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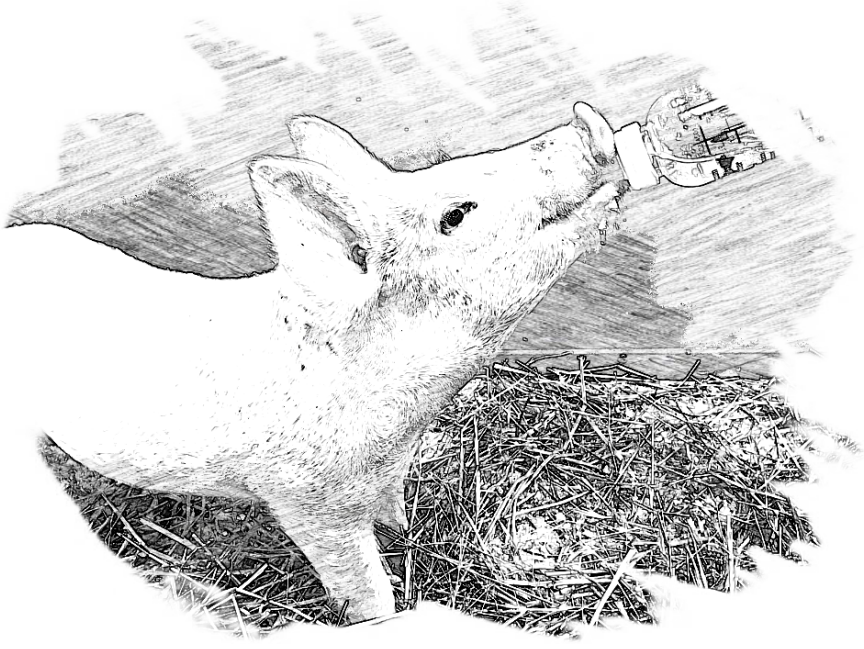
Refinement of porcine models in diabetes and transplantation research

Elin Manell



Refinement of porcine models in diabetes and transplantation research

Elin Manell
Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences
Uppsala

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Cover: Pig bottle-feeding glucose solution
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Abstract

Animal models are widely used in biomedical research aiming to prevent and improve treatments of diseases. The 3Rs (replace, reduce, refine) are considered when working with laboratory animals. Socialisation and training of pigs in research are important to avoid stress responses that could potentially affect research data. Domestic pigs were subjected to a structured training programme before inclusion in renal transplantation studies. The training programme enabled blood and urine sampling, and ultrasound examinations in conscious pigs without restraint.

The porcine diabetes model was further characterised with regards to hormonal responses to oral glucose, metabolic changes due to insulin deficiency, distribution of glucagon-like peptide-1 receptors (GLP-1R) in pancreas and gastrointestinal tract, and GLP-1R occupancy *in vivo* during oral glucose tolerance test (OGTT). Both similarities and differences between pigs and humans were identified, both of which are important to keep in mind when designing animal studies. Furthermore, a refined model for OGTT in pigs, which reduces experimental variation and facilitates comparisons between experiments, was established. An insulin treatment protocol for streptozotocin diabetic pigs was developed to enable long-term studies and minimise risk of serious complications.

Well characterised animal models and calm animals are important to acquire relevant and reliable research data, and to minimise the number of animals needed. Using refined techniques to minimise stressful situations is also important from an animal welfare perspective. Results presented in this thesis contribute to the 3Rs.

Keywords: Pig, 3Rs, training, diabetes mellitus, streptozotocin, oral glucose tolerance test, insulin, glucagon-like peptide-1, glucagon-like peptide-1 receptor, fatty acid, triglyceride, amino acid, cystatin-C, renal transplantation.

Author's address: Elin Manell, Swedish University of Agricultural Sciences, Department of Clinical Sciences, P.O. Box 7054, 750 07 Uppsala, Sweden

Förbättringar av grismodeller för forskning kring diabetes och transplantation

Sammanfattning

Djurmodeller används ofta inom biomedicinsk forskning som syftar till att förebygga eller förbättra behandlingar av sjukdomar. När man använder försöksdjur tar man hänsyn till 3R (replace, reduce, refine). Socialisering och träning av grisar i forskning är viktigt för att undvika stress som kan påverka forskningsdata. Konventionella grisar genomgick ett strukturerat träningsprogram innan de gick in i njurtransplantationsstudier. Träningsprogrammet möjliggjorde blod- och urinprovstagning samt ultraljudundersökningar hos vakna grisar utan fasthållning.

Grismodellen för diabetes karaktäriserades djupare med avseende på hormonellt svar till oral glukos, metabola förändringar på grund av insulinbrist, distribution av glucagon-like peptide-1 receptorer (GLP-1R) i pankreas och magtarmkanalen, och bindning till GLP-1R *in vivo* under oralt glukosbelastningstest (OGTT). Både skillnader och likheter mellan gris och människa identifierades, vilka båda är viktiga att beakta när man designar djurstudier. Vidare togs en förfinad modell för OGTT fram vilken minskar experimentell variation och underlättar jämförelser mellan studier. Ett insulinbehandlingsprotokoll för streptozotocin-diabetiska grisar etablerades vilket möjliggör långtidsstudier och minimerar risken för allvarliga komplikationer.

Väl karaktäriserade djurmodeller och lugna djur är viktiga för att få relevant och tillförlitlig forskningsdata, och för att minimera antalet djur som behövs. Användandet av förfinade tekniker för att minimera stressande situationer är också viktigt ur djurvälfärdssynpunkt. Resultaten i avhandlingen bidrar till 3R.

Nyckelord: Gris, 3R, träning, diabetes mellitus, streptozotocin, oral glukosbelastning, insulin, glukagon, GLP-1, GLP-1R, fettsyra, triglycerid, aminosyra, cystatin-C, njurtransplantation.

Författarens adress: Elin Manell, Sveriges Lantbruksuniversitet, Institutionen för kliniska vetenskaper, Box 7054, 750 07 Uppsala, Sweden

Dedication

To Hannes

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Manell E, Rydén A, Hedenqvist P, Jacobson M, Jensen-Waern M (2014). Insulin treatment of streptozotocin-induced diabetes re-establishes the patterns in carbohydrate, fat and amino acid metabolisms in growing pigs. *Laboratory animals*, 48(3), pp 261-269.

II. Manell E, Hedenqvist P, Svensson A, Jensen-Waern M (2016). Establishment of a Refined Oral Glucose Tolerance Test in Pigs, and Assessment of Insulin, Glucagon and Glucagon-Like Peptide-1 Responses. *PLoS ONE*, 11(2):e0148896,

III. Manell E, Puuvuori E, Svensson A, Velikyan I, Hulsart-Billström G, Hedenqvist P, Holst JJ, Jensen-Waern M, Eriksson O. Exploring the GLP-1 – GLP-1R axis in porcine pancreas and gastrointestinal tract *in vivo* by *ex vivo* autoradiography. *(submitted)*

IV. Ryden A*, Manell E*, Biglarnia A, Hedenqvist P, Strandberg G, Ley C, Hansson K, Nyman G, Jensen-Waern M. (2020) Nursing and training of pigs used in renal transplantation studies. *Laboratory animals,* 54(5), pp 469-478. *Denotes equal contribution.

Papers I, II and IV are reproduced with the permission of the publishers.

The contribution of Elin Manell to the papers included in this thesis was as follows:

I. Took part in the execution of the experimental study. Analysed the data, drafted the manuscript and finalised it together with co-authors.

II. Involved in planning and organising the study. Performed the experimental study together with co-authors. Carried out the laboratory work and analysed the data. Drafted the manuscript and finalised it with input from co-authors. Corresponding author.

III. Took major part in planning and organising the study. Performed the experimental study together with co-authors. Analysed the autoradiography images and carried out some of the laboratory work. Took major part in analysing the data. Drafted the manuscript and finalised it with input from co-authors. Corresponding author.

IV. Took major part in planning and organising the study. Performed the experimental study together with co-authors. Interpreted the data and drafted the manuscript together with AR (shared first author).

Abbreviations

3Rs	Replace, reduce, refine
BW	Body weight
CT	Computed tomography
DKA	Diabetic ketoacidosis
DM	Diabetes mellitus
DPPIV	Dipeptidyl peptidase-4
GFR	Glomerular filtration rate
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
GLUT2	Glucose transporter 2
GTTs	Glucose tolerance tests
HE	Hematoxylin and eosin
IHC	Immunohistochemistry
IVGTT	Intravenous glucose tolerance test
NEFA	Non-esterified fatty acid
OGTT	Oral glucose tolerance test
PET	Positron emission tomography
SLU	Swedish University of Agricultural Sciences
SPF	Specific pathogen free
STZ	Streptozotocin
TG	Triglyceride
WHO	World Health Organization

1. Introduction

Animal models are widely used in biomedical research with the overall aim to improve prevention and treatment of diseases affecting humans or animals. Within the European Union, around 9.5 million animals are used for scientific purposes (research, testing, routine production, education) each year (EU, 2020). With the use of animals for scientific purposes comes the responsibility to treat the animals in the best possible way and to assure that accurate and relevant data is collected. In 1959, Russel and Burch published 'The Principles of Humane Experimental Techniques' in which they introduced the terms replace, reduce and refine (3Rs) (Russel & Burch, 1959). While it is desirable to replace animal models in research, they are still necessary and therefore it is essential to use the best methods possible to reduce the numbers of animals needed, and to refine procedures to minimise pain, suffering and distress. The 3Rs have become internationally recognised and are considered in, for example, the EU legislation on the protection of animals used for scientific purposes (EU, 2010) and US guidelines for the care and use of laboratory animals (NRC, 2011). The overall aim of this thesis was to work with refinement of porcine models in diabetes and renal transplantation research.

2. Background

2.1 Pigs as animal models in biomedical research

Pigs are commonly used as animal models in biomedical research when large animal models are needed since pigs share a number of anatomical and physiological characteristics with humans (as reviewed by Swindle & Smith, 2016). Within the European Union, around 70 000 pigs are used for scientific purposes each year (EU, 2020). The corresponding numbers for dogs and non-human primates, which historically have been important animal models, are 14 000 and 8 000 respectively, i.e. these species are used to a much lesser extent (EU, 2020).

Pigs were domesticated some 7000 years ago (as reviewed by Miller & Ullrey, 1987) and are easy to keep and breed in a laboratory setting. The pig has a short reproduction cycle compared to many other large animals, and produce large litters. In addition to scientific advantages of using pigs over dogs to model humans, it is also more accepted by the general population to use pigs in research (Vetenskapsrådet, 2019). Although non-human primates often represent models of high fidelity, it is legally prohibited within the European Union to use non-human primates if another species can be used to reach the same goal (EU, 2010).

A range of different breeds and crossbreeds of pigs are used in biomedical research, figure 1. Pigs do not need to be purpose-bred (EU, 2010) and commonly used domestic breeds include Yorkshire (large white), landrace, Hampshire, Duroc and Piétrain (as reviewed by Bollen *et al.,* 2010). Because of the high growth rate of domestic pigs, minipigs are more commonly used in long-term studies. In Europe, Göttingen minipig is the only commercially available minipig. In the United States, Hanford,

Yucatan, Yucatan micro and Sinclair minipigs are also commonly used (as reviewed by Swindle *et al.,* 2012).

Figure 1. Two breeds and two crossbreeds of pigs commonly used in biomedical research. Photo: Elin Manell

2.2 Training of pigs for research studies

Distress is an obvious animal welfare concern, but stress responses can also affect a range of physiological parameters (as reviewed by NRC, 2008). Stress responses in laboratory animals can affect research results and might lead to increased variation in experiments, which in turn cause an increase in the number of animals needed. Alterations in physiology and behaviour might even confound research data to the extent that incorrect conclusions are drawn. Blood sampling is a potentially stressful procedure in pigs (Jensen-Waern & Nyberg, 1993) and must be carried out with methods avoiding a stress response. Of special interest to diabetes research, blood glucose levels increase rapidly in response to acute stress (Rand *et al.*, 2002).

Some general guidelines for handling pigs in a laboratory setting have been published (Sorensen, 2010; Kaiser *et al.*, 2006; Smith & Swindle, 2006), but detailed information about methods and training for specific procedures is very limited. Pigs can be accustomed to petting and brushing (Nicholls *et al.*, 2012) and will interact more frequently with people familiar to them (Terlouw & Porcher, 2005). In the laboratory setting it is possible to reach and train all individual pigs. This is an advantage over non-human primates that are not domesticated to same extent, and develop strict hierarchies (as reviewed by Magden *et al.,* 2015), which makes it very challenging to reach individuals that are low in rank.

2.3 Diabetes mellitus

Diabetes mellitus (DM) is a chronic disease characterised by chronic hyperglycaemia. In the late 19th century, a scientific breakthrough was made when it was discovered that pancreatectomised dogs developed DM (Mering & Minkowski, 1890). The pancreatic endocrine tissue, islets of Langerhans, are highly involved in diabetes development. The islets are scattered throughout the pancreas, and contain glucagon producing alpha cells, insulin producing beta cells and somatostatin producing delta cells.

The number of people affected by DM globally is increasing. When this PhD project was commenced in 2014, WHO estimated that 347 million people were affected by DM (WHO, 2014) and now in 2020 this number is 422 million people (WHO, 2020). Patients with DM suffer from long-term complications due to high blood glucose concentrations, including cardiovascular disease, renal failure and vision impairment (Franco *et al.*, 2007; Stratton *et al.*, 2000; The Diabetes Control and Complications Trial Research Group, 1993); and DM is the seventh most common cause of death (WHO, 2018). Better treatment methods are needed to improve quality of life for people suffering from long-term complications, and to increase life expectancy.

DM is classified based on aetiology, type 1 and type 2 being the most common forms of DM. Type 1 DM is caused by an autoimmune destruction of pancreatic beta-cells due to unknown triggers (as reviewed by Berne, 2018). In type 1 DM, absolute insulin deficiency causes hyperglycaemia, and before the discovery of insulin in the 1920s (Banting & Best, 1922), DM was a fatal disease. In type 2 DM, decreased insulin sensitivity prevent insulin

from exerting its biological effect. Early during the cause of the disease plasma insulin concentrations are high, but decline over time and more than half of the patients require insulin treatment after 10-15 years, (as reviewed by Berne, 2018). The aetiology behind type 2 DM is not fully understood, but as a consequence of lifestyle changes in the modern society with less physical exercise, high consumption of food rich in energy, obesity and stress, prevalence of type 2 DM is increasing (as reviewed by Berne, 2018). Type 1 DM often has its onset in childhood (DiMeglio *et al.*, 2018) and long-term complications are common since these patients live with the disease for most of their lives. Type 2 DM used to be recognised as a disease in the adult population but is now affecting younger people. The disease has increased in children and adolescents during the recent years (Mayer-Davis *et al.*, 2017; Dabelea *et al.*, 2014), and in the years to come it is expected to see more long-term complications also in this population.

2.3.1 Metabolic alterations due to insulin deficiency

Insulin is a hugely important hormone for coordination of the use of fuels by tissues. Insulin promotes glucose uptake in tissues (Macleod, 1922) and inhibit gluconeogenesis in the liver (Kaldor *et al.*, 1964). During insulin deficiency, hyperglycaemia develops. Glucagon counteracts insulin and is the most important hormone to correct hypoglycaemia by promoting glycolysis and gluconeogenesis (Rizza *et al.*, 1979). Insulin inhibit glucagon secretion and insulin deficiency causes hyperglucagonaemia (as reviewed by Champe *et al.,* 2008).

Insulin inhibit hormone sensitive lipase in adipose tissue (Nilsson *et al.*, 1980) and insulin deficiency cause rapid mobilisation of non-esterified fatty acids (NEFA) (Laurell, 1956). Furthermore, insulin deficiency cause decreased activity of lipoprotein lipase in adipose tissue and consequently decreased removal of triglycerides (TG) from the circulation (Bagdade *et al.*, 1968). Glucagon stimulates lipolysis in adipocytes which contributes to increased NEFA concentrations in the circulation (Hagen, 1961). Glucagon also act on the liver to stimulate ketogenesis (Heimberg *et al.*, 1969).

Amino acids are building blocks in protein synthesis and some amino acids can be used for gluconeogenesis and ketogenesis (as reviewed by Champe *et al.,* 2008). Insulin exerts an anabolic effect on muscle tissue and insulin deficiency cause muscle protein to break down (Odedra *et al.*, 1982) and amino acids, predominantly alanine, are released into the circulation

(Felig *et al.*, 1970). However circulating glucogenic amino acids, such as glutamate, glutamine, alanine, prolineglycine, serine and threonine, are taken up by the liver and their concentrations in the circulation decrease while circulating branched chain amino acids increase (Blackshear & Alberti, 1975).

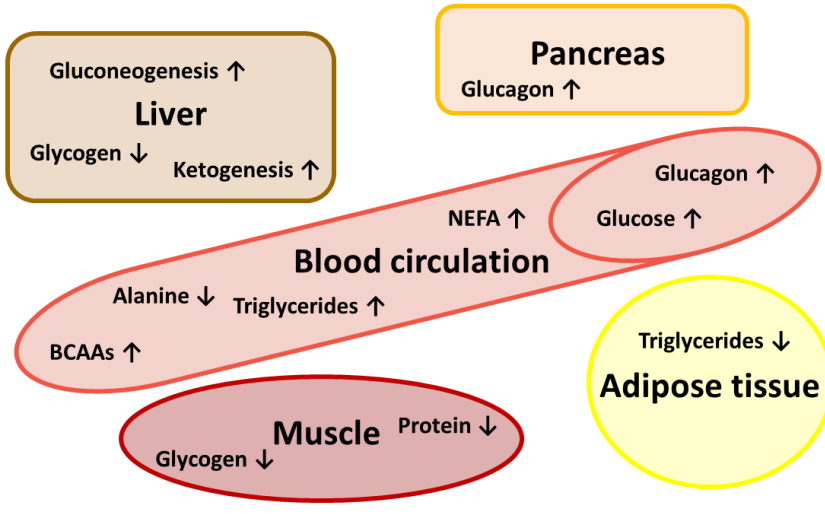

Figure 2. Some metabolic alterations seen during insulin deficiency.

2.3.2 Incretin effect and the GLP-1 receptor

Oral intake of glucose elicit a greater insulin release compared to intravenous infusion (Elrick *et al.*, 1964), this is termed the incretin effect (**in**testinal se**cret**ion of **in**sulin). The incretin effect is impaired in both type 1 (Greenbaum *et al.*, 2002) and type 2 DM (Bagger *et al.*, 2011; Nauck *et al.*, 1986), however the mechanisms are not fully understood. Already in the beginning of the 20th century, before the discovery of insulin, it was observed that intestinal factors were involved in glucose metabolism and DM (Moore, 1906). Later it was demonstrated that gastric inhibitory polypetide and glucagon-like peptide-1 (GLP-1) are responsible for the incretin effect by potentiating insulin release from pancreatic beta-cells under hyperglycaemic conditions (Drucker *et al.*, 1987; Kreymann *et al.*, 1987; Dupre *et al.*, 1973). Endogenous GLP-1 has a very short half-life in plasma of only a few minutes

(Deacon *et al.*, 1996) due to degradation by the enzyme dipeptidyl peptidase-4 (DPPIV) (Deacon *et al.*, 1995). GLP-1 receptor (GLP-1R) agonists, more resistant to degradation, are widely used as treatment for type 2 DM and has recently also been introduced as adjunct treatment in type 1 DM (Wang *et al.*, 2017).

L-cells are enteroendocrine cells located between other cells in the intestinal epithelium (Buffa *et al.*, 1978). GLP-1 is secreted from L-cells (Orskov *et al.*, 1986) and binds to the G-protein coupled GLP-1R (Thorens, 1992). The peptide consists in two forms, equally biologically active, GLP-1(7-37) and GLP-1(7-36NH_2) (Orskov *et al.*, 1993). The extent of amidation of stored GLP-1 varies between species (Kuhre *et al.*, 2014), however, predominantly amidated peptide is secreted in both humans and pigs (Hansen *et al.*, 1999; Orskov *et al.*, 1994). GLP-1R location have been studied in various tissues, however immunohistochemical (IHC) methods have been questioned due to limitations in specificity of generated anti GLP-1R antibodies (Pyke & Knudsen, 2013). IHC staining with an antibody extensively validated for primate tissues, showed that GLP-1Rs are present in pancreatic beta-cells and acinar cells, parietal and muscle cells in the stomach, Brunner's gland epithelial cells in duodenum, myenteric plexus neurons throughout the gastrointestinal tract, pulmonary artery smooth muscle cells, kidney arterial smooth muscle cells, and sinoatrial node myocytes (Pyke *et al.*, 2014). However, detailed quantifications of receptors are not possible with IHC methods. Furthermore, replacement of primates in biomedical research should be a priority (EU, 2010). Lack of methods for quantitative assessments of GLP-1R density means that it is difficult to assess occupancy of GLP-1Rs by endogenous GLP-1 or GLP-1R agonists. The required proportion of engaged receptors for a significant physiological effect is thus unknown.

2.3.3 Oral glucose tolerance test

To study the incretin effect, and to evaluate the effect of different treatments on glucose tolerance, the oral glucose tolerance test (OGTT) is widely used. OGTT is a valuable tool in research but is also used in the clinic to diagnose type 2 DM based on WHO recommendations (WHO, 2006). In humans, the test is carried out by the person drinking glucose (1.75g glucose/kg BW, max 75g) dissolved in water within 5 minutes, followed by blood sampling.

2.4 Porcine models of diabetes

As mentioned above, pigs share many anatomical and physiological features with humans. Properties of pancreas, gastrointestinal tract and integumentary system are of special interest in diabetes research.

The porcine pancreas resembles the human pancreas in size and position (as reviewed by Larsen & Rolin, 2004), but the number and distribution of the pancreatic lobes differ (Ferrer *et al.*, 2008), figure 3. Islet size distribution fall into similar range in humans and pigs, however pigs have relatively more beta-cells within the islets (Kim *et al.*, 2009), and the porcine islets are not encapsulated (van Deijnen *et al.*, 1992). Porcine and human insulin differ by only one amino acid (Nicol & Smith, 1960; Brown *et al.*, 1955).

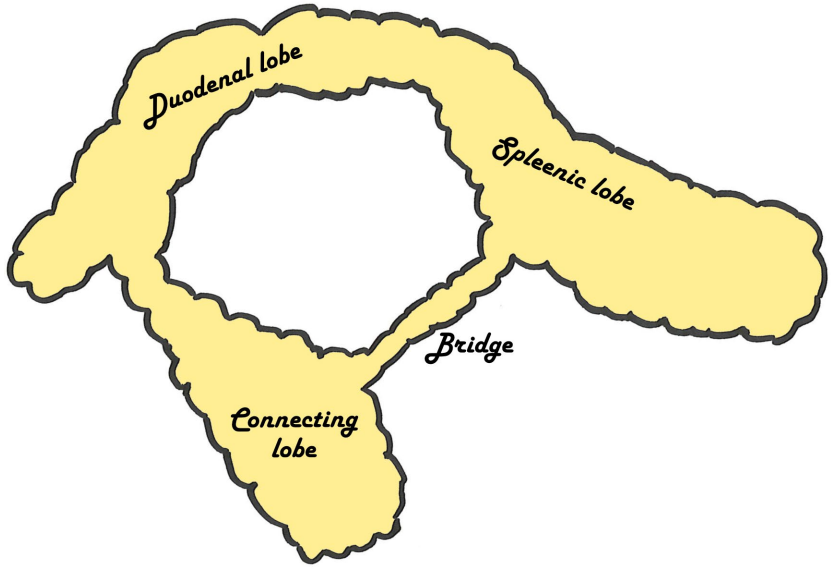

Figure 3. Original illustration of normal anatomy of porcine pancreas according to the anatomy described in Ferrer *et al.*, 2008.

DM is a metabolic disorder and the gastrointestinal tract is highly involved in the pathophysiological processes. Both pigs and humans are classified as omnivorous. Although there are some significant differences in the anatomy of the gastrointestinal tract of pigs and humans, the physiology of digestion in pigs is remarkably like that in humans (as reviewed by Swindle *et al.,* 2012). Pigs represent a good animal model to study nutrition

and are a much closer match than for example rodents (as reviewed by Roura *et al.,* 2016). Pigs and humans have the same requirements of essential amino acids, and both species are highly dependent on dietary quality since symbiotic microorganisms within the gut play a relatively minor role in modifying the nutrients that are ingested (Miller & Ullrey, 1987). Of special interest to this thesis, the amino acid sequence of porcine GLP-1, secreted from intestinal L-cells, is identical to that of human GLP-1 (Orskov *et al.*, 1989).

The high similarity between porcine and human skin and subcutaneous tissue (Qvist *et al.*, 2000; Rose *et al.*, 1977) and pharmacokinetics of subcutaneous injections (Zheng *et al.*, 2012) are of special interest in diabetes research, since several substances such as insulin and GLP-1 analogues are injected subcutaneously.

2.4.1 Type 1 diabetes mellitus

Spontaneous DM has been described in one pig (Biester, 1925), but in an experimental setting DM needs to be induced. A type 1 DM like condition, with ablation of beta cells in the pancreas, can be induced by strepotozotocin (STZ) in pigs (Gäbel *et al.*, 1985). STZ was first studied due to its antibacterial action (Vavra *et al.*, 1959). However, it was soon discovered that STZ had diabetogenic actions *in vivo* (Junod *et al.*, 1969; Rakieten *et al.*, 1963). STZ enters cells via glucose transporter 2 (GLUT2) (Elsner *et al.*, 2000; Schnedl *et al.*, 1994) where it causes DNA strands to break (Yamamoto *et al.*, 1981b; Yamamoto *et al.*, 1981a). High doses of STZ causes NAD depletion due to strong activation of DNA repair mechanisms, and consequently cell death (Pieper *et al.*, 1999; Yamamoto *et al.*, 1981a). GLUT2 is not only present on pancreatic beta-cells but also in liver and kidney (as reviewed by Champe *et al.,* 2008), and STZ can also cause damage to those organs (Voss *et al.*, 1988; Yamamoto *et al.*, 1981a; Loftus *et al.*, 1974). However, at dosage that would give almost complete DNA fragmentation in pancreatic beta-cells, DNA in the liver is fragmented to a lesser extent and NAD stores are not depleted (Yamamoto *et al.*, 1981a).

DM can be induced by high dose STZ in domestic pigs (Jensen-Waern *et al.*, 2009; Gäbel *et al.*, 1985) and miniature breeds such as Göttingen minipig (Liu *et al.*, 1998), but the animals need to be carefully monitored the first days after diabetes induction. STZ treatment cause insulin to leak out into the blood circulation and consequent hypoglycaemia need to be treated with

intravenous glucose (Gäbel *et al.*, 1985). There is also a risk of complications due to STZ uptake in liver and kidney (Jensen-Waern *et al.*, 2009) even though the effects are often small and transient at dosage used to induce DM (Hara *et al.*, 2008; Gäbel *et al.*, 1985). When a diabetic phenotype has developed, ketone body production might pose a risk to the health of the animal. Pigs with no or minor endogenous insulin production need insulin treatment in long-term studies to avoid diabetic ketoacidosis (DKA) (Dreschfeld, 1886).

2.4.2 Type 2 diabetes mellitus

Methods and results described in this thesis are relevant for research on type 2 DM. However, no models of type 2 DM were used in this PhD project, and below is a very brief description of some porcine models for type 2 DM.

Göttingen minipigs can be fed a high-fat high-energy diet to induce obesity accompanied by increased plasma concentrations of glucose and insulin, insulin resistance and adipose tissue inflammation (Renner *et al.*, 2018; Larsen *et al.*, 2002a). To reduce insulin secretory capacity to model type 2 DM, STZ can be given in combination with nicotinamide to produce partial destruction of pancreatic beta-cells (Larsen *et al.*, 2002b).

Ossabaw miniature pigs have lived isolated on Ossabaw Island since deposited there in the 1500s by Spanish explorers. When fed a high caloric diet the pigs develop obesity, insulin resistance, dyslipidemia, hypertension and coronary artery disease (Dyson *et al.*, 2006), and in later stages progress to DM (as reviewed by Swindle & Smith, 2016).

2.4.3 Oral glucose tolerance test

The most commonly used method for OGTT in pigs is to feed glucose (2g/kg) along with a small amount of feed, although some studies use different amounts of glucose or administer glucose by gavage (as reviewed by Bellinger *et al.,* 2012). Bypassing the mouth by oral gavage is not comparable to the standard human OGTT since oral sensory stimulation improves glucose tolerance (Teff & Engelman, 1996). With the mixed meal approach, the diet itself may alter glucose absorption. For example, fibres affect post-prandial absorption of glucose, attenuating blood glucose and insulin concentrations and decelerate gastric emptying (Torsdottir *et al.*, 1989; Jenkins *et al.*, 1978). The use of different amounts and content of feed in previous studies have resulted in variations in the glucose tolerance curves

(Liu *et al.*, 2018; Renner *et al.*, 2010; Christoffersen *et al.*, 2009; Larsen *et al.*, 2003; Larsen *et al.*, 2002b). In translational medicine where pigs are used as models for humans, a more standardised OGTT model that is closer to the human standard OGTT is desirable.

2.5 Renal transplantation

There are many different aetiologies for end-stage renal disease in humans, microvascular complications in DM being one of them (as reviewed by Braunwald, 2019). Renal transplantation is a life-saving procedure for patients with end-stage renal disease (Wolfe *et al.*, 1999). However, despite improved short- and long-term survival of grafts during the last decades, around 50% of kidneys from deceased donors and 30% of living donor organs are lost within 10 years (Hart *et al.*, 2020). Some patients die waiting for transplantation due to shortage of donated organs (Hart *et al.*, 2020) and more research is needed to improve survival of transplanted kidneys and consequently decrease the demand for retransplantation.

2.6 Porcine models of renal transplantation

Pigs are commonly used in renal transplantation studies due to many similarities in structure and function between human and porcine kidneys (as reviewed by Swindle & Smith, 2016). In contrast to rodents, dogs and cynomolgus monkeys which have unipapillate kidneys, pigs like humans have multipapillate kidneys (as reviewed by Chamanza *et al.,* 2019 and Swindle & Smith, 2016). The adult porcine and human kidneys have similar number of nephrons (Rytand, 1938; Vimtrup, 1928), and there are similarities in arterial structure between pigs and humans (Evan *et al.*, 1996). Swine leucocyte antigens have been characterised (as reviewed by Chardon *et al.,* 1999), and by swine leucocyte antigen-typing potential study animals before inclusion, matched or mismatched transplantations can be created deliberately.

Post-operatively, interventions such as urine sampling, ultra sound examinations and estimation of glomerular filtration rate (GFR) might be of interest. In humans, plasma levels of cystatin C are used to estimate GFR (as reviewed by Ferguson *et al.,* 2015), but potential value of cystatin C as a biomarker in pigs has not been thoroughly evaluated.

3. Aims of the thesis

The overall aim of this thesis was to work with refinement of porcine models in diabetes and renal transplantation research.

The specific aims were to:

- Establish an insulin treatment protocol for STZ induced diabetic Yorkshire x Swedish Landrace pigs.
- Examining whether the metabolic changes in carbohydrate, protein and fat metabolism induced by STZ in pigs could be reversed by insulin treatment.
- Refine and standardise the porcine OGTT model, and describe hormonal responses to an oral glucose load with respect to insulin, glucagon and GLP-1 in growing pigs as a model for human children and adolescents.
- Quantitatively describe the distribution of GLP-1Rs in pancreas and gastrointestinal tract of pigs, and assess the receptor occupancy of endogenous GLP-1 *in vivo* during OGTT in pigs.
- Subject pigs in renal transplantation studies to systematic socialisation and training during the pre-operative acclimatisation period, to enable post-operative blood sampling, urine sampling and ultrasound examinations of the urinary bladder and transplanted kidney without restraint.
- Investigate whether plasma concentrations of cystatin C can be used for estimation of GFR in recently renal transplanted pigs.

4. Materials and methods

Detailed methods are described in the respective papers. This section provides a brief summary of and comments on the materials and some of the most important methods.

4.1 Animals and housing

In all studies, domestic SPF pigs (Vallgårda & Wallgren, 2011) were used, Yorkshire x Swedish Landrace (paper I and II), Yorkshire x Swedish Landrace x Hampshire (paper II) or Yorkshire x Hampshire (paper III and IV). High-health animals were selected to minimise risk of infections affecting animals, research protocols and/or results.

The pigs were housed at the Department of clinical sciences, SLU, Uppsala, figure 4. All pigs were fitted with indwelling vein catheters to enable repeated blood sampling without any discomfort for the animals, consequently the pigs had to be housed individually (pens measuring 3-4.3 m^2) but were within sight and sound of one another. The pigs were also allowed to come out in the corridor between the pens once in a while, and could then have physical contact with other pigs through the front of the pens. Straw and wood shavings were provided as bedding. In paper IV, animals were provided with synthetic fleece instead of bedding postoperatively. A light/dark schedule (12:12 h in study 1, 10:14 h in study II-IV) was used and an infrared lamp was provided in the corner of each pen. The room temperature was kept at 18 ± 2°C. The pigs were fed commercial finisher diet (SOLO 330 P SK, Lantmännen, Sweden) twice daily, amount depending on body weight according to the SLU regimen for growing pigs (Göransson & Lindberg, 2011). Water was provided *ad libitum*.

Figure 4. Housing of pigs at the Department of Clinical Science, SLU, Uppsala. Straw and wood shavings provided as bedding. An infrared lamp located in the corner of each pen. Photo: Elin Manell.

4.2 Acclimatisation and training

In paper I-III, all pigs were given two weeks to acclimatise to the new environment before going into experiment. This time allows for the microbiota of the pigs to stabilise in the new environment (Katouli *et al.*, 1999) and for clinical examinations to ensure that the pigs are healthy. During the acclimatisation period, the pigs were also socialised and trained for specific procedures. Each pig was given at least 15 min of socialisation/training, 5 times per week. The trainer sat down in the pen, offered pieces of fruit, and petted and brushed the pigs once they allowed it. In all studies, the pigs were trained to step onto an electronic scale while given fruit rewards, and trained to allow clinical examination. A structured evaluation of the training programme used at the Division of comparative medicine at the Department of clinical sciences, SLU, and its adaption to renal transplantation studies is described in paper IV.

4.2.1 Bottle-feeding training

In paper II, pigs were trained to bottle-feed glucose dissolved in water with the aim to establish a refined OGTT model. The experiment was run in two blocks with two different litters of pigs. With the first litter (L1), bottle-feeding training was initiated on day ten after arrival, and the pigs were bottle-fed Mondays-Fridays. The second litter (L2) was bottle-fed seven days per week from the day of arrival. The more frequent bottle-feeding in

L2 was carried out because a decline in bottle-feeding ability was observed over time in L1.

4.2.2 Structured training programme for renal transplantation studies

In paper IV, the pigs were subjected to a structured four-step socialisation and training programme, summarised in table 1. Five persons with experience of training pigs took equal part in the training programme. In step 1, pigs were allowed to adapt to their new environment for three days. Staff entered the housing area for feeding and cleaning of the pens. In step 2, the trainer sat in the pen for 15 minutes per day, allowing the pig to get accustomed to the person. Once the pig was close enough, the trainer started to gently touch and brush the animal and offered it pieces of fruit from the hand. The pigs were also trained to allow touching and palpation of the ears as preparation for blood sampling from the auricular vein. In step 3, the training from step 2 continued and further included touching with an ultrasound transducer dummy over the abdomen to tolerate ultrasound examination of the urinary bladder and transplanted kidney post-operatively. Pigs were accustomed to urine collection by a trainer who held a paper kidney dish for free-flow sampling. In step 4, the training from step 2 and 3 continued, and the pigs were also trained to undergo a clinical examination including auscultation of heart and lungs. For each individual pig, a new step was introduced once the pig had been completely accustomed to the procedures in the previous step.

Table 1. Socialisation and training of pigs in a four steps during a two week acclimatisation period.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Intervention by the trainer	Feeding the animals Pigs left to settle down	Trainer sits in the pen Touches and brushes Offer fruits Trainer talks	Ultrasound of abdomen with dummy Collection of free-flow urine samples	Clinical examination

4.3 Indwelling vein catheters and blood sampling

To facilitate stress free blood sampling, all pigs were fitted with indwelling vein catheters, into *vena jugularis*, under aseptic conditions and general anaesthesia. Vein catheters were either surgically implanted, tunnelled subcutaneously and exteriorised at the back between the scapulas (paper I-III), or inserted via *vena auricularis* by minimally invasive Seldinger technique (paper IV). All vein catheters must be meticulously managed to prevent catheter related infections, and aseptic technique was used when handling the catheters for blood sampling or injection. Uncoated catheters were flushed with saline and filled with heparinised saline between uses to prevent fibrin flap formation and thrombosis. MPC-coated catheters (paper IV) were flushed and filled with saline only.

Blood samples were collected in serum, EDTA or BDP800 tubes as appropriate. BDP800 tubes contain DPPIV-inhibitors and other enzyme inhibitors, and preserves active GLP-1 and glucagon in plasma.

4.4 Diabetes induction

Streptozotocin diabetes was induced in study I. STZ (Sigma S0130, Stockholm, Sweden) was dissolved in 100 mmol/L disodium citrate buffer solution, pH 4.5, at a concentration of 80 mg/mL. Due to the short half-life of STZ, the solution was prepared while the animals were under general anaesthesia for vein catheter surgery, and administered intravenously immediately after preparation. Each pig received a total amount of 150 mg/kg BW of STZ.

4.5 Post-operative care

Pigs that had been under general anaesthesia were always monitored until they were standing up and alert.

4.5.1 Diabetes induction

The pigs were monitored for 13 hours after STZ injection. Blood glucose concentrations below 3 mmol/L were promptly treated with an intravenous bolus of glucose (Glukos APL, 500 mg/mL, Apoteksbolaget, Sweden) of 0.5 g/kg BW.

4.5.2 Renal transplantation

In paper IV, pigs underwent major surgery with renal transplantation and removal of native kidneys. Post-operatively, the animals were placed in a cage in a room adjacent to the operating room and oxygen supplementation was delivered by a face mask. Heart rate, respiratory rate, oxygen saturation and body temperature were monitored until the pigs regained consciousness. Once the pigs were back in their home pen, if needed, they were hand-fed pieces of fruit to stimulate appetite, supported to drink water, and assisted to stand and walk.

4.6 Glucose tolerance tests

Prior to glucose tolerance tests (GTTs), straw and wood shavings were removed from the pen and the pigs were provided synthetic fleece during fasting and GTTs. Glucose tolerance was tested after an 18 hour overnight fast. Water was withdrawn one hour before OGTT. Blood samples were collected before and up to 3 hours after glucose intake/infusion.

OGTT: The pigs were bottle fed an oral glucose load (Glukos APL Pulver till oral lösning, APL, Stockholm, Sweden) of either 1.75g/kg BW (paper II) or 2.5 g/kg BW (paper II (one pig) and III). Glucose was dissolved in 2 ml water/g glucose. The glucose solution had to be consumed within 5 minutes.

4.7 Ultrasound examinations

Ultrasound examinations were performed pre-operatively in non-trained, anaesthetised donor pigs to screen for renal cysts. Postoperatively, transplanted kidneys were thoroughly examined a few days after surgery. The urinary bladder of renal transplanted pigs was examined one or two times per day with a portable ultrasound machine (paper IV - study I, II, IV).

4.8 Euthanasia and post-mortem examinations

At the end of the studies, the pigs were euthanised with pentobarbital sodium either intravenously or intracardially under anaesthesia. The pigs underwent gross examinations post mortem to ensure that no abnormalities that could affect research results were present.

4.9 Blood analyses

4.9.1 Blood glucose

Blood glucose concentrations were measured with test strips (Accu-Check, Roche Diagnostics, Basel, Switzerland; validated for porcine blood at the Department of clinical chemistry, SLU).

4.9.2 NEFA, TG and amino acids

Serum NEFA concentrations were measured with 96-well Serum/plasma Fatty Acid Kit Non-Esterified Fatty Acid Detection 100 point kit (Zen-bio, Durham, NC). Serum TG concentrations were measured with automated equipment (EKTACHEM DT60II, Medinor, Stockholm, Sweden). Blood ketone bodies concentrations were measured with test strips (FreeStyle Precision β-ketone, Abbot/ADC, Chicago, IL). Serum concentrations of free amino acids and the amino acid-like compound taurine were measured with reverse-phase high-performance liquidchromatography (Syncronis C18 159 x 4.0 5µm Syncronis Column, Gothenburg, Sweden).

4.9.3 Insulin, C-peptide, glucagon, active and total GLP-1

Plasma insulin concentrations were measured with Porcine Insulin ELISA (10-1200-01, Mercodia, Uppsala, Sweden) In paper II, results were converted from µg/L to pmol/L by a conversion factor of 174, as recommended by the manufacturer. Mercodia changed the calibration of Porcine Insulin ELISA in 2017, and in paper III results were converted from mU/L to pmol/L by a conversion factor of 6, as recommended by the manufacturer. Plasma C-peptide concentrations were measured with Porcine C-peptide ELISA (Mercodia, Uppsala, Sweden). Plasma glucagon concentrations were measured with Glucagon ELISA (10-1281-01, Mercodia, Uppsala, Sweden). Plasma concentration of active GLP-1 were measured with Glucagon-Like Peptide-1 (Active) ELISA (EGLP-35K, Merck Millipore, St Charles, MO). Plasma concentrations of total GLP-1 were measured by radioimmunoassays after extraction of plasma with 70 % ethanol (vol/vol, final concentration). Carboxy-terminal GLP-1 immunoreactivity was determined using antiserum 89390 which has an absolute requirement for the intact amidated carboxy-terminus of GLP-1 7-36amide and crossreacts less than 0.01% with carboxy-terminally truncated

fragments and 89% with GLP-1 9-36amide, the primary metabolite of dipeptidyl-peptidase IV mediated degradation. The sum of the two components (total GLP-1 concentration) reflects the rate of secretion of the L-cell.

4.9.4 Creatinine and cystatin-C

Plasma creatinine concentrations were analysed with an enzymatic method (8L24-01, Abbot Laboratories, USA). Plasma cystatin-C concentrations were analysed with a particle-enhanced immunoassay (reagent: 1014; Gentian, Moss, Norway).

4.10 *Ex vivo* autoradiography

In paper III, pigs were injected with radiolabelled ^{177}Lu-Exendin-4. The study was divided in two experiments. In the OGTT group in the first experiment, an oral glucose load was given 10 minutes before ^{177}Lu-Exendin-4 injection. Experiment 2 was designed after *ex vivo* autoradiography results from experiment 1 had been analysed, and the oral glucose load was given immediately before ^{177}Lu-Exendin-4 injection and combined with an earlier euthanasia time-point, in order to see if that would capture a GLP-1R receptor occupancy in all intestinal segments.

Blood samples were collected from all pigs at predetermined time points after ^{177}Lu-Exendin-4 injection, and the pigs were euthanised at either 60 min (experiment 1) or 30 min (experiment 2) after ^{177}Lu-Exendin-4 injection. Immediately after euthanasia, tissue biopsies were collected from pancreas, stomach, proximal duodenum (5 cm from pyloric orifice), jejunum (50 cm from pyloric orifice), distal ileum (5 cm from ileocaecal valve), colon and spleen (negative control), and frozen in liquid nitrogen and kept in -80°C until sectioned.

Biopisies were processed into serial sections for autoradiography and hematoxylin and eosin (HE) staining for histology. In experiment 1, four slides were prepared for autoradiography, and results from each pig averaged. The results from different slides showed high similarity, hence in experiment 2, one slide was prepared for autoradiography. The tissue slides were exposed against digital phosphorimager screens, together with known references of ^{177}Lu, cross calibrated against a gamma counter to enable quantification of the autoradiograms. The phosporimaging plates were then

developed by a Typhoon autoradiography reader (GE Healthcare, Chicago, IL).

Image J (NIH) was used to analyse the autoradiography images. Segmentations were delineated on all tissues, and for duodenum also on internal sub-regions with clearly elevated binding. The references were similarly segmented. The binding in each tissue were corrected by the background of the plate as assessed by separate rectangular segmentations. All radioactivity measurements values were decay-corrected to the end of synthesis of ^{177}Lu-Exendin-4 for each experiment, to enable direct quantification. The known reference (Bq/counts) was used to convert autoradiography binding values in counts/mm^2 to Bq/mm^2. The known thickness of the sections (20 μm) was used to convert the binding values to Bq/mm^3. Finally, the specific radioactivity (GBq/μmol) of each batch ^{177}Lu-Exendin-4 at the end of synthesis was used to convert the binding in each tissue from Bq/mm^3 to fmol/mm^3. In experiment 1, the binding of ^{177}Lu-Exendin-4 in all slides of the respective tissue in each animal were averaged for the final reported value.

4.11 Retrospective analyses of Exendin-4 PET/CT in pigs

Results from *ex vivo* autoradiography showed clear differences in GLP-1R densities in different tissues, and data collected in a previous study (Nalin *et al.*, 2014) was used in order to investigate if the same pattern could be seen *in vivo* with ^{68}Ga-Exendin-4. A retrospective qualitative visual assessment was performed of the *in vivo* distribution of GLP-1R as assessed by PET. Relevant tissues (pancreas, stomach, duodenum, small intestine, large intestine/colon and spleen) were identified on co-registered PET and CT images by the software PMOD 3.7 (PMOD Technologies, Zürich, Switzerland). All images were normalised to standardised uptake value (SUV)=6 to enable direct comparison.

5. Results and discussion

5.1 Socialisation and training

After social training, all pigs were interested to interact with people involved in the studies and were calm during handling, figure 5. In diabetes research, it is crucial to avoid a stress response during blood sampling, a potentially stressful procedure in pigs that can rapidly increase blood glucose concentrations (Rand *et al.*, 2002; Jensen-Waern & Nyberg, 1993).

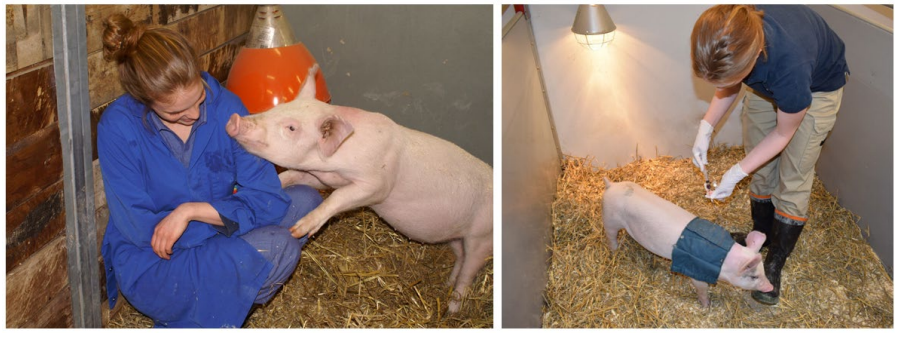

Figure 5. Socially trained pigs are calm and interested to interact with people. Photo: Hannes Manell

5.1.1 Structured training programme for renal transplantation studies

In paper IV, a structured socialisation and training programme enabled post-operative examinations without sedation or restraint in renal-transplanted pigs. In survival studies, a number of examinations and other interventions may be necessary. Restraint can induce a stress response in pigs (Becker *et al.*, 1985) and sedation or anaesthesia should be avoided since the drugs may

interfere with research results. For example, global and regional kidney perfusion is compromised by anaesthesia (Iguchi *et al.*, 2019) and blood flow might be a parameter evaluated after renal transplantation. Furthermore, the administration of sedative or anaesthetising agents may induce stress, even though there are methods to keep such stress to a minimum.

All 36 pigs fulfilled the 14 days training programme. There was an inter-individual difference in how fast the pigs progressed from one step in the training program to the next, figure 6. The first day, all pigs showed signs of fear, such as vocalisation or running away from the front of the pen, when people entered the stable, but became less afraid during the following two days. Step 2 was the most time consuming part of the training program, with some pigs requiring 6 days of training before they allowed petting and brushing. After step 2, the pigs adapted more quickly to the remaining interventions. Establishing the initial contact and being able to pet and brush the pig has also previously been recognise as the most important, but also challenging step (Nicholls *et al.*, 2012). Regardless of type of study, step 1 and 2 are relevant as first steps.

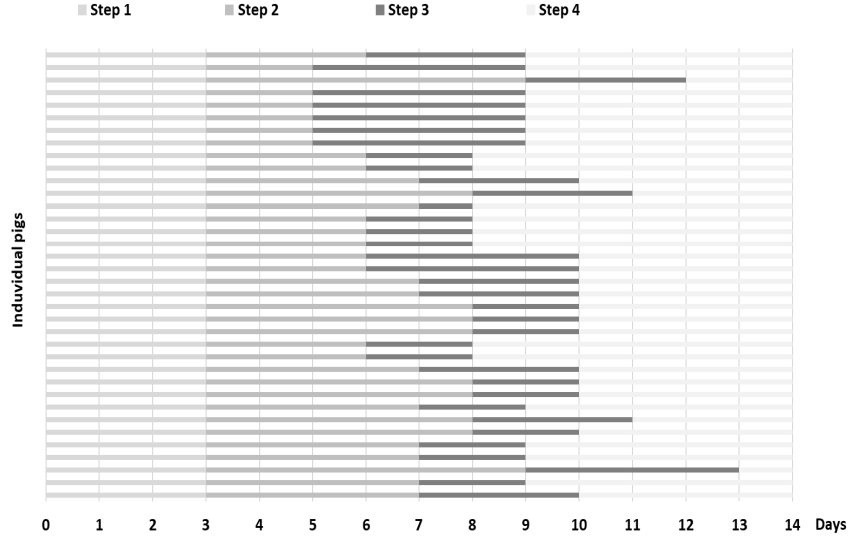

Figure 6. Individual progress of pigs in the socialisation and training programme prior to inclusion in renal transplantation studies. Horizontal lines indicate the time (days) for each individual to fulfil the different steps.

Out of the 36 pigs included in the training program, all pigs learnt to accept all interventions. Of the 25 pigs included in renal transplantation studies (11 pigs were recruited to other studies), blood samples and urine samples could be collected, and ultrasound examinations of urinary bladder and transplanted kidney could be carried out, all without any restraint or sedation. Blood and urine could be collected from the pigs without giving any rewards. For ultrasound examinations, the pigs had to stand or lie still for a longer period of time and were then rewarded with either pieces of fruit or being brushed, depending on the preference of the individual pig, in order to complete the examinations, figure 7. Waiting for pigs to urinate in order to collect free-flow urine samples is a potentially time consuming task. However, when pressing the water nipple in the pen to create a sound of running water, pigs started to urinate most times.

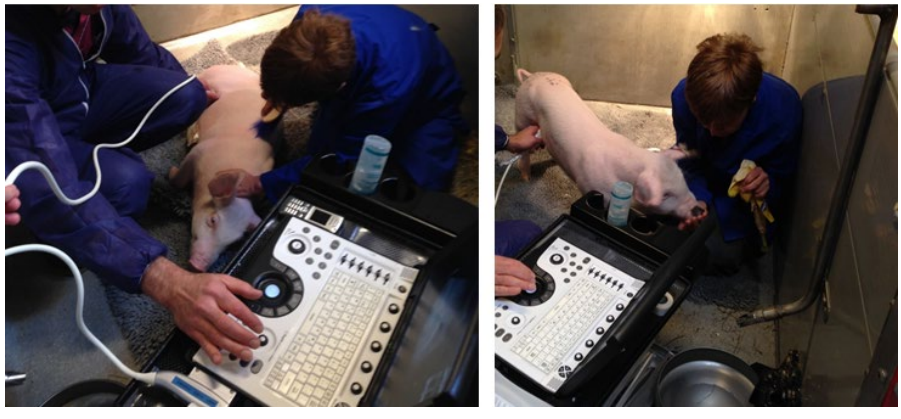

Figure 7. Ultrasound examinations of urinary bladder and renal graft, facilitated by brushing or offering pieces of fruit to renal transplanted pigs. Photo: Elin Manell

The 100% success of the training in paper IV is important for the 3Rs since no animals had to be excluded and the studies could be carried out without any stress for the pigs. Detailed descriptions of methods for training pigs are very limited. Nicholls *et al.,* 2012 described the effect of non-standardised training of pigs for retinal transplantation studies on ease of postoperative handling. However, 27% of the pigs would not allow petting or brushing, all pigs were sedated for post-operative examinations, and the pigs were manually restrained or confined in the corner of the pen by a board, in order to facilitate injection of sedatives. In the author's experience, pigs

that are comfortable around people can easily be reached and injected with a butterfly needle without need for restraint. In another study, pigs were handled individually for 3 minutes per day, and 85-90% did not allow petting even after training for up to 10 weeks (Terlouw & Porcher, 2005). These examples illustrates that there is not enough knowledge in the research community about how to successfully socialise and train pigs, and dissemination of successful protocols are important.

Pigs are curious animals and are motivated to interact with humans even if the persons ignore them (Terlouw & Porcher, 2005). Pigs interact more frequently with people familiar to them (Terlouw & Porcher, 2005), and preoperative training is best performed by the same people that will do the postoperative interventions. In general, the more time spent with the pigs, the easier it is to handle them (Nicholls *et al.*, 2012). Once a certain level of socialisation has been reached, further time investment might be unnecessary. How long the socialisation and training period needs to be depend on several factors such as breed, previous experiences, and which and how many interventions that the pigs need to be accustomed to. Results from paper IV showed that domestic pigs can be socialised to allow petting and brushing within 6 days, with a prior 3 days of acclimatisation.

5.1.2 Bottle-feeding training

In paper II, a technique for bottle-feeding an oral glucose load was established. While most pigs were curious to investigate the feeding bottle and interested to taste the glucose solution, some pigs were hesitant. The hesitant pigs were given meat broth for a couple of days, thereafter the glucose content in the bottle was gradually increased and the broth content gradually decreased. Two out of 18 pigs refused to drink both glucose and meat broth; these two were excluded from OGTT but underwent IVGTT. Out of the 16 pigs that learnt to bottle-feed glucose solution, nine pigs consumed the solution within the time limit (5 min) during OGTT. In L1 (not bottle-fed on weekends) the sucking from the feeding-bottle declined somewhat over time and the pigs started to bite more on the rubber teats. In L2 (bottle-fed every day) the pigs continued to suck from the feeding-bottle and improved their suckling behaviour throughout the study.

Since the experiment in paper II was carried out, we have more experience of training pigs for OGTT at the Department of clinical sciences, SLU. We now know that some pigs need more than two weeks to learn an

efficient bottle feeding technique, hence a longer training period can be applied to be able to include more pigs. Pigs unwilling to drink both glucose and meat broth have successfully been thought to first bottle-feed apple juice, and then glucose by gradual substitution. Bottle-feeding every day is important for the pigs to learn an efficient bottle-feeding technique. However, once they have learnt it, they do not need to be bottle-fed every day to maintain the skill. Domestic pigs that were left without bottle-feeding for a month could still bottle-feed perfectly. Previously trained Göttingen minipigs that were not bottle-fed for 4.5 months needed only 1-2 training session before they bottle-fed perfectly again.

5.2 Refined model for OGTT in pigs

The bottle-feeding training technique described above enable oral glucose tolerance testing in a similar manner as is done in humans. The OGTT model is a refinement of the mixed meal OGTT used in pigs, and has major advantages compared to the previously described method. With the refined method, the effect of glucose alone could be studied as the pigs drank only glucose (dissolved in water). Furthermore, the results were not affected by any diet, and comparisons between studies can easily be done. A comparison of human standard OGTT, the refined porcine OGTT model and porcine mixed meal OGTT is summarised in table 2.

Table 2. Comparison of human standard OGTT, refined porcine OGTT model and porcine mixed meal OGTT.

	Human OGTT	Refined porcine OGTT method	Traditional porcine mixed meal OGTT
Nutritional content	Glucose + water	Glucose + water	Glucose + feed (content varies between studies)
Time of glucose intake	Standardised, within 5 minutes	Standardised, within 5 minutes	Seldom reported, not standardised (?)
Intestinal glucose absorption		Similar to human OGTT	Attenuated due to fibre content of meal
Shape of glucose curves	Monophasic and biphasic described	Monophasic and biphasic described	Only monophasic described
Comparison between different studies	Easy due to standardised test	Easy due to standardised test	Difficult due to the use of different feed in different studies

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Fasting concentrations of blood glucose and plasma glucagon, table 3, were in the same range as levels measured in human adolescents (Manell *et al.*, 2016). Plasma insulin concentrations vary with age and stage of puberty in both humans and pigs (Peplies *et al.*, 2014; Jeffery *et al.*, 2012; Renner *et al.*, 2010; Grant, 1967). In paper II, young pre-pubertal pigs were used.

Plasma insulin concentrations reported were measured by Porcine Insulin ELISA 10-1200-01 (Mercodia, Uppsala, Sweden) and the conclusion drawn in paper II was that pigs have lower plasma insulin concentrations than pre-pubertal children. However, it was recently brought to the author's attention that Mercodia changed the calibration of the Porcine Insulin ELISA 10-1200-01 claiming that the new calibration is correct (personal communication with Mercodia). The new calibration produces insulin concentrations 4 times higher than previously. When the new calibration is used for the samples from paper II, fasting insulin concentrations are mean 22.8 pmol/L (range 10.4-48.0) for litter 1 and mean 20.3 pmol/L (range 2.1-47.3) for litter 2. These concentrations are actually very similar to those seen in young pre-pubertal children (Peplies *et al.*, 2014).

Table 3. Fasting concentrations of glucose, insulin, glucagon and GLP-1.

Litter 1			
	All pigs (n = 8)	**Females (n = 4)**	**Males (n = 4)**
Weight (kg)	18.5 (17.2–20.1)	18.9 (18–20.1)	18.2 (17.2–19.3)
Fasting glucose (mmol/L)	5.8 (5.3–6.7)	5.8 (5.3–6.7)	5.9 (5.4–6.3)
Fasting insulin (pmol/L)	5.7 (2.6–12.0)	5.9 (2.6–9.7)	5.5 (3.0–12.0)
Litter 2			
	All pigs (n = 10)	**Females (n = 5)**	**Males (n = 5)**
Weight (kg)	39.3 (35–43)	40.5 (36.5–43)	38.1 (35–40.5)
Fasting glucose (mmol/L)	5.7 (5.2–6.3)	5.5 (5.2–6.2)	6.0 (5.4–6.3)
Fasting insulin (pmol/L)	5.1 (0.5–11.8)	5.5 (1.7–11.8)	4.7 (0.5–11.3)
Fasting glucagon (mmol/L)	7.7 (5.7–10.9)	7.6 (5.7–10.9)	7.7 (5.8–9.0)
Fasting active GLP-1 (mmol/L)	8.8 (3.5–15.9)	7.9 (3.5–15.9)	9.7 (6–12.8)

Weight and fasting concentrations of blood glucose and plasma insulin, glucagon and active GLP-1 before IVGTT in litter 1 (Yorkshire x Swedish Landrace, 2 months old) and litter 2 (Yorkshire x Swedish Landrace x Hampshire, 3 months old). Values are mean (range).

doi:10.1371/journal.pone.0148896.t001

During OGTT, blood glucose concentrations were increased 10-30 minutes after glucose intake, figure 8. The time from oral glucose intake to peak blood glucose levels was similar to OGTT in humans, however, the pigs were more efficient in clearing glucose from the circulation (Matsuo *et al.*, 2014; Bagger *et al.*, 2011). This is also in line with results from IVGTT in paper II, where the pigs were more glucose tolerant compared to humans. Plasma insulin concentrations were gradually increased with peak values within 10-30 minutes in all pigs, and then gradually decreased. In four out of

the five pigs analysed for glucagon, an initial early increase in plasma glucagon concentrations was followed by a gradual decrease. Within 30 minutes after glucose intake, plasma glucagon concentrations were ~70% lower than fasting levels. At the 20-60 min sampling occasion, the decrease was close to reach statistical significance (p=0.063). Since blood samples from only five pigs were available for glucagon analysis, it is possible that the glucagon inhibition would have reached statistical significance if more pigs had been included. Even though one should not take for granted that biological actions are the same across species, it is still likely that there was a true decrease in plasma glucagon concentrations given the appearance of the glucagon curve, and the numerous previously published scientific evidence of such effect during OGTT.

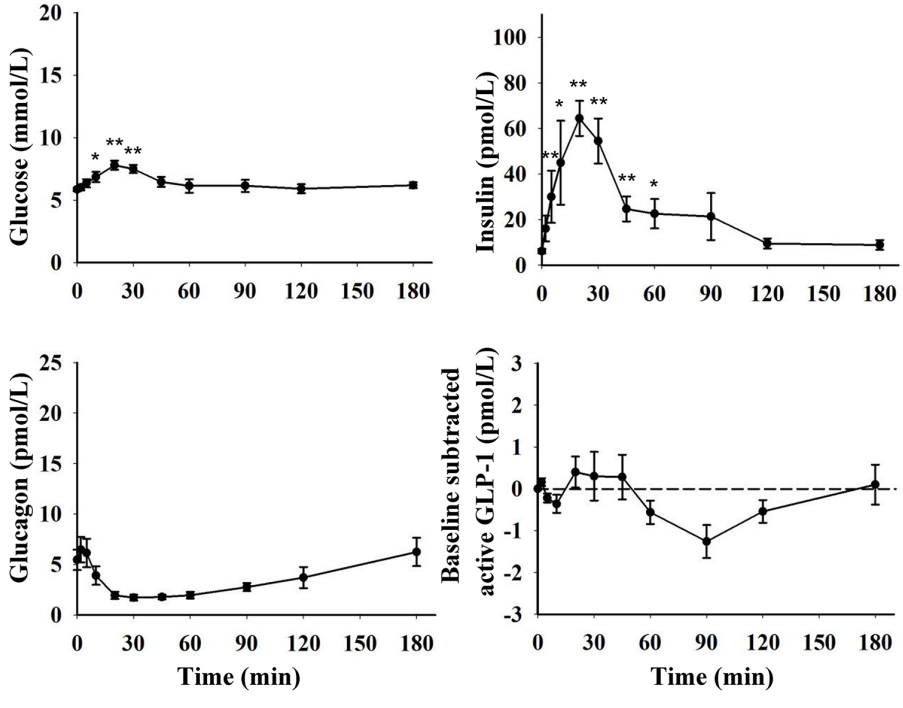

Figure 8. Blood glucose concentrations, and plasma concentrations of insulin, glucagon and basline subtracted (change from fasting levels) active GLP-1 during OGTT (1.75g/kg BW, n=9 for blood glucose and plasma insulin, n=5 for plasma glucagon and active GLP-1) in growing pigs. *indicate a significant increase compared to fasting levels (* = $p<0.05$, ** = $p<0.01$).

Two patterns of glucose curves were identified during OGTT, figure 9, monophasic (n=5) and biphasic (n=4). In the biphasic curves, two peaks were observed. Blood glucose concentrations increased to a range of 7.1-8.8 mmol/L within 30 min after glucose intake, and returned to values comparable to fasting before a second peak, range 7.5-8.1 mmol/L, was seen within 60-120 min after glucose intake. Biphasic curves have been described in humans with a strong association to the female sex (Tschritter *et al.*, 2003). Interestingly, all pigs with biphasic curves were females. In humans, biphasic glucose curves have been associated with lower BMI and better glucose tolerance, insulin sensitivity and beta cell function (Bervoets *et al.*, 2015; Tschritter *et al.*, 2003). Although the underlying biological mechanisms remain to be elucidated, this phenomenon is important to keep in mind. One pig showed a second peak with blood glucose concentration of 8.1 mmol/L at the 120 min sampling occasion, i.e. above the limit for impaired glucose tolerance (WHO, 2006). The 2-h glucose value is commonly used to diagnose impaired glucose tolerance and diabetes mellitus. If only 2-h glucose concentration is measured, individuals with biphasic curves may be captured on their second peak and diagnosed with impaired glucose tolerance although they represent a healthier phenotype.

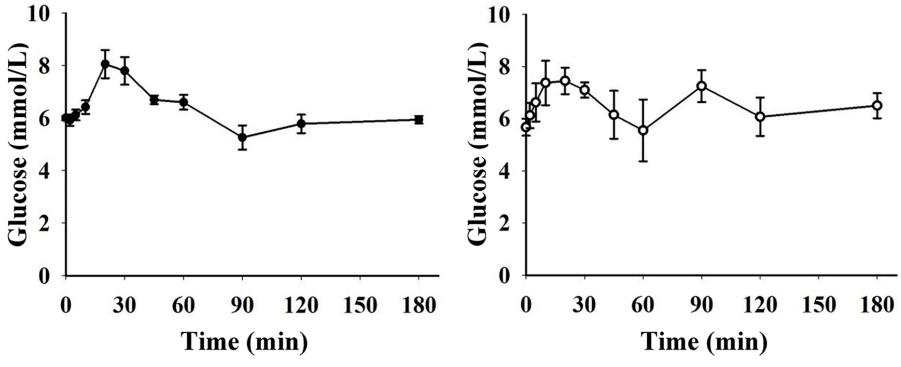

Figure 9. Blood glucose concentrations during OGTT (1.75g/kg BW) in growing pigs displaying monophasic (n=5; filled circles) and biphasic (n=4; open circles) curves.

There were large inter-individual variations in fasting concentrations of active GLP-1 among the pigs. Even though the pigs were the same age, lived under the same housing conditions and were fed the exact same meal size

and content, plasma fasting concentrations of active GLP-1 ranged from 3.5 to 15.9 pmol/L. Also in humans there are large inter-individual variations in fasting plasma concentrations of GLP-1 (Faerch *et al.*, 2015). When comparing GLP-1 concentrations across species and studies, one must keep in mind that GLP-1 is formed by post-translational cleavage of the precursor proglucagon (Orskov *et al.*, 1986), and assays used to measure GLP-1 concentrations must be highly specific to avoid cross-reaction with other proglucagon-derived peptides. Bak *et al.,* 2014 reported that commercially available kits vary considerably in their specificities and sensitivities, and that variability of concentrations measured in identical samples were alarmingly high. It can be anticipated that a number of published studies could contain data obtained with inappropriate assays. Glucagon-Like Peptide-1 (Active) ELISA (EGLP-35K, Merck Millipore) used to analyse porcine samples in paper II detect both isoforms of active peptide with high sensitivity and specificity (Bak *et al.*, 2014). Fasting plasma concentrations of GLP-1 were in the same range as seen in humans (Vilsboll *et al.*, 2001).

There was essentially no increase in plasma concentrations of active GLP-1 during OGTT, and one pig underwent a second OGTT with a higher glucose load (2.5g/kg BW) in order to see if that would induce GLP-1 secretion, figure 10. After glucose intake, a first peak with 80% increase in plasma concentration of active GLP-1 was seen at 10 min, and a second peak with 80-90% increase was observed at 60-90 min. Also in humans, active GLP-1 is released in a biphasic pattern with an early phase within 10-15 min and a second phase within 30-60 min (as reviewed by Baggio & Drucker, 2007). Although a 90% increase of plasma concentrations of active GLP-1 was seen, the actual increase was only 3.1 pmol/L. Plasma concentrations of active GLP-1 are low due to the rapid degradation by DPPIV (Deacon *et al.*, 1995) and in retrospect, it would have been interesting if also total GLP-1 concentrations had been analysed to see the total secretion of GLP-1 in response to oral glucose intake in pigs.

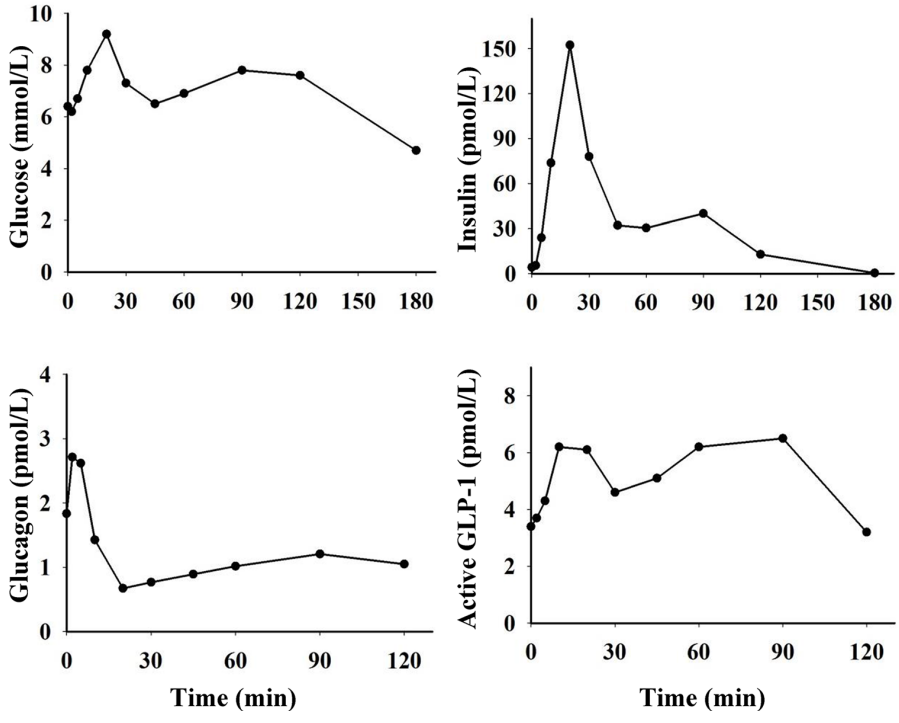

Figure 10. Blood glucose concentrations, and plasma concentrations of insulin, glucagon and active GLP-1 during OGTT (2.5g/kg BW) in one male 10 week old pig.

5.3 GLP-1 receptors in the gastrointestinal tract

The quantitative distribution of GLP-1Rs in the pancreas, gastrointestinal tract and spleen (negative control), measured in control pigs in paper III, is shown in figure 11. The highest densities of GLP-1Rs were seen in pancreas and duodenum, which is in line with previous reports on human tissues examined by *in vitro* radiography (Körner *et al.*, 2007) and non-human primate tissues where highest IHC staining intensity was also seen in pancreas and duodenum (Pyke *et al.*, 2014). An advantage of the experiments in paper III was that detailed quantification of receptor binding *in vivo* was possible by *ex vivo* methods.

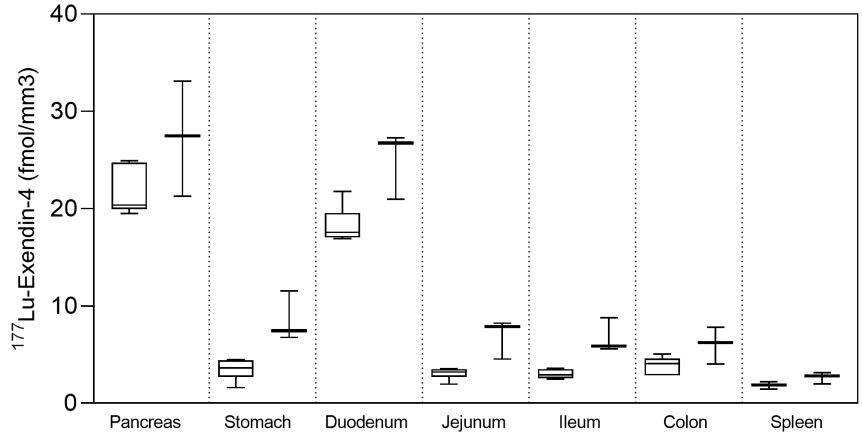

Figure 11. ^{177}Lu-Exendin-4 in pancreas, stomach, duodenum, jejunum, lieum, colon and spleen measured by *ex-vivo* autoradiography in control pigs in experiment 1 (left, n=6) and experiment 2 (right, n=3). Values are presented in boxplots with first quartile, median and third quartile. Whiskers indicate min to max.

It is important to allow injected radiolabelled substances time to circulate to minimise background signal. In experiment 1 (paper III), ^{177}Lu-Exendin-4 was allowed to circulate for 60 minutes before euthanasia and at that time concentration of ^{177}Lu-Exendin-4 in plasma was low, figure 12. In experiment 2, the pigs were euthanised 30 minutes after ^{177}Lu-Exendin-4 injection and analyses of ^{177}Lu-Exendin-4 in plasma showed 2-3 times higher background compared to experiment 1. This is likely the reason for consistently higher ^{177}Lu-Exendin-4 in tissues in experiment 2. However, results from control pigs in experiment 1 and experiment 2 showed a similar distribution pattern and showed that this method to assess target distribution of ^{177}Lu-Exendin-4 by *ex vivo* autoradiography in pigs is highly reproducible.

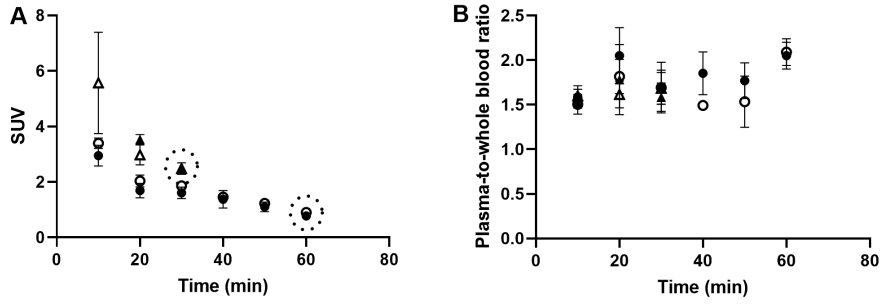

Figure 12. ^{177}Lu-Exendin-4 in blood (A) and plasma (B) in pigs in experiment 1, control group (n=6) filled circles, OGTT group (n=4) open circles, and experiment 2, control group (n=3) filled triangles, OGTT group (n=3) open triangles). Values are mean ± SD. Dotted circles represent the end-point in each experiment.

In duodenum, there were confined areas with very strong radioactive signal in the *ex vivo* autoradiography images. Since the method used enabled detailed quantification of receptor density, it was revealed that the high uptake areas in duodenum had higher receptor density than that measured within the pancreas, figure 13D-E. When compared to HE stained sections, the area with high ^{177}Lu-Exendin-4/mm3 tissue corresponded to submucosa with Brunner's glands. GLP-1Rs have been found to be numerous in Brunner's gland epithelial cells in non-human primates and humans (Pyke *et al.*, 2014; Körner *et al.*, 2007), and this is a likely location also in pigs given the results from *ex vivo* autoradiography. Brunner's glands secretions, including bicarbonate, glycoproteins and pepsinogen II, protects the proximal duodenum from acid chyme (Cornaggia *et al.*, 1987; Kirkegaard *et al.*, 1984; Smits & Kramer, 1984), and treatment with GLP-1 analogues in rodents increases production of substances involved in pathogen defence, barrier layer protection and mucosal healing (Bang-Berthelsen *et al.*, 2016).

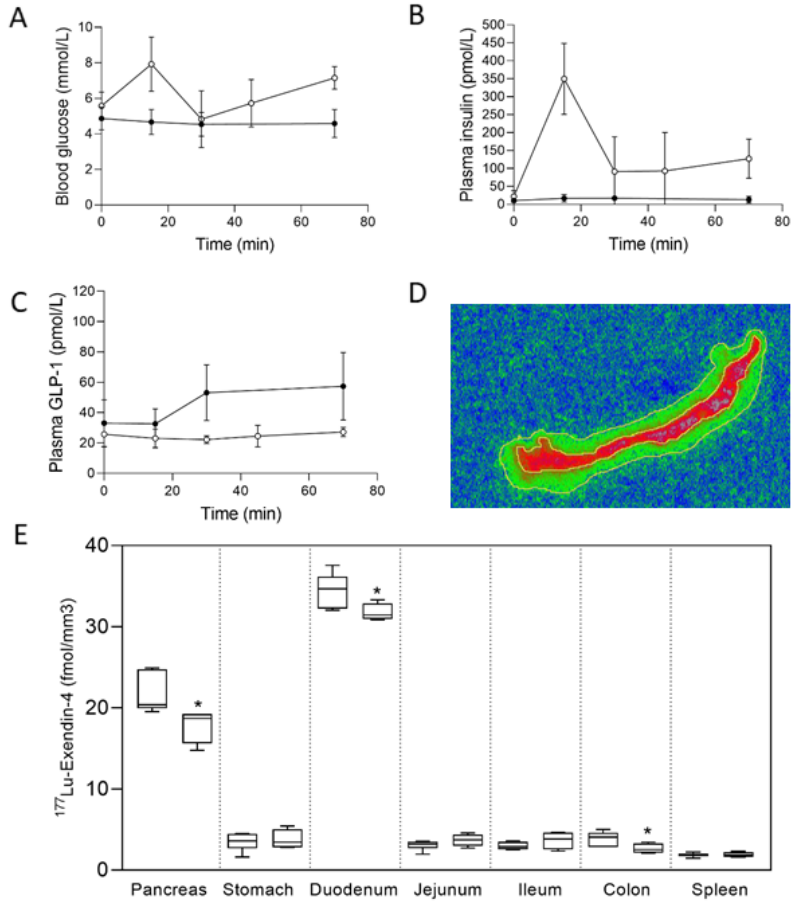

Figure 13. A) Blood glucose, B) plasma insulin and C) plasma total GLP-1 concentrations in pigs during experiment 1, control group (n=6) filled circles, OGTT group (n=4) open circles. Values are mean ± SD. ^{177}Lu-Exendin-4 was injected at time=10 min. D) Example of duodenum autoradiography image. Outer line represents entire section, inner line represents strong signal area. E) ^{177}Lu-Exendin-4 in pancreas, stomach, duodenum, jejunum, ileum, colon and spleen measured by autoradiography in experiment 1 in pigs, control (left, n=6) and OGTT (right, n=4). *indicate a significant ($p<0.05$) decrease in ^{177}Lu-Exendin-4 in the OGTT group compared to controls. Values are presented in boxplots with first quartile, median and third quartile. Whiskers indicate min to max.

In the other gastrointestinal segments, the radioactive signal was lower and dispersed compared to duodenum. When compared to negative control (spleen), the amount of ^{177}Lu-Exendin-4 was still higher in stomach,

jejunum, ileum and colon, demonstrating GLP-1R expression in those tissues. GLP-1 is known to decrease gastric motility and decrease gastric acid secretion (Wettergren *et al.*, 1993), and has been identified in muscle cells and parietal cells of the stomach in non-human primates (Pyke *et al.*, 2014). GLP-1R was also identified in myenteric plexus neurons (Pyke *et al.*, 2014) and GLP-1 affect intestinal motility (as reviewed by Holst, 2007). These receptor localisations are also possible localisations of porcine GLP-1R.

In pancreas, the ^{177}Lu-Exendin-4 signal was equally distributed across the entire autoradiography image. This result is in line with previous *ex vivo* autoradiography experiment where pig islets showed negligible contrast above the exocrine background (Eriksson *et al.*, 2017), and a previous PET experiment where uptake of ^{68}Ga-Exendin-4 was not affected by STZ induced beta-cell ablation (Nalin *et al.*, 2014). However, this is an area where porcine tissues differ from humans and non-human primates where GLP-1Rs are present in high density in beta-cells, and lesser expression seen in acinar cells within the pancreas (Pyke *et al.*, 2014; Körner *et al.*, 2007). Further experiments are needed to describe detailed receptor localisations on cellular levels in pigs, but these results point to a different and species-specific role of GLP-1 – GLP-1R axis in the pancreas and glucose metabolism in pigs.

The pigs in paper III were given a higher oral glucose load than commonly given to humans, and interestingly plasma GLP-1 concentrations did not increase in response to oral glucose, figure 13C. Consistently GLP-1R occupancy by endogenous GLP-1 was estimated as relatively low by the *ex vivo* autoradiography measurements. ^{177}Lu-Exendin-4/mm^3 tissue was 17.6% lower in pancreas, 7.9% lower in duodenum and 31.8% lower in colon, in the OGTT group compared to controls, figure 13E. GLP-1 is well known to increase insulin secretion from pancreatic beta-cells under hyperglycaemic conditions in humans (as reviewed by Baggio & Drucker, 2007). There are very limited data in the literature of GLP-1 secretion in response to oral glucose in pigs. The results in paper III are in line with results from paper II where increase in plasma concentrations of active GLP-1 was low or absent during OGTT. Also, in another study where pigs were given a meal challenge, plasma concentrations of active GLP-1 did not increase (Pluschke *et al.*, 2018). GLP-1 containing L-cells are few in the upper small intestines and plentiful in the distal part and large intestines (Kuhre *et al.*, 2014; Eissele *et al.*, 1992). Porcine small intestines are relatively long, 30-40 times the length of the pig's body (as reviewed by

Swindle *et al.,* 2012), consequently it will take time for nutrients to reach L-cells. Thus, since the human small intestines are shorter (as reviewed by Swindle *et al.,* 2012), the rapid increase in plasma concentrations of GLP-1 seen in humans after nutrient ingestion is likely to reflect early contact of glucose with proximal L-cells (Svendsen *et al.*, 2015). Perhaps GLP-1 is not an important incretin in pigs. On the other hand, presence of porcine GLP-1 containing L-cells (Orskov *et al.*, 1986), available receptors and the fact that the amino acid sequence of GLP-1 is conserved between species (Orskov *et al.*, 1989) indicate presence of a physiological function. Furthermore, local infusion of glucose into the lumen of porcine ileum stimulates GLP-1 release (Hansen *et al.*, 1999). Due to selective breeding of domestic pigs, they are very efficient in converting feed into muscle tissue, and are more glucose tolerant than humans. Perhaps a larger oral glucose load than that used in the present experiment would induce substantial GLP-1 release in pigs, and better model the GLP-1 response to oral glucose seen in humans. On the other hand, additional differences in the GLP-1 – GLP-1R axis have also been identified. Results in paper III confirmed previous results that GLP-1Rs are equally distributed in the pancreas and not predominantly found in the islets (Eriksson *et al.*, 2017), which is not the case for humans (Pyke *et al.*, 2014). Furthermore, when administering high doses of Exendin-4 intravenously to pigs, severe tachycardia >200 bpm develops (Rydén *et al.*, 2016). Although Exendin-4 increase heart rate also in other species such as non-human primates, the tachycardia is not as severe (Rydén *et al.*, 2016). Pigs might not be as well adapted to high plasma concentrations of GLP-1 as other species. While pigs and humans share many physiological characteristics, and there are several advantages of using animal models in situations where experiments cannot be carried out in humans, potential differences in GLP-1 regulation and GLP-1R distribution must be kept in mind when using pigs as animal models.

The *ex vivo* autoradiography results in paper III demonstrated high similarity to the biodistribution of ^{68}Ga-Exendin-4 in pigs previously scanned by PET/CT, figure 14. Clear uptake of ^{68}Ga-Exendin-4 was seen in pancreas and duodenum. Uptake was due to binding of GLP-1R as it was inhibited by co-injection with unlabelled Exendin-4 in excess. Diffuse binding was seen in small and large intestines, but it was unclear whether it was influenced by administration of unlabelled Exendin-4. No uptake was seen in spleen. Exendin-4 is well known to be excreted in urine (Nalin *et al.*,

2014) and high uptake of ^{68}Ga-Exendin-4 was seen in kidneys and urinary bladder, but since it was not receptor mediated it was uninfluenced by co-injection with unlabelled Exendin-4. PET has a lower spatial resolution than *ex vivo* autoradiography, but analogous studies investigating GLP-1R target distribution and occupancy can probably be performed *in vivo* using ^{68}Ga-Exendin-4, both in pigs and humans.

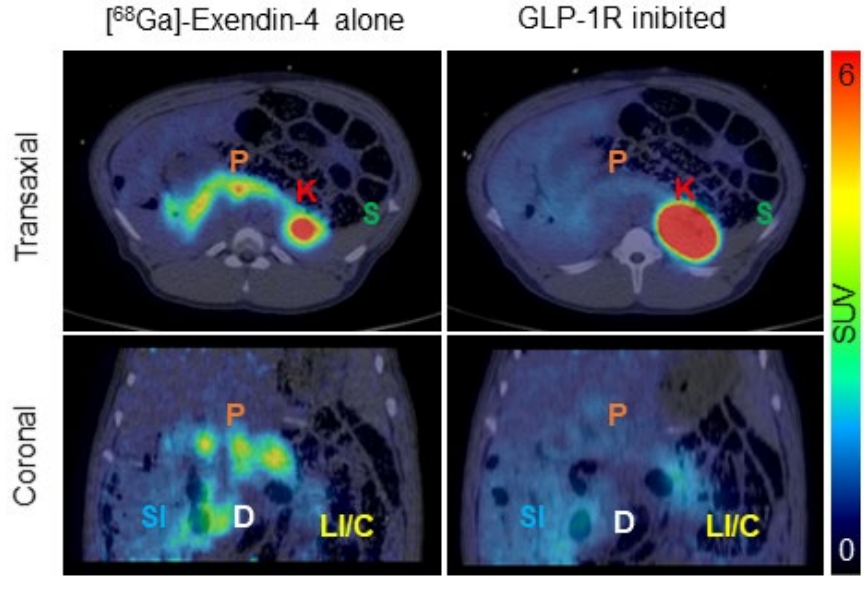

Figure 14. *In vivo* PET/CT images after injection of ^{68}Ga-Exendin-4 in pigs. The examination in each pig was performed as baseline scans, i.e. ^{68}Ga-Exendin-4 alone (left panels) or blocking scans, where unlabelled Exendin-4 in excess was co-injected to occupy the GLP-1R and inhibit the binding of ^{68}Ga-Exendin-4 (right panels). All images are normalised to SUV = 6 to enable direct comparison. Letters denote the location of pancreas (P), kidney (K), spleen (S), duodenum (D), small intestine (SI) and large intestine/ colon (LI/C).

5.4 Metabolic alterations and insulin treatment of STZ-diabetic pigs

In paper I, pigs became hyperglycaemic after STZ treatment with blood glucose concentrations above 23 mmol/L within 48 hours. In human patients with type 1 DM, plasma c-peptide concentrations below 100 pmol/L are seen. Similarly in the pigs, plasma c-peptide concentrations were below 100 pmol/L after beta-cell ablation and through the rest of the study period. Before initiation of insulin treatment, the pigs were hyperglycaemic with fasting blood glucose >17 mmol/L, and displayed clinical signs of diabetes mellitus including polyuria/polydipsia, and decreased daily weight gain.

Five days after STZ injection, one pig showed inappetence. Blood ketone body concentrations indicated hyperketonemia and the pig was treated with short-acting insulin (Actrapid®, 100 IU/mL, Novo Nordisk A/S) and treatment with Caninsulin®, described below, was initiated. Like in untreated or poorly controlled DM in humans, ketone body production in the liver can be accelerated in STZ diabetic pigs and frequent clinical observations of pigs with diabetes is important. One pig was used as untreated control during the 5 week study since the indwelling catheter stopped functioning. The general appearance of this pig was good, however it had a decreased daily weight gain (average 186 g) and post mortem examination revealed skeletal muscle wasting, subcutaneous fat reduced to a minimum, and no visible fat reserves in the abdominal cavity. Eventually, lack of insulin lead to DKA (Dreschfeld, 1886) and pigs used in long-term studies must be treated with insulin.

Insulin treatment was initiated one week after STZ injection. The initial dose of 2/3 IU/kg BW of intermediate acting insulin (Caninsulin® vet, 40 IU/mL, Intervet) was gradually increased over nine days to 1 IU/kg BW, divided equally between two doses per day. Fasting blood glucose concentrations were lowered over time with insulin treatment, however there were large variations with blood glucose concentrations around 4 mmol/L some days and concentrations above 20 mmol/L other days in the same pig. However, clinical signs of disease were resolved by insulin treatment. The daily weight gain of insulin treated pigs was 532 ± 96 g, which corresponds to the weight gain of conventional high health herd pigs of the same age.

After STZ treatment, serum concentrations of TG, NEFA and BCAA increased and alanine decreased, figure 15. These changes are consistent with changes seen in humans during insulin deficiency (Odedra *et al.*, 1982; Blackshear & Alberti, 1975; Felig *et al.*, 1970; Bagdade *et al.*, 1968; Hagen,

1961; Laurell, 1956). In humans, serum TG and NEFA concentrations vary with age (Kudo, 1969; Heald *et al.*, 1967; Munkner, 1959). The serum TG concentrations seen before STZ treatment in paper I (0.22 ± 0.06 mmol/L) correspond to those of healthy children and adolescents (Kudo, 1969). Serum NEFA concentrations in healthy pigs (0.23 ± 0.09 mmol/L) corresponded to levels seen in human adolescents, while prepubertal children have somewhat higher serum NEFA concentrations (Heald *et al.*, 1967).

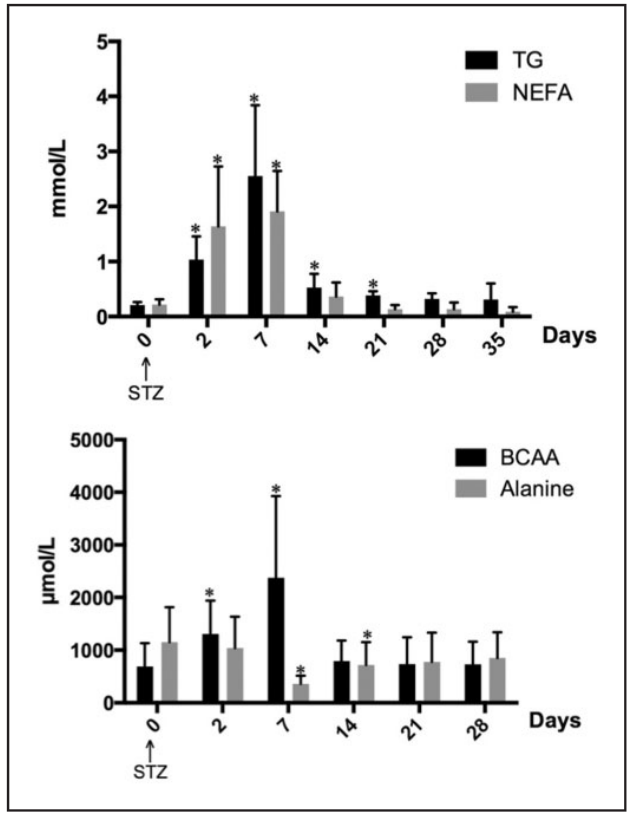

Figure 15. Serum triglyceride (TG) and non-esterified fatty acids (NEFA), upper panel, branched-chain amino acids (BCAA) and alanine, lower panel, concentrations in pigs Nos. 1,2,3,4,5,7,8 before and after streptozotocin (STZ) treatment. From day 7 onwards, the animals were treated with insulin twice daily. *indicates a significant difference ($p<0.05$) from before insulin treatment.

Gaining perfect glycaemic control is difficult for patients with DM which is the reason why long term complications due to high blood glucose concentrations is still a big problem in the society and DM is the seventh most common cause of death in the world (WHO, 2018; Franco *et al.*, 2007; Stratton *et al.*, 2000). Hence, it is not surprising that the pigs in paper I did not gain good glycaemic control, and that is not necessarily desired either. With a daily dose of 1 IU/kg BW divided equally by two doses per day, the pigs were free from clinical signs of disease, which is important from an animal welfare perspective. Working with diabetes models, hyperglycaemic animals are often desired, but severe complications avoided. In paper I, alterations in fasting plasma concentrations of NEFA, TG and amino acids were restored with insulin treatment, which means that the risk of progression to DKA is low. Domestic pigs are usually not used in long-term studies due to their large increase in body weight, hence long-term complications due to poor glycaemic control is usually not a concern. Blood glucose concentrations of the STZ diabetic pigs did fluctuate a lot during the day, this characteristic may be used as a model for poor glycaemic control. The insulin dose was not increased above 1 IU/kg BW since fasting blood glucose concentrations below 4 mmol/L were detected on a few occasions, although without clinical signs of hypoglycaemia. The insulin treatment regimen described is satisfactory for many types of studies. However, efforts could be made to try to improve glycaemic control if that would be important for the research protocol.

5.5 Interventions in renal transplantation studies

Pigs included in paper IV, underwent post-operative interventions such as blood sampling, ultra sound examinations and urine sampling. Plasma concentrations of creatinine were followed in animals where native kidneys had been removed, figure 16, as an indicator of renal function. GFR was not calculated, but increased plasma creatinine concentrations indicate a gradual decrease in GFR (as reviewed by Sjaastad *et al.,* 2003). Pre-operatively all pigs had plasma creatinine concentrations < 100 μmol/L. It is interesting to note that plasma creatinine concentrations could be above 1000 μmol/L without affecting the animal's general health status. Pigs have a large relative muscle mass compared to other species such as mice, dogs and humans, and plasma creatinine concentrations can therefore become relatively high.

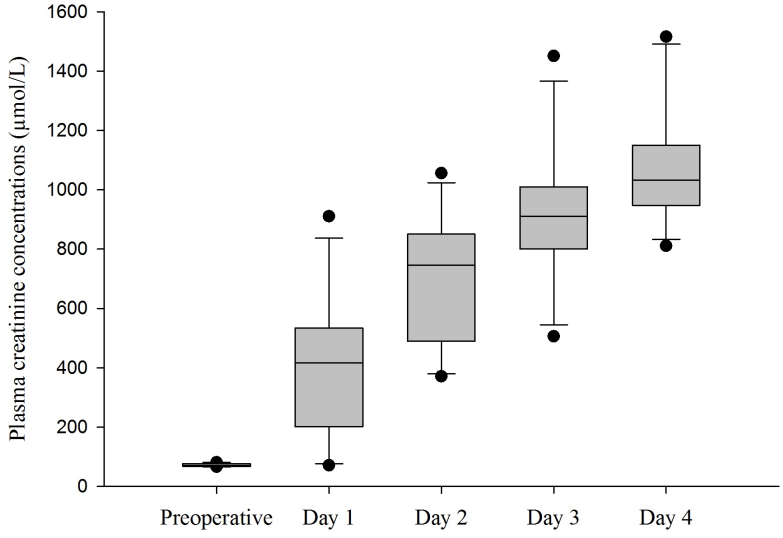

Figure 16. Plasma creatinine concentrations in samples taken before and 1, 2, 3 and 4 days after renal transplantation in pigs (n=11). Values are presented in boxplots with first quartile, median and third quartile. Whiskers indicate 10th and 90th percentile. Dots represent points that lie outside the 10th and 90th percentile.

Plasma cystatin C concentrations are not affected by muscle mass or dietary protein intake (Tangri *et al.*, 2011; Stevens *et al.*, 2006), and were measured in paper IV since it is considered superior to creatinine for estimation of GFR in humans (Ferguson *et al.*, 2015). In the samples from the renal transplantation studies, cystatin C concentrations were below detectable levels at all sampling occasions despite markedly increased plasma creatinine concentrations after transplantation. This finding indicates that cystatin C is not a reliable biomarker for GFR estimation in pigs, which is in line with a previous study that concluded that cystatin C concentrations in pigs with endotoxemic shock did not correlate with GFR measured by inulin clearance and should be interpreted with great care (Eriksson *et al.*, 2012). In dogs, cystatin C might be used as an adjunct to creatinine for the diagnosis of decrease GFR but is considered inferior to creatinine when used alone (Pelander *et al.*, 2019). Plasma concentrations of cystatin C demonstrates yet another example where species differences must be considered.

In the renal transplantation studies, minimally invasive Seldinger technique was used to insert indwelling vein catheters into the jugular vein through the auricular vein. The technique worked very well and the pigs accepted blood sampling throughout the study. Minimally invasive techniques are preferred from a 3R perspective as it will have minor effects on the animal. One limitation though is that pigs move their head quite a lot for rooting, investigating objects and looking around in their surroundings, which makes it more difficult to follow the pigs movements during blood sampling compared to when the vein catheter has been surgically implanted and exteriorised on the back between the scapulas. In the renal transplantation studies, blood samples were collected once per day and then it can be accepted that blood samples differ a little in time from day to day. However, in other circumstances such as during GTTs, when blood need to be withdrawn at exact time points, surgically implanted catheters are preferred to make sure that important research data are not missed and that the animals are used in the best possible way.

Three litters of potential kidney donor pigs were examined by ultrasound to screen for renal cysts before inclusion in the studies. The presence of renal cysts ≥10mm led to exclusion of 71%, 50% and 0% of the potential organs from the three litters. The high frequency of renal cysts found in two of the litters underscores the importance of examining donors before inclusion in renal transplantation studies. Even though renal cysts may not cause alterations in renal function (as reviewed by Newman *et al.,* 2007), outcomes in renal transplantation studies might be affected. Congenital renal cysts are frequently seen in pigs (as reviewed by Drolet, 2012). A study investigating pigs at slaughter reported a prevalence of 12.5% (Jansen & Nordstoga, 1992), but in a single herds as much as 47.5% prevalence has been reported (Wells *et al.*, 1980). In paper IV, prevalence varied a lot between litters, which is in line with previous observations that renal cysts are sometimes inherited (Wijeratne & Wells, 1980). The majority of the cysts are medullary and not visible by ocular inspection (Jansen & Nordstoga, 1992), i.e. inspection during surgery is not sufficient and potential donors need to be examined by ultrasound.

6. Concluding remarks

This thesis describes a number of refinements of porcine models in diabetes and renal transplantation research. Refinement is closely linked to reduction. By applying refined techniques, often reductions can be made, and in practice, this PhD project has also involved methods for reduction. For example, the insulin treatment protocol described in paper I will minimise the risk of serious complications such as DKA or pronounced hypoglycaemia in STZ diabetic pigs, which is an important refinement from an animal welfare perspective. In addition, reducing the risk of serious complications means that the risk of having to remove animals from the study is decreased, hence reducing the numbers of animals needed.

By further characterising the pig as an animal model for diabetes, it was shown that domestic prepubertal pigs are good models for human children and adolescents in many aspects, and that glucose tolerance can be tested in pigs in a similar manner as done in humans by bottle-feeding the pigs glucose dissolved in water. Metabolism and hormonal secretion in relation to diabetes research showed high similarity between pigs and humans, although some important differences were identified such as the pigs being more glucose tolerant, and that there are differences in GLP-1 regulation and GLP-1R distribution. An additional species difference identified was that cystatin C is not an appropriate biomarker for GFR in renal transplanted pigs. An animal model does not necessarily need to mimic the modelled species in all aspects. Major medical advances have been made by research using different laboratory animal species. However, limitations must be kept in mind to avoid unnecessary use of animals in inappropriate models.

Training and socialisation of pigs in research avoid stressful situations and the need for restraint. Calm and cooperative animals are crucial for good

animal welfare and to acquire reliable research data. The initial training where the pigs get accustomed to the trainers and allow contact such as petting and brushing is somewhat time consuming, but the most important step. Once the pigs allow petting and brushing, they can easily get accustomed to different types of examinations and other interventions. Applying a structured training program ensures that all pigs are effectively socialised and trained.

Photo and image processing: Elin Manell

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Popular science summary

Animal models are used in biomedical research in situations where studies cannot be carried out in humans. Pigs share a number of anatomical and physiological characteristics with humans and are therefore often a suitable species when a large animal model is needed. From an ethical point of view, it is important that the animals are treated well, but it is also essential to prevent stress responses that might affect research results. The aim of this thesis was to work with refinements of porcine models in diabetes and renal transplantation research.

In the thesis, a structured socialisation and training programme for pigs that will take part in renal transplantation studies is presented. After completed training, the pigs were social and calm. It was possible to collect blood samples and urine samples, and to examine the pigs by ultrasound without any need for restraint.

Insulin treatment is essential for diabetic pigs without insulin production, and an insulin treatment protocol that minimise the risk of clinical signs of disease was developed. A refined method for testing glucose tolerance is also presented. This is an important test, for example to be able to evaluate effects of different treatments. The physiology regarding metabolism and hormones related to diabetes was investigated in pigs and both similarities and differences between pigs and humans were identified. This type of information is important to have to be able to design animal studies in a good way so that as few animals as possible are included and so that each animal is used in the best possible way.

Populärvetenskaplig sammanfattning

I forskning som syftar till att förebygga och behandla sjukdomar används djurmodeller i situationer där studier inte kan utföras på människor. Grisar och människor delar många anatomiska och fysiologiska egenskaper och därför lämpar sig grisen ofta för användning när en stordjursmodell behövs. Det är viktigt ur ett etiskt perspektiv att försöksdjuren är trygga och behandlas väl, men det är också av betydelse att förhindra stress som annars kan påverka försöksresultaten. Målet med den här avhandlingen var att arbeta med förbättringar av grismodeller som används för forskning kring diabetes och njurtransplantation.

I avhandlingen presenteras ett strukturerat socialiserings- och träningsprogram för grisar som ska ingå i njurtransplantationsstudier. Efter genomgången träning var grisarna tama och det gick att samla blodprover och urinprover samt utföra ultraljudsundersökningar på grisarna utan att de behövde någon form av fasthållning.

Insulinbehandling är nödvändigt för diabetiska grisar utan egen insulinproduktion och ett insulinbehandlingsprotokoll som minimerar risken för att grisarna ska visa symtom på sjukdom togs fram. Här presenteras också en förbättrad metod för att undersöka grisarnas förmåga att ta hand om socker, ett viktigt test för att kunna utvärdera effekter av behandlingar. Fysiologin kring metabolism och hormoner relaterat till diabetes undersöktes hos grisar och specifika skillnader och likheter mellan grisar och människor identifierades. Den typen av information är viktigt för att kunna planera djurstudier på bästa sätt så att så få djur som möjligt används och så att varje djur används på bästa sätt.

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The aim of this thesis was to work with refinement of porcine research models. A structured training programme for pigs in renal transplantation studies, which enabled postoperative interventions in conscious pigs without restraint, is presented. Furthermore, a refined model for porcine oral glucose tolerance test was established, hormonal responses to an oral glucose load described and glucagon-like peptide-1 receptor localisations investigated. Metabolic alterations due to insulin deficiency in streptozotocin diabetic pigs are described, and an insulin treatment protocol was established.

Elin Manell received her postgraduate education at the Department of Clinical Sciences. Her undergraduate degree in veterinary medicine was obtained at the Swedish University of Agricultural Sciences (SLU).

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