A porcine type 1 Diabetes Mellitus model, for non-invasive \textit{in vivo} imaging of the glucagon-like peptide-1 receptor in the pancreas, using $[^{68}\text{Ga}]{\text{Ga}}$-DO3A-VS-Cys40-conjugated synthetic exendin-4 in PET-CT

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Kan betacellsmassan i pankreas bestämmas med hjälp av PET-CT? En studie av GLP-1R/exendin-4-komplexet hos gris

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SUMMARY

Diabetes mellitus is a rising epidemic throughout the world and there is currently great interest in quantifying the beta-cell mass (BCM) in vivo non-invasively. In the present experiment, the feasibility of in vivo imaging of the glucagon-like peptide-1 receptor (GLP-1R) in beta-cells was examined, using the positron emission tomography (PET) tracer $^{68}$Ga-DOTA-VS-Cys40-exendin-4 as a marker, in native pancreatic beta-cells of a porcine diabetic animal model and healthy controls.

Eight Swedish high-health domestic pigs were randomly assigned to be either controls or made diabetic using streptozotocin (STZ). The experiment proceeded during eight weeks, starting with an acclimatisation period. Once the pigs had been socialised they underwent surgery for the insertion of a jugular vein catheter, allowing induction of diabetes with STZ, intravenous (i.v.) injections and stress-free blood sampling. Development of diabetes was confirmed by clinical examinations, blood glucose values and insulin-staining of pancreatic sections post mortem.

The diabetic pigs were insulin treated and responded well. PET-CT (PET-computed tomography) examinations were performed on healthy controls and insulin-treated diabetic pigs. At the beginning of the PET-CT scan, oxygen-15 labelled water ($^{15}$O)WAT) was injected i.v. to measure tissue perfusion in the pancreas and kidneys. The specific binding of $^{68}$Ga-DOTA-VS-Cys40-exendin-4 to the GLP-1R in vivo was assessed by i.v. administration of the tracer compound giving a baseline image. This was followed by the administration of a competing high dose of synthetic exendin-4 and a new imaging sequence. The pigs were humanely euthanised 0–6 days after the PET-CT examination and full post mortem examinations were performed in all pigs.

Diabetes was successfully induced, confirmed by immunohistochemical (IHC) staining for insulin. An important incidental finding, during PET-CT examination, was that the tracer and synthetic exendin-4 immediately induced a significant tachycardia in all pigs both at low and high dose. PET scans showed a reduced tissue perfusion in the pancreas and kidneys of the diabetic pigs. GLP-1R-mediated uptake of the tracer was detected in the pancreas of both healthy controls and diabetic pigs and surprisingly, the uptake of the tracer did not differ between the two groups. Thus, the pancreatic tracer uptake of $^{68}$Ga-DOTA-VS-Cys40-exendin-4 was not significantly reduced by selective destruction of beta-cells in diabetic pigs and the GLP-1R is not a suitable target for imaging of native pancreatic beta-cells in pigs. Additionally, this experiment shows how the pig can be made diabetic, insulin-treated and properly anaesthetised for several hours, which makes the pig a suitable animal model for further diabetic research.
**SAMMANFATTNING**


Åtta svenska serogrisar (av rasen Yorkshire x svensk lantras x Hampshire) från samma kull randomiserades till att antingen vara kontroll eller bli diabetiker med hjälp av streptozotocin (STZ). Experimentet pågick under ett år och inkluderedes med en acklimatiseringsperiod. När grisarna hade blivit socialiserade genomgick de kirurgi då en jugulär venkateter placerades, för att möjliggöra diabetesinduktion med STZ samt intravenösa (i.v.) injektioner och blodprovstagnings under stressfria förhållanden. Genom klinisk undersökning, blodglukosvärden och insulinfärgning av bukspottskörtelvävnad post mortem konfirmerades att grisarna blivit diabetiska.

De diabetiska grisarna insulinbehandlades, med gott resultat. PET-CT-undersökning utfördes på både friska och insulinbehandlade diabetiska grisar. Först genomfördes en PET-CT-scan med radioaktivt vatten ($^{15}$O)WAT för att mäta vävnadsperfusionen i bukspottskörteln och njurarna. För att påvisa specificiteten in vivo av tracern $^{68}$Ga-Ga-DODA-VS-Cys40-exendin-4 till GLP-1R injicerades först tracern ensamt varpå en omgång bilder togs, följt av i.v. administration av en kompetitiv högdos syntetiskt exendin-4 och en ny sekvens bilder. Grisarna avlivades några dagar efter PET-CT-undersökningen.

Ett viktigt bifynd under PET-CT-undersökningarna var att samtliga grisar drabbades av kraftig takykardi omedelbart efter injektion av tracer och syntetiskt exendin-4. Vävnadsperfusionen i bukspottskörteln och njurarna var signifikant lägre hos de diabetiska grisarna jämfört med de friska kontrollerna. Vidare visade PET-CT-undersökningarna receptorspecifik bindning av tracern till GLP-1R, men upptaget skiljde sig inte väsentligt mellan de två grupperna. Detta visar att upptaget av $^{68}$Ga-Ga-DODA-VS-Cys40-exendin-4 inte påverkas av selektiv destruktion av betaceller hos diabetiska grisar och att GLP-1R inte är en biomarkör för levande betaceller i pankreas hos gris. Studien visar dock att grisen med gott resultat kan göras diabetisk och insulinbehandlas, för att i sitt diabetiska insulinbehandlade tillstånd sövas under flera timmar i sträck. Detta gör grisen till ett lämpligt modelldjur för vidare diabetesforskning.
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INTRODUCTION

Diabetes mellitus is a rising epidemic throughout the world. Prevalence studies show that diabetes is a growing burden for the global society and there is currently no peak in sight (Whiting et al., 2011; King et al., 1998; Amos et al., 1997; King & Rewers, 1993).

This master degree project is part of a larger collaboration between various professions (medical doctors, mathematicians, chemists and physicists) from the PET preclinical platform at Uppsala University and veterinarians from the Department of Clinical Sciences at the Swedish University of Agricultural Sciences.

Studies to quantify the glucagon-like peptide-1 receptor (GLP-1R) in the pancreas using positron emission tomography-computed tomography (PET-CT) have previously been performed on a rodent diabetic model and healthy cynomolgus primates (Selvaraju et al., 2013), but there is currently no published study on the pig as an animal model using this technology and tracer.

The aim of this study was to examine the feasibility of in vivo imaging of the GLP-1R in beta-cells, using the PET tracer $[^{68}\text{Ga}]\text{Ga-DO3A-VS-Cys40-exendin-4}$ as a marker, in native pancreatic beta-cells of a porcine diabetic animal model. The hypothesis was that this could be a possible method for quantifying the beta-cell mass.

LITERATURE REVIEW

Prevalence of Diabetes Mellitus

Humans

There are two types of diabetes mellitus described in humans – type 1 diabetes mellitus (T1D) and type 2 (T2D). Since data sources from the World Health Organisation (WHO) do not separate these two types, the presented prevalence numbers include both T1D and T2D (Wild et al., 2004).

Wild et al. (2004) estimated that the prevalence of diabetes, in all age-groups worldwide, was 2.8% in year 2000 and predicted it would be 4.4 % in 2030. The total number of people with diabetes was estimated to 171 million in year 2000 and it was predicted to be 366 million in year 2030. Another study, including prevalence numbers from 91 countries, estimated that the world prevalence of diabetes among adults were 286 million (6.4%) in 2010 and would be 439 million (7.7%) in 2030 (Shaw et al., 2010). In the most recently published article, data from 1980 to April 2011 was reviewed. The authors say that previous estimates of the global burden of diabetes have been too conservative and Whiting et al. say that in 2011 there were 366 million people with diabetes, a number which they expect to increase to 522 million people by 2030 (Whiting et al., 2011).

Based on data from WHO the global mortality, that can be attributed to diabetes, in year 2000 was estimated to be 2.9 million deaths, corresponding to 5.2% of all deaths that year (Roglic et al., 2005).
Cats, dogs and pigs

Prevalence estimates of diabetes in cats vary between 0.25 to 2.0% (Rand et al., 2004). A U.K. study that included 14,030 insured cats found a prevalence of 0.43%, with Burmese cats significantly more likely (odds ratio 3.7) to become diabetic (McCann et al., 2007). In cats, diabetes mellitus is one of the endocrinopathies with highest prevalence (Zini et al., 2010).

The incidence of diabetes in a Swedish study in 180,000 dogs was 13 cases per 10,000 dog-years at risk with significant breed-specific sex- and age-differences (Fall et al., 2007).

In pigs, spontaneous diabetes is extremely rare, and only one case has been reported. However, the pig is a suitable animal model for diabetes in humans, since they are similar in terms of physiology and metabolism. Thus, for studies in the diabetic pig, diabetes needs to be chemically induced (Jensen-Waern et al., 2009; Larsen & Rolin, 2004).

Non-invasive imaging of the beta-cell mass

Diabetes mellitus is a serious illness with major implications to the patient as well as the global economy. There are several imaging techniques, which may be used for examining the pancreatic tissue as a whole. Currently, the beta-cell function and glycaemic control may be analysed through different metabolic tests. The pancreatic tissue can only be examined post mortem and by in vivo biopsy sampling for partial tissue analysis. However, a non-invasive in vivo method to examine and quantify the endocrine pancreatic beta-cells has not yet been developed (Leibiger et al., 2012; Malaisse & Maedler, 2012; Nauck, 2009; Robertson, 2007; Souza et al., 2006a).

Having the possibility to in vivo image the beta-cell mass (BCM) would provide further insight into the pathogenesis and changes of BCM throughout different diabetic stages. It would be a helpful diagnostic tool and useful when distinguishing the two types of diabetes as well as a valuable tool for monitoring medical therapy or to follow-up islet transplantation. Furthermore, the ability to accurately image and quantify the insulin-producing beta-cells is of high importance to further islet transplantation and therapeutic research (Yang et al., 2013; Andralojc et al., 2012; Arifin & Bulte, 2011; Virostko & Powers, 2009; Paty et al., 2004).

Imaging methods

Positron emission tomography – computed tomography and single photon emission computed tomography

Positron emission tomography (PET) is a nuclear imaging method that uses a radiolabelled biological compound (tracer). The examined subject is placed in the PET scanner tunnel and is surrounded by detectors. The tracer is injected and is then distributed following its pharmacological properties in the body of the subject, where the tracer results in high energy $\gamma$-photons being emitted. The detectors convert the high-energy $\gamma$-photons emitted from the patient into an electronic signal. Repeated image sequences are acquired to measure the radioactive concentration in specific tissues as a function of time. Based on the photons that are emitted as a result of the tracer and using mathematical correction algorithms, a three-dimensional image showing the quantity of radioactivity in a specific region is provided.
(Andralojc et al., 2012; Saha, 2010; Alessio & Kinahan, 2006). To relate the tracer signal with a correct anatomical localisation in the patient a CT is used in combination with the molecular imaging technique. The addition of a CT greatly enhanced the PET technology, but also resulted in increased radiation dose to the patient (Dobrucki & Sinusas, 2005; Beyer et al., 2000).

Single photon emission computed tomography (SPECT) and PET are based on different physical methods. In PET, the radioactive isotope decays with the emission of a positron. The positron collides with atomic electrons of the surrounding tissues; these collisions cause the positron to lose energy and it finally annihilates with an electron. This annihilation results in two high-energy photons being emitted in opposite directions, which are then simultaneously registered by the detectors of the PET. The position of origin of the photons in the volume of the examined subject can then be calculated. On the other hand, in SPECT, the technology is based on using a radioactive isotope which emits single photons instead (Chatziioannou, 2005). SPECT-CT is not as widely used as PET-CT, due to high cost in relation to the number of clinical indications for SPECT-CT (Rahmim & Zaidi, 2008). The implications of the difference in the basics of image technology between PET and SPECT will be discussed below.

SPECT has several similarities with PET. PET-CT and SPECT are both highly sensitive nuclear imaging modalities, measuring picomolar radioactivity. However, PET has an advantage over SPECT in terms of sensitivity, i.e. PET detects a higher proportion of emitted tracer signals (~ two to three times as many) (Andralojc et al., 2012; Rahmim & Zaidi, 2008). Furthermore, PET can quantify the tracer signal in absolute terms (Selvaraju et al., 2013), which is not possible with SPECT (Rahmim & Zaidi, 2008; Chatziioannou, 2005).

Through multiple-energy windows dual tracers can be used in SPECT; this cannot be performed using PET (Rahmim & Zaidi, 2008). Moreover, with SPECT, there is a possibility to widen the observational time window to follow biological events over a longer period of time (hours to days) compared to PET, due to longer half-lives of single photon emitters used in SPECT (Meikle et al., 2005). However, this also prolongs the radiation load to the patient. Moreover, the short half-life of radionuclides used in PET imaging improves sensitivity, since these tracers may be injected at higher radioactivity (of course to a certain extent), consequently increasing the detectable radiation over a shorter period of time to the same radiation load as a tracer with longer half-life but lower radioactivity (Rahmim & Zaidi, 2008).

An important issue with PET and SPECT is photon attenuation. This refers to the fact that emitted radiation interacts with the tissue when it passes through the body of the subject, which makes the photon change direction, ultimately leading to loss of energy and scattering and thus reducing the signal in the image. There are different approaches to perform photon attenuation correction, one of which is to use a CT to map the attenuation of a body region and use this data to correct for attenuation in the PET image. Attenuation correction is easier achieved with PET than with SPECT, due to the fact that the origin of the tracer signal can be acquired in PET since the photons are emitted in pairs, while in SPECT the origin of the
signal needs to be well known since it cannot be calculated. This is a great challenge for SPECT and results in lower sensitivity compared to PET (Saha, 2010; Rahmim & Zaidi, 2008; Bailey et al., 2005; Chatziioannou, 2005). However, there may be inaccuracies in attenuation correction followed by discrepancies between the PET and CT images (Mawlawi & Townsend, 2009; Pan et al., 2005). Additionally an imaging artefact called random coincidences occurs in PET (not an issue in SPECT). This occurs when two photons are detected within the same coincidence time frame but the photons do not come from the same origin. This results in incorrect location of the origins of those photons in the PET-image. Trying to accurately correct for these random coincidences is subject for ongoing research in PET imaging (Rahmim & Zaidi, 2008).

The spatial resolution (i.e. the detail of the image) of both PET and SPECT is relatively low, and visualising individual pancreatic islets is not possible. PET has superior spatial resolution compared to SPECT (2 – 4 mm versus 8 – 10 mm), but even so a highly specific tracer which emits a great amount of radioactivity or a dense amount of target on the beta-cells is required for imaging of the BCM due to the small size of the islets (Andralojc et al., 2012). Higher spatial resolution causes less spread of the tracer signal. The spatial resolution is determined both by the features of the scanner (crystal and pixel size, for example) as well as the mathematical algorithms used for image reconstruction (Soret et al., 2007).

Furthermore, both PET-CT and SPECT underestimate tracer signals from targets smaller than the scanner’s spatial resolution. This is called the “partial-volume-effect” (PVE) (Andralojc et al., 2012). The PVE is an issue when imaging targets that are < 2.5 times the spatial resolution of the imaging system used and causes inaccurate images due to blurring. Other factors that may affect the PVE are the shape of the target, the background activity concentration and the mathematical algorithm used to reconstruct the PET image (Mawlawi & Townsend, 2009; Soret et al., 2007). The PVE ultimately leads to underestimation of the standardized uptake (SUV) of the target due to dilution of tracer signals in the background/surrounding signals (Blomberg et al., 2013), consequently leading to underestimation of the BCM (Eriksson & Alavi, 2012).

It has been estimated that the uptake of a tracer signal needs to be 100 times higher in beta-cells compared to surrounding cells in a healthy individual to overcome the shortcomings of PET/SPECT spatial resolution. The purpose of imaging the BCM is to detect small changes in BCM. Since the proportion of beta-cells in a diabetic patient may be as low as 0.2% of the pancreatic tissue (Weir et al., 1990), as to normal 2%, the tracer uptake has to be 1000–fold in diabetics. Finding a tracer that meets those demands is not an easy task (Sweet et al., 2004a; Sweet et al., 2004b).

However, quantifying the BCM does not necessarily require identifying individual beta-cells, but could instead be performed by detecting the radioactive signal associated with the total amount of beta-cells throughout the pancreas, which can be possible since the pancreas is larger than the spatial resolution of the PET system (Selvaraju et al., 2013). Still, when imaging a structure as small as the beta-cells, the target-to-background-ratio has to be very high (Di Gialleonardo et al., 2012).
**Magnetic resonance imaging**

Magnetic resonance imaging (MRI) is a less sensitive technique than PET or SPECT, but has higher spatial resolution and exquisite soft tissue contrast. High-field MRI has high resolution which is needed for individual islet imaging, but makes it more susceptible to motion artefacts due to longer acquisition times. Breathing motion artefacts, but not gastrointestinal motion, can be corrected for (Andralojc *et al.*, 2012; Lamprianou *et al.*, 2011; Smirnov *et al.*, 2008).

There is no radiation exposure caused by the MRI, which makes it suitable for repeated imaging sessions, although examination times can be long and the environment noisy. Moreover, patients with pacemakers and some types of metal implants cannot be examined (Gotthardt *et al.*, 2013).

Using MRI technology to non-invasively image native beta-cells is not yet possible. This is due to it being a technology with lower sensitivity compared to PET and lack of available tracers meeting the requirements of beta-cell-to-background contrast (Andralojc *et al.*, 2012; Medarova & Moore, 2009). When using MRI, a specific contrast reagent is needed to distinguish exocrine from endocrine pancreas, which can at this point in time only be performed through *ex vivo* staining before performing islet transplantation. Recent studies using MRI for BCM imaging, are still in the very early stages and further investigation is needed to provide appropriate tracers for MRI technology (Yang *et al.*, 2013; Lubag *et al.*, 2011; Antkowiak *et al.*, 2009).

**PET/MRI**

Combining the qualities of PET and MRI could be a promising tool for examination of the BCM. There is ongoing research on hybrid PET/MR imaging (Torigian *et al.*, 2013; Pichler *et al.*, 2010; Wu & Kandeel, 2010).

**Requirements of the imaging method, tracers and targets**

There are several requirements of an appropriate imaging method for *in vivo*, non-invasive imaging of the BCM. The beta-cells are dispersed throughout the pancreatic tissue, which only consists of 1–2% of beta-cells in a healthy pancreas. In a diabetic state, this proportion of beta-cells is most likely decreased. Moreover, healthy pancreatic islets are very small (~ 50–400 µm) (Katsumichi & Pour, 2007). To overcome the difficulties in accurately imaging a target as sparse as the beta-cells, the imaging method needs to have a high spatial resolution and requires a highly specific tracer molecule as well as a highly specific target for the beta-cells only. The tracer must not bind to the exocrine pancreas or the surrounding tissues (high signal-to-background ratio and high signal-to-noise ratio) and the imaging method needs to discriminate endocrine from exocrine tissue. The target needs to stay unaffected in the diabetic state (Andralojc *et al.*, 2012; Brom *et al.*, 2010; Ley, 2006).

The result is also dependent of the expression rate of the target, which has to be highly expressed by the beta-cells to be detectable by the techniques available. To reach detectable tracer signal rates, some tracer doses needs to be relatively high. This is an issue if using a biologically active compound or when considering the radiation to the patient. The stability of the tracer molecule is also important. *In vivo*, the tracer has to avoid degradation by...
endogenous peptidases, to stay active throughout the whole imaging session (Andralojc et al., 2012; Kwee et al., 2011).

Furthermore, there are issues concerning the size of the tracer molecule. *In vitro* imaging may prove good results showing a molecule with high affinity and specific binding to the beta-cells comparing to the exocrine tissue, although if the tracer molecule is too large it may remain in the circulation *in vivo* rather than migrating to the beta-cell target, consequently leading to a low target-to-background ratio and difficulties in detecting the beta-cells due to the slow clearance. A similar problem occurs if a large amount of tracer is accumulated in the excretion route, i.e. the kidneys, liver or intestinal organs (Andralojc et al., 2012).

At this point in time, there is still no tracer and imaging method meeting all the above mentioned needs for imaging the BCM.

**Suggested targets and ligands**

To quantify the BCM, several receptors and biological molecules have been proposed as targets. Some of the targets and ligands (binding to the actual target) that are or have been under evaluation for imaging of the BCM are mentioned below.

The vesicular monoamine transporter type 2 (VMAT2) has been proposed being a promising target, since it is expressed by beta-cells but not by exocrine pancreatic cells (Maffei et al., 2004; Anlauf et al., 2003). Several studies have been published, where VMAT2 has been targeted with dihydrotetabenazine (DTBZ) as a PET tracer, with the aim to quantify the BCM. However, VMAT2 is not an optimal target, due to nonspecific binding in exocrine pancreatic tissue (Singhal et al., 2011; Virostko et al., 2011; Eriksson et al., 2010; Fagerholm et al., 2010; Goland et al., 2009; Kung et al., 2008; Souza et al., 2006b). It has been observed that VMAT2 is expressed in pancreatic polypeptide cells and hence, is not beta-cell specific (Saisho et al., 2008).

The dopamine D$_2$-like receptor was suggested an appropriate target, since it was detected on beta-cells and mediated insulin-secretion (Rubí et al., 2005). However, BCM could not be determined using this target since exocrine pancreas uptake also occurred (de Lonlay et al., 2006).

The neurotransmitter acetylcholine seems to play a central part in the insulin secretion via muscarinic receptors expressed by the beta-cells (Gilon & Henquin, 2001; Ahrén, 2000). Preliminary data from a small study indicate excellent uptake in the pancreatic tissue of mice, human and monkeys, using [18F]4-fluorobenzylatrozamicol as a tracer targeting muscarinic receptors. However, a significant uptake could also be detected in salivary gland, gall bladder and liver (Clark et al., 2004). High uptake in these tissues may hamper signals coming from tracer uptake in the pancreas.

Another target that has been considered for beta-cell imaging is the sulfonylurea receptor 1 (SUR1), which is an ATP-regulated K$^+$-channel (Wängler et al., 2004a; Bernardi et al., 1988). The SUR1 has been targeted using sulfonylureas, such as glibenclamide and repaglinide, which are glucose-lowering drugs used in T2D patients (Brom et al., 2010; Hansen et al., 2005;
Henquin, 1992), but a high uptake has been observed in the liver, kidneys and small intestines, while the uptake in the pancreas was rather low (Schmitz et al., 2004; Wängler et al., 2004b). In a study using immunostaining, SUR1 was shown to be expressed all over the pancreatic islet, i.e. in alpha-, beta- and delta-cells, which makes it an unsuitable target for BCM determination (Suzuki et al., 1999).

D-mannoheptulose is transported into the beta-cells mainly through GLUT2 transporters, which has been confirmed in vitro. However, this target is not completely beta-cell-specific (Ladrière et al., 2001). Moreover, D-mannoheptulose elevates blood glucose levels by inhibition of insulin secretion, which makes this substance inappropriate for use in diabetic patients (Yang et al., 2013).

Since beta-cells have high zinc content which is co-released with insulin (Søndergaard et al., 2003; Dodson & Steiner, 1998), it has been attempted to stain beta-cells using $^{125}$I-labelled dithizone, a zinc chelator which binds to beta-cells. Unfortunately, the compound is rapidly degraded in vivo (Andralojc et al., 2012; Brom et al., 2010), thus it was conjugated with histamine (dithizone-$^{[131]}$I]-histamine) to enhance the stability of the tracer. The stability was increased, but resulted in high background activity in liver and kidneys (Garnuszek et al., 2000; Garnuszek et al., 1998). It has also been shown that dithizone may be toxic to beta-cells (Clark et al. 1994; Toroptsev & Eschchenko, 1983), which gives it a limited use as a tracer for in vivo imaging. Another tracer, gadolinium-DOTA-diBPEN, has also been used to detect the Zn$^{2+}$-release by the beta-cells in vivo using MRI, with promising results (Lubag et al., 2011; Esqueda et al., 2009).

Manganese-enhanced MRI is another technique presently under evaluation. Instead of targeting the beta-cell surface, the technique aims to detect intracellular manganese incorporated into the beta-cells by Ca$^{2+}$-channels activated by glucose. Only beta-cells with a proper glucose metabolism will incorporate the Mn$^{2+}$, since Mn$^{2+}$-uptake is glucose-dependent (Malaisse & Maedler, 2012; Gimi et al., 2006). Studies with promising result have been performed in vivo in mice (Antkowiak et al., 2012; Antkowiak et al., 2009), but there are concerns about cytotoxicity caused by manganese (Dobson et al., 2004; Olanow, 2004; Roth & Garrick, 2003). It has been shown that manganese concentrations > 300 µM causes apoptosis in neuronal cells (Latchoumycandane et al., 2005), which may limit its use in vivo. However, much lower doses are applied for the use in MRI, and further studies using Mn$^{2+}$-enhanced MRI are currently ongoing (Malaisse & Maedler, 2012; Lamprianou et al., 2011; Silva et al., 2004).

The monoclonal islet cell surface IgM antibody IC2 has been used as a tracer targeting beta-cells to image the BCM in a T1D mouse model. The tracer was highly specific to the beta-cells (Moore et al., 2001; Aaen et al., 1990; Buschard et al., 1988), but the large size of this tracer molecule, being an immunoglobulin, lead to a slow clearance, a low beta-cell-to-background ratio and may limit its role as a tracer (Schneider, 2008). Furthermore, this IgM antibody could lead to an immune reaction in humans since it is of rat origin (Di Gialleonardo et al., 2012) and it has been proposed that the corresponding beta-cell antigen is not expressed constantly by the beta-cells (Buschard et al., 1988). Another suggestion has been single-chain
antibodies which are highly specific to beta-cells and exhibit fast blood clearance. The tracer signal has shown a linear correlation with the BCM in vitro and in vivo in rats (Ueberberg et al., 2010; Ueberberg et al., 2009). Another monoclonal antibody (8/9-mAb), targeting transmembrane protein 27 (TMEM27) has also been used to detect BCM in vivo in mice using PET. TMEM27 is observed to be beta-cell specific in both mouse and human pancreas using fluorescence. This makes TMEM27 a promising target for further research (Vats et al., 2012; Akpinar et al., 2005).

Previous studies have also mentioned the glucagon-like peptide-1 receptor (GLP-1R) as a promising target for in vivo imaging, for example tumour imaging such as insulinomas and pheochromocytomas (Kiesewetter et al., 2012; Gao et al., 2011; Wild et al., 2008; Körner et al., 2007; Wild et al., 2006). The GLP-1R is expressed on the beta-cell surface (Tornehave et al., 2008) and an antagonist of the GLP-1R has been used to quantify the BCM in rats, but it was shown that kidney and liver uptake was relatively high, which may hamper the image in this small animal model (Mukai et al., 2009). The glucagon-like peptide-1 (GLP-1), an agonist of the GLP-1R, is rapidly inactivated in vivo, why it was conjugated with albumin to prolong its activity (Kim et al., 2003). Unfortunately, this lead to a slower clearance and ultimately an attenuated pancreas-to-background ratio (Andralojc et al., 2012).

Synthetic exendin-4 (exenatide), a GLP-1 mimetic which is less prone to rapid degradation (Kazafeos, 2011), has been used for targeting GLP-1R in insulinomas (Sowa-Staszczak et al., 2013; Christ et al., 2009; Wicki et al., 2007; Wild et al., 2006), on beta-cells in mice and rats (Selvaraju et al., 2013; Reiner et al., 2011; Gotthardt et al., 2006), in a human and mice beta-cell graft (Wu et al., 2011; Pattou et al., 2010) and in nonhuman primates (Selvaraju et al., 2013), with promising result. By conjugating exenatide with DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) or DO3A (1,4,7,10-tetraazacyclododecan-1,4,7-triacetic acid), it is possible to label the molecule with a positron emitter, which is needed for PET imaging (Brom et al., 2010).

In rats and mice, by using IHC and autoradiography exendin-4 has shown to bind specifically to insulin-producing cells (Wu et al., 2013; Connolly et al., 2012). The high tracer excretion through the kidneys causes heavy spill-over and makes it impossible to properly analyse the acquired images in a small animal model, such as the rodent. This was, however, not considered an issue in nonhuman primates (Selvaraju et al., 2013). Further studies have been requested, with the pig as a suggested animal model (Selvaraju et al., 2013; Simonsen et al., 2006), thus the present study was performed.

The glucagon-like peptide-1 receptor

The receptor and its expression

The GLP-1R is a transmembrane G-protein coupled receptor (GPCR) in family B of GPCR (Doyle & Egan, 2007; Josefsson, 1999). It has sequence homologies with other GPCR, i.e. the receptors for secretin, calcitonin and parathyroid hormone (Thorens, 1992).

In human tissue, GLP-1R protein expression was detected, by autoradiography, at low concentration in the brain, intestine, pancreas, kidney, blood vessels in the lung and in the
breast tissue, whilst no receptors could be found in the spleen, liver, adrenal glands, heart, skeletal muscle, prostate, hypophysis, adipose tissue or lymph nodes. The absolute highest amounts of receptors were found in the neurohypophysis. In the pancreas, GLP-1 receptors were observed in both islets and acini, although with a higher receptor density in the islets (about twice as high). Islets were receptor positive at lesser extent if the tissue had endured chronic pancreatitis (Körner et al., 2007). In another study, using another technique, mRNA from the GLP-1R was found in the human heart, brain, kidney, lung and stomach, where it had the same amino acid sequence as the pancreatic form (Wei & Mojsov, 1995). Furthermore, in humans, in vitro GLP-1R autoradiography shows that GLP-1R mRNA is present in many parts of the brain, such as the cerebral cortex, hypothalamus, hippocampus and thalamus (Alvarez et al., 2005).

In the human pancreas, it was strongly indicated by Tornehave et al. (2008) that GLP-1R has the highest expression on the surface of the beta-cells, facing the endothelium. Despite the use of a more sensitive technique than previous studies, according to the authors, they were unable to observe GLP-1R activity in alpha- and delta-cells. However, immunoreactivity was found in some of the large pancreatic ducts (Tornehave et al., 2008). In vitro, GLP-1 can stimulate delta-cells to secrete somatostatin, which may indicate that delta-cells also express GLP-1R, although it has been suggested that the somatostatin-secretion following GLP-1 exposure may be due to paracrine effects (Tornehave et al., 2008; Fehmann et al., 1995; Fehmann & Habener, 1991).

In human cell lines, GLP-1 receptors have been found on bone marrow stromal cells (Sanz et al., 2010) and on immature osteoblasts (Pacheco-Pantoja et al., 2011; Walsh & Henriksen, 2010). No GLP-1R have been found on mature osteoblasts (Bollag et al., 2000).

In rats, GLP-1 receptors are expressed in the lungs and brain. The binding affinity by GLP-1 is rapid, reversible and high, specifically in the hypothalamus and brain stem (Rodriquez de Fonseca et al., 2000; Kanse et al., 1988). mRNA transcripts for GLP-1R have also been found in the pancreas, intestine, stomach, liver, lung, brain, kidney, smooth muscle cells and cardiomyocytes of mice/rats, (Pyke & Knudsen, 2013; Ban et al., 2008; Bullock et al., 1996; Campos et al., 1994) and in tubular cells of the porcine kidney (Schlatter et al., 2007), although it was proposed by Bullock et al. (1996) that the receptor found in rodent kidneys and heart may be a structural variant of the pancreatic GLP-1R (i.e. not being completely identical). Furthermore, in rats, GLP-1R gene expression was found in the nodose ganglion, an important junction for nervus vagus (Nakagawa et al., 2004). Through light microscopic autoradiography in rat brain, GLP-1R was observed in the subfornical organ and area postrema (AP), which are in close neuroanatomical connection with vagal nerves and important hypothalamic regions involved in water and food intake as well as the sensation of emesis. Through its anatomical situation, AP is an interface between the brain and the peripheral blood. Furthermore, AP has an insufficient blood-brain barrier to large polar molecules (Orskov et al., 1996a; Miller & Leslie, 1994).

Significant species differences have been demonstrated, with high expression of GLP-1R in rodent thyroid c-cells while human and primates had low expression of GLP-1R in this tissue.
Similar results were demonstrated concerning lung tissue, where high receptor density was found in rodent lung tissue while it was remarkably low in human lung tissue in comparison (Bjerre Knudsen et al., 2010; Körner et al., 2007).

**Ligands**

The binding of the peptide GLP-1 to its receptor is highly specific. Glucagon also binds to the GLP-1R, although with much less affinity than GLP-1. This has low relevance since glucagon never reaches plasma levels at which it would bind to the receptor instead of GLP-1 (Doyle & Egan, 2007; Graziano et al., 1993). A study in dogs has shown that a metabolite of truncated GLP-1, the glucagon-like peptide-1-(9-36)-amide, is an antagonist of the GLP-1R but has low binding affinity to the receptor (Knudsen & Pridal, 1996).

As mentioned, exendin-4 is also an agonist of the GLP-1 receptor and truncated exendin-(9-39)-amide is an antagonist (Schirra et al., 1998; Göke et al., 1993; Thorens et al., 1993).

**Intracellular effect of GLP-1 receptor binding**

The receptor is stimulated by GLP-1 binding when glucose is elevated. This causes an intracellular reaction to take place, where adenylyl cyclase is being activated and consequently cAMP formatted (Graziano et al., 1993; Drucker et al., 1987). The increase of cAMP induces the activation of protein kinase A and cAMP-regulated guanine nucleotide exchange factor II (Epac2) (Shibasaki et al., 2007; Renström et al., 1997), which in turn leads to membrane potential being shifted and intracellular Ca$^{2+}$ rising inside the beta-cells (Tsuboi et al., 2003). This reaction sequence ultimately leads to an increase of readily releasable insulin secretory vesicles and their exocytosis (Vilsbøll, 2009; Holst & Gromada, 2004; Renström et al., 1997).

**The glucagon-like peptide-1**

**Production of GLP-1**

The glucagon-like peptide-1 (7-36)-amide (GLP-1) is a gastrointestinal hormone in the group of incretin hormones. GLP-1 is produced by, and secreted from, intestinal endocrine L-cells (Doyle & Egan, 2007; Holst, 2007) which are mainly located in the distal parts of the small intestine (Theodorakis et al., 2006; Mortensen et al., 2003). They are epithelial endocrine cells with direct contact with the lumen of the intestine and thereby in contact with the nutrients passing by their apical surface (Baggio & Drucker, 2007).

The GLP-1 secretion is stimulated by several factors, meal ingestion in particular (Orskov et al., 1996b; D'Alessio et al., 1995; Herrmann et al., 1995). Studies have shown that oral administration of glucose stimulates secretion of incretin hormones, as a function of the glucose load (Nauck et al., 1986b; Unger et al., 1968). It has also been demonstrated that leptin, an adipose tissue hormone involved in the regulation of satiety, stimulate the secretion of GLP-1 (Anini & Brubaker, 2003; Friedman & Halaas, 1998). There is a rapid GLP-1 increase 10–15 minutes following meal intake, and since the majority of the L-cells are located in the distal part of the small intestines it is unlikely that the GLP-1 secretion is due to stimulation by nutrients in contact with the L-cells only (Baggio & Drucker, 2007; Herrmann et al., 1995).
et al., 1995). There is most likely a neuroendocrine stimulation involved in the GLP-1 secretion as well (Balkan & Li, 2000; Rocca & Brubaker, 1999; Balks et al., 1997) and it has been proposed that GLP-1 is being locally produced within the CNS, thereby modulating neurotransmission (Holst et al., 2011).

Regulatory mechanisms of GLP-1 levels

Once GLP-1 is secreted it is rapidly inactivated. The half-life of its bioactive form in the circulation is < 2 minutes (Baggio & Drucker, 2007; Simonsen et al., 2006; Deacon et al., 1995). The mechanism of the rapid inactivation of GLP-1 remained unknown until dipeptidyl-peptidase 4 (DPP-IV) was found on the surface of the endothelial cells in the capillaries that supply and drain the intestinal mucosa, adjacent to the L-cells. DPP-IV, also known as CD26, is a serine protease with specific action, cleaving dipeptides at the N-terminal of peptides containing an alanine or proline at a certain site (Baggio & Drucker, 2007; Hansen et al., 1999; Deacon et al., 1996; Mentlein et al., 1993). GLP-1 is rapidly cleaved and metabolised into GLP-1(9-36) amide, and thereby inactivated, by DPP-IV (Hansen et al., 1999; Mentlein et al., 1993). As a consequence, half of the GLP-1 entering the vena portae has already been inactivated before reaching the systemic circulation (Hansen et al., 1999). DPP-IV is omnipresent and can be found in several tissues throughout the body including the kidney, lung, adrenal gland, liver, spleen, testis, CNS, pancreas, intestine and also on the surface of lymphocytes and macrophages (Baggio & Drucker, 2007; Elovson, 1980). In rats, over 50% of GLP-1 amounts injected by intravenous (i.v.) infusion was degraded within two minutes, while in knock-out mice, lacking the functional gene producing DPP-IV, GLP-1 remained intact (Baggio & Drucker, 2007; Marguet et al., 2000).

In rats, the cleaved metabolite of GLP-1 is excreted through the kidneys within approximately five minutes (Ruiz-Grande et al., 1993) In a study, comparing renal failure patients with healthy controls, it was shown that GLP-1 metabolites were elevated in the renal patients, while the levels of the bioactive form of GLP-1 did not differ from the controls. This indicates that the kidneys are important for the elimination of GLP-1 metabolites in humans as well (Meier et al., 2004). However, in pigs, Simonsen et al. (2006) shows that the liver and peripheral tissues also play a role in the excretion of GLP-1.

There are limited studies regarding the inhibition of GLP-1 production, although there are a few studies that indicate that insulin, somatostatin as well as the neuropeptide galanin may inhibit GLP-1 secretion from the L-cells (Baggio & Drucker, 2007; Lim & Brubaker, 2006; Chisholm & Greenberg, 2002; Hansen et al. 2000; Fehmann et al., 1995).

The function of GLP-1

The incretin effect

Oral administration of glucose is associated with a greater increase in insulin levels, than when given as an i.v. glucose infusion, which has brought upon the expression “the incretin effect” (Holst, 2007; Nauck et al., 1986b; Perley & Kipnis, 1967; Elrick et al., 1964).

Being an incretin hormone, GLP-1 promotes the synthesis of insulin, increases glucose-dependent insulin secretion and improves beta-cell responsiveness to glucose. GLP-1
stimulates an elevation of insulin mRNA levels as well as insulin content in the beta-cells (Vilsbøll, 2009; Farilla et al., 2003; Zander et al., 2002; Wang et al., 1995; Holz et al., 1993; Nathan et al., 1992; Drucker et al., 1987; Mojsov et al., 1987). In one study, diabetic rats were injected with GLP-1 during two days. The rats’ insulin secretion increased and consequently the plasma glucose levels were reduced (Farilla et al., 2002). This has also been shown in humans, following subcutaneous (s.c.) injection of GLP-1. At high dose GLP-1, the human subjects felt unwell and 50% experienced nausea/vomiting (Ritzel et al., 1995).

It has been proposed that an intact sensory afferent system is essential to GLP-1-mediated insulin secretion, since low doses of GLP-1 administered i.v. to mice increase glucose-dependent insulin secretion in control subjects but not in sensory denervated mice (Ahrén, 2004).

In obese adults and patients with T2D levels of GLP-1 seem to be attenuated and the incretin effect diminished (Kjems et al., 2003; Toft-Nielsen et al., 2001; Verdich et al., 2001; Vilsbøll et al., 2001; Ranganath et al., 1996; Nauck et al., 1986a; Perley & Kipnis, 1967), although elimination rates of GLP-1 are equal compared to healthy individuals (Vilsbøll et al., 2003a). Therefore it has been proposed that the reduction in GLP-1 levels in obese and T2D patients may be due to lowered secretion of GLP-1 (Vilsbøll et al., 2003b; Ranganath et al., 1996) or due to leptin resistance, since obese people often exhibit leptin resistance (Anini & Brubaker, 2003; Friedman & Halaas, 1998; Halaas et al., 1997; Considine et al., 1996). However, there are T2D patients exhibiting a proper incretin effect following GLP-1 exposure (Vollmer et al., 2008; Elahi et al., 1994; Nauck et al., 1993) and GLP-1 levels have been shown to not differ between diabetic subjects and healthy controls (Knop et al., 2007) thus this is a debated topic. Some authors mean that a deterioration of the incretin effect is more likely secondary to other metabolic causes, such as hyperglucagonaemia (Nauck et al., 2011; Vollmer et al., 2008); this hypothesis could perhaps be confirmed by a study where four weeks of intensive insulin treatment significantly improved beta-cell responsiveness to GLP-1 threefold in T2D subjects (Højberg et al., 2009) and these results are in line with a study showing that GLP-1R expression is down-regulated by hyperglycaemia (Xu et al., 2007). Concluding, this topic is complex, with several factors that may affect the observed attenuated incretin response, for example high BMI, genetic background and duration of the T2D, thus further studies are required (Gjesing et al., 2012; Herzberg-Schäfer et al., 2012; Nauck et al., 2011; Vollmer et al., 2008).

GLP-1 also reduces post-prandial glycaemic excursions. This has been shown in T2D patients following both short-term (Rachman et al., 1997) as well as after three and six weeks of GLP-1 exposure (Zander et al., 2002; Todd et al., 1998). A study in T1D patients demonstrated that GLP-1 reduces glycaemic excursions in this group as well (Dupre et al., 1995).

**Delivering gastric emptying and body weight reduction**

Delayed gastric emptying has been observed in response to GLP-1 (Meier et al., 2006; Meier et al., 2003; Zander et al., 2002; Flint et al., 2001; Näslund et al., 1999; Wishart et al., 1998; Nauck et al., 1997; Willms et al., 1996). Willms et al. (1996) suggested that the delayed gastric emptying in itself most likely contributes to the glucose lowering effect of GLP-1. In a
6-week pilot study of T2D human subjects, treatment with GLP-1 resulted in an average weight loss of 1.9 kg (Zander et al., 2002).

**Regulating beta-cell mass**

GLP-1 inhibits beta-cell apoptosis *in vitro*, in human islets of Langerhans. GLP-1 preserved morphology and function of the beta-cells and prevented apoptosis when the beta-cells were exposed to oxidative stress by glucotoxicity, lipotoxicity and both in combination (Buteau et al., 2004; Robertson et al., 2004; Farilla et al., 2003; Hui et al., 2003).

Pancreatic ductal cells from rats cultured during GLP-1 exposure underwent re-distribution of the cell cycle and differentiation towards endocrine insulin producing cells (Bulotta et al., 2002). A similar result was demonstrated when pancreatic tumour (AR42J) cells (Zhou et al., 1999) and progenitor cells in pancreatic islets and ducts were exposed to GLP-1 (Abraham et al., 2002) and proliferation of acinar and ductal rat cells has been observed in another study (Perfetti et al., 2000). Diabetic rats were injected with GLP-1 during two days. Four days later autopsy and immunostaining concluded there was an increase in cell proliferation of both endocrine and exocrine segments of pancreas and in parallel a decrease in apoptotic processes (Farilla et al., 2002). Thus, GLP-1 seems to regulate BCM by stimulating proliferation and by inhibiting apoptotic processes.

**Other endocrine effects**

It has been demonstrated that GLP-1 inhibits the secretion of glucagon in both healthy and T2D human patients. The exact mechanism(s) for this effect remains undetermined (Meier et al., 2003; Nauck et al., 2002; Nauck et al., 1997; Creutzfeldt et al., 1996; Ritzel et al., 1995; Nauck et al., 1993; Fehmann & Habener, 1991).

GLP-1 can also stimulate secretion of somatostatin from delta cells. This effect may either be due to direct stimulation of the pancreatic delta cells or possibly through paracrine pathways (Chisholm & Greenberg, 2002; Hansen et al., 2000; Fehmann et al., 1995; Fehmann & Habener, 1991).

In one study, mesenchymal human bone marrow stem cells were exposed to GLP-1, which promoted the cell proliferation and inhibited the differentiation into adipocytes. Since GLP-1 receptors have been located on bone marrow stromal cells and osteoblasts, this topic demands further studies on long-term effects of incretin-based therapies (Phillips & Prins, 2011; Sanz et al., 2010).

Another study has demonstrated that GLP-1 eliminates postprandial elevation of triglyceride concentrations in humans. The authors suggested these findings may be explained by delayed gastric emptying as well as inhibition of lipolysis due to enhanced insulin secretion (Meier et al., 2006).

It has been demonstrated that GLP-1 has a natriuretic effect in both healthy and obese, insulin-resistant men, showing reduced H\(^+\)-secretion and enhanced Na\(^+\)-excretion (Gutzwiller et al., 2004). Similar results have been seen in rats (Crajoinas et al., 2011; Yu et al., 2003).
Cardiovascular effects

It has been proposed that GLP-1 may have cardiovascular benefits, such as cardioprotective and vasodilatatory effects (Ban et al., 2008; Basu et al., 2007; Sokos et al., 2007; Meier et al., 2006). In a study consisting of T2D patients with coronary heart disease, GLP-1 improved endothelial function (Nyström et al., 2004). In mice, GLP-1 increased the recovery of heart function cardiomyocyte viability and reduced reperfusion injury following ischemia of the myocardium (Ban et al., 2008).

In rats, GLP-1 protected the myocardium against infarction and reperfusion injury. It also increased left ventricular (LV) function and myocardial glucose uptake (Zhao et al., 2006; Bose et al., 2005). In dogs with paced-induced dilated cardiomyopathy, GLP-1 increased cardiac output, improved left ventricular and systemic haemodynamics, in combination with increased myocardial insulin sensitivity and glucose uptake (Nikolaidis et al., 2004a). GLP-1 seems to improve LV function of the human myocardium as well. When GLP-1 was administered as a 72-hour infusion following an acute myocardial infarction in humans, LV function was improved (Nikolaidis et al., 2004b), and beneficial cardiovascular results have also been reported in three other studies (Read et al., 2012; Müssig et al., 2008; Sokos et al., 2006). Interestingly, beneficial cardiac effects have also been observed in GLP-1R knockout mice models when exposed to GLP-1, indicating there being a pathway not mediated through the GLP-1R (Ban et al., 2008). However, no beneficial cardiac result could be seen in a small study of non-diabetic patients with compensated chronic heart failure following 48h GLP-1 infusion (Halbirk et al., 2010).

In rats, GLP-1 increased arterial blood pressure and heart rate (HR) significantly when administered i.v. as well as intracerebroventricularly (i.c.v.). The stimulating effect on arterial blood pressure and HR could be blocked by exendin-(9-39)-amide, administered i.v. or i.c.v. (Barragán et al., 1999). Similar results were shown in another study in rats, where both peripheral and central administration of GLP-1 resulted in increased arterial blood pressure and HR (Yamamoto et al., 2002) and increased HR has also been shown in calves following i.v. GLP-1 infusion (Edwards et al., 1997). Barragán et al. (1994) concluded that the stimulating effect on cardiovascular parameters did not seem to be mediated by catecholamines through the α- or β-adrenergic receptors, while Yamamoto et al. (2002) suggested the opposite – that sympathetic innervation is involved. Thus, further research is required.

Central and peripheral neuroendocrine effects

GLP-1 promotes satiety and reduces food and water intake (Gutzwiller et al., 1999a; Gutzwiller et al., 1999b; Toft-Nielsen et al., 1999; Flint et al., 1998; Navarro et al., 1996; Turton et al., 1996). It has been proposed that GLP-1 is locally produced within the CNS (Holst et al., 2011), that peripheral GLP-1 is able to cross the blood-brain barrier (Kastin et al., 2002), as well as that there being vagal-brainstem-hypothalamic pathways involved in the regulation of satiety. The role of GLP-1 in the regulation of feeding is still not fully understood (Phillips & Prins, 2011; Baggio & Drucker, 2007; Abbott et al., 2005; Tang-Christensen et al., 2001; Rodriguez de Fonseca et al., 2000; Näslund et al., 1999). During hyperglycaemic conditions, stimulation of GLP-1R in the CNS has an important role in
whole-body glucose homeostasis by inhibiting muscle glucose use, increasing insulin secretion as well as enhancing storage of hepatic glycogen, through peripheral neural pathways (Knauf et al., 2005).

One study of human subjects demonstrated that GLP-1 induced a significant increase in cortisol and ACTH levels (Ryan et al., 1998). Similar results were achieved in another study, consisting of healthy and T1D subjects who were exposed to GLP-1, resulting in elevated cortisol levels, no matter the glycaemic state of the subject, proposing that GLP-1R agonists may affect the hypothalamus pituitary axis in humans. Although, the pathway of this effect needs to be investigated further (Gil-Lozano et al., 2010).

In vitro, GLP-1 has shown to protect rat hippocampal neurons from apoptosis when exposed to degenerative substances. This indicates that GLP-1 may have a neuroprotective and/or a neurotrophic function (Perry et al., 2002a; Perry et al., 2002b) and similar results were demonstrated in vivo in mice. In GLP-1R deficient mice, learning deficits were observed, while improved learning and memory skills were observed in rats over-expressing GLP-1R (During et al., 2003). Furthermore, GLP-1 has been observed to reduce amyloid accumulation in vivo in the mouse brain (Perry et al., 2003).

Exenatide – synthetic exendin-4

History

Exendin-4 is a natural GLP-1 receptor agonist, isolated from the venom produced in the salivary glands of the Gila monster (Nielsen et al., 2004; Eng et al., 1992).

Pharmacology of exenatide and field of application

Exenatide, synthetic exendin-4, is a synthetic peptide with an amino acid sequence that is homogenous with the natural GLP-1 to 53%. It is also a GLP-1R agonist and has similar glucoregulatory and insulinotropic effects as endogenous GLP-1, but is ~5 500 times more potent in blood glucose lowering (Vilsbøll, 2009; Alarcon et al., 2006; Nielsen et al., 2004; Kolterman et al., 2003; Young et al., 1999; Göke et al., 1993; Eng et al., 1992). Exenatide has an extended half-life compared to physiological GLP-1, due to amino acid differences at position two of the peptide; exendin-4 has a glycine instead of alanine, which makes it a less suitable substrate for DPP-IV to truncate (Kazafeos, 2011; Vilsbøll, 2009).

Exenatide improves glycaemic control, induces significant weight loss and attenuate haemoglobin A1c (HbA1c) levels, which may be analysed to monitor glycaemic control (Higgins, 2012; Moretto et al., 2008; Amori et al., 2007; Buse et al., 2007; Blonde et al., 2006; DeFronzo et al., 2005; Kendall et al., 2005; Buse et al., 2004; Dupré et al., 2004; Fineman et al., 2003; Edwards et al., 2001; Szayna et al., 2000). There are several studies reporting that GLP-1 and exendin-4 are involved in reducing food intake (affecting satiety) and delaying gastric emptying (Linnebjerg et al., 2008; Abbott et al., 2005; Talsania et al., 2005; Dupré et al., 2004; Kolterman et al., 2003; Edwards et al., 2001; Szayna et al., 2000) and it has been reported that exenatide, as short-term treatment, also results in weight loss in obese, non-diabetic women (Dushay et al., 2012).
Exenatide was approved in 2005 by the Food and Drug Administration (FDA) in the U.S for use in T2D patients that are unable to achieve adequate glycaemic control using metformin and/or sulfonylurea and where weight loss or hypoglycaemia is of concern. It was labelled as Byetta® and is administered by s.c. injections twice daily. It is recommended to discontinue after six months of the therapy if HbA1c or the weight has not decreased by at least 1% and 3% respectively during this time (Kazafeos, 2011; Madsbad et al. 2011; NICE, 2011; Arnolds et al., 2010; Nathan et al., 2009). In the European Union Byetta® was approved in 2006 (EMA, 2013).

In a small study in healthy cats, exenatide stimulated insulin secretion with a mild lowering of blood glucose, although, not to the degree that it greatly increased the cats’ abilities to normalise serum glucose levels following an i.v. injection. The authors request further studies to evaluate exenatide treatment in diabetic cats (Gilor et al., 2011).

Several studies in vitro and in vivo indicate that exendin-4, like GLP-1, affects beta-cell mass and function (Yusta et al., 2006; Nielsen et al., 2004; Fineman et al., 2003; Xu et al., 1999). Furthermore, it has been demonstrated that exendin-4 protects rat hippocampal neurons from apoptosis when exposed to degenerative substances, which indicates that exendin-4 may have a neuroprotective and/or a neurotrophic function. Similar results were seen in a study using a rat animal model for Parkinson’s disease, where treatment with exendin-4 promoted adult neurogenesis both in vitro and in vivo. In Huntington’s disease mice treated with exendin-4, motor function was improved and survival time prolonged. These results may be of interest for further research in neurodegenerative processes in the CNS (Martin et al., 2009; Bertilsson et al., 2008; Perry et al., 2002a; Perry et al., 2002b).

**Side effects**

**General side effects**

The most reported side effect following treatment with exenatide is nausea, with higher incidence during the first eight weeks of treatment (up to 30% at 10 µg dosage and 15–25% at 5 µg dosage). There has been no correlation between degree of weight loss and duration of nausea, and people not experiencing nausea lost weight as well. Other frequent side effects were vomiting and diarrhoea, which also were reported to a higher extent at the beginning of the treatment (Blonde et al., 2006; DeFronzo et al., 2005; Kendall et al., 2005; Buse et al., 2004).

There have been none or few reported incidences of severe hypoglycaemia following exenatide-treatment during trials. The hypoglycaemic events reported were mild or moderate in severity, although occurring at a significant incidence (from 5.3%, up to 35% at high dose, i.e. 10 µg). Only one withdrawal occurred due to hypoglycaemia. Other reported adverse effects are upper respiratory tract infection and headache (DeFronzo et al., 2005; Kendall et al., 2005; Buse et al., 2004).

The frequency of serious and severe adverse events during trials were low (3–10 %) and evenly distributed between categories (10 µg, 5 µg and placebo) (DeFronzo et al., 2005; Buse et al., 2004). There has been no evidence indicating cardiovascular, pulmonary, hepatic or
renal toxicity during the 30 week trials of exenatide treatment (DeFronzo et al., 2005; Kendall et al., 2005).

**Antibody formation**
At week 30 of three different exenatide-trials, anti-exenatide antibody titres were detected in 41–49% of subjects. The majority of the antibody titres were in the low range and the presence of antibodies had no connection with outcome or adverse events (Amori et al., 2007; DeFronzo et al., 2005; Kendall et al., 2005; Buse et al., 2004).

**Renal side effects**
The U.S. FDA has received 78 reports of kidney related problems in patients using exenatide from April 2005 through October 2008. This is a small percentage of the total number of users – nearly 7 million prescriptions of Byetta® were dispensed during this period, although, safety information regarding possible kidney problems has been featured the Byetta® label information. Health care professionals are recommended not to initiate treatment with Byetta® in patients with severe renal impairment and caution should be applied when considering dose increase in patient with moderate renal impairment (Tuttle et al., 2013; U.S. Food and Drug Administration, 2009a; U.S. Food and Drug Administration, 2009b).

**Risk of pancreatitis**
In non-diabetic rats treated with exenatide, pancreatic acinar inflammation and pyknosis were observed (Nachnani et al., 2010). There have been concerns regarding an excess of pancreatitis cases in exenatide-treated patients, since there have been several case reports published in this matter (Iyer et al., 2012; Ayoub et al., 2010; Tripathy et al., 2008; Denker & Dimarco, 2006). However, patients with T2D have in general nearly a threefold higher risk of pancreatitis compared with non-diabetics (Girman et al., 2010; Noel et al., 2009) and there is conflicting evidence if there is a true increased risk for pancreatitis associated with the use of exenatide (Tatarkiewicz et al., 2013; Elashoff et al., 2011; NICE, 2011; Spranger et al., 2011). A large safety surveillance report, including 27 966 subjects, did not identify any increased risk in patients treated with exenatide (Dore et al., 2009). Similar results were seen in a large cohort-study, including 25 719 subjects (Dore et al., 2011). Despite these results, a warning has been added to the exenatide patient information leaflet not to use exenatide if there is a history of pancreatitis or severe hypertriglyceridaemia, and further studies on this matter are requested (Phillips & Prins, 2011; Spranger et al., 2011).

**Pancreatic cancer**
Concerns have also been raised regarding a possibly increased risk for pancreatic cancer in patients using exenatide, based on the suggested higher risk of pancreatitis and since chronic pancreatitis has been proposed to increase the risk of pancreatic cancer (Elashoff et al., 2011; Jura et al., 2005). There are also several studies indicating that exendin-4 affects proliferation of pancreatic cells. In one study pancreatic tumour cells (AR42J cells), which were negative for islet hormones and their mRNAs, were exposed to exendin-4. The results demonstrated that the intracellular cAMP levels rose and cells started becoming positive for islet hormones (Zhou et al., 1999). Furthermore, it has been demonstrated that exendin-4 stimulates differentiation of beta-cells in rat ductal progenitor cells, and in a partial pancreatectomy rat
model 15 days of exenatide treatment stimulated regeneration of beta-cell mass and prevented the development of diabetes (Xu et al., 1999). In mice, it was seen that exendin-4 suppressed beta-cell apoptosis in the pancreas after streptozotocin (STZ) injection (Li et al., 2003). However, in a 13 week study in rats exposed to exenatide, no signs of ductal proliferation was observed (Tatarkiewicz et al., 2013). In a recently published review, the authors request further evidence of the drug safety of incretin-based therapies (Butler et al., 2013).

**Thyroid effects**

In rodents, long-term exposure to another GLP-1R agonist (called liraglutide) resulted in calcitonin release, proliferation of the rodent c-cells and tumour formation. In cynomolgus monkeys, 20 months of exposure to liraglutide did not lead to hyperplasia of the c-cells. Both humans and monkeys demonstrated low expression of GLP-1R in the thyroid, comparing to the rodent model. This strongly indicates species differences, although long-term effects of the usage of GLP-1R agonists in human beings, with regards to the thyroid, demands further investigation (Bjerre Knudsen et al., 2010). Elashoff et al. (2011) stated that there was an increased risk of thyroid cancer in humans using exendin-4, although this study has been questioned by other authors (Phillips & Prins, 2011).

**Cardiovascular effects**

No increased cardiovascular risk has been observed in humans using exenatide, rather a reduction in cardiovascular events has been reported (Best et al., 2011; Ratner et al., 2011). It has been proposed that exendin-4 may have a beneficial effect regarding the development of atherosclerosis (Arakawa et al., 2010). In a porcine animal model of ischemia and reperfusion injury, treatment with exenatide reduced myocardial infarct size (Timmers et al., 2009).

In some studies exendin-4 appears to reduce the systolic blood pressure in mice and humans, with a weak correlation between reduction in blood pressure and weight loss in humans (Gill et al., 2010; Okerson et al., 2010; Hirata et al., 2009; Moretto et al., 2008), although, there is conflicting evidence in this matter. Yamamoto et al. states that stimulation of the GLP-1R using exendin-4 increases blood pressure in rats (Yamamoto et al., 2002). In healthy humans no differences in blood pressure was seen following a s.c. injection of 10 µg exenatide, but a heart rate increase of 8.2 beats per minute was observed (Mendis et al., 2012).

In mice, i.c.v. administration of exendin-4 depressed heart rate variability. An increase in heart rate was seen following both acute and chronic i.c.v. administration of exenatide. It was also observed that the stimulation of central GLP-1R reduced the parasympathetic modulation of the heart rate, which led to an increased heart rate (Griffioen et al., 2011). Recently, a systematic review and meta-analysis was published, including 32 studies, concerning the effects of exenatide on heart rate and blood pressure, concluding that there is a connection between GLP-1 analogues and a small increase in heart rate as well as modest lowering of the blood pressure. The authors are welcoming further studies using more accurate means of measuring the heart rate (Robinson et al., 2013).
Other side effects

It has been demonstrated in one study that exendin-4 may affect the hypothalamus-pituitary-axis in humans, resulting in elevated cortisol levels (Gil-Lozano et al., 2010). Furthermore, exenatide has been shown to significantly reduce the plasma concentration and production rate of triglyceride-rich lipoprotein-apolipoprotein B-48, RLP-cholesterol, RLP-triglyceride and apolipoprotein C-III in humans (Xiao et al., 2012; Schwartz et al., 2010; Schwartz et al., 2008), although there is conflicting evidence in this matter (Amori et al., 2007).

The future of exenatide

At this point a long-acting exenatide, with administration once a week, is under evaluation. The overall result until this point is that exenatide given once weekly offers superior glycaemic control, greater reduction in HbA1c-levels and there are less reports regarding nausea. Similar reductions in weight were seen no matter once weekly or twice a day administration (Russell-Jones et al., 2012; Blevins et al., 2011; Drucker et al., 2008; Kim et al., 2007). There may be limitations in once weekly exenatide due to longer time to reach steady state (4-5 weeks). Additionally, a higher rate of antibody formation has been reported and in case of adverse events, such as pancreatitis, it will take weeks for the levels to decline (Madsbad et al., 2011).

To conclude, the long-term effects of incretin-based therapies, such as exenatide, has not yet been explored completely, and continued evaluation regarding long-term adverse events is required (Amori et al., 2007).

AIM OF THE PRESENT EXPERIMENT

The aim of the present experiment was to examine the feasibility of in vivo imaging of the GLP-1R in beta-cells, using the PET tracer $^{68}$GaGa-DO3A-VS-Cys40-exendin-4 as a marker, in native pancreatic beta-cells of a porcine diabetic animal model. There is at this point no published study on the pig as an animal model using this technology and tracer.

Previous studies have stated that Swedish high-health herd-certified domestic pigs (Yorkshire x Swedish Landrace) can be made diabetic by i.v. injection of STZ and that pigs have a similar diabetic metabolism as humans. It is therefore a suitable animal model for further diabetic studies (Jensen-Waern et al., 2009; Gäbel et al., 1985).

Quantifying the BCM is of major interest in several areas of diabetic research and medicine. Finding a way to quantify the BCM, in vivo and non-invasively, is essential to islet transplantation research and would provide further insight into the pathogenesis and changes of BCM throughout different diabetic stages of both T1D and T2D. It would also be useful at a time of subsequent examination after having initiated a medical treatment and to follow-up viability of transplanted islets (Arifin & Bulte, 2011; Virostko & Powers, 2009; Souza et al., 2006a; Paty et al., 2004). The GLP-1 receptor is known to be highly expressed on the beta-cell surface (Tornehave et al., 2008), thus quantifying the GLP-1 receptor in the pancreas could be an opening towards quantifying the BCM.
In this study, we also wanted to assess the perfusion in the pancreas and kidneys in healthy controls and STZ-induced diabetic pigs. During diabetic disease progression, changes in the islet vasculature can be seen. Currently, in vivo studies reflecting the change in perfusion of the pancreatic islets are scarce (Medarova & Moore, 2009).

MATERIAL AND METHODS

Experimental design

The experiment was approved by the Ethical Committee for Animal Experimentation, Uppsala, Sweden.

In this study, eight Swedish high-health herd-certified domestic pigs (Yorkshire x Swedish Landrace x Hampshire) from the same litter were used. The pigs were born February 21st, 2013 and bred at Lövsta, SLU, Sweden. Four pigs of each sex were randomly assigned to be either controls (#2 castrate, #4 female, #6 female, #7 castrate) or made diabetic (#1 castrate, #3 female, #5 female, #8 castrate) using STZ. The experiment proceeded during eight weeks, please see Figure 1 below.

![Experimental design of the present experiment. The pigs were acclimatised and socially trained. Diabetes was induced by STZ-injection and insulin treatment was initiated five days post STZ. PET examination was performed on control pigs and insulin treated diabetic pigs.](image)

Animals and housing

The pigs were housed individually, within sight and sound of one another, in pens of approximately 3 m², at the Department of Clinical Sciences (Swedish University of Agricultural Sciences, SLU, Uppsala, Sweden). In the pen every pig was provided straw and wood shavings as bedding and an infrared lamp in one corner (24h). The pens were cleaned twice a day. The room temperature was 18 ± 2 °C. The pigs were fed a commercial finisher diet with no growth promoters (Solo 330 P SK, Lantmännen Sweden) twice a day (at 07:00 and 18:00), apple as treats and they had free access to water. The pigs were 40 days old upon arrival on April 2nd, 2013.

Acclimatisation and social training
Upon arrival at the Department of Clinical Sciences the pigs were immediately moved into their own pen. Initially they were frightened and unsecure in their new environment; this is why they were given two days to adapt. Social training began on day three. The trainer sat down inside the pen, allowing the pigs to get used to humans. At first the pigs were very stressed, but after a few minutes the pigs accepted pieces of apple offered by the trainer. Every day for at least fifteen minutes the pigs were trained to get used to social contact, which included accepting pieces of apple, brushing and cuddling. The pigs were also accustomed to being touched around their ears as preparation for injections in that area. After a week they were greeting us by the door of the pen. Seven days after arrival we also started performing daily clinical examinations, taking their temperature and auscultating heart and lungs.

Gradually the pigs were introduced to a piece of canvas with Velcro, since the jugular vein catheter, later on placed, would be protected by a canvas pocket sealed with Velcro. At first the pigs were allowed to smell the canvas and later on the Velcro was opened a couple of times at 1-2 meters of distance making the pigs used to the sound. A majority of the pigs were curious about the Velcro but a couple were scared at the sound of it. After a few days one could open the Velcro next to the head of each pig without the pig getting bothered the least.

The pigs were also trained to step on an electronic spring scale (Ecco 101, Farmer Tronic Industries A/S, Vamdrup, Denmark) and were then weighed three times a week throughout the study. At arrival the pigs weighed an average of 15.3 ± 1.4 kg (mean ± SD).

**Surgery and anaesthesia – Insertion of a jugular vein catheter**

*The operational procedure*

Once the pigs had been socialised they underwent surgery, (controls on April 17th and the diabetics on April 25th) to insert a jugular vein catheter which later on would be used to perform i.v. injections and sample blood in a stress-free way throughout the study.

The pig was starved over night with water provided *ad lib*. Analgesia and general anaesthesia was induced inside the pen. The pig was moved to a preparation room where an auricular vein catheter was placed and intubation with an endotracheal tube was performed. Throughout the operational procedure the pig was inhaling 30% oxygen in nitrogen and an i.v. infusion of Ringer’s acetate (Ringer-acetat, Fresenius Kabi AB, Uppsala, Sweden) was administered. Anaesthesia was maintained either by iterating the same medicaments as for induction or by using a total-intravenous-anaesthesia-protocol (TIVA), as noted below.

The area of surgery was shaved and cleaned with soap and chlorhexidine solution and the hoofs were covered with socks to minimise heat loss. The pig was placed in dorsal recumbency and straps were used for fixation of the legs. An incision was made over the right jugular vein. A silicon catheter (SIL-C70 with rounded tip, Instec Solomon, PA, USA) was
placed in a sterile manner and the incision site was sutured. Thereafter the pig was placed in lateral recumbency and a subcutaneous tunnel was made for the silicone catheter to appear between the scapulae. Canvas was sutured to the back of the pig between the scapulas, to protect the catheter inside of a pocket sealed by Velcro. A catheter (Venflon®, Becton-Dickinson, Helsingborg, Sweden) was inserted at the proximal end of the silicon catheter to facilitate i.v. injections. The duration of the surgical procedure was approximately one hour.

Procaine benzyl penicillin 30 mg/kg (Penovet® vet. 300mg/ml, Boehringer Ingelheim Vetmedica, Malmö, Sweden) was administered by an intramuscular (i.m.) injection once a day for three days, starting at the day of surgery.

**Anaesthesia**

Anaesthesia was induced in the controls (pig #2, #4, #6 and #7) and in two of the destined to be diabetic pigs (#5 and #8) by an i.m. injection of 5 mg/kg tiletamin and zolazepam (Zoletil Forte® vet. 250mg/ml, Virbac, Carros, France), 0.05 mg/kg medetomidine (Domitor® vet. 1 mg/ml, Orion Pharma Animal Health, Sollentuna, Sweden) and 0.1 mg/kg butorphanol (Dolorex® vet. 10 mg/ml, Intervet AB, Sollentuna, Sweden). General anaesthesia was maintained by iterating 25% of the initial dose Zoletil Forte® vet. i.v. when needed. Anaesthesia was induced in the two other destined to be diabetic pigs (#1 and #3) by an i.m. injection of 4 mg/kg alfaxalone (Alfaxan® 10 mg/ml, Vétoquinol UK Limited, Buckingham, U.K.) and 2 mg/kg midazolam (Dormicum® 5mg/ml, Roche AB, Stockholm, Sweden). Before intubation analgesia was ensured through an i.v. injection of 2 mg/kg fentanyl (Fentanyl B. Braun® 50 µg/ml, B. Braun Medical AB, Danderyd, Sweden).

In one of the control pigs (#6) and the other two destined to be diabetic pigs (#1 and #3) anaesthesia was maintained using a TIVA protocol of the medicaments used for the induction. Infusion rate was adjusted finely whenever needed.

Throughout the anaesthesia the pigs were being monitored with regards of respiration rate (RR), HR, oxygen saturation (SpO₂), end-tidal carbon dioxide (EtCO₂), rectal body temperature, ECG, non-invasive blood pressure, tidal volume, minute ventilation and inspired oxygen concentration (FiO₂).

**Induction of diabetes**

Diabetes was induced under anaesthesia in pig #1, #3, #5 and #8 on April 25th with an i.v. injection of STZ (150 mg/kg, Sigma S0130, Stockholm, Sweden), according to (Jensen-Waern et al., 2009; Gåbel et al., 1985). STZ has a short half-life why it was dissolved in 100 mmol/L disodium citrate buffer solution (pH 4.5), at 80 mg/ml concentration. The injection was performed within five minutes, with an injection rate of approximately 1 ml/second.

**Postoperative care**

The pigs were moved back to their own pen after surgery and extubated. The pigs were monitored and given food once they were fully awake and could walk without difficulty.
Analgesia was given if required by an i.m. injection of 0.01 mg/kg buprenorphine (Temgesic® 0.3 mg/ml, RB Pharmaceuticals, Berkshire, U.K.).

The indwelling jugular vein catheters were flushed twice daily with 10 ml sterile saline (Natriumklorid Fresenius Kabi® 9 mg/ml, Fresenius Kabi AB, Uppsala, Sweden) and 5 ml 2% heparinised saline solution (5000 IU/ml, LEO Pharma AB, Malmö, Sweden) throughout the study. The daily clinical examination now included looking at the incision wound.

Streptozotocin may initially cause hypoglycaemia (Gäbel et al., 1985), thus the affected pigs (#1, #3, #5 and #8) were closely monitored until they were hyperglycaemic. Blood glucose was measured at 3, 5, 7 and 9 hours post STZ injection. If any pig was hypoglycaemic it was given an i.v. glucose infusion.

On the morning of April 30th (five days post STZ) pig #5 was found dead in its pen, thus this pig was not included in any further stages of the experiment. The post mortem diagnosis was acute circulatory distress, with acute liver and lung stasis. Focally in the myocardium, a small area of fibrosis was seen, which may be due to a chronic infarct or previous focal myocarditis. The body was pale, but no aetiology to anaemia could be found. Degenerative changes seen in the hepatocytes were estimated to be of reversible nature. The pancreas could not be examined due to autolysis.

Insulin treatment

On May 1st the diabetic pigs (i.e. #1, #3 and #8) were given 3.5 IU Actrapid® Penfill® (100 IU/ml, Novo Nordisk Scandinavia AB, Malmö, Sweden) s.c., which is a fast-acting humane insulin with rapid onset of acting and short duration. Since there is a risk of antibody formation in the pigs against the humane insulin, these injections were a onetime event due to hyperketonaemia.

In the evening of May 2nd (seven days post STZ) insulin treatment was continued, now injecting intermediate-acting porcine insulin s.c. (Caninsulin® vet. 40 IU/ml, Intervet AB, Sollentuna, Sweden), with starting dose 0.8 IU/kg x 2. The dosage was adjusted if needed, aiming towards a blood glucose concentration of 6–15 mmol/L. Injections were performed whilst the pigs were eating.

Blood analyses

Glucose measurements

All glucose values throughout the study were obtained with test strips using a glucometer (Aviva Accu-Chek, Roche Diagnostics, Basel, Switzerland) that had been validated for porcine blood previously at the Department of Clinical Sciences, SLU, Sweden.

Throughout the study blood glucose was measured at least twice daily. Blood was sampled from the jugular vein catheter, which was flushed with sterile saline (Natriumklorid Fresenius Kabi® 9 mg/ml, Fresenius Kabi AB, Uppsala, Sweden) before and after sampling. Before sampling, 3 ml blood was drawn and thrown away.
**Ketone bodies**

Blood samples were taken for analysis of β-ketone bodies if clinically indicated, using test strips (FreeStyle Precision β-ketone, Abbot/ADC, Alameda, CA, U.S.). Ketone bodies were analysed on May 1st – (see results below in Table 2).

**C-peptide and glucagon stimulation test**

C-peptide is widely used in human trials and is a means of analysing insulin secretion (Souza et al., 2006a). Several blood samples were drawn to analyse serum C-peptide concentrations during the study. The samples were analysed at Rudbeck Laboratory (Uppsala University, Sweden) using Porcine C-peptide ELISA (Mercodia Uppsala, Sweden). Samples were drawn from all eight pigs, on the day of catheterization/STZ. In the STZ-pigs samples were also drawn on day 4, 15 and 27 post STZ.

A glucagon stimulation test (GST) was performed either on day 28 or 29 post STZ (May 23rd and 24th) by injection of 1 mg glucagon (Glucagon Novo Nordisk 1 mg, Novo Nordisk Scandinavia AB, Malmö, Sweden) s.c. to the diabetic pigs to cause hyperglycaemia and, in case of remaining functional beta-cells in the pancreas, induce release of C-peptide. Blood for analysis of serum C-peptide was sampled 30 minutes after the injection of glucagon.

**Haematology and biochemistry**

On April 17th and April 25th, i.e. the days of surgery, blood was sampled from all eight pigs to analyse haematology (Hb, EPK, EVF, MCV, MCHC, reticulocytes and morphology) using an electronic cell counter (Advia 2120, Siemens, Erlangen, Germany) and total and differential white blood cell counts using EDTA-preserved blood. Using automated equipment (Architect C4000, Abott, Diagnostics, North Ryde, Australia), serum was analysed for values of total bilirubin, electrolytes (sodium, potassium and chloride) and enzyme activity of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), gamma-glutamyltransferase (GT) and glutamate dehydrogenase (GLDH).

On April 29th (four days post STZ) and May 22nd (27 days post STZ) blood was sampled from the diabetic pigs for the analyse of ALAT, ASAT, GLDH and creatinine.

All blood parameters were analysed at an accredited laboratory at SLU, using methods validated for porcine blood.

**Pet-CT and anaesthesia**

**Preparations**

On the day of the examination the pigs were fed and, if diabetic given their regular morning dose of insulin, at least over an hour prior to anaesthesia. Anaesthesia was induced in their own pen at the Department of Clinical Sciences (SLU, Uppsala). A peripheral catheter was inserted into an auricular vein (all pigs) for injections and infusions, and into an ear artery (pig #2) to be able to measure the arterial blood pressure. Two different anaesthetic protocols were used. Anaesthesia was maintained using a TIVA-protocol. Please see a detailed description of the anaesthesia below.
The anaesthetised pig was intubated with an endotracheal tube and transported approximately 15 minutes by car and wired up to a portable monitor device (Mindray) measuring RR, HR, SpO₂ and EtCO₂, to the Uppsala University Hospital, where the PET-CT examination was performed. All diabetic pigs were connected to a portable ventilator (Hamilton C2) during the transport or ventilated using a manual resuscitator.

At the Uppsala University Hospital the pig was placed in dorsal recumbency, connected to a Kion ventilator, 30% oxygen in nitrogen was administered and the pig continued being monitored now measuring RR, HR, SpO₂, EtCO₂, rectal body temperature, ECG, non-invasive blood pressure, tidal volume, minute ventilation and FiO₂. The pig was mechanically ventilated to ensure EtCO₂ was maintained at approximately 5.5 kPa.

**Anaesthesia during PET**

Two anaesthetic protocols were used, one for controls and one for diabetic pigs. This was due to the fact that medetomidine (used for controls) is not suitable for diabetics, since it increases blood glucose levels and decreases insulin secretion (Ambrisko et al., 2005). The anaesthetic protocol used for controls was evaluated by the anaesthesiologist prior to this study and is the regular protocol used for healthy pigs at the research facility.

The controls were anaesthetised by an i.m. injection of 5 mg/kg tiletamin and zolazepam (Zoletil Forte® vet. 250mg/ml, Virbac, Carros, France), 0.05 mg/kg medetomidine (Domitor® vet. 1 mg/ml, Orion Pharma Animal Health, Sollentuna, Sweden) and 0.1 mg/kg butorphanol (Dolorex® vet. 10 mg/ml, Intervet AB, Sollentuna, Sweden) as analgesia.

Anaesthesia and analgesia was induced in the diabetic pigs using 4 mg/kg alfaxalone (Alfaxan® 10 mg/ml, Vétolux Limited, Buckingham, U.K.), 2 mg/kg midazolam (Dormicum® 5 mg/ml, Roche AB, Stockholm, Sweden) and 2 µg/kg fentanyl (Fentanyl B. Braun® 50 µg/ml, B. Braun Medical AB, Danderyd, Sweden), given i.v. (#3) or i.m. (#1 and #8). Anaesthesia was induced to pig #1 and #8 by i.m. injections, since their catheters were malfunctioning. In the pigs with malfunctioning catheters, access for venous sampling was then granted through invasive (on site) surgery to the jugular vein (#1) or a catheter reaching the jugular through the auricular vein.

General anaesthesia and analgesia was maintained using two different TIVA-protocols, administered through the auricular vein catheter, where control pigs were given a constant rate infusion (CRI) of 5 mg/kg/h tiletamin and zolazepam, 0.05 mg/kg/h medetomidine and 0.1 mg/kg/h butorphanol. The diabetic pigs were given a CRI of 3 mg/kg/h alfaxalone, 1 mg/kg/h midazolam and 1 µg/kg/h fentanyl at infusion starting dose, continuously adjusted to achieve adequate anaesthetic depth. The anaesthesia was administered through the auricular vein catheter. The duration of the general anaesthesia was approximately five hours.

To maintain the intravenous fluid homeostasis, an i.v. infusion of Ringer’s acetate (Ringer-acetat, Fresenius Kabi AB, Uppsala, Sweden) was administered using the indwelling jugular vein catheter.
**Perfusion study**

At the beginning of the PET examination, oxygen-15 labelled water ([^15]O)WAT) was injected i.v. in the auricular vein as a bolus (200 MBq) using a contrast pump (Medrad) 0.8 ml/s for ten seconds, followed by 15 ml NaCl and 1.0 ml/s[^15]O)WAT for 15 seconds. This was performed to measure the perfusion in the pancreas and kidneys by a 10 minute dynamic PET scan (27 frames).

**The PET-CT examination**

Each pig was positioned with the pancreas in the centre of the 15 cm axial field of view. A full body CT, low dose scout view (140 kV, 10 mAs), was performed to detect the pancreatic area, using a Discovery ST PET-CT scanner (GE Healthcare, Milwaukee, MI, U.S.) and a photon attenuation correction scan was acquired.

The tracer[^68]GaGa-DO3A-VS-Cys40-exendin-4 was injected i.v., 8.08 ± 3.83 MBq (mean ± SD), corresponding to a low dose of 0.025 ± 0.01 µg exenatide peptide/kg, to obtain a baseline PET image (31 dynamic frames). In two controls and two diabetics a full body PET-CT was repeated, to study the full body uptake of the tracer signal. Thereafter, the tracer[^68]GaGa-DO3A-vs-Cys40-exendin-4 (69.01 ± 26.6 MBq) was injected i.v. together with a high dose of unlabelled exenatide (3.98 ± 1.33 µg/kg), to make the unlabelled high dose exenatide compete with the tracer for the GLP-1R and outnumber the tracer, to obtain a blocking image (31 dynamic frames).

The tracer and unlabelled exenatide were administered through the auricular vein catheter and the indwelling jugular vein catheter was used to analyse blood glucose during PET and to measure the radioactivity of the tracer, for PET image mathematical calculations.
Cystocentesis was performed if the urinary bladder needed to be emptied during the examination. This procedure resulted in a change in patient position, thus a new attenuation correction CT was made after cystocentesis and images were checked, to make sure the pancreas was in the focal area.

PET-CT examination was performed on each pig in the following order (see Table 1). The diabetic pigs were examined three to four weeks after insulin treatment had been initiated.

Table 1. Dates of PET examinations

<table>
<thead>
<tr>
<th>Date</th>
<th>Controls</th>
<th>Diabetics</th>
<th>Time during the day</th>
</tr>
</thead>
<tbody>
<tr>
<td>April 22\textsuperscript{nd}</td>
<td>#2</td>
<td></td>
<td>Afternoon</td>
</tr>
<tr>
<td>April 23\textsuperscript{rd}</td>
<td>#4</td>
<td></td>
<td>Afternoon</td>
</tr>
<tr>
<td>April 24\textsuperscript{th}</td>
<td>#6</td>
<td></td>
<td>Morning</td>
</tr>
<tr>
<td>April 29\textsuperscript{th}</td>
<td>#7</td>
<td></td>
<td>Afternoon</td>
</tr>
<tr>
<td>May 22\textsuperscript{nd}</td>
<td>#3</td>
<td></td>
<td>Afternoon</td>
</tr>
<tr>
<td>May 24\textsuperscript{th}</td>
<td>#1</td>
<td></td>
<td>Morning</td>
</tr>
<tr>
<td>May 28\textsuperscript{th}</td>
<td>#8</td>
<td></td>
<td>Afternoon</td>
</tr>
</tbody>
</table>

The PET-CT examination lasted for approximately four to five hours. The pig was transported back to the Department of Clinical Sciences in the same manner as it was transported to the Uppsala University Hospital. Extubation was performed in the pen, once the pig could swallow, and the ear vein catheter was removed.

An additional PET examination was performed on the brain of a ninth pig (slightly older than the previous eight pigs), to assess if the tracer could pass the blood-brain-barrier and bind to GLP-1R in the brain.

**Euthanasia, post mortem examination and tissue sampling**

The pigs were euthanised, 0–6 days after PET, with an overdose i.v. injection of pentobarbital sodium (Pentobarbital\textsuperscript{®} vet. 100 mg/ml, Apoteksbolaget, Sweden). Additionally, an intracardiac injection of 20 ml Pentobarbital\textsuperscript{®} was performed, once the pig was deeply anaesthetised.

In all pigs a full post mortem examination including histopathology was performed by a veterinary pathologist at SLU or the Veterinary Institute, Uppsala. The autopsy was performed on the same day as the pig was euthanised, except pig #5 where autopsy was performed two days later. Tissue samples from three different regions of the pancreas (duodenal, connective and splenic lobe) were fixed in formalin to perform insulin-staining by IHC.

**Statistical analyses**

Data is given as mean ± SD. Nonparametric Mann-Whitney rank sum test (GraphPad Prism 5 and GraphPad Instat) was used to test for significance in the perfusion study and for MAP and HR values, considering $p < 0.05$ to be significant.
RESULTS

Surgery and anaesthesia – Insertion of a jugular vein catheter

All pigs were clinically examined before surgery and the general condition was good. The jugular vein catheter was placed without complications in the right side in all the pigs except in pig #1. In pig #1 the catheter was placed into the left jugular vein. Both anaesthetic protocols worked satisfactorily.

Postoperative care

The pigs recovered well and quickly after the anaesthesia and surgery. They were hungry and fed within a couple of hours after being extubated. Twelve hours post surgery the pigs were brisk and curious as usual. Additional analgesia was not required.

The days after the operation all of the pigs had subcutaneous swelling and oedema around the incision site, but no pain reaction was observed when the oedema was palpated. The swelling was reduced substantially within a couple of days. The wounds healed without signs of infection, although the incision wound of pig #1 did not heal completely, which is why the catheter was removed on May 7th (twelve days post surgery).

General appearance, induction of diabetes and insulin treatment

The general appearance was mainly good throughout the study for all pigs, and the control pigs in particular. The pigs showed a daily weight gain of 0.7 ± 0.05 kg/pig during a two week period prior to STZ.

![Weight gain chart](chart1.png)

Chart 1. Weight gain the two weeks prior to diabetes induction by STZ-injection.

The first 24 hours after the STZ-injection are critical, due to risk of hypoglycaemia (Gäbel et al., 1985), thus blood glucose was closely monitored. Nine hours post STZ all pigs were hyperglycaemic (blood glucose > 11 mmol/L) and after 24h blood glucose was > 25 mmol/L.
Within a couple of days, all three pigs exhibited clinical signs such as polydipsia, polyuria, hyperglycaemia and weight loss, showing diabetes mellitus was successfully induced. The average weight loss was $0.52 \pm 0.02$ kg/pig/day during the untreated diabetic period, which is shown by Chart 2 below.

![Chart 2. Weight loss after diabetes induction by STZ-injection on April 25th.](chart2.png)

On the morning of May 1st insulin treatment was initiated using the humane insulin Actrapid® Penfill® (100 IU/ml, Novo Nordisk Scandinavia AB, Malmö, Sweden) since β-ketone bodies were elevated, please see results below in Table 2. The blood concentration of β-ketone bodies was still elevated in one pig three hours later, thus the Actrapid® was iterated. Additional β-ketone tests were then negative.

The following day, May 2nd (seven days post STZ), insulin treatment was continued twice a day injecting 0.8 UI/kg intermediate-acting insulin instead (Caninsulin® vet. 40 IU/ml, Intervet AB, Sollentuna, Sweden), since this is a porcine insulin. The dosage was thereafter adjusted several times, according to blood glucose and weight, and doses varied from 0.3 IU/kg to 1.0 IU/kg twice a day.

The insulin injections were performed whilst the pigs were busy eating and pieces of apples were used as rewards. At the beginning of the insulin treatment the pigs were scared or shaking their head when the injection was given, but after a few days of treatment they had stopped reacting to the injection and it could be carried out quickly and easily.

Proper glycaemic control was hard to achieve, which already is well known (Manell et al. 2014), thus slight hyperglycaemia was preferred to avoid hypoglycaemia during night. However, the therapeutic response was good, inasmuch the pigs did not show polydipsia and polyuria to the same extent and started gaining weight ($0.53 \pm 0.02$ kg/pig/day the first week after insulin treatment was initiated) and accumulate subcutaneous fat again (please see Chart 2).
3 below). The weight gain corresponds to that of commercial high-health herd-certified pigs, which is about 0.5 kg/day (Manell et al. 2014).

![Weight gain chart]

Chart 3. Weight gain from STZ and after initiated insulin treatment on May 1st until end of study.

**Blood analyses**

**Glucose measurements**

The STZ successfully induced diabetes, resulting in persistent hyperglycaemia in all pigs. Blood glucose was analysed daily to adjust the insulin dose. Proper glycaemic control was hard to achieve which led to slight hyperglycaemia throughout the study. However, the pigs were insulin-treated aiming towards a blood glucose concentration of 6–15 mmol/L, to avoid acute hypoglycaemia.

**Ketone bodies**

β-ketone bodies in the blood were analysed on May 1st due to poor general condition, and considering the acute death of pig #5. Please see the results below (Table 2). No blood test was performed on pig #1 in the afternoon, due to problems with its indwelling catheter.

<table>
<thead>
<tr>
<th>Pig</th>
<th>Ketone value before noon (mmol/L)</th>
<th>Ketone value in the afternoon (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig #1</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>Pig #3</td>
<td>1.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Pig #8</td>
<td>3.7</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Results indicated ketoaemia in the morning in two of the diabetic pigs (i.e. elevated β-ketone
bodies) and in the afternoon in one pig. Following tests were β-ketone negative and no more testing was clinically indicated.

**C-peptide and glucagon stimulation test**

The C-peptide levels remained low in all pigs (fasting levels up to ~ 60 pmol/L), including the controls. The glucagon stimulation test resulted in a slight increase (but still < 120 pmol/L) in one diabetic pig (#3).

**Haematology and biochemistry**

The test results from April 17th and April 25th, from all eight pigs, were within reference range. Blood samples four days post STZ (April 29th) were within reference range, except for pig #1 which had elevated ASAT and GLDH activities, which most likely was due to toxic effects of the STZ (Dufrane *et al.*, 2006). The same parameters were analysed 27 days post STZ (May 22nd) and all values were then restored to normal.

**Complications**

On April 23rd pig #2 was squinting with its left eye, most likely due to the fact that it had not been given eye lubricant during surgery the previous day. It was given fucidic acid eye drops (Fucithalmic® vet. 1%, Dechra Veterinary Products A/S, Uldum, Denmark) twice that day in the affected eye. The following day the eye appeared normal and treatment was discontinued.

Pig #1, #3 and #8 had fever and/or affected general condition on April 30th (five days post STZ), and also considering the death of pig #5, they were given procaine benzyl penicillin 30 mg/kg (Penovet® vet. 300mg/ml, Boehringer Ingelheim Vetmedica, Malmö, Sweden) i.m. once daily during one week.

On May 2nd pig #1 still had fever, chills and poor general condition. It was then given 0.1 mg/kg butorphanol (Dolorex® vet. 10 mg/ml, Intervet AB, Sollentuna, Sweden) i.m. once for analgesia at onset of illness and 2.5 mg/kg enrofloxacin (Baytril® vet. 100mg/ml, Bayer A/S, Animal Health Division, Copenhagen, Denmark) i.m. once daily during six days. Pig #1 also showed stereotypical behaviour (persistent licking on a wall and other objects), which could have been a sign of hepatic encephalopathy, since the pig at that point also had elevated activities of liver enzymes (ASAT and GLDH).

The catheters were flushed twice a day, which could be performed easily initially but after a few days it became more difficult to flush them until they stopped functioning one by one. Towards the end of the experiment pig #3 was the only pig with a functioning catheter. This pig was euthanised on May 23rd and the last five days of the study we could not sample blood glucose from the remaining pig (#8), although blood glucose from pig #8 was measured on the day of PET (May 29th).

We experienced problems with the indwelling jugular vein catheter of control pig #6 during PET. It is possible that the catheter was blocked by a thrombus and that forced flushing led to it being flushed into the blood stream. The day after PET the pig’s personality and behaviour had changed. Now, it did not want to eat apple, appeared not to understand how to drink from
the water nipple and was very calm (from earlier having been a crazy pig playing and biting on your shoes).

**Anaesthesia during PET**

Overall, the anaesthetic protocols of both types worked adequately. No muscle relaxant was needed and the pigs lay perfectly still as required for the performance of the PET-CT examination.

During the whole PET anaesthesia, from start, the diabetic pigs had lower mean arterial blood pressure (MAP) than the control pigs. Diabetic pigs had mean MAP values of 54.9 ± 10.8 mmHg and median MAP value of 53.0 mmHg whilst controls had mean MAP values of 93.0 ± 17.8 mmHg and median MAP value of 93.0 mmHg during PET, which is a significant difference ($p < 0.0001$).

**Perfusion study**

There was a significantly ($p < 0.05$) lower perfusion of Oxygen-15 labelled water ([15O]WAT) in the pancreas and the kidneys in the diabetic pigs compared to controls. Please see Figure 4 below. The perfusion was decreased by 46% in the pancreas and 40% in the kidneys of the diabetic pigs.

![Figure 4](image)

*Figure 4. Perfusion in the pancreas and kidneys in Non-Diabetic (controls) and Diabetic pigs. The asterisk indicates $p < 0.05$. The short horizontal bars represent interquartile range and the long horizontal bars represent median values. The figure is acquired by Marie Berglund at the PET preclinical platform.*

**PET-CT results**

In the control pigs, the PET results showed specific tracer binding to GLP-1R located in the pancreas, by a high uptake of the compound at low dose (baseline), which could be blocked by the unlabelled high dose exenatide as competition. However, the pancreatic uptake of the tracer did not differ significantly ($p = 0.43$) between the control pigs and the diabetic pigs.
Figure 5. Transverse PET-CT images of the pancreas using the tracer $[^{68}\text{Ga}]\text{Ga-DO3A-VS-Cys40-exendin-4}$, showing the duodenal lobe (D), connective lobe (C) and splenic lobe (S), with two controls to the left (panels A – D) and two diabetics to the right (panels E – H). The figure is acquired by Ram K. Selvaraju at the PET preclinical platform.

In the thoracic view of tracer distribution at low dose, a higher uptake was observed in the lungs of the diabetic pigs compared to control pigs, please see Figure 6. This uptake persisted following high dose exenatide, thus the uptake was not receptor-mediated.

Figure 6. Dorsal PET images, thoracic and partly abdominal view, showing the high $[^{68}\text{Ga}]\text{Ga-DO3A-VS-Cys40-exendin-4}$ tracer uptake in a diabetic pig lung compared to a control pig. Site of injection (S), pancreas (P), kidneys (K), urinary bladder (B) and lungs (L). The figure is acquired by Ram K. Selvaraju at the PET preclinical platform.

There was a high uptake in the kidneys and urinary bladder (the main route of tracer excretion) in all pigs. The uptake in other tissues, such as liver and muscle, was negligible in both groups and no detectable receptor binding was seen in the heart. The additional PET examination, concluded there was no tracer uptake in the brain.
**Tachycardia**

In all pigs, administration of the tracer compound resulted in significant increases in HR, both at low dose (92 ± 14 beats/min to 102 ± 10 beats/min (mean ± SD), median HR of 89 to 100, \( p = 0.0337 \)) and high dose (115 ± 17 beats/min to 217 ± 25 beats/min (mean ± SD), median HR of 108 to 228, \( p = 0.0022 \)). A \( \beta \)-blocker, metoprolol (Seloken\(^\circledR\) 1 mg/ml, AstraZeneca AB, Södertälje, Sweden) was administered i.v. at a dosages of 1–5 mg in three of the control pigs, but the severe tachycardia persisted.

Pig #4 suffered cardiac arrest during the transport back to SLU. The pig was given 0.3 mg epinephrine (Adrenalin 0.1 mg/ml, Martindale Pharmaceuticals, Romford, Essex, U.K.) i.v. and CPR was performed. The heart started beating after a couple of minutes and the pig began to breathe again. Once extubated at SLU the pig woke up quite quickly and wanted to eat only one hour later.

Three days post PET (April 25\(^{\text{th}}\)) occasional arrhythmias were auscultated in pig #2. Four days after PET (April 27\(^{\text{th}}\)) arrhythmia at a frequency of one extra beat approximately every eight regular beats was auscultated in pig #4.

Chart 4 below shows the significant HR increase in all pigs immediately after both low and high dose administration of exendin-4 and tracer (significance calculated based on HR values at 45 minutes compared to 60 and 105 compared to 120 minutes). The HR started to increase after 60 minutes from the start of the PET examination.

![Chart 4. Heart rate increase following intravenous injection of high dose exendin-4 and tracer. Low dose (LD) given at 60 minutes and high dose (HD) administered at 120 minutes from start of the PET examination.](image)

The additional pig, on which the PET examination of the brain was performed, had also an elevation of HR following high dose i.v. exenatide injection (maximum HR 160 beats per
minute). This pig was older than both controls and the diabetic pigs. The control pigs, which were the youngest, had a maximum HR of 250–260 beats per minute, whilst the diabetic pigs, which were three to four weeks older than the controls, had a maximum HR of 190–200 beats per minute, after high dose exenatide.

**Blood glucose during PET**

During PET, two pigs were hypoglycaemic (control #6 and diabetic #1) towards the end of the PET examination, after both low and high dose exenatide. The other five pigs were normo- or hyperglycaemic during PET.

**Post-mortem examination and tissue sampling**

No post mortem findings affecting the quality of the animal model were seen in any of the pigs. The only findings related to the heart were caused by the euthanasia.

The IHC of the pancreas with insulin-staining, showed a normal staining pattern in control pigs, whereas there was a poor to no staining in the STZ-treated pigs.

**DISCUSSION**

**Animal model**

The porcine animal model was successful. Following STZ-injection, clinical signs of diabetes were observed, such as hyperglycaemia, polydipsia, polyuria, hyperketonaemia and weight loss. Upon insulin treatment the clinical signs were alleviated, and the pigs started gaining weight and accumulate subcutaneous fat, which is also evidence of a well functioning diabetic animal model. Furthermore, the IHC with insulin-staining of the pancreas showed a markedly low level of staining in the STZ-treated pigs, in comparison to control pigs, thus, confirming that STZ effectively destroyed the vast majority of the beta-cells in the STZ-treated pigs.

The C-peptide values were low in all pigs, indicating that analyse of C-peptide may not be a suitable test for hypoinsulinaemia in pigs and has to be further evaluated.

The anaesthetic protocols worked satisfactorily as 1) the animals lay absolutely still during PET-CT examinations, as required, without the use of muscle relaxants, and 2) the same anaesthesia could be used during transportation and examinations.

**Perfusion study**

A significantly reduced perfusion (almost half) was seen in the pancreas and kidneys of the diabetic STZ-pigs compared to controls. The STZ-pigs had also a lower MAP than the control pigs during PET. It is possible that this lowering of MAP is the cause of the difference in perfusion. The reason why the diabetic STZ-pigs had a lower MAP is not known; however, different anaesthetic protocols, hyperglycaemia and STZ-toxicity may be potential explanations. Furthermore, it cannot be excluded that the age difference between controls and diabetic pigs (three to four weeks) has affected the result.

An anaesthetic study in pigs, shows that Alfaxan®, which was a substantial part of the TIVA used in the diabetic STZ-pigs, causes peripheral vasodilatation (Pfeiffer et al., 2013), while
Zoletil®, an important part of the TIVA used in the control pigs, instead causes an increase in peripheral blood pressure (Lee & Kim, 2012). Thus, the significant difference in pancreatic and kidney perfusion between control and STZ-treated pigs could be due to net MAP differences caused by the different anaesthetic protocols used.

In a rat diabetic model, tissue perfusion was significantly lower in non-insulin-treated diabetic rats compared to insulin-treated rats, two weeks post STZ (Kashiwagi et al., 2012). In another study of STZ-treated rats, a significantly lower systolic blood pressure was seen eight weeks post STZ-treatment. The lowered blood pressure was associated with up-regulation of cardiac muscarinic M2-receptors. The blood pressure and the M2-receptor mRNA levels, were restored once glycaemic control was achieved (Liu et al., 2008). Since the pigs of the current experiment were not under optimal glycaemic control, hyperglycaemia could explain the lower MAP seen in the diabetic pigs. Hyperglycaemia causes secondary osmotic diuresis and increased water loss, which together with anaesthesia, might have led to hypovolaemia during the PET examination, and thereby causing the lower MAP and subsequently reduced tissue perfusion (Oliver et al., 2010). Furthermore, hypotension is seen in the initial phase of diabetic disease progression (Maeda et al., 1995).

A known side effect of chronic hyperglycaemia is vascular damage (Son et al., 2004), thus theoretically, hyperglycaemic stress may have caused the difference in perfusion. Vascular dysfunction and oxidative stress parameters were seen in STZ-treated rats six weeks post STZ but could be reversed by insulin treatment (Oelze et al., 2011). In the current experiment, the pigs were only diabetic for three to four weeks and they were insulin treated. Even though they were not under optimal glycaemic control, it is unlikely that vascular damage occurred in that short period of time causing that significant difference in perfusion and there were no signs of vascular damage post mortem.

Another explanation to the difference in perfusion is STZ-toxicity, which can be seen in the liver and kidneys (Dufrane et al., 2006). However, the STZ-treated pigs in this experiment had no elevated blood parameters indicating kidney damage and there were no post mortem findings indicating STZ-toxicity. This strongly indicates that the reduction in blood pressure and reduced tissue perfusion was not due to the STZ-treatment (Oelze et al., 2011).

Still, the reason to the significant difference in perfusion is only speculative and therefore remains unknown. However, in future metabolic studies, it would be wise to use one single anaesthetic protocol in all subjects. Regardless, the PET method to measure perfusion was successful since it was able to detect a significant difference.

**PET-CT results**

A high uptake of the labelled \[^{68}\text{Ga}]\text{Ga}-\text{DO3A-VS-Cys40-exendin-4}\) tracer was seen in the pancreas of the control pigs at low dose, which could be successfully abolished after administration of competitive unlabelled exenatide at high dose, resulting in a blocking image, demonstrating that the uptake in the pancreas was receptor-mediated. This implies that \[^{68}\text{Ga}]\text{Ga}-\text{DO3A-VS-Cys40-exendin-4}\) may become an important tool for non-invasive evaluation of the GLP-1R system and the pharmacodynamic effects of GLP-1R agonists.
Furthermore, since no uptake of the tracer was seen in the liver, it is plausible that this compound may be useful for in vivo imaging of GLP-1R expressed by vivid beta-cells transplanted to the liver.

However, the pancreatic uptake of the tracer at baseline did not differ significantly between the control pigs and the diabetic pigs, suggesting that GLP-1 receptors are expressed in significant amounts in other parts of the pancreas than just beta-cells, which consequently disproves GLP-1R being a suitable target for imaging of native beta-cells and accurate quantification of the BCM.

There are several possible explanations to why a significant difference in tracer uptake is lacking between control and diabetic pigs. It could be due to the fact that beta-cells constitute such a small portion of the total pancreatic tissue, that the PET technology is not sensitive enough to detect a significant difference. Or, theoretically, the GLP-1R could have been up-regulated, i.e. other cells starting to express GLP-1 receptors, in the diabetic pigs. Another explanation may be that the difference in BCM could not be detected due to a more abundant, non-beta-cell-specific uptake in other pancreatic cells, in both controls and diabetic pigs, thereby masking the difference in BCM. The presence of GLP-1R on pancreatic ductal cells has been reported (Tornehave et al., 2008). There are also studies showing that GLP-1R may be present on delta-cells of the pancreas (Fehmann et al., 1995; Fehmann & Habener, 1991) and that GLP-1 can bind to somatostatin subtype 2 receptors on alpha-cells in a perfused rat pancreas (de Heer et al., 2008).

Furthermore, an unspecific uptake (i.e. not receptor-bound) was seen in the lungs of the diabetic pigs. Anaesthetics may affect the uptake and distribution of PET tracers (Hildebrandt et al., 2008; Momosaki et al., 2004), thus the unspecific uptake in the lungs could be due to the different anaesthetic protocols. Alfaxan®, used in the diabetics, is partly metabolised in the lungs (Nicholas et al., 1981), which theoretically implies that a metabolite of Alfaxan® may have bound to the tracer. To be sure of the role of different anaesthetics, healthy pigs should be anaesthetised with alfaxalone and the lungs evaluated by PET after tracer administration. Another theory is that the STZ or the intermittent hyperglycaemia of the diabetic pigs might have damaged the vessels in the lung, leading to leakage and tracer accumulation. However, this is not likely since no signs of vascular injury were found at gross or histopathologic examination post mortem.

Towards the end of the PET examination, two pigs were hypoglycaemic. The other five pigs had been normo- or hyperglycaemic during the whole PET examination. The PET of the two hypoglycaemic pigs had been performed before noon, whereas the other pigs after noon. Also, the control pig had not received any breakfast and the diabetic pig had been given insulin just prior to the anaesthesia and following PET. This, together with the known insulinotropic and glucose-lowering effects of exenatide, may have caused the hypoglycaemia. Furthermore, no difference in heart rate or blood pressure was seen during PET between the hypoglycaemic and the other normo-/hyperglycaemic pigs. Thus, the hypoglycaemia observed is considered unlikely to have affected the results in the experiment.
**Tachycardia**

During PET, all pigs suffered severe tachycardia immediately following high dose i.v. exenatide injection. No detectable receptor binding was seen in the heart. The tachycardia was mediated by the tracer compound, since the HR was affected in all pigs, immediately after tracer administration, regardless of age, STZ-treatment or anaesthetic protocol.

The degree of tachycardia somewhat differed between control pigs, diabetic pigs and the ninth pig on which the brain PET scan was performed, with the highest degree of tachycardia in the youngest pigs and the lowest degree of tachycardia in the oldest, thus it cannot be excluded that the age difference may have affected the result. Still, there was a significant increase in heart rate following i.v. injection of exenatide in all pigs.

Similar effects of exenatide have been seen in other previous studies. In rats, GLP-1 increased arterial blood pressure and heart rate significantly, when administered i.v. as well as i.c.v. The stimulating effect on arterial blood pressure and heart rate could be blocked by exendin-(9-39)-amide, administered through the same route and also i.c.v. if the GLP-1 had been administered i.v. (Barragán et al., 1999). The stimulating effect on cardiovascular parameters seem not to be mediated by catecholamines through the α- or β-adrenergic receptors (Barragán et al., 1994), which is in line with the result in the current experiment, where the tachycardia persisted even though a β-blocker was administered. Similar results were also seen in another study in rats, which were conscious and unrestrained. Both peripheral and central administration of GLP-1 resulted in increased arterial blood pressure and heart rate (Yamamoto et al., 2002). Furthermore, increased heart rate has been observed in calves following i.v. GLP-1 infusion (Edwards et al., 1997). In healthy humans no differences in blood pressure was seen following a s.c. injection of 10µg exenatide, but a heart rate increase of 8.2 beats per minute was observed (Mendis et al., 2012). A systematic review and meta-analysis published recently concluded there is a connection between GLP-1 analogues and a small increase in heart rate as well as modest lowering of the blood pressure. The authors are welcoming further studies using more accurate means of measuring the heart rate (Robinson et al., 2013).

One study shows how GLP-1 inhibits blood-brain glucose transfer in humans (Lerche et al., 2008), thus a possible theory causing the tachycardia is brain hypoglycaemia in the pigs. Another way to explain the tachycardia could possibly be by the region area postrema (AP), involved in nausea sensations. GLP-1 receptors are seen in AP (Orskov et al., 1996a), which much likely explains the nausea seen in subjects exposed to GLP-1 or exenatide (Ritzel et al., 1995). Area postrema is in close neuroanatomical connection with vagal nerves and important hypothalamic regions involved in water and food intake as well as the sensation of emesis. Through its anatomical situation, AP is an interface between the brain and the peripheral blood. Furthermore, AP has an insufficient blood-brain barrier to large polar molecules (Miller & Leslie, 1994). In the additional pig no uptake of the tracer could be detected in the brain, indicating that the tachycardia was not mediated by direct effect on receptors located inside the CNS. Still, in mice, i.c.v. administration of exendin-4 depressed heart rate variability. An increase in heart rate was seen following both acute and chronic i.c.v. administration of exenatide. It was also observed that the stimulation of central GLP-1R
reduced the parasympathetic modulation of the HR, which led to an increased HR (Griffin et al., 2011).

Explaining the reason behind the tachycardia is an intriguing task and additional studies are required to determine the aetiology. The effect on the HR has to be considered if this tracer compound is to be used in a clinical setting.

CONCLUSIONS

The pig is a well functioning animal model for human T1D. In this study we were able to anaesthetise the pigs for long periods of time, with an anaesthetic depth, which was demanded for PET examination. The pigs rapidly recovered from anaesthesia and quickly showed normal behaviour.

The [\(^{68}\)Ga]Ga-D03A-VS-Cys40-conjugated synthetic exendin-4 was not a selective tracer for native beta-cells. Thus, the GLP-1R is not a suitable target for BCM imaging. Furthermore, the pigs showed severe tachycardia as a side-effect of the tracer and exenatide injection, thus this compound should be used with great caution in pigs.

Moreover, a significantly reduced perfusion was seen in the pancreas and kidneys of the STZ-treated pigs. The reason for this remains unknown but it is possible it was a result of the two different anaesthetic protocols, thus only one anaesthetic protocol should be used in future PET-CT studies, to eliminate this factor.

Concluding, the pig is a promising animal model for further diabetic research in the endeavour to quantify the BCM. It would be of high interest to know if the actual insulin production of a patient relies on a few beta-cells with high insulin-productivity or a larger amount of beta-cells with attenuated efficacy, thus research towards the estimation of BCM should strive towards a methodology where both mass and function can be observed.

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