo Nordisk 2010). One recent study reported that mean body weight in type 2 diabetic adolescent subjects at a mean age of 14.8 years after a 5-week liraglutide treatment with dosages up to 1.8 mg remained stable (Batellino 2012). In contrast to the present study the participants of the study of Batellino seemed to be obese as they showed a mean body weight of 113.2 kg. Additionally, it has to be taken into account that in comparison to the 90-day treatment period of the present study in adolescent pigs, that can be seen as a model of accelerated growth and development, a 5-week treatment in slower growing adolescent humans may not have been long enough to detect influences on body weight gain (Litten-Brown et al. 2010; Puiman et al. 2008). Adequate pharmacotherapy for the treatment of adolescent type 2 diabetic patients is urgently needed given the facts that the prevalence of younger people suffering from type 2 diabetes is steadily increasing and that only few pharmacological agents are approved for this indication (Bloomgarden 2004; Flint et al. 2011; George et al. 2013). Furthermore, GLP-1R agonists like liraglutide and exenatide are gaining more and more interest to be evaluated as possible drugs for the treatment of pediatric obesity because of their weight-reducing effect (Kelly et al. 2012; Kelly et al. 2013; Sherafat-Kazemzadeh et al. 2013; Wald et al. 2009). Due to the findings in the present study, caution is warranted during prospective long-term treatment trials in adolescent subjects and liraglutide dosages should be carefully evaluated and adjusted as the treatment may not only have a much more pronounced effect on body weight than in adult patients but also impair body and organ growth.

3. Effects of liraglutide treatment on glucose control in GIPR^{dn} transgenic pigs

The effect of liraglutide on glucose control in GIPR^{dn} transgenic pigs was evaluated by performing oral as well as intravenous glucose tolerance tests in animals after an 18-hour fasting period prior to and after the treatment period. Furthermore, fasting glucose levels were investigated regularly during the treatment period.

3.1. Improved glucose tolerance and insulin sensitivity in liraglutide-treated GIPR^{dn} transgenic pigs

During oral glucose tolerance testing after the 90-day treatment period liraglutide-treated GIPR^{dn} transgenic pigs showed 22-27% reduced AUC glucose compared to placebo-treated GIPR^{dn} transgenic pigs. AUC glucose was also significantly reduced by 15-22% during intravenous glucose tolerance tests. Thus, liraglutide treatment led to significant improvement of oral as well as intravenous glucose tolerance. During the oral glucose tolerance test pigs were fed a mixed meal consisting of commercial pig fodder and 50% glucose solution. Therefore the lower glucose values in the liraglutide-treated GIPR^{dn} transgenic pigs compared to the placebo-treated ones can partly be explained by the known ability of liraglutide to delay gastric emptying with further delayed intestinal absorption and appearance of glucose in the circulation (Baggio et al. 2007; Ribel et al. 2002). However, intravenous glucose tolerance is not influenced by gastric emptying time and still was enhanced. Therefore it is likely that the improvement in glucose tolerance was additionally mediated by improved insulin sensitivity. The calculation of different insulin sensitivity indices including HOMA-IR, QUICKI and ISI $_{(Matsuda)}$ clearly showed that liraglutide treatment significantly improved insulin sensitivity compared to placebo treatment, which was also a frequent finding of studies in rodent and pig models (Cummings et al. 2010; Guo et al. 2013; Li et al. 2011; Raun et al. 2007a; Ribel et al. 2002; Sturis et al. 2003; Yang et al. 2012). Increased fat mass is known to be associated with augmented release of fatty acids as well as inflammatory cytokines which all together supports the development of insulin resistance (Kahn et al. 2006). Therefore, the improvement in insulin sensitivity may partly be explained by the ability of liraglutide to decrease food intake and cause weight reduction going along with a loss of fat

mass (Raun et al. 2007a). Consequently, liraglutide treatment in rodents was also shown to be associated with significantly lower triglyceride levels in serum as well as in the liver (Cummings et al. 2010; Guo et al. 2013). It was also suggested that liraglutide treatment may increase lipid oxidation, as liraglutide-treated rats showed similar body weight compared to restricted fed rats but nevertheless had a lower amount of body fat (Cummings et al. 2010). Some studies also reported that liraglutide decreased some inflammatory biomarkers, although the results were not completely consistent (Courreges et al. 2008; Cummings et al. 2010; Guo et al. 2013; Parthsarathy et al. 2013). The mechanism of liraglutide to improve insulin sensitivity is complex and seems to include further effects than just the consequences of a reduced food intake and body weight, as it was e.g. shown that liraglutide-treated rats show lower fasting plasma insulin concentrations in comparison to non-treated restricted fed rats while both groups showed similar fasting glucose levels (Cummings et al. 2010). Further mechanisms influencing insulin sensitivity could involve direct effects of liraglutide on the liver, adipocytes and muscle cells that have also been described for native GLP-1 and would result in decreased hepatic glucose production and increased peripheral glucose uptake and storage (Abu-Hamdah et al. 2009; Burcelin et al. 2001; Cummings et al. 2010). This would be in line with a recent study that showed the ability of liraglutide to influence gene and protein expression in the liver as well as adipose tissue and thereby to improve the state of insulin resistance in a murine animal model (Li et al. 2011).

3.2. Reduced insulin secretion in liraglutide-treated GIPR^{dn} transgenic pigs

In the present study liraglutide treatment caused a significant reduction of insulin secretion during a mixed meal oral glucose tolerance test in GIPR^{dn} transgenic pigs compared to placebo treatment. At first sight it might be confusing that liraglutide, as an analogue of the potent insulinotropic hormone GLP-1, decreases insulin secretion. However, it has to be taken into account that the insulinotropic action of GLP-1 is strictly dependent on a certain threshold of glucose concentration and that insulin secretion is not or only slightly stimulated if blood glucose is around the normal fasting range (Nauck et al. 1993c; Qualmann et al. 1995). Regarding the marginal elevation of blood glucose during oral glucose tolerance testing observed in liraglutide-treated GIPR^{dn} transgenic pigs after the

treatment period with maximum concentration of only up to 124 mg/dl it is comprehensible that liraglutide did not act insulinotropic in this case. Furthermore, low blood glucose levels themselves lower the demand for insulin within the organism, independent of liraglutide. The fact that lower postprandial glucose levels mainly caused by the delayed gastric emptying are going along with also reduced postprandial insulin secretion has already been shown after administration of native GLP in humans (Meier et al. 2003; Nauck et al. 1997). A study of Ribel et al. reported an improvement of oral glucose tolerance after liraglutide treatment in hyperglycemic minipigs going along with no significant changes in insulin secretion, but this is not completely comparable to the present study as these minipigs showed an overall streptozotocin-related impaired insulin secretion and glucose was administered mixed with a smaller amount of pig fodder (Ribel et al. 2002). During oral glucose tolerance tests in rodents, it has to be taken into account that glucose is usually administered by gavage without any food, which may in turn influence glucose levels and the following insulin response by bypassing a delayed gastric emptying. Various results of liraglutide treatment on insulin secretion were detected during oral glucose tolerance tests in diabetic and non-diabetic rodents, underlining that effects of liraglutide on insulin secretion depend on glucose concentrations (Larsen et al. 2008; Raun et al. 2007a; Sturis et al. 2003). During the intravenous glucose tolerance test performed in the therapeutic group after liraglutide treatment, AUC insulin was decreased by 46% in comparison to placebo treatment. Together with the finding of also significantly lower blood glucose levels during the test this may be caused by the improved insulin sensitivity. However this is not in accordance with the results seen in the prophylactic group during the intravenous glucose tolerance test after the treatment period, because in this case glucose levels were also significantly lower but insulin secretion was not changed in liraglutide-treated compared to placebo-treated $GIPR^{dn}$ transgenic pigs. Altogether it has to be mentioned that individual insulin values during intravenous glucose tolerance tests showed high variance and are therefore difficult to interpret. During the initial characterization of the $GIPR^{dn}$ transgenic pig model Renner et al. observed that these animals *inter alia* show reduced insulin secretion during glucose tolerance testing, which was confirmed in the present study, as insulin secretion was strongly reduced compared to wild-type pigs investigated in a previous study (Renner et al. 2010). This demonstrates consistent phenotypic changes in this animal model over

several generations (F4 in the present study).

3.3. Unchanged fasting blood glucose in liraglutide-treated GIPR^{dn} transgenic pigs

During the whole treatment period there were no significant differences seen in fasting blood glucose levels between liraglutide- and placebo-treated GIPR^{dn} transgenic pigs. Additionally, fasting blood glucose did not exceed the normoglycemic range (Kixmöller 2004; Kraft 2005). During the characterization of the GIPR^{dn} transgenic pig model it was already shown that these pigs show unaltered fasting blood glucose levels at least during the determined time range up to the age of 2 years (Renner et al. 2010). An effect of liraglutide on fasting blood glucose levels in the GIPR^{dn} transgenic pig model was therefore not expected because its insulinotropic effect is glucose-dependent (Nauck et al. 1993c; Qualmann et al. 1995). This is also in accordance with studies that show a potent basal blood glucose lowering effect of liraglutide in hyperglycemic rodent models, but not in normoglycemic rodent models (Bock et al. 2003b; Larsen et al. 2008; Rolin et al. 2002; Shimoda et al. 2011; Sturis et al. 2003; Vrang et al. 2012).

4. Decreased total beta-cell volume in liraglutide-treated GIPR^{dn} transgenic pigs

In the present study, liraglutide-treated GIPR^{dn} transgenic pigs showed decreased volume density and total volume of beta-cells in the pancreas compared to placebo-treated GIPR^{dn} transgenic pigs. Although this finding was not significant in animals of the prophylactic group at an age of 5 months, a strong tendency was determined. Within the therapeutic group at an age of 8 months the reduction of volume density as well as total volume of beta-cells in the pancreas was significant. Nuclear profile counting within pancreatic sections suggested that the reduced beta-cell volume in liraglutide-treated animals is rather due to hypotrophy than hypoplasia compared to placebo-treated animals. The total volume of isolated beta-cells in the pancreas, a potential marker for islet neogenesis (Bouwens et al. 1998; Xu et al. 1999), did not show significant differences between liraglutide- and placebo-treated animals. Additionally, HOMA-β that can be interpreted as an index for beta-cell function (Matthews et al. 1985) was not significantly different between liraglutide- and placebo-treated GIPR^{dn} transgenic

pigs and no histological differences were seen regarding insulin staining intensity and pattern in the pancreas. It is difficult to compare these results to other studies that used porcine models, as only one study partly evaluated the *in vivo* effect of liraglutide on the beta-cells in pigs and this study was performed under completely different conditions compared to the present study: After pancreatico-duodenectomized Yucutan miniature pigs received a graft of isolated pancreatic islets into the portal circulation they were treated with liraglutide for 60 days and the proportion of beta-cells, determined only one year after transplantation, was not different between liraglutide- and placebo-treated pigs (Emamaullee et al. 2009). In contrast to the present study, an increase of HOMA-β was frequently observed during clinical trials in type 2 diabetic patients and therefore an improvement of beta-cell function was suggested (Blonde et al. 2009; Garber 2011). Beside these findings, data about the actual *in vivo* effect of liraglutide and other GLP-1R agonists on the beta-cells volume in humans are lacking because there are still no appropriate non-invasive imaging techniques for the quantification and evaluation of the human beta-cell volume available (Malaisse 2005). However, the results of the present study regarding the beta-cell volume are not in accordance with *in vivo* studies that showed the ability of liraglutide to increase the beta-cell volume in rodent models, associated with enhanced beta-cell proliferation and/or decreased beta-cell apoptosis rate (Rolin et al. 2002; Shimoda et al. 2011; Sturis et al. 2003; Vrang et al. 2012). *In vitro* studies using rodent or porcine islet cells also determined a pro-proliferative and anti-apoptotic effect of liraglutide and showed that it can improve the survival of human islets in culture (Bregenholt et al. 2005; Emamaullee et al. 2009; Friedrichsen et al. 2006; Miao et al. 2013; Toso et al. 2010). While translating the results from basic research of *in vivo* studies with rodent models to human clinical application two important points have to be kept in mind: First of all, the pancreas of rodents shows a much higher capacity for regeneration and proliferation compared to the human pancreas (Butler et al. 2007; Menge et al. 2008). Therefore it can be doubted if the liraglutide mediated increases of the total beta-cell volume in rodents are representative for the human organism. Second, it is well known that liraglutide shows a much shorter half-life in rodents (4-8 h) compared to humans (13-15 h) (EMEA 2009a). That is the reason why liraglutide is administered twice daily in most rodent studies compared to the suitability of a once daily administration in humans, which all together results in different exposure times and plasma

liraglutide levels that may additionally influence the effect of liraglutide (Knudsen et al. 2000; Raun et al. 2007a; Sturis et al. 2003). Regarding these facts the pig that shows similar liraglutide half-life (14 h) compared to the human and additionally higher physiologic and pathophysiologic similarities can be seen as a more representative model (EMEA 2009a; Swindle et al. 2012). Furthermore, liraglutide did not increase the beta-cell volume in all studies using rodent models as there are also published reports where beta-cell volume was unaltered or even lowered after liraglutide treatment (Brand et al. 2009; Guo et al. 2013; Larsen et al. 2008; Raun et al. 2007a; Rolin et al. 2002; Sturis et al. 2003). Some authors suggested that the trophic effect of liraglutide on beta-cells may be dependent on the individual metabolic status of the animals and only occurs when hyperglycemia is concomitantly evident within the organism (Knudsen 2010; Sturis et al. 2003). Indeed, in most of the studies an increase of beta-cell volume could be proven in hyperglycemic rodent models like diabetic ZDF rats or diabetic *db/db* mice (Rolin et al. 2002; Sturis et al. 2003; Vrang et al. 2012). However, also non-diabetic Sprague Dawley rats showed greater beta-cell volume after liraglutide treatment and liraglutide additionally increased beta-cell volume in normoglycemic *m/m* mice. It has to be mentioned that the duration of these two studies was rather short-term (1 week and 2 weeks) and that the effect seen in the Sprague Dawley rats was only temporary as it was vanished after 6 weeks of liraglutide treatment (Bock et al. 2003b; Shimoda et al. 2011). Furthermore, in other studies liraglutide treatment of hyperglycemic rodent models like severely diabetic ZDF rats failed to increase beta-cell volume as no differences were seen in comparison to placebo treatment (Brand et al. 2009; Larsen et al. 2008). Therefore the hypothesis that liraglutide continuously increases beta-cell volume in hyperglycemic rodent models but not in normoglycemic ones is partly disproved, maybe also underlined by the fact that it has been shown in studies of Buteau et al. for native GLP-1 in Ins-1 cell lines that the stimulation of cell proliferation, in comparison to its insulinotropic effect, seems not to be glucose dependent (Buteau et al. 2001; Buteau et al. 1999). The various results of the *in vivo* effect of liraglutide on beta-cells in rodent models suggest that a lot of factors like age, different metabolic status, duration of treatment and dosage may all together influence its presence and extent (Rolin et al. 2002). Additionally, liraglutide is able to potently improve glucose control as it acts insulinotropic in hyperglycemic subjects and decreases food intake also in normoglycemic subjects

(Blonde et al. 2009; Bock et al. 2003b; Larsen et al. 2008; Sturis et al. 2003). The improvement of glucose control could alleviate the destructive impact of glucotoxicity and lipotoxicity on beta-cells in diabetic subjects and this would in turn reduce the rate of beta-cell apoptosis and thereby save beta-cell volume (Federici et al. 2001; Leibowitz et al. 2001; Shimabukuro et al. 1998). This would mean that the beneficial impact of liraglutide on beta-cells shown in many studies is not completely mediated by direct trophic effects but also indirectly by improvements of glycemic control, which is difficult to differentiate. As the degree of liraglutide mediated improvement of glycemic control can vary between different diabetic rodent models and also within the same diabetic animal model dependent on the initial metabolic state this may partly explain the inconsistent results regarding its effect on the beta-cell volume observed in rodents (Larsen et al. 2008; Rolin et al. 2002; Vrang et al. 2012). For the calculation of the total beta-cell volume in the present study, pancreas weight was included, which showed a tendency to be decreased in liraglutide-treated $GIPR^{dn}$ transgenic pigs compared to placebo-treated ones in both groups, but no significance was reached. As liraglutide-treated $GIPR^{dn}$ transgenic pigs showed a drastic reduction of body weight gain compared to placebo-treated ones, the total beta-cell volume was also divided by the individual body weight of the pigs and this parameter ($V_{(\beta\text{-cell,Pan})}$/BW) neither showed significant differences in the prophylactic group nor in the therapeutic group. This is in accordance with previous reports describing a linear correlation between the beta-cell volume and the body weight in both rats and pigs (Bock et al. 2003a; Montanya et al. 2000). Increased body weight is only one factor among others like pregnancy or increased glucose and fat supply that amplify the demand for insulin within the organism and therefore can cause a compensatory increase of beta-cell volume to a certain extent (Bock et al. 2003a; Bonner-Weir et al. 1989; Bouwens et al. 2005; Steil et al. 2001). As liraglutide-treated $GIPR^{dn}$ transgenic pigs showed strongly reduced food intake up to 50% during *ad libitum* feeding in comparison to placebo-treated animals they also received less nutrients including glucose and fat. Therefore it might be considered that their demand for insulin as well as the stimulation of beta-cells is lower. This could serve as an explanation for the tendency of a decreased HOMA-β as an indicator for beta-cell function (Matthews et al. 1985) observed in liraglutide-treated animals in both groups compared to placebo-treated animals. Furthermore, it is likely that liraglutide did not have direct inhibiting effects on the beta-cells

but that the indirect effect mediating the reduced food intake and body weight is responsible for the decreased total beta-cell volume in liraglutide-treated GIPR^{dn} transgenic pigs. This would be in accordance with the fact that liraglutide-treated GIPR^{dn} transgenic pigs in the present study show very similar total beta-cell volume when compared to restrictive fed untreated GIPR^{dn} transgenic pigs in the study of Renner et al. (2010).

5. Concluding remarks and perspectives

In conclusion, a 90-day treatment period of adolescent GIPR^{dn} transgenic pigs with the long-acting GLP-1R agonist liraglutide provoked principle clinical effects observed in type 2 diabetic humans: Enhanced intravenous as well as oral glucose tolerance, an improvement of several insulin sensitivity indices as well as a reduction of food intake and body weight. However, the effect on body weight was much more dramatic than after treatment of adult type 2 diabetic subjects. Further investigation should be kept on to accurately clarify the reasons for this marked inhibition of weight gain. Underlying mechanisms might involve a reduced secretion of the anabolic hormone insulin. To address this hypothesis, transcriptome profiling of skeletal muscle tissue could be useful to detect alterations in insulin-stimulated gene expression pattern, which would underline a reduced insulin secretion as an important factor for the different body weight gain seen in the present study. Skeletal muscle would be a preferred tissue because it is a primary target for insulin actions and represents a major proportion of the body weight (Klip et al. 1990; Wang et al. 1992; Wang et al. 1996). The use of the human recommended dosage of liraglutide combined with the treatment of adolescent pigs could also have contributed to the strong body weight reducing effect. Therefore special care is warranted for prospective long-term liraglutide treatment trials involving adolescent patients and accurate dosages have to be evaluated carefully. Liraglutide treatment decreased the total beta-cell volume in GIPR^{dn} transgenic pigs maybe indirectly by decreasing food intake and thereby mediating a reduced nutrient intake. *In vitro* studies using rodent or porcine islets determined a pro-proliferative and anti-apoptotic effect of liraglutide and *in vivo* studies using rodent models are inconsistent in their findings regarding the effect of liraglutide on beta-cells (Brand et al. 2009; Bregenholt et al. 2005; Friedrichsen et al. 2006; Guo et al. 2013; Larsen et al. 2008; Miao et al. 2013; Raun et al. 2007a; Rolin et al. 2002; Shimoda et al. 2011; Sturis et al. 2003; Toso et al. 2010;

Vrang et al. 2012). Therefore it would be interesting to gather further insights into the *in vitro* effect of liraglutide on isolated islets of GIPR^{dn} transgenic pigs, mainly regarding the information if liraglutide is able to stimulate proliferation or inhibit apoptosis in islets of this pig model. It has already been described that the GIPR^{dn} transgenic pig model shows key findings of a prediabetic stage but does not show a clinically overt diabetes in a determined range of 2 years (Renner et al. 2010). However, the feeding of a high fat diet could be used to promote obesity as well as insulin resistance and thereby challenge a clinical manifestation of diabetes mellitus in this pig model going along with the development of hyperglycemia. If this worked, another 90-day treatment period with liraglutide could bring useful information about the impact of the initial metabolic state in the GIPR^{dn} transgenic pig model on the effect of liraglutide. Additionally, the effects of liraglutide could be tested in other pig models like e.g. the *INS*^{C94Y} transgenic pig model that *inter alia* exhibits hyperglycemia already soon after birth (Renner et al. 2012). At the moment, numerous DPP-4 inhibitors and GLP-1R agonists are not approved yet but under development (Khan et al. 2012). Additionally, a great point of interest for the future can be seen in the development of dual agonist drugs like GLP-1/gastrin agonists or in molecules that can activate the GLP-1R and concomitantly are antagonists of the glucagon receptor (Claus et al. 2007; Fosgerau et al. 2013). Therefore, the GIPR^{dn} transgenic pig model could further be used for the preclinical evaluation of appropriate therapeutic dosages as well as for testing the efficacy and safety of these new drugs.

VII. SUMMARY

Effects of the long acting glucagon-like peptide-1 receptor (GLP-1R) agonist liraglutide in adolescent pigs with impaired glucose-dependent insulinotropic polypeptide receptor (GIPR) function

Pancreatic beta-cell failure and decline are major pathogenetic principles for the development and progressive course of type 2 diabetes mellitus. Thus, GLP-1R agonists are a promising option for the treatment of type 2 diabetes as they seem to have the potential to increase the beta-cell volume in rodent models. However, data are lacking about their *in vivo* effect on human beta-cells because of the absence of appropriate non-invasive imaging techniques for quantification of the beta-cell volume. Large animal models like the pig that show strong physiological and pathophysiological similarities to humans are a useful and necessary addition to further clarify the effects of GLP-1R agonists on the organism.

Therefore, the effect of the long-acting GLP-1R agonist liraglutide on glycemic control, body weight, food intake and especially the total beta-cell volume was evaluated in transgenic pigs that express a dominant-negative glucose-dependent insulinotropic polypeptide receptor ($GIPR^{dn}$) in the pancreatic beta-cells. This large animal model seemed to be especially suitable for the present study as it shows key findings of a prediabetic state: An impaired function of the incretin hormone glucose-dependent insulinotropic polypeptide (GIP) going along with impaired glucose tolerance and progressive reduction of the beta-cell volume. Two different age groups of $GIPR^{dn}$ transgenic pigs (prophylactic group: 2 months of age, no reduction of beta-cell volume; therapeutic group: 5 months of age, 35% reduction of beta-cell volume) were treated with subcutaneous injection of liraglutide (dose range 0.6 mg – 1.8 mg, depending on body weight) or placebo once daily for 90 days. Prior to and after the treatment period glucose tolerance was evaluated by performing oral as well as intravenous glucose tolerance tests. Finally animals were subjected to necropsy and the total beta-cell volume was determined by quantitative-stereological analyses.

In both age groups of $GIPR^{dn}$ transgenic pigs, body weight of liraglutide-treated pigs was lower by 30-40% in comparison to placebo-treated animals, which may at least partly be explained by the concomitant 20-50% reduced food intake.

Intravenous as well as oral glucose tolerance was improved by liraglutide treatment. In the mixed meal oral glucose tolerance test at the end of the treatment period liraglutide-treated animals exhibited only a very moderate increase of blood glucose, probably due to the known effect of liraglutide on gastric emptying. This was associated with significantly smaller AUC insulin in liraglutide- vs. placebo-treated animals. Further, liraglutide treatment reduced the HOMA-IR and increased several insulin sensitivity indices. The total beta-cell volume of liraglutide-treated animals was decreased in comparison to placebo-treated ones, most probably due to the reduced food intake and delayed release of glucose from the stomach. The total beta-cell volume related to body weight did not differ between liraglutide- and placebo-treated $GIPR^{dn}$ transgenic pigs.

In conclusion, the $GIPR^{dn}$ transgenic pig model recapitulates principle clinical effects of liraglutide observed in type 2 diabetic humans like improved glucose tolerance and insulin sensitivity as well as a reduction of body weight and food intake. However, the reduction of body weight gain observed in adolescent pigs was much more dramatic than the body weight loss of adult patients. Thus, special care is warranted in prospective long-term treatment trials involving adolescent patients. In contrast to rodent models, there was no evidence for an increasing effect of liraglutide treatment on the total beta-cell volume in the $GIPR^{dn}$ transgenic pig model.

VIII. ZUSAMMENFASSUNG

Auswirkungen des langwirksamen Glukagon-ähnlichen Peptid-1 Rezeptor (GLP-1R) Agonisten Liraglutide auf heranwachsende Schweine mit gestörter Funktion des Glukose-abhängigen insulinotropen Polypeptid Rezeptors (GIPR)

Das Versagen und der Verlust von pankreatischen Beta-Zellen gehören zu den wichtigsten Faktoren die für die Entwicklung und den progressiven Verlauf des Typ 2 Diabetes mellitus verantwortlich sind. GLP-1R Agonisten stellen eine vielversprechende Option für die Behandlung des Typ 2 Diabetes mellitus dar, da sie in einigen Studien in Nagermodellen das Beta-Zellvolumen erhöhen konnten. Über den *in vivo* Effekt auf humane Beta-Zellen ist allerdings noch nichts bekannt, was vor allem damit zusammenhängt dass beim Menschen bislang noch keine nicht-invasiven Methoden zur Quantifizierung des Beta-Zellvolumens *in vivo* vorhanden sind. Großtiermodelle wie das Schwein sind dem menschlichen Organismus bezüglich physiologischer und pathophysiologischer Abläufe sehr ähnlich und stellen deshalb eine sinnvolle und notwendige Ergänzung zu den Nagermodellen dar, um die Auswirkungen von GLP-1R Agonisten auf den Organismus genauer zu untersuchen. Aus diesem Grund wurden in der vorliegenden Studie die Auswirkungen des langwirksamen GLP-1R Agonisten Liraglutide auf die Glukosekontrolle, das Körpergewicht, die Futteraufnahme sowie besonders auf das Gesamt-Beta-Zellvolumen in transgenen Schweinen untersucht, die einen dominant-negativen GIPR ($GIPR^{dn}$) in den pankreatischen Beta-Zellen exprimieren. Dieses Großtiermodell erschien besonders geeignet für diese Studie weil es wesentliche Merkmale eines prädiabetischen Zustands zeigt: Eine gestörte Funktion des Inkretinhormons GIP und außerdem altersabhängig eine verminderte Glukosetoleranz und Insulinsekretion, die begleitet wird von einem progressiven Beta-Zellverlust. Zwei verschieden Altersgruppen von $GIPR^{dn}$ transgenen Schweinen wurde 90 Tage lang einmal täglich subkutan Liraglutide (Dosisbereich 0.6 mg – 1.8 mg, abhängig vom Körpergewicht) oder Placebo gespritzt (prophylaktische Gruppe: 2 Monate alt, unverändertes Beta-Zellvolumen; therapeutische Gruppe: 5 Monate alt, bereits 35% reduziertes Beta-Zellvolumen). Die Glukosetoleranz vor und nach der Behandlung wurde durch orale sowie intravenöse Glukosetoleranztests untersucht. Am Ende des

Behandlungszeitraums wurden die Tiere euthanasiert und das Beta-Zellvolumen wurde mittels quantitativ-stereologischer Analysen bestimmt.

Die Behandlung mit Liraglutide führte in beiden Altersgruppen zu einer massiven Hemmung der Körpergewichtsentwicklung (30-40% Reduktion des Körpergewichts im Vergleich zu Placebo behandelten Tieren am Ende der Behandlungsperiode), was zumindest teilweise durch eine 20-50% reduzierte Futteraufnahme zu erklären war. Außerdem führte die Behandlung mit Liraglutide zu einer verbesserten oralen und intravenösen Glukosetoleranz. Während des oralen Glukosetoleranztests am Ende der Therapie zeigten Liraglutide behandelte Tiere einen nur sehr moderaten Anstieg der Glukosespiegel. Da die Glukose während dieses Tests gemischt mit Futter verabreicht wurde, könnte dieses Ergebnis auf den bekannten Effekt von Liraglutide auf die Magenentleerung zurückzuführen sein. Auch die AUC für Insulin war während diesem Test in Liraglutide behandelten Tieren signifikant niedriger als bei Placebo behandelten Tieren. Des Weiteren senkte die Behandlung mit Liraglutide den HOMA-IR-Index und erhöhte einige Insulinsensitivitäts-Indices. Das Gesamt-Beta-Zellvolumen war im Vergleich zu Placebo behandelten Schweinen bei Liraglutide behandelten Tieren niedriger, wahrscheinlich aufgrund der reduzierten Futteraufnahme und der stark protrahierten Freisetzung von Glukose aus dem Magen. Bezogen auf das Körpergewicht konnte zwischen Liraglutide und Placebo behandelten Tieren kein Unterschied im Gesamt-Beta-Zellvolumen festgestellt werden.

Das $GIPR^{dn}$ transgene Schweinemodell zeigte nach Liraglutide Behandlung viele klinische Effekte die auch beim humanen Typ 2 Diabetiker beobachtet wurden. Dazu gehören die Verbesserung der Glukosetoleranz und der Insulinsensitivität sowie das geringere Körpergewicht und die reduzierte Futteraufnahme im Vergleich zur Placebo Behandlung. Allerdings war die geringere Körpergewichtszunahme der heranwachsenden transgenen Schweine in dieser Studie viel stärker ausgeprägt als sie beim erwachsenen Menschen zu beobachtende Körpergewichtsreduktion. Deshalb sollten zukünftige Langzeit-Behandlungsstudien von heranwachsenden humanen Patienten mit erhöhter Vorsicht durchgeführt werden. Im Gegensatz zum Nagermodell konnte kein Hinweis darauf gefunden werden, dass Liraglutide einen vermehrenden Effekt auf das Beta-Zellvolumen im $GIPR^{dn}$ transgenen Schweinemodell hat.

IX. INDEX OF FIGURES

X. INDEX OF TABLES

XI. INDEX OF ABBREVIATIONS

AC	adenylate cyclase
AcOH	acetic acid
ACTB	beta-actin
ADA	American Diabetes Association
ALS	acid labile subunit
AP	alkaline phosphatase
AST	aminotransferase
ATP	adenosine triphosphate
AUC	area under the curve
BW	body weight
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
C-terminal	carboxy-terminal
CV	coefficient of variance
DAB	3´3 diaminobenzodine tetrahydrochloride
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleotide triphosphate
DPP-4	dipeptidyl peptidase-4
DTT	dithioreitol
dTTP	deoxythimidine triphosphate

ELISA	enzyme linked immunosorbent assay
EDTA	ethylendiaminetetraacetic acid
EMEA	European Medicine Agency
Epac 2	guanine nucleotide exchange factor 2
FDA	U.S. Food and Drug Administration
FPG	fasting plasma glucose
µGT	gammaglutamyltransferase
GH	growth hormone
GIP	glucose-dependent insulinotropic polypeptide
$GIPR^{dn}$	dominant-negative glucose-dependent insulinotropic polypeptide receptor
GIPR	glucose-dependent insulinotropic polypeptide receptor
GLP-1	glucagon-like peptide-1
GLP-2	glucagon-like peptide-2
GLP-1R	glucagon-like peptide-1 receptor
$GLP1r^{-/-}$	glucagon-like peptide-1 receptor knockout
G-protein	guanine nucleotide-binding protein
GRPP	glicentin-related polypeptide
GTT	glucose tolerance test
HbA_{1c}	glycated hemoglobin
HCl	hydrochloric acid
HOMA-IR	homeostasis model assessment of insulin resistance
HOMA-β	homeostasis model assessment of beta-cell function
IAPP	islet amyloid polypeptide
Ig	immunglobulin

IGF-I	insulin-like growth factor-I
IGFBP	insulin-like growth factor binding protein
i.m.	intra muscular
Ins2	insulin 2
Irs 2	insulin receptor 2 substrate
$ISI_{(Matsuda)}$	insulin sensitivity index according to Matsuda
IU	international units
IVGTT	intravenous glucose tolerance test
LEAD	Liraglutide Effect and Action in Diabetes
MAPK	mitogen-activated protein-kinase
ME	metabolizable energy
MPGF	major proglucagon peptide
N-terminal	amino-terminal
OGTT	oral glucose tolerance test
PCR	polymerase chain reaction
Pdx1	pancreatic and duodenal homeobox gene 1
PEU	plasma equivalent unit
PG	postprandial glucose
pH	potential of hydrogen
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
QUICKI	quantitative insulin sensitivity check index
RIA	radioimmunoassay
RIP2	rat insulin 2 promotor
SBP	systolic blood pressure

SDS	sodium dodecyl sulfate
SEM	standard error of means
SGLT-2	sodium glucose cotransporter-2
TAE	tris-acetate buffer
TBS	tris-buffered saline
TIU	trypsin inhibitory unit
TRIS	tris-(hydroxymethyl)-aminomethan
UCD-T2DM	University of California, Davis, type 2 diabetes mellitus
UV	ultraviolet
ZDF	Zucker diabetic fatty

XII. REFERENCE LIST

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