Evaluation of radiotracers for beta cell imaging

Inge van der Kroon
About the cover
The cover shows isolated islets of Langerhans under a light microscope. Healthy isolated islets of Langerhans have a smooth spherical surface and are golden-brown, with a diameter between 50 and 400 µm. For the research presented in this thesis numerous islets of Langerhans were isolated from pancreata and before transplantation these isolated islets of Langerhans were checked and counted under the microscope.
Evaluation of radiotracers for beta cell imaging

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen,
op gezag van de rector magnificus prof. J. H. J. M. Van Krieken,
volgens besluit van het college van decanen,
in het openbaar te verdedigen op maandag 22 mei 2017
om 14.30 uur precies

door

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geboren op 9 december 1986
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RATIONALE
Approximately 382 million people worldwide are affected by diabetes and this number is expected to increase with more than 200 million in the next twenty years (1).

Although the pathogenesis of diabetes has been investigated for decades, the relation between beta cell mass (BCM) and function in the development and progression of diabetes is not well understood. Currently, the progression of diabetes and the follow up after islet transplantation (a method to improve glucose control in patients with frequent hypoglycaemia) is assessed by functional measurements of glucose levels, insulin, HbA_{1c} and C-peptide, while information on BCM is limited and mainly relies on information from autopsy data.

A non-invasive method to follow BCM over time during the development of the disease and after islet transplantation could provide more insight in the relation between BCM and function and could help to improve therapeutic options. One of the most promising approaches to visualize the beta cells in vivo is radionuclide imaging after injection of radiolabeled tracers. The very high sensitivity of these imaging modalities allows detection of very low radiotracer concentrations.

OUTLINE
In this thesis several potential radiotracers for beta cell imaging are characterized. In chapter 1, the challenges involved in the imaging of beta cells and several potential tracers to visualize and quantify the beta cells are discussed.

One of the most promising tracers to visualize the beta cells in the islets of Langerhans is exendin which binds to the glucagon-like-peptide-1 receptor (GLP-1R). In chapter 2, the potential to visualize islets transplanted in the calf muscle with ^{111}In labeled exendin is shown. In chapter 3 this tracer is further characterized and its potential to quantify the BCM by in vivo SPECT imaging in islet transplants is demonstrated.

The potential of another tracer to visualize transplanted islets, radiolabeled iodobenzamide (IBZM) targeting the dopamine 2 receptor (D2R) is investigated in chapter 4, followed by further characterization in chapter 5.

The potential of radiolabeled exendin to visualize islets in the native pancreas was already shown by Brom et al (2). They showed an excellent correlation between beta cell mass and SPECT signal, although accurate delineation of the pancreas was
challenging since surrounding tissue, like the duodenum and stomach also express the GLP-1R, which can lead to an under- or overestimation of the actual BCM. Therefore a method to visualize the whole pancreas would simplify the delineation of the pancreas, potentially leading to a more accurate quantification of the BCM. A potential tracer to visualize the exocrine pancreas, $^{99m}$Tc-demobesin, and its potential to simplify quantification of the $^{111}$In-exendin SPECT signal is investigated in chapter 6.

The use of radiotracers to visualize the beta cells leads to concerns about potential radiation-induced damage to the islets of Langerhans. Estimation of the risk on radiation-induced damage to the islets requires a method to calculate the radiation dose to the islets. In chapter 7 a model is developed combining both clinical and preclinical data, and different dosimetry methods (whole organ and small scale dosimetry). This model is used to calculate the radiation dose to the islets of Langerhans after administration of radiolabeled exendin.

In the general discussion, the main results and future applications of the described tracers are discussed.

REFERENCES
General introduction – Evaluation of radiotracers for diabetes imaging
INTRODUCTION

Diabetes mellitus is characterized by hyperglycemia, resulting from loss in beta cell function or mass, or loss in insulin sensitivity. In type 1 diabetes (T1D) the insulin producing beta cells are destructed by autoimmunity, whereas in type 2 diabetes (T2D) the body is resistant to insulin (1). In the end stages of both diseases significant amounts of beta cells are lost. However, information on the beta cell mass (BCM) before the actual onset of diabetes (pre-diabetic state) and the progression of beta cell loss during diabetes is limited. Most of this knowledge relies on autopsy data, which are logically single time point evaluations.

A non-invasive imaging method to follow the BCM during the course of diabetes would help to get more insight in the role of BCM in diabetes. Such a method would also allow following islets after transplantation, which is a potential therapeutic option for T1D patients.

SPECIFIC REQUIREMENTS FOR TRACERS USED IN DIABETES IMAGING

Development of a tracer for beta cell imaging comes with challenges. The beta cells are located in the islets of Langerhans, have a size of only 50-400 µm and are scattered throughout the pancreas accounting for only 1-2% of the total pancreatic tissue (2), whereas after islet transplantation even a much smaller BCM should be imaged. Beside beta cells other cell types are present in the islets of Langerhans. Therefore, a potential tracer for beta cell imaging should be specific for the beta cells, with no or very low uptake in the exocrine tissue and other cell types within the islets. Furthermore, the target should be abundantly expressed and preferably not change during the progression of the disease. The tracer should retain in the beta cells to obtain high target-to-background ratios. Malaisse et al (3) even mentioned a thousand fold higher accumulation in the beta cells compared to the other pancreatic cells to be suitable as a beta cell marker. Whereas according to another paper a suitable target should have 100,000 receptors per cell and at least a fivefold difference between specific and unspecific labeling (4).

This small islet size and low amount of beta cells require imaging modalities with either very high spatial or chemical resolution to accurately visualize the beta cells. Imaging modalities such as computed tomography (CT) and magnetic resonance imaging (MRI) have a high spatial resolution, although not high enough to distinguish individual islets. Whereas imaging modalities like positron emission tomography (PET) and single photon emission computed tomography (SPECT) have sensitivities in the nanomolar range but a very poor spatial resolution (>1 mm). Even though the spatial
resolution of PET and SPECT is limited, radiotracers targeting receptors specifically expressed on beta cells seem to be the most promising approach for BCM imaging.

**POTENTIAL TARGETS AND APPROPRIATE LIGANDS**

As mentioned in the previous section, the potential target should be abundantly expressed in the beta cells, and no or only low expression should be present in the other (exocrine and endocrine) cells of the pancreas. After the selection of potential targets, a suitable ligand should be selected. For radionuclide imaging; peptides, small molecules and antibodies are used, each with their own advantages and disadvantages. For example natural peptides, which can be used as a ligand, are often instable. In the following section the most promising targets and their ligands are described in detail.

**Glucagon-like peptide-1 receptor (GLP-1R)**

The GLP-1R is highly expressed on beta cells (5-7), but not present on the alpha, delta and pp cells, and is therefore a potential target for beta cell imaging. Activation of the receptor by GLP-1 stimulates insulin secretion. Unfortunately, the natural receptor agonist, GLP-1 is rapidly degraded by the dipeptidyl peptidase-IV (DPP-IV) and has an in vivo half-life of only a few minutes (8). Due to this short half-life the natural GLP-1 is not suited for imaging. As an alternative to the natural GLP-1 the more stable GLP-1 analogs, exendin-3 and exendin-4 (9, 10) which are resistant to DPP-IV degradation, can be used.

Initially radiolabeled exendin was used for the visualization of insulinomas, which are neuroendocrine tumors derived from beta cells overexpressing the GLP-1R (11, 12). Gotthardt et al successfully showed a subcutaneous insulinoma in a mouse with scintigraphic imaging after injection of $^{123}$I-exendin. Later other radionuclides, like $^{111}$In, $^{68}$Ga, $^{99m}$Tc and $^{18}$F were used (13-19) for the visualization of insulinomas. Nowadays this method is successfully applied for the visualization of insulinomas in patients (13, 20-24) and it even shows potential for the treatment of insulinomas (11, 25).

Radiolabeled exendin can not only be used for insulinoma imaging, but also to visualize the islets of Langerhans in the native pancreas. Mikkola et al showed specific binding of both $^{64}$Cu-exendin and $^{68}$Ga-exendin in the islets of Langerhans in the pancreas with autoradiography, but they were unable to show the uptake of radiolabeled exendin with PET (26). However, Brom et al was able to show pancreatic uptake after injection of $^{111}$In-exendin with SPECT. They were even able to show a
linear correlation between BCM, determined with histology and quantitative analysis of the SPECT signal (27). A subsequent study showed that exendin uptake was not influenced by the alpha cell mass, which supports the statement that exendin is specific for the beta cells (28). Furthermore, the potential of this tracer was also shown in a clinical study, where the uptake of $^{111}$In-exendin was clearly decreased in T1D subjects compared to healthy subjects (27).

Besides imaging of islets in the native pancreas, radiolabeled exendin is also used for the visualization of transplanted islets. Intraportally transplanted islets could successfully be visualized with both $^{18}$F-exendin (29) and $^{64}$Cu-exendin (30), although the difference in liver signal between animals with and without an islet transplantation was small. Furthermore, islets transplanted in the fore-arm, one year after autologous transplantation, could be visualized with radiolabeled exendin (31).

**Dopamine receptor**
Dopamine, a neurotransmitter, plays an important role in many brain functions and is involved in neurodegenerative disorders like Parkinson’s disease (32). Already since the 1980’s the dopamine receptors are used as a target for brain imaging (33). In 2005 Rubi et al showed that the D2 receptor was also expressed in human and rodent islets and the receptor expression showed excellent co-localization with insulin expression (34). The identification of the D2 receptor lead to the idea of using the dopamine receptor as a target for BCM imaging. Initially, $^{18}$F-DOPA was used for insulinoma imaging (35), and to diagnose congenital hyperinsulinism (36, 37). Although it is possible to use $^{18}$F-DOPA for these applications, the relatively high uptake in the exocrine pancreas limits the application for BCM determination in the native pancreas. However, the tracer might be suitable for the imaging of transplanted islets as shown by Eriksson et al in 2014 (38). A different tracer specifically binding to the beta cells via the dopamine receptor is $^{18}$F-fallypride, which shows specific uptake in islets, but also relatively high amount of non-specific binding in the exocrine pancreas (39, 40), which might limit the application in the native pancreas.

**Vesicular monoamine transporter 2 (VMAT2)**
VMATs are ATP dependent transporters regulating the uptake of monoamines from cytoplasm into secretory granules in neuroendocrine cells. Two isoforms of these transporters are known; VMAT1 and VMAT2 (41). $^{11}$C-dihydrotatrabenazine ($^{11}$C-DTBZ) binds to the VMAT2 and was originally used for imaging of the VMAT2 in patients with neurodegenerative diseases (42).
In 2003 Anlauf reported expression of VMAT2 in beta cells but not in other endocrine cells (43). Later the potential of $^{11}$C-DTBZ for imaging the BCM in streptozotocin (STZ) induced diabetic animals (44) and in a spontaneous diabetes model (45) was shown. Furthermore, a first proof of concept in humans showed a lower uptake in diabetic compared to healthy subjects (46); however the uptake in the diabetic subjects was higher than expected. Despite these promising results there is some debate about the specificity of VMAT2 for beta cells (47, 48). In 2010 Fagerholm et al showed lower uptake in diabetic animals, although according to autoradiography images this was due to a difference in exocrine uptake and not due to specific uptake in the islets (49). These findings support that VMAT2 is not specific for the beta cells. However, autoradiography images of human pancreatic tissue in the same study, showed specific uptake in the human islets of Langerhans next to high unspecific binding to the exocrine pancreatic tissue. Even if VMAT2 is specific for the beta cells, the high exocrine binding of $^{11}$C-DTBZ hampers absolute quantification of the BCM with DTBZ. Besides $^{11}$C-DTBZ other radiolabels like $^{18}$F are used for BCM quantification (50, 51), unfortunately these tracers also showed high exocrine uptake (51). In 2013 the VMAT2 expression in pancreatic beta cells in different species was investigated (52). Human and pig beta cells showed VMAT2 expression, but VMAT2 expression was absent in rodent beta cells (52).

**Serotonin**

Serotonin is a neurotransmitter which is not only found in the central nervous system but is also present in the beta cells in the islets of Langerhans (53, 54), and not in the exocrine pancreas of mouse, rat and human (55). Serotonin in the pancreas is suggested to be involved in insulin secretion (53). 5-hydroxytryptophan (5-HTP), a precursor of serotonin is suggested as potential beta cell marker, despite taken up by not only the beta cells, but also the alpha cells. Fortunately, the retention is 5-10 times higher in beta cells than in alpha cells (56), making it suitable as a potential beta cell marker. $^{11}$C-5-HTP is already successfully used in the imaging of neuroendocrine tumors (57, 58). Recently, Di Gialleonardo investigated the $^{11}$C-5-HTP uptake in an exocrine and endocrine cell line. In both cell lines $^{11}$C-5-HTP was taken up rapidly, but the exocrine cell line showed rapid washout of the tracer. Additionally, they showed that inhibition of monoamine oxidase A (MOA-A) with clorgyline increased the signal in the endocrine cells (59).

In a preclinical study with rats, $^{11}$C-5-HTP accumulation in the pancreas was significantly decreased in diabetic animals. Target to background ratios increased by inhibition of MOA-A (60). $^{11}$C-5-HTP was also evaluated in a clinical study with 9 healthy volunteers and 10 T1D patients. In this study a 66% decrease in uptake in T1D
subjects compared to the healthy controls was observed, with the most apparent decrease in the corpus and caudal region of the pancreas. However a large variation between the different subjects was observed (61).

**Exocrine tracers**

Accurate delineation of the pancreas in rodents on *in vivo* images is difficult due to the random shape of the pancreas and the low contrast of the pancreas on CT images. It has been suggested to use pancreatic tracers to accurately determine the pancreatic region and thereby simplify the evaluation of beta cell tracers (62). Potential pancreatic tracers are radiolabeled amino acids, which rapidly accumulate in the exocrine pancreas. For example $^{11}$C-methionine is used as a pancreatic marker (26, 63, 64) but also other amino-acids, like iodinated phenylalanine (62, 65-67) and iodinated tyrosine (66) can be used as pancreatic markers.

Beside amino acids, receptors specifically expressed on pancreatic tissue, like the gastrin releasing peptide receptor (GRP-R) can be used as an exocrine tracer. The natural analog of the receptor has only a short half-life and therefore the more stable analogue demobesin is developed, which accumulates highly in the pancreas (27, 68, 69). A third alternative is $^{11}$C-acetate, a tracer originally developed to monitor cardiac metabolism, showing high uptake in the pancreas (70).

Beside the discussed tracers in the previous section, several more tracers, like IC2 (71) and mitiglinide derivatives binding to sulphonylurea receptors (72) are proposed for beta cell imaging and discussed in the numerous reviews written on beta cell imaging (3, 4, 73-75)

**TRACER CHARACTERIZATION**

Potential tracers should be extensively characterized first *in vitro* and subsequently *in vivo*. In an *in vitro* setting the affinity, specificity and binding kinetics for the target receptors are tested. Natural peptides are often stabilized by modifications to prevent enzymatic degradation. These modifications might influence the binding affinity of the ligands. After this initial *in vitro* characterization the tracer is more extensively characterized *in vivo* to get more insight in, not only the *in vivo* stability and specificity of the tracer, but also on the excretion route, and accumulation and retention in the target tissue. Furthermore, the optimal time point for imaging and the optimal tracer dose leading to the highest target-to-background ratios need to be determined.
After complete characterization of the radiotracer in healthy animals, the potential of the radiotracer in animal models for diabetes should be investigated.

**DIABETES MODELS**

Preferably the characterization of the tracer should first be performed in a well-controlled diabetes model. Chemically induced diabetes models are an excellent option, since the severity of diabetes can be regulated. After administration of the chemical a high percentage of the beta cells is destroyed, resulting in hyperglycemia. This method can be used in most rat and mice strains, although the sensitivity for the chemicals varies (76).

Alloxan and STZ are the two most commonly used compounds to induce diabetes. Their chemical structure is similar to glucose and is recognized by the glucose transport 2 receptor. The diabetogenic action of these compounds and their application are well described in literature (76-78), although the severity of diabetes and the mortality rate in these studies is not always accurately described.

More realistic diabetes models can be used to investigate the true potential of the tracer in a clinical setting. Several animal models for diabetes are available, some resembling T1D and others similar to T2D. Commonly used models are NOD mice, BB rats, ob/ob mice, db/db mice and Zucker rats (78, 79).

**DOSIMETRY**

It is widely known that exposure to ionizing radiation can lead to tissue damage. However, the amount of tissue damage depends on the type of radiation (e.g. α, β, γ), energy of the radiation, accumulation in the tissue and radiosensitivity of the tissue. To estimate the potential damage to a tissue (target) the absorbed dose can be calculated. Beside the activity in the target tissue, also activity in other tissues (sources) can contribute to the absorbed dose in the target tissue. Calculation of the absorbed dose, which is the amount of absorbed energy in tissue per unit mass, expressed in gray (Gy = J/kg) is called dosimetry.

There are several methods available to calculate the absorbed organ doses. Monte Carlo (MC) simulations are considered the most robust method, however this method is time consuming and sometimes complicated. In these MC simulations tissue density, type of radiation and energy of radiation are included and many simulations are performed, resulting in an estimation of the energy deposition in a tissue.
A second, more commonly used method to calculate the absorbed organ dose is by using dose conversion factors, or so called S-values (80), as implemented in OLINDA/EXM (81). OLINDA/EXM is a software program which can be used to calculate organ absorbed doses after administration of radiopharmaceuticals. An S-value describes the contribution of the activity in one organ (source) to the dose in another organ (target). Source and target can be the same; in this case the self-dose is calculated. For calculation of the absorbed dose, not only the S-value ($S_{\text{target-source},j}$) but also the time integrated activity coefficient in the source organ needs to be known. The time integrated activity coefficient ($\tau_{\text{source},j}$ in MBq.h/MBq = h) is obtained by integration of the time-activity curve, where the activity in the organ is represented by the fraction of administered activity. To obtain the absorbed dose in the target ($AD_{\text{target}}$) in Gy, the contributions of all source organs are summed and multiplied by the administered activity ($A_{\text{adm}}$):

$$AD_{\text{target}} = A_{\text{adm}} \sum_{\text{source},j} \tau_{\text{source},j} \cdot S_{\text{target-source},j}$$  \hspace{1cm} (1)

Unfortunately, the S-value method can only be used to calculate macroscopic doses and except for the kidneys (82) no models are available to simulate an inhomogeneous activity distribution within an organ. Radiolabeled exendin accumulates specifically in the insulin producing beta cells in the islets of Langerhans in the pancreas. Estimation of the absorbed pancreas dose would underestimate the islet absorbed dose. For calculation of sub-organ doses, handling non-uniform activity accumulation mainly MC simulations and dose point-kernels are used (e.g. STRATOS, dosimetry package of Philips).

**Application of dosimetry**

Dosimetry is mainly used to predict the potential of a new radiopharmaceutical and to assess the risk of radiation induced damage to healthy tissues. For example, before treatment with a beta emitter, a gamma emitting surrogate of the tracer can be used to estimate the potential of the therapeutic radionuclide. The maximum amount of administered activity can be estimated to prevent toxicity to healthy tissue while maximizing the amount of activity to the target tissue.

Dosimetry can also be used to simulate the absorbed dose due to different radionuclides and selecting the optimal radionuclide for imaging (e.g. maximizing signal in target tissue, minimizing absorbed dose other organs).
Chapter 1

**Dosimetry of exendin**

Radiolabeled peptides are regularly cleared via the kidneys and are therefore often the dose limiting organ. Several studies have investigated the absorbed kidney dose due to imaging with radiolabeled exendin ($^{68}$Ga, $^{111}$In, $^{99m}$Tc, $^{64}$Cu) (15, 26, 83, 84). Even the potential of radiolabeled exendin as a therapeutic agent to treat neuroendocrine tumors was investigated (25). In this study they considered a maximum absorbed kidney dose of 23 Gy safe, resulting in renal side effects in 1-5% of the patients after 5 years (85). In order to ensure that imaging with exendin is safe also pancreatic doses were calculated (15, 83, 84). Based on dosimetric data from external beam radiation an absorbed dose of 10 Gy resulted in diabetes in 16% of the patients >20 years after external beam irradiation (86). However, these studies did not take into account the inhomogeneous activity distribution within the pancreas and therefore largely underestimate the islet absorbed dose.

**REFERENCES**


Non-invasive imaging of islet transplants with $^{111}$In-exendin-3

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Adapted from: Journal of Nuclear Medicine 2016;57:799-804
ABSTRACT
Islet transplantation is a promising treatment for type 1 diabetic patients. However, there is acute as well as chronic loss of islets after transplantation. A non-invasive imaging method that could monitor islet mass might help to improve transplantation outcome. In this study, islets were visualized after transplantation in a rat model with a dedicated small animal SPECT scanner by targeting the glucagon-like peptide-1 receptor (GLP-1R), specifically expressed on beta cells, with $^{111}$In-labeled exendin-3.

Methods: Targeting of $^{111}$In-exendin-3 to the GLP-1R was tested in vitro on isolated islets of WAG/Rij rats. For in vivo evaluation, WAG/Rij rats (6-8 weeks) were transplanted in the calf muscle with 400 or 800 islets. Four weeks after transplantation, SPECT/CT images were acquired one hour post injection of $^{111}$In-labeled exendin-3. After SPECT acquisition, the muscles containing the transplant were analyzed immunohistochemically and by autoradiography.

Results: The binding assay, performed on isolated islets, showed a linear correlation between the number of islets and $^{111}$In-exendin-3 accumulation (Pearson r=0.98). In vivo a 1.70 ± 0.44 fold difference in tracer uptake between 400 and 800 transplanted islets was observed. Ex vivo analysis of the islet transplant showed co-localization of tracer accumulation, as shown by autoradiography, with insulin-positive cells and GLP-1R expression, as determined immunohistochemically.

Conclusion: $^{111}$In-exendin-3 accumulates specifically in the beta cells after islet transplantation and is a promising tracer to monitor the islet mass non-invasively.

Keywords: exendin-3; GLP-1R; islet transplantation; SPECT; islet imaging
INTRODUCTION

For type 1 diabetic patients with poor glycemic control, pancreatic islet transplantation is a promising but still experimental treatment. The standard transplantation procedure is infusion of pancreatic islets in the portal vein, where islets are trapped in the sinusoidal capillaries of the liver. Most patients become insulin independent for more than one year after transplantation. However, insulin independency drops to approximately 10% five years after transplantation (1). Even though these insulin dependent patients still profit from this treatment, because instability of blood glucose levels and risk of hypoglycemia is reduced, further improvement of transplant survival and islet function is warranted. This is of special importance in view of the side effects of the immunosuppressive treatment that is required to prevent rejection of the transplanted islets (1-3).

Current methods to monitor islets after transplantation, such as measurement of HbA1c, C-peptide, and insulin levels, only provide functional information and cannot accurately predict the number of surviving islets. Medical imaging methods may offer the possibility to monitor the number of surviving islets after transplantation, which could provide information (complementary to functional capacity) about the effect of interventions on transplantation outcome. This might help to further improve this new therapeutic approach in order to achieve longer lasting insulin independency.

Numerous studies have investigated the feasibility to image transplanted islets non-invasively. Islets were transfected to express luciferase (4), or islets from transgenic animals expressing luciferase were used (5). Although feasibility has been proven in preclinical transplantation models, these methods require transfection of beta cells or the use of transgenic animals and are therefore relatively difficult to translate to humans. In several studies, pancreatic islets pre-labeled with iron oxide contrast agents, were transplanted allowing monitoring with MRI (6,7). These methods can show the presence of islets, however, it remains a matter of debate to which degree the presence of iron oxide contrast agents represents viable and functional islets. Determination of the relation between viable beta cell mass and beta cell function might therefore be difficult to achieve.

Transplanted islets also have been visualized using tracers targeting receptors specifically expressed on beta cells in the islets, such as the glucagon-like peptide-1 receptor (GLP-1R) (8). Wu et al (9) have demonstrated the feasibility to visualize islets transplanted in the liver by targeting the GLP-1R by PET with radiolabeled exendin-4. Targeting a receptor specifically expressed on beta cells with a radiotracer allows serial imaging in vivo. In order to fully exploit the potential of this imaging method,
quantification of true beta cell mass in islet transplants should be established for validation of in vivo imaging results.

Establishing and characterizing quantitative beta cell imaging by targeting the GLP-1R requires a simple transplantation model, which allows accurate evaluation of tracer accumulation and histological verification of imaging findings. A suitable location for islet transplantation is the muscle. The muscle is already used in clinical islet transplantation (10-13) and since all islets are clustered in one location tracer uptake can be determined by quantitative analysis of PET and SPECT images, while islet survival can easily be verified histologically.

In this study, we demonstrate the feasibility of non-invasive in vivo imaging of different numbers of islets, transplanted in the calf muscle, by targeting the GLP-1R with $^{111}$In-labeled exendin-3.

**MATERIALS AND METHODS**

**Radiolabeling**

[Lys$^{40}$(DTPA)]exendin-3 (Peptides Specialty Laboratories, Heidelberg, Germany) (Figure 1) (referred to as exendin-3 in the remainder of the text) was radiolabeled as described previously (14). Briefly, 150 MBq $^{111}$InCl$_3$ was added to 1 µg exendin-3 dissolved in 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5 (Sigma Aldrich, St. Louis, MO, USA) and incubated for 20 minutes at room temperature. After incubation, Ethyleendiaminetetra-acetic acid (Sigma Aldrich) and Polysorbate 80 (Sigma Aldrich) were added to a final concentration of 5 mM and 0.1%, respectively.

![Figure 1: Chemical structure of [Lys$^{40}$(DTPA)]exendin-3](image-url)
The radiochemical purity of $^{111}$In-exendin-3 was determined by instant thin-layer chromatography (ITLC) (ITLC-SG, Agilent Technologies, Lake Forest, CA, USA), using 0.1M NH$_4$Ac (Sigma Aldrich) in 0.1 M Ethyleendiaminetetra-aceticacid, pH 5.5. The reaction mixture was purified by solid-phase extraction using hydrophilic-lipophilic balance (HLB) cartridge (30 mg, Water Oasis, Milford, MA, USA), to remove unlabeled $^{111}$In. The cartridge was activated with 1 mL ethanol, washed with 2 mL water and conditioned with 1 mL of 0.1 M MES, pH 5.5. Subsequently, the labeling mixture was loaded on the cartridge and washed with 1 mL 0.1 M MES and 2 mL water. $^{111}$In-exendin-3 was eluted from the cartridge with 200 µL 100% ethanol. For injection, the purified labeling solution was diluted in 0.5% Phosphate buffered saline (PBS)/Bovine Serum Albumin (BSA) (w/v) to obtain a final ethanol concentration of less than 10%.

Animals
Six to eight weeks old female WAG/Rij rats were purchased from Charles River Laboratories (Erkrath, Sulzfeld, Germany). WAG/Rij rats were used both as islet donor and recipient. The animal experiments were approved by the animal welfare committee of the Radboud University Nijmegen.

Islet isolation
Pancreatic islets were isolated from female WAG/Rij rats by collagenase digestion. Rats were suffocated with CO$_2$/O$_2$, the abdominal cavity was opened and a canula was placed in the common bile duct. The pancreas was perfused with 8 mL Roswell Park Memorial Institute (RPMI) (R0883, GIBCO, BRL Life Sciences Technologies, Bleiswijk, The Netherlands), containing 1 mg/mL collagenase Type V (Sigma Aldrich). Subsequently, the pancreas was dissected and kept in ice-cold RPMI containing collagenase until digestion. The pancreata were digested for 11.5 minutes at 37 °C. The digestion was stopped by adding complete RPMI medium (RPMI medium supplemented with 10% fetal calf serum (HyClone, Celbio, Logan, UT, USA), 2 mM L-glutamine (Sigma Aldrich), 100 U/mL penicillin and 100 U/mL streptomycin (Sigma Aldrich). The digested tissue was washed twice in complete RPMI medium (2 min at 210 x g) and passed through a 500 µm mesh. Subsequently, the islets were purified on a discontinuous Ficoll gradient (densities 1.108, 1.096, 1.037 g/mL, Cellgro by Mediatech Inc., Manassas, VA, USA) by centrifugation at 625 x g for 16 minutes (without brake). The islets were collected from the interface of the second and third layer. Finally, the remaining Ficoll was removed by washing twice with complete RPMI medium (2 min at 210 x g). The purified islets were cultured overnight at 37 °C with 5% CO$_2$ in complete RPMI medium. Both islet viability and purity exceeded 90% after the isolation procedure.
**In vitro function assay**

Islet function after isolation was assessed by a static glucose incubation assay. After isolation and overnight recovery, islets were collected, counted and resuspended in complete RPMI. Islets were seeded in a 24 well plate (Corning Inc, Tewksbury, MA, USA), 30 islets per well (n=5), and pre-incubated for one hour (37 °C, 5% CO₂) in 600 µL Krebs buffer (115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 2.2 mM CaCl₂, 1 mM MgCl₂, 20 mM C₆H₁₈N₂O₄S and 0.2% BSA (Sigma Aldrich)) supplemented with 1.67 mM D-glucose (Sigma Aldrich). After 1 hour, the incubation buffer was replaced by 600 µL fresh Krebs buffer supplemented with 1.67 mM D-glucose. Subsequently, islets were incubated in Krebs buffer supplemented with 16.7 mM D-glucose and 1.67 mM D-glucose respectively, 1 hour for both conditions. After each incubation step, the supernatant was collected and samples were stored at -20°C until insulin determination by an insulin ELISA immunoassay (Mercodia AB, Uppsala, Sweden) performed according to the manufacturer’s protocol.

**In vitro binding assay**

After overnight recovery, islets were collected, counted and resuspended in complete RPMI. After transwell saturation, using binding buffer (RPMI containing 0.5% BSA (v/w)), islets were transferred to 24 well transwell plates (50, 100, 200 or 400 islets per transwell, n=3 per condition) (Corning Inc.) and incubated in 500 µL Krebs buffer for 30 min at 37 °C. Subsequently, islets were washed with 500 µL binding buffer and approximately 2 kBq of ¹¹¹In-exendin-3 was added, followed by incubation for 4 h at 37 °C. To determine whether the binding was GLP-1R-mediated, 1 µg unlabeled exendin-3 was added together with the radiolabeled exendin-3 in separate wells (n=3 per condition). After incubation, islets were washed five times using binding buffer and islet associated radioactivity was measured in a well type gamma counter (Wallac 1480 wizard, Perkin Elmer, Waltham, MA, USA).

**Transplantation**

After overnight recovery, isolated islets were collected, spun down at 15 x g for 2 min and resuspended in RPMI without fetal calf serum. Recipient rats were anesthetized with isoflurane (induction 4-5%, maintenance 1.5-2% in 50% O₂ and 50% air) (Abbott Laboratories, Toronto, Canada) and the right hind leg of the rat was shaved and disinfected with Betadine® (Meda Pharma B.V., Amstelveen, The Netherlands) before islet inoculation. Islets were aspirated into a catheter line and infused manually in the calf muscle at low speed (injected volume 100 ± 10 µL) using a Hamilton syringe (Hamilton Company, Reno, NV, USA). Rats were transplanted with either 400 (n=6) or 800 (n=6) islets.
SPECT acquisition and biodistribution

Four weeks after transplantation, rats were injected intravenously with 15 ± 0.4 MBq $^{111}$In-exendin-3 via the tail vein (peptide dose 0.1 µg in 200 µL PBS, 0.5% BSA). One hour after injection of the radiolabeled exendin-3, rats were anesthetized with isoflurane (induction 4-5%, maintenance 1.5-2% in O$_2$ and air) and SPECT/CT was acquired using a dedicated small animal SPECT/CT scanner (U-SPECT-II, MILabs, Utrecht, The Netherlands). SPECT images were acquired with a 1.0 mm pinhole general purpose rat and mouse collimator, 36 bed positions with a total acquisition time of 50 minutes. CT images were acquired for anatomical information (65 kV, 615 µA, 1 bed position, spatial resolution 160 µm).

After the SPECT/CT acquisition (2 h p.i.), rats were euthanized by CO$_2$/O$_2$ suffocation and the muscle containing the transplant and other relevant tissues were dissected, weighed and measured in a gamma counter (Wallac 1480 wizard, Perkin Elmer, Waltham, MA, USA). Muscles were fixed in 4% formalin (w/v).

SPECT reconstruction and quantitative SPECT analysis

The SPECT images were reconstructed using the U-SPECT reconstruction software (U-SPECT-Rec, MILabs, Utrecht, The Netherlands) with the following settings: OSEM, 1 iteration, 16 subsets, voxel size 0.75 mm$^3$.

The uptake of $^{111}$In-exendin-3 in the transplant was quantified using Inveon Reconstruction Workplace (IRW) (Siemens Healthcare, Den Haag, the Netherlands). A volume of interest was placed over the transplant. Total voxel intensity in the islet transplant was corrected for the background by subtracting the background signal in the contra-lateral control muscle using an identical volume of interest and the injected activity.

Immunohistochemical analysis

The formalin-fixed muscle, containing the transplant, was dehydrated and embedded in paraffin. Four µm sections were cut and stained with Hematoxilin and Eosin for localization of the transplant. Consecutive sections were stained for the presence of insulin, as described previously (15) and the presence of the GLP-1R.

The GLP-1R staining was performed as follows: antigen retrieval was performed in 10 mM sodium citrate, pH 6.0 for 10 minutes at 99 °C. Subsequently, sections were incubated for 10 minutes with 3% H$_2$O$_2$ in PBS at RT in the dark, to block endogenous peroxidase activity. Non-specific binding was blocked by incubation for 30 minutes with 5% swine serum. The primary anti-GLP-1R antibody (ab39072, Abcam,
Cambridge, UK) was diluted in PBS, 1% BSA (1:1,000). Primary antibody incubation for 90 minutes was followed by incubation with swine-anti-rabbit peroxidase (1:100) (p0271, DAKO, Copenhagen, Denmark). Finally, Bright DAB (BS04500, Immunologic BV, Duiven, The Netherlands) was used to visualize peroxidase activity. Microautoradiography, to visualize tracer accumulation, was performed on four µm sections as described by Brom et al (15).

Sections, stained for insulin and GLP-1R, were analyzed using a Leica DM5000 microscope and images were obtained with a color camera (Evolution MP; Leica using Axio Vision 4.4 software). To determine transplant volume, the insulin positive area of the transplant per section was multiplied with the inter-section distance. Three animals were excluded from the analysis since no transplant was found immunohistochemically.

**Statistical analysis**
All mean values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using unpaired two-tailed t-test, the correlation described in this paper was calculated using the Pearson correlation coefficient (r) using GraphPad Prism v5.03 (GraphPad Software, Inc., San Diego, CA, USA). The level of significance was set at p<0.05.

**RESULTS**

**Radiolabeling**
Exendin-3 was labeled with $^{111}$In with a specific activity of 700 GBq/µmol. Radiochemical purity was >99% as determined by ITLC.

**In vitro function assay**
The results of the *in vitro* islet function assay are summarized in Figure 2. Islets responded to high glucose stimulation with an increase in insulin production from 41.1 ± 18.6 ng/mL/hour to 792 ± 176 ng/mL/hour. After high glucose stimulation, insulin levels decreased to basal levels when incubated in low glucose buffer: 80.3 ± 31.4 ng/mL/hour, indicating normal function. The stimulation index, defined as a ratio of stimulated to basal insulin secretion, was 10.4 ± 1.98 (n=5).
In vitro binding of $^{111}$In-exendin-3 to isolated islets

Figure 3 shows the binding of $^{111}$In-exendin-3 to rat islets in vitro. After 4 hours $31 \pm 3.6$ amol of the $1.28$ fmol added $^{111}$In-exendin-3 accumulated in 50 islets. Accumulation of $^{111}$In-exendin-3 increased with increasing number of islets (100 islets: $39 \pm 7.6$ amol, 200 islets: $80 \pm 1.2$ amol and 400 islets: $112 \pm 2.9$ amol). This resulted in excellent linear correlation between $^{111}$In-exendin-3 accumulation and the number of islets (Pearson r=0.98, p=0.02). Co-incubation with $1 \mu g$ (0.2 nmol) of unlabeled exendin-3 blocked the accumulation of $^{111}$In-exendin-3 >80% in all conditions indicating GLP-1R mediated accumulation.

SPECT/CT imaging

Islets, transplanted in the right calf muscle were clearly visible in SPECT/CT images 4 weeks after transplantation, one hour after injection of $^{111}$In-exendin-3 (Figure 4), with hardly any uptake in the surrounding muscle tissue. Besides accumulation in the
transplant, signal was also observed in the bladder (Figure 4A), kidneys and the lungs. Furthermore, a clear difference in SPECT signal was observed between rats transplanted with 400 (n=4) and 800 islets (n=5) (Figures 4B and 4C). Three animals were excluded from the analysis since no transplant was found immunohistochemically.

The mean uptake in the group transplanted with 800 islets was 177 ± 59 Bq (signal to noise ratio (SNR): 13 ± 3.5) and the uptake in the group transplanted with 400 islets was 104 ± 4.5 Bq (Figure 5B) (p<0.05), with a SNR of 8.8 ± 1.0. On average the SPECT signal in the 800 group was 1.70 ± 0.44 times higher than in the 400 group. All SPECT images were fused with CT images for anatomical reference.

Figure 4: A) Maximum intensity projection of a SPECT/CT image of islets transplanted in the right calf muscle of a rat (red arrow). Images were acquired 1 hour post injection of 15 MBq $^{111}$In-exendin-3. Besides the signal from the transplant, radioactivity was observed in the bladder (green arrow). A clear difference in SPECT signal was observed between rats transplanted with either B) 400 islets (white arrow) or C) 800 islets (blue arrow) (coronal slices).
Biodistribution

The results of the biodistribution are summarized in Figure 6. $^{111}$In-exendin-3 showed low accumulation in blood, heart, spleen and liver (maximum 0.1% ID/g). Uptake was observed in stomach, duodenum and lungs (0.74 ± 0.45, 0.51 ± 0.32 and 10.0 ± 5.28% ID/g, respectively) which corresponds with observations described previously (15). Kidney accumulation was very high (31.9 ± 11.5% ID/g), which was not GLP-1R mediated. No difference in uptake was observed between muscles containing the transplant and control muscles, due to the small transplant size compared to the muscle tissue (± 2 g).
Immunohistochemical analysis

*Ex vivo* autoradiography showed $^{111}$In-exendin-3 tracer accumulation in the islets transplanted in the muscle (Figure 7C). Hardly any background activity was observed in the muscle. Tracer accumulation showed co-localization with insulin and GLP-1R expression (Figure 7A-C). The positive insulin staining indicates viable and insulin-producing beta cells in the transplant. The average transplant volume in the group transplanted with 400 islets was $2.02 \pm 0.16 \times 10^5 \text{ µm}^3$ and the transplants in group transplanted with 800 islets was $4.30 \pm 0.47 \times 10^5 \text{ µm}^3$ (Figure 5A) ($p<0.05$). On average, the transplant volume in the 800 group was $1.92 \pm 0.24$ times larger than that in the 400 islets group.

DISCUSSION

In the present study, the feasibility to *in vivo* visualize islets of Langerhans, transplanted in the calf muscle, by non-invasive SPECT using $^{111}$In-exendin-3 as a tracer is demonstrated. Binding of $^{111}$In-exendin-3 to isolated islets *in vitro* showed a linear correlation between uptake and the number of islets. Furthermore, differences in the amount of transplanted islets could be detected by *in vivo* SPECT. Therefore, this non-invasive imaging method is suitable to provide real-time information about beta cell mass in the islet transplant.

Targeting the GLP-1R specifically expressed on the beta cells of the pancreatic islets offers an attractive alternative to methods using islets pre-labeled with USPIOs as described by Evgenov *et al* (7) and others (6,16-18). Targeting a receptor, such as the GLP-1R, may potentially offer higher specificity and does not require pre-labeling of islets. Furthermore, radiotracer imaging allows quantification of tracer uptake.
It has previously been demonstrated that targeting the GLP-1R with radiolabeled exendin is an elegant method for the imaging of beta cells, mainly in insulinoma models (14,19) and the native pancreas (15). Wu et al applied this strategy to visualize islets transplanted in the liver by PET after injection of exendin-4 labeled with copper-64 or fluorine-18 (9,20). In these studies relatively high background signal was observed, caused by nonspecific uptake of the tracer in the liver (20), which might hamper accurate quantification of islet-mediated accumulation of the tracer.

We believe that the transplantation model described here may offer significant advantages for determination of the number of viable islets in the transplant, because 1. the complete transplant can be visualized in the muscle with low background signal and 2. the complete transplant can be evaluated immunohistologically, because of the focal localization of the islets in a predefined area of the calf muscle. Another commonly used focal islet transplantation site, in preclinical setting, is the kidney capsule; however, this transplantation site is less suitable than the muscle when using radiotracers, due to their often high kidney uptake (31.9 ± 11.5% ID/g for exendin).

SPECT/CT images showed clear uptake of the radiolabeled exendin-3 with an average SNR of 13 ± 3.5 after transplantation of only 800 islets. Furthermore, a difference between 400 and 800 islets in SPECT signal was observed, with the signal of 800 islets being 1.70 ± 0.44 fold higher than the uptake in 400 islets. Based on histological analysis, a 1.92 ± 0.24 fold difference in transplant volume between the 400 and 800 group was observed. An explanation for this difference between the imaging and histological data might be the partial volume effect. The transplant volume is small compared to the resolution of the SPECT system. This in combination with the low uptake might result in a difference between actual and quantified signal in the transplant. Considering that 400 islets are not sufficient to restore normoglycemia in a clinical setting, this method shows great promise for detecting even small amounts of islets. This would also allow to detect small changes in beta cell mass due to rejection or recurrent autoimmunity. Due to low uptake of our tracer in the liver (0.1% ID/g), this tracer might also be suited to follow islets transplanted in the liver. However, dispersion of the islets throughout the organ might impair the detection capacity of this tracer and histological evaluation of the islet transplant size in the liver is highly challenging.

Immunohistochemical analysis of the transplants showed excellent co-localization of the tracer as determined with autoradiography, insulin and GLP-1R staining. These findings in combination with the results of the in vitro accumulation of the tracer demonstrate specific binding of the tracer to the beta cells in the transplanted islets.
Moreover, these histological findings confirm insulin production of the transplanted islets four weeks after transplantation.

Further evaluation of the correlation of tracer uptake in islet transplants of different sizes over time should be considered for validation of the method for longitudinal imaging of islet transplants. Since this tracer is already used in clinical trials (15), this application can easily be translated and would have the potential to longitudinally measure the effect of therapeutic interventions on the islet mass.

CONCLUSION
In this study, islets transplanted in the muscle of rats were visualized using $^{111}$In-exendin-3. Both in vitro and histological data support specific tracer uptake and we propose the muscle transplantation model as a highly suitable controlled model for validation of this imaging technology. In vitro, a linear relation between the number of islets and exendin uptake was observed. Furthermore a significant difference in uptake between 400 and 800 islets was observed in vivo. Since the radiotracer can be injected repeatedly, this method will allow longitudinal monitoring of islet mass in vivo. Because the tracer molecule is already under clinical evaluation (15), clinical studies in patients transplanted with islets appear to be feasible.

ACKNOWLEDGEMENTS
The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union’s Seventh Framework Programmes FP7/2007-2013/ under REA grant agreement n° 289932 and FP7/2007-2013 under grant agreement n° 222980 and from the Institute of Genetic and Metabolic Disease, Radboud University Nijmegen. No potential conflict of interest relevant to this article was reported.
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Non-invasive *in vivo* determination of viable islet graft volume by $^{111}$In-exendin-3

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Submitted
ABSTRACT
Pancreatic islet transplantation is a promising therapy for patients with type 1 diabetes. However, the duration of long-term graft survival is limited due to inflammatory as well as non-inflammatory processes and routine clinical tests are not suitable to monitor islet survival. $^{111}$In-exendin-SPECT (single photon emission computed tomography) is a promising method to non-invasively image islets after transplantation and has the potential to help improve the clinical outcome. Whether $^{111}$In-exendin-SPECT allows detecting small differences in BCM and measuring the actual volume of islets that were successfully engrafted has yet to be demonstrated. Here, we evaluated the performance of $^{111}$In-exendin-SPECT using an intramuscular islet transplantation model in C3H mice. In vivo imaging of animals transplanted with 50, 100, 200, 400 and 800 islets revealed an excellent linear correlation between SPECT quantification of $^{111}$In-exendin uptake and insulin-positive area of islet transplants, demonstrating that $^{111}$In-exendin-SPECT specifically and accurately measures BCM. The high sensitivity of the method allowed measuring small differences in graft volumes, including grafts that contained less than 50 islets. The presented method is reliable, convenient and holds great potential for non-invasive monitoring of BCM after islet transplantation in humans.

Keywords: exendin, SPECT imaging, islet transplantation
INTRODUCTION

Transplantation of islets of Langerhans is a promising treatment for patients with type 1 diabetes (T1D). The short-term results of islet transplantation in normalizing blood glucose levels are encouraging (1). However, the rate of insulin-independency drops to less than 15% after 5 years (2). Although the loss of transplanted beta cells quickly renders patients insulin-dependent in a life-time perspective, the remaining graft function still exerts a positive effect on glucose homeostasis, reducing late complications and further progression of micro-vascular diseases (3). Furthermore, the islet grafts lower the amount of insulin required to maintain normoglycemia preventing potentially lethal, severe hypoglycemia. However, in view of the considerable side effects caused by the immunosuppressive therapy required to prevent graft rejection, improved survival of the transplanted islet is desirable. In order to optimize islet replacement therapy and to prevent loss of graft function, numerous approaches are currently under investigation, including modified immunosuppressive treatments (4, 5) and islet encapsulation strategies (6, 7) treatment with growth factors and other hormones, as well as alternative sources for beta cells (i.e. stem cells, tissue bioengineering) (8-11). In order to monitor the graft volume and optimize new strategies for beta cell replacement a non-invasive technology to visualize viable transplanted islets \textit{in vivo} is warranted. Such a method should be quantitative and sensitive in order to allow the detection of small changes in the number of surviving islets.

A promising approach to visualize transplanted islets \textit{in vivo} has been demonstrated by Saudek et al, using Magnetic Resonance Imaging (MRI). The group described MRI of islets that had been labeled with super-paramagnetic iron oxide particles (SPIOs) prior to transplantation (12). The feasibility of longitudinal non-invasive monitoring of islet transplants was further demonstrated in animal models by Evgenov and co-workers, successfully monitoring islet transplants for up to 188 days after surgery (13). Pre-labeling of the islets with SPIOs is therefore a promising method with clinical potential (14).

Alternatively, radionuclide imaging modalities were used because of their high detection sensitivity of transplanted islets. First results of PET imaging of an islet graft were published in 2006, using islets which were transfected with an insulin promoter-dependent reporter gene that leads to trapping of the PET probe $^{18}$F-FHBG in islet grafts (15). In another experiment, islets were pre-labeled with $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) and post-transplantation events could be monitored for up to 6 hours after transplantation (16).
More recently, specific targeting of beta cells after \textit{in vivo} injection of radiolabeled exendin followed by SPECT imaging was reported as a promising strategy to non-invasively visualize and quantify BCM in the pancreas of rodents, as well as in healthy and diabetic individuals [17, 18]. Similar exendin-based radiotracers were applied for non-invasive imaging of islet grafts in rodent transplantation models as well as in human skeletal muscle [19-21]. Although the use of such tracers in a clinical setting of islet transplantation is highly warranted, establishing the correlation between true beta cell mass and the uptake of the radiotracer \textit{in vivo} is the essential validation step before such studies should be conducted in humans. Such decisive studies have not been performed for GLP-1R imaging of islet transplants.

In the present study, we measured the uptake of $^{111}$In-exendin-3 in transplants consisting of different amounts of islets in the calf muscles of C3H mice by non-invasive SPECT imaging \textit{in vivo} and validated $^{111}$In-exendin as a quantitative biomarker for assessment of transplanted beta cell volume.

\textbf{RESEARCH DESIGN AND METHODS}

\textbf{Animals}

Female C3H/HeNCrl mice (22–30 g) were purchased from Charles River (Calco, Italy). Experiments were approved by the Animal Ethical Committee of the Radboud University, Nijmegen, The Netherlands.

\textbf{Pancreatic islet isolation and transplantation}

Pancreatic islets were isolated from 6-8 weeks old mice by a collagenase digestion method. Briefly, mice were euthanized and 2 mL of cold RPMI 1640 (Invitrogen, Carmarillo, CA, USA) containing collagenase type V (1 mg/mL; Sigma Aldrich, St Louis, MO, USA) were infused into the pancreatic duct \textit{in situ}. Perfused pancreata were collected in serum-free RPMI medium and kept on ice until enzymatic digestion at 37°C for 12 min. Islets were purified on a discontinuous Ficoll gradient of following densities: 1.118, 1.096 and 1.037 g/mL (Cellgro by Mediatech Inc., Manassas, VA, USA) and islets were collected between the second and the third fraction. Islets were cultured overnight in a humidified 5% CO$_2$ atmosphere at 37°C in RPMI 1640 medium supplemented with L-glutamine (Sigma Aldrich, St. Louis, MO, USA), penicillin-streptomycin (10 mg/mL; Sigma Aldrich) and 10% (v/v) fetal calf serum (HyClone, Cellbio, Logan, UT, USA). Islets were counted and hand-picked under bright field microscope and 50, 100, 200, 400 or 800 islets were transplanted in the calf muscle, parallel to the fibula, using needles with a 0.8 mm diameter. The exact number of
transplanted islets was determined by subtracting the remaining islets in the tube from the initially counted number.

**Radiolabeling of exendin-3**

[Lys\(^{40}\) (DTPA)]-exendin-3 was purchased from Peptide Specialty Laboratories (Heidelberg, Germany). Tracer labeling with In-111 was performed as previously described (17). Radiochemical purity was determined by ITLC and radiolabeled exendin-3 was purified by solid-phase extraction using a HLB-column as previously reported (17).

**SPECT acquisition**

Mice with 50, 100, 200, 400 and 800 islets were scanned after 4 weeks. All mice (n=4-5) were injected with approximately 15 MBq of \(^{111}\)In-exendin-3 (peptide dose 0.1 µg in 200 µL PBS, 0.5% BSA) in the tail vein. SPECT scans were acquired 1 h post-injection on a U-SPECT-II/CT dedicated small-animal scanner (MILabs, Utrecht, Netherlands) for 50 min with a high sensitivity mouse collimator (1.0 mm pinholes). Computed tomography (CT) was performed subsequently for anatomical reference. Standards of 74 kBq, 55 kBq, 37 kBq and 18 kBq in 50 µL volume each, were scanned under the same parameters as reference for quantification. Images were reconstructed with voxel size of 0.4 mm, 3 iterations and 16 subsets, using U-SPECT-II reconstruction software (MILabs, Utrecht, The Netherlands). The VOI was drawn over the islet transplant region, total voxel intensity registered in the islet graft was corrected by the mean of 3 measurements of contra-lateral control muscle, to subtract the background signal originating from the muscle tissue. The absolute activity (in kBq) was calculated by multiplying the corrected voxel intensity value with the calibration factor determined by quantitative analysis of standards with known radioactivity and data were normalized by the injected dose.

**Morphometric analysis of the transplant**

Immediately after SPECT acquisitions, mice were euthanized, muscles were fixed in 4% paraformaldehyde and embedded in paraffin, then sectioned into 4 µm slices for autoradiography analysis or for determination of insulin volume by immunohistochemistry. For autoradiography analysis, muscle sections were exposed to an imaging plate (Fuji Film BAS-SE 2025, Raytest, Straubenhardt, Germany) for 7 days and images were visualized with a radioluminography laser imager (Fuji Film BAS 1800 II system, Raytest, Straubenhardt, Germany) and were finally stained with hematoxylin-eosin (HE) to confirm the presence of pancreatic islets. For determination of insulin volume, insulin staining was performed in muscle sections. Antigen retrieval was done using 10 mM sodium citrate buffer, pH 6.0, for 10 min
Blocking of endogenous peroxidase activity was performed by incubation with 0.6% H$_2$O$_2$ in 40% methanol/60% PBS for 30 min at RT in the dark. An additional blocking step was done with 5% swine serum in PBS for 30 min at RT. Primary anti-insulin antibody (cat. sc 9168, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was applied at a dilution of 1:50 (in PBS containing 1% BSA w/v). Subsequently, sections were washed with PBS and incubated with secondary horseradish peroxidase-conjugated swine-anti-rabbit IgG (1:50) (cat. P0217, Dakopatts, Copenhagen, Denmark) in PBS containing 1% BSA w/v for 30 min at RT. The staining was visualized with diaminobenzidine (PowerVisionTM DAB substrate system, Immunologic, Duiven, The Netherlands) and nuclei were counterstained with haematoxylin. To determine the volume of the transplant, sections were scanned with Pannoramic250 Flash II scanner (3D Histech, Budapest, Hungary), beta cell surface was manually drawn around insulin positive region using Photoshop CS6, and the surface was multiplied by the distance separating each analyzed section, which is 40 µm.

**Statistical analysis**

Correlation studies were expressed as Pearson r. Data processing was performed with Graphpad Prism 5 (San Diego CA, USA).
RESULTS

\(^{111}\text{In}\)-exendin accumulation in the muscle co-localizes with islet transplants. To check whether \(^{111}\text{In}\)-exendin-3 signal originates from transplanted islets, C3H mice were transplanted with 800 islets in the calf muscle and were injected with \(^{111}\text{In}\)-exendin-3 four weeks after transplantation, where accumulation of the radiotracer in the islets becomes reproducible (22). Autoradiographical analysis of muscle sections showed tracer accumulation in well localized regions of the tissue (Figure 1A) and immunostaining for insulin confirmed that the radioactive signal originated from the islets (Figure 1B).

\(^{111}\text{In}\)-exendin uptake by transplanted islets correlates linearly with BCM. \(^{111}\text{In}\)-exendin-3 uptake in grafts containing various amounts of islets was detected and clearly delineated by SPECT signal (Figure 2A). Quantitative analysis of SPECT signal originating from the transplant revealed differences in \(^{111}\text{In}\)-exendin-3 accumulation depending on the number of initially transplanted islets, where the uptake was 5.9 kBq ± 2.4, 22.9 kBq ± 4.8, 30.1 kBq ± 10.1, 60.9 kBq ± 9.8 and 88.7 kBq ± 11.5, in muscles transplanted with 50, 100, 200, 400 and 800 islets, respectively (Figure 2B). Immunohistochemical determination of graft volume was performed in all groups of mice (Figure 2C). Plotting of SPECT data against the insulin staining volume revealed an excellent linear correlation between \(^{111}\text{In}\)-exendin uptake and transplant size (pearson r=0.89).

DISCUSSION

The aim of this study was to evaluate the relation between \(^{111}\text{In}\)-exendin uptake in islet transplants compared to actual BCM in a muscle model of islet transplantation. Tracer uptake and beta cell volume showed an excellent linear correlation. In addition, reliable visualization of transplants consisting of low numbers of islets indicates towards high sensitivity of this method. This high sensitivity in combination with the excellent correlation of beta cell mass and tracer uptake demonstrate the potential of this method to quantify small differences in viable beta cell volume.

Previously, imaging of liver islet transplants using \(^{64}\text{Cu}\)-labelled exendin has been demonstrated in NOD/SCID mice (19). Twelve days follow-up of the grafts revealed significantly higher uptake of \(^{64}\text{Cu}\)-labeled exendin-4 in the liver of transplanted animals when compared to the control group. Here, we used the skeletal muscle transplantation as a highly controlled model for histological verification of the insulin positive area (which is challenging in the liver transplantation model given that islets are distributed throughout a large part of the organ), a prerequisite enabling us to, for
the first time, reveal an excellent linear correlation between the actual numbers of beta cells that were successfully engrafted, and the uptake of the tracer in the islet grafts.

Recently, it was reported that injection of $^{123}$I-IBZM enables the quantification of BCM in islet grafts, where tracer uptake could be linearly correlated with graft volume (23,

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**Figure 2. Uptake of $^{111}$In-exendin by islet transplants is in linear correlation with transplant size.** A) SPECT imaging detected the grafts initially transplanted with 50, 100, 200, 400 or 800 islets with high sensitivity, 4 weeks after transplantation (white arrows). B) $^{111}$In-exendin uptake (expressed in kilobecquerels, kBq) was dependent on transplant size (n=4-5). C) SPECT signal (expressed in kBq) correlated linearly with graft insulin-positive volume ($\mu m^3$) as determined by morphometric analysis of transplant sections (Pearson r=0.89).
24). However, a far lower correlation was observed between $^{123}$I-IBZM uptake and insulin positive graft volumes when compared to $^{111}$In-exendin, indicating that GLP-1R could allow more accurate assessments of islet graft survival. Moreover, our data indicate that $^{111}$In-exendin has a much higher sensitivity for detection of islet grafts when compared to $^{123}$I-IBZM as we could easily visualize islet grafts after transplantation of 50 islets while 1000 islets were needed for in vivo visualization by IBZM. In fact, more than 50% of the islets could be lost in the first days after transplantation (25), indicating that $^{111}$In-exendin-SPECT was able to detect grafts containing far less than the 50 islets being initially transplanted. The superior detection sensitivity of $^{111}$In-exendin-SPECT could be explained by the higher abundance of the GLP-1R on the surface of the beta cells when compared to the dopamine 2 receptor (21, 23). Hence, $^{111}$In-exendin-SPECT has the potential to detect small grafts even after post transplantation beta cell loss. This enables the possibility to evaluate and optimize treatment to preserve the remaining islets in patients that became insulin dependent after islet transplantation, helping to preserve their positive effect on glucose homeostasis.

In summary, we were able to, for the first time demonstrate that $^{111}$In-exendin-3 accumulation in islet grafts shows an excellent linear correlation with the amount of living beta cells, and allows the detection of islet grafts consisting of less than 50 islets by SPECT imaging. Our data clearly indicate that this approach holds great potential for accurate and sensitive quantification of viable beta cells. Clinical studies evaluating the potential of this promising radiotracer for imaging of islets grafts in humans are under preparation.

**AUTHOR CONTRIBUTIONS**

W.A.E. contributed to the design of the study, conducted experiments, researched data and wrote the manuscript. I.v.d.K., S.W., D.B., L.J and C.F. conducted experiments and K.A. contributed in the design of the study and the writing of the manuscript. O.B contributed to the design of the study and the writing of the manuscript. M.B. and M.G. contributed to the discussion and the design of the study. All authors reviewed/edited the manuscript. M.G. is responsible for the overall integrity of the study. All authors agreed upon the submission version of the manuscript.
ACKNOWLEDGEMENTS
We kindly thank Bianca Lemmers, Kitty Lemmens, Iris Lamers-Elemans (Central Animal Facility, Radboud University Medical Center) for their excellent technical assistance in the animal experiments.

The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme (FP7/2007-2013), project BetaTrain, under REA grant agreement n° 289932, and project Betalimage, under grant agreement n° 222980.

DUALITY OF INTEREST
The authors declare no competing financial interests

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SPECT of transplanted islets of Langerhans by dopamine 2 receptor targeting in a rat model

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Adapted from: Molecular Pharmaceutics 2016;13:85-91
ABSTRACT

Objective: Pancreatic islet transplantation can be a more permanent treatment for type 1 diabetes compared to daily insulin administration. Quantitative and longitudinal non-invasive imaging of viable transplanted islets might help to further improve this novel therapy. Since islets express dopamine 2 (D2) receptors, they could be visualized by targeting this receptor. Therefore, the D2 receptor antagonist based tracer $[^{125/123}]$IBZM was selected to visualize transplanted islets in a rat model.

Methods: BZM was radioiodinated and the labeling was optimized for position 3 of the aromatic ring. $[^{125}]$-3-IBZM was characterized in vitro using INS-1 cells and isolated islets. Subsequently, 1,000 islets were transplanted in the calf muscle of WAG/Rij rats and SPECT/CT images were acquired six weeks after transplantation. Finally, the graft containing muscle was dissected and analyzed immunohistochemically.

Results: Oxidative radioiodination resulted in three IBZM isomers with different receptor affinities. The use of 0.6 mg/mL chloramine-T hydrate resulted in high yield formation of predominantly $[^{125}]$-3-IBZM, the isomer harboring the highest receptor affinity. The tracer showed D2 receptor mediated binding to isolated islets in vitro. The transplant could be visualized by SPECT six weeks after transplantation. The transplants could be localized in the calf muscle and showed insulin and glucagon expression, indicating targeting of viable and functional islets in the transplant.

Conclusion: Radioiodination was optimized to produce high yields of $[^{125}]$-3-IBZM, the isomer showing optimal D2R binding. Furthermore, $[^{123}]$IBZM specifically targets the D2 receptors on transplanted islets. In conclusion, this tracer shows potential for non-invasive in vivo detection of islets grafted in the muscle by D2 receptor targeting.

Key Words: iodination, IBZM, SPECT, islets of Langerhans, transplantation
INTRODUCTION
For patients suffering from type 1 diabetes, insulin is never a cure, only a treatment. Pancreatic islet transplantation could be a permanent solution for these patients. Unfortunately, insulin independency drops to less than 15% five years after transplantation (1). Currently, graft function is monitored by measuring functional biomarkers, such as C-peptide and HbA1c levels, which show large inter-individual differences and are delayed markers for islet loss (2). Furthermore, it has been shown that beta cell function is not always a representative measure for the remaining beta cell mass (3, 4). Therefore, visualization of the remaining viable graft volume might provide important information on the metabolic state of the islet graft (5). A possible method to accurately monitor islet loss is longitudinal quantitative imaging. Non-invasive imaging modalities like Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET) could provide important insights into the fate of transplanted islets (6) and could help to improve the outcome of this promising therapeutic approach.

Several attempts have been made to visualize transplanted islets, using different imaging modalities. Transplanted islets, pre-labeled with a paramagnetic contrast agent, could be visualized using magnetic resonance imaging (MRI) (7, 8). Besides MRI, transplanted islets can also be imaged using PET or SPECT. The first results of imaging the graft by PET date from 2006, where islets, transfected with a reporter gene or an adenoviral vector, could be visualized using $^{18}$F-Fluorohydroxybutylguanine ($^{18}$F-FHBG) as a tracer (9, 10). Furthermore, $^{18}$F-Fluorodeoxyglucose ($^{18}$F-FDG) showed potential to image immediate events following transplantation (11, 12). Transplanted islets were also imaged by SPECT and PET using $^{111}$In-labeled exendin-4 (13). In fact exendin appears to be useful for longitudinal graft imaging (14). Nevertheless, the use of alternative tracers, in addition to exendin, might help to gain important complementary information about the islet graft, such as metabolic stress, inflammatory processes, dedifferentiation and rejection. In other words, alternative tracers might allow more complete characterization of islet grafts.

One of these potential alternative tracers is iodobenzamide (IBZM, Figure 1), a molecule with strong anti-dopaminergic activity and very high specificity for the dopamine 2 (D2) receptor. Labeled with Iodine-123, this molecule is clinically used for D2 receptor imaging in neurodegenerative diseases (15, 16). Furthermore, D2 receptors are expressed in pancreatic islets, where they mediate insulin secretion upon dopamine binding (17, 18). Consequently, $[^{123}]$IBZM may be a promising candidate to target transplanted islets via the D2 receptor.
In this study, we determined the potential of iodinated benzamide (BZM) for imaging of transplanted islets of Langerhans. The radioiodination of BZM was optimized for iodination of the 3 position of the aromatic ring, since different substitutions on this ring resulted in molecules with different affinity for the receptor. The \textit{in vitro} binding properties of the tracer were characterized and finally, islets engrafted in the calf muscle of WAG/Rij rats were visualized by SPECT after injection of $^{123}$IIBZM.

**MATERIALS AND METHODS**

**Compounds and radionuclides**

BZM and IBZM were purchased from ABX (advanced biochemical compounds, Radeberg, Germany). $^{123}$IIBZM and sodium$^{125}$Iodide were obtained from GE healthcare (Eindhoven, Netherlands) and Perkin Elmer (Boston, MA, USA), respectively.

**Radiolabeling**

BZM was iodinated using oxidative iodination, as described previously (15). The effect of the chloramine-T concentration on the reaction product was determined. Briefly, chloramine-T hydrate (15 µL; 0.6 mg/mL; 0.9 mg/mL or 1.2 mg/mL) was added to a mixture of BZM (25 µL, 10 mg/mL), 10 µL 0.5 M phosphate buffer and 5 MBq Na$^{125}$I. After incubation for 2 min at RT, the reaction was quenched using 200 µL metabisulphite (1 mg/mL). Reaction products were purified by reversed-phase high performance liquid chromatography (RP-HPLC) using an Eclips XDB C18 column (Agilent Technologies, Santa Clara, CA, USA). The column was eluted with 0.1% trifluoroacetic acid (TFA) in H$_2$O (0-5 min) followed by a linear gradient from 3% to 100% acetonitrile with 0.1% TFA over 30 min (flow rate: 1 mL/min). The fractions containing $^{125}$IBZM were collected and diluted with RPMI 1640 medium (Invitrogen, Camarillo, CA, USA) containing 0.5% (w/v) bovine serum albumin (BSA).
Cell culture
The rat insulinoma cell line INS-1 was cultured as described previously (19). In short, cells were maintained in RPMI supplemented with 10% fetal bovine serum (FCS) (HyClone, Cembio, Logan, UT, USA), 2 mM L-glutamine (Sigma Aldrich, St Louis, MO, USA), 10 mM HEPES (Invitrogen), 50 μM β-mercaptoethanol (ICN Biomedicals Inc, Aurora, OH, USA), 1 mM sodium pyruvate (Invitrogen), 100 U/mL penicillin (Sigma Aldrich) and 100 μg/mL streptomycin (Sigma Aldrich), in a humidified atmosphere containing 5% CO₂ at 37°C.

In vitro binding to INS-1 cells
INS-1 cells were harvested by trypsinization, transferred to Eppendorf tubes (3 x 10⁶ cells per tube) and washed twice with binding buffer (RPMI containing 0.5% BSA (w/v)). Approximately 1,600 Bq [¹²⁵I]IBZM (20.5 fmol) was added followed by incubation for 4 h at 37°C. After incubation, cells were washed twice with binding buffer and cell-associated radioactivity was measured in a well type gamma counter (Wallac 1480 wizard, Perkin Elmer).

In vitro internalization
The internalization kinetics of [¹²⁵I]IBZM were determined as described previously (20) using suspensions of INS-1 cells (3 x 10⁶ cells per tube). The cells were resuspended in binding buffer along with approximately 1,600 cpm [¹²⁵I]IBZM (20.5 fmol) and incubated for 1, 2 or 4 h at 37°C. After incubation, the cells were washed twice with binding buffer. To determine the surface-bound radiolabeled fraction, ice-cold acid buffer (0.1 M acetic acid, 154 mM NaCl, pH 2.5) was added and cells were incubated for 10 min at 4°C. After washing the cells twice with binding buffer, the cell-associated activity was measured in a gamma-counter. The internalized fraction (cell-associated activity after acid wash) and the receptor-bound fraction (activity removed by acid wash) were determined.

Animals
Female Wistar Albino Glaxo (WAG)/Rij rats, 6-8 weeks old (Charles River Laboratories, Erkrath, Sulzfield, Germany) were used as islet donors and recipients. Animals were provided with food and water ad libitum. Animal experiments were approved by the animal welfare committee of the Radboud University Nijmegen.

Islet isolation
In order to validate the results obtained using INS-1 cells, D2 receptor binding of the tracer was further analyzed using isolated islets. Pancreatic islets were isolated from WAG/Rij rats by collagenase digestion. Rats were euthanized by CO₂/O₂ suffocation.
and 8 mL of ice cold RPMI 1640 containing 1 mg/mL collagenase type V (Sigma Aldrich) were infused via the pancreatic duct in situ. After dissection, the perfused pancreata were collected in serum free medium containing collagenase and kept on ice until digestion. Pancreata were digested for 11 min and 30 sec at 37°C. Digestion was stopped by adding RPMI medium containing 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (complete RPMI). After washing, digested pancreata were passed through a mesh. Afterwards, islets were purified on a discontinuous Ficoll gradient (densities: 1.108; 1.096; 1.037; Cellgro by Mediatech Inc, Manassas, VA, USA) by centrifugation at 625 x g for 16 min without brake. Islets were collected from the intersection of the second and third layer and remaining Ficoll was removed by washing with complete RPMI. Isolated islets were cultured overnight in complete RPMI medium in a humidified atmosphere containing 5% CO₂ at 37°C.

**In vitro binding to isolated islets**

After overnight recovery, islets were collected, counted and resuspended in complete RPMI. Islets were divided in 24 well trans-well plates (600 islets per trans-well) (Corning Inc, Tewksbury, USA) and washed with binding buffer. Subsequently, approximately 1,600 cpm of [¹²₅I]-3-IBZM was added followed by incubation for 4 h at 37°C. To examine D₂ receptor mediated uptake, 5 µg of unlabeled IBZM was added together with [¹²₅I]-3-IBZM to separate wells. After incubation, islets were washed with binding buffer and islet associated radioactivity was determined in a well type gamma counter.

**Islet transplantation**

Isolated islets were collected, resuspended and counted in RPMI medium without supplements. Healthy recipient rats were anesthetized using isoflurane (induction 4-5%, maintenance 2% in 80% O₂ and 20% N₂O) (Abbott Laboratories, Toronto, Canada) and the right hind leg was shaved and disinfected with Betadine® (Meda Pharma B.V., Amstelveen, The Netherlands) prior to islet injection. Thousand islets were aspirated into a catheter line and infused manually at a constant, low speed into the calf muscle using a Hamilton syringe (100 ± 17.3 µL) (Hamilton Company, Reno, NV, USA).

**SPECT acquisition, reconstruction and biodistribution**

Six weeks after transplantation, the rats (n=3), transplanted with 1,000 islets, were injected with 53.7 ± 6.1 MBq of [¹²³I]IBZM (IBZM dose 1.7 ng in 500 µL injection fluid) in the tail vein. One hour after injection, SPECT images were acquired under inhalation (isoflurane) anesthesia on a USPECTII/CT small animal scanner (MiLabs, Utrecht, Netherlands) for 50 minutes using a 1 mm pinhole general purpose rat and mouse collimator. CT was performed as an anatomical reference with the following
settings: spatial resolution 160 µm; 65 kV and 615 µA. SPECT scans were reconstructed using the USPECT reconstruction software (MILabs, Utrecht, Netherlands) with following settings: selection of the $^{123}$I photopeak (143-175 keV) corrected for background (175–200 keV), pixel based OSEM, voxel size 0.75 mm, 2 iterations and 16 subsets. After SPECT/CT acquisition, rats were euthanized by CO$_2$/O$_2$ suffocation and the muscle containing the transplant and other relevant tissues (blood, muscle, heart, lung, spleen, pancreas, kidney, liver, stomach and duodenum) were dissected, weighed and measured in a well type gamma counter to determine the distribution of the tracer over the different organs. Unfortunately, in vivo competition studies could not be performed due to the lethal effects of such high IBZM dose.

**Immunohistochemistry**

Graft containing muscles were fixed in 4% formalin, dehydrated and embedded in paraffin. Sections (4 µm) of the calf muscle were prepared at levels 50 µm apart from each other and used for Haematoxin and Eosin (HE) staining to localize the transplant. Levels containing the transplant were stained immunohistochemically for the presence of insulin, as described previously, (21) and glucagon. The primary antibody against glucagon (#2760, Cell Signaling, Danvers, MA, USA) was diluted in PBS containing 1% BSA (1:500). Antigen retrieval was performed in 10 mM sodiumcitrate buffer, pH 6.0 for 10 minutes at 99°C. Subsequently, sections were incubated for 10 minutes with 3% H$_2$O$_2$ in PBS at RT in the dark, to block endogenous peroxidase activity. Non-specific binding was blocked by incubation for 30 minutes with 5% goat serum. Primary antibody incubation for 60 minutes was followed by incubation with the secondary goat-anti-rabbit biotin (1:200)(BA-1000, Vector Laboratories, Burlingame, CA, USA) and subsequently with the ABC complex (Vectastain, ABC kit, Elite). Finally, Bright DAB (BS04500, Immunologic BV, Duiven, Netherlands) was used for visualization.

**RESULTS**

**Radiolabeling**

After purification, $[^{125}]$IBZM with a specific activity of 81.4 GBq/µmol was obtained with a radiochemical purity exceeding 99% as determined by RP-HPLC. Iodination using 1.2 mg/mL chloramine-T hydrate resulted in moderate regioselectivity: 1) 7.8% $[^{125}]$-5-IBZM, 2) 59.7% $[^{125}]$-3-IBZM and 3) 32.5% $[^{125}]$-3,5-di-IBZM, respectively. The second peek of the elution profile was identified as $[^{125}]$-3-IBZM because it showed equal HPLC retention time as $[^{123}]$-3-IBZM. Increasing amounts of the oxidant resulted in higher yields of the di-iodo analogue, $[^{125}]$-3,5-di-IBZM. The binding properties of
the different fractions to INS-1 cells are shown in Figure 2. After four hours, 9.51 ± 1.13 fmol of $^{125}\text{I}$-3-IBZM was bound to the INS-1 cells, whereas only 0.69 ± 0.07 fmol $^{125}\text{I}$-5-IBZM and 1.20 ± 0.14 fmol of the di-iodo analogue $^{125}\text{I}$-3,5-di-IBZM was bound to the INS-1 cells. These results show that $^{125}\text{I}$-3-IBZM had optimal D2 receptor binding properties. To increase regioselectivity for $^{125}\text{I}$-3-IBZM, the concentration chloramine-T hydrate was lowered to 0.9 mg/mL and 0.6 mg/mL. The iodination using 0.6 mg/mL chloramine-T hydrate, shown in Figure 2, resulted in 86.4% $^{125}\text{I}$-3-IBZM, 9.9% $^{125}\text{I}$-5-IBZM and 3.7% $^{125}\text{I}$-3,5-di-IBZM. Iodination using 0.9 mg/mL chloramine-T resulted in only 77% $^{125}\text{I}$-3-IBZM.

![Figure 2: A) Cell binding of the different IBZM analogues to INS-1 cells B) Elution profile of the reaction products at various concentrations of chloramine-T hydrate.](image)

**Internalization kinetics**

Figure 3 summarizes the internalization kinetics of $^{125}\text{I}$-3-IBZM by INS-1 cells as determined *in vitro*. As expected for an antagonist, there was no or very little internalization (0.65 ± 0.03 fmol, 3.2%) observed after 1 h. At this time point, 13.0 ± 0.7 fmol (63.4%) was bound to the D2 receptor. After 2 and 4 h respectively, no significant differences in binding or internalization were observed.

**Pancreatic islet isolation and transplantation**

After the islet isolation procedure, islet viability and islet purity exceeded 90%. During overnight recovery in complete RPMI medium, islets regained their original morphology, as determined microscopically. Transplantation did not cause unexpected events in the recipient rats.
Binding capacity of $[^{125}\text{I}]$IBZM to isolated islets

Figure 4 shows the *in vitro* binding characteristics of $[^{125}\text{I}]$-3-IBZM to 600 isolated islets of WAG/Rij rats. After 4 h, $5.19 \pm 0.51$ fmol $[^{125}\text{I}]$-3-IBZM was bound to the islets. Addition of 10 µg unlabeled IBZM reduced the binding to $0.95 \pm 0.12$ fmol, indicating specific binding of the tracer to the D2 receptor on the islets of Langerhans.

![Figure 3: In vitro internalization kinetics of $[^{125}\text{I}]$-3-IBZM. The black line represents the surface bound fraction of $[^{125}\text{I}]$-3-IBZM. The grey line represents the internalized fraction of $[^{125}\text{I}]$-3-IBZM.]

![Figure 4: Binding capacity of $[^{125}\text{I}]$-3-IBZM to isolated islets. The solid bar represents the binding of $[^{125}\text{I}]$-3-IBZM. The white bar represents the binding of $[^{125}\text{I}]$-3-IBZM in the presence of an excess $[^{\text{Nat}}\text{I}]$IBZM.]

SPECT/CT imaging
The transplanted islets of Langerhans in the hind leg were visualized by SPECT, 1 hour after injection of $^{[123]}$IIBZM. Six weeks after transplantation, a clear SPECT signal of $^{[123]}$IIBZM was observed in the transplanted islets in the calf muscle (Figure 5).

Biodistribution study
Figure 6 summarizes the results of the biodistribution study. Biodistribution in WAG/Rij rats two hours post injection showed tracer uptake in various organs such as the lung, spleen, liver, kidney and duodenum. The relatively high uptake in the stomach ($4.43 \pm 0.57\% \text{ ID/g}$) was caused by the presence of free iodine after hepatic breakdown of the tracer. Due to the small volume of the transplant, as compared to the volume of the calf muscle, no significant difference in tracer uptake was observed between the muscle containing the transplant and the control muscle.
Immunohistochemistry
All transplants could be localized within the muscle using HE staining. The transplants location determined by HE staining co-localized with insulin staining and glucagon staining of consecutive sections, as shown in Figure 7. Furthermore, all transplants (n=3) showed clear insulin and glucagon staining, indicating viable, insulin and glucagon producing beta and alpha cells in the transplant.

**DISCUSSION**
Ever since the presence of D2 receptors on rodent and human islets was revealed, it was pointed out as a possible target for beta cell imaging. (22) Accordingly, IBZM imaging of transplanted islets has been proposed but the true potential of this tracer for beta cell imaging remained to be elucidated. Here, we have clearly demonstrated
the possibility to visualize transplanted islets \textit{in vivo} by specific targeting of the D2 receptor with $[^{123}\text{I}]$IBZM in a rat model.

In the 1980s, a variety of benzamide derivatives, all harboring strong anti-dopaminergic effects and high specificity for the D2 receptor, have been discovered. (23) In 1988 Kung \textit{et al.} described the synthesis of $[^{125}\text{I}]$IBZM and its application for D2 receptor mapping in the CNS (15). In the labeling procedure, some “minor impurities” were mentioned. Here, for the first time, we identified these “impurities” as the para-isomer and the di-iodo analogue. Furthermore, we determined the D2 receptor binding capacity of each labeled analogue \textit{in vitro} and optimized the labeling to produce predominantly the ortho-isomer, $[^{125}\text{I}]-3$-IBZM. At optimized conditions, carrier free $[^{125}\text{I}]-3$-IBZM was synthesized at a specific activity of 81.4 GBq/µmol with a radiochemical purity exceeding 99% after HPLC purification. The high specificity for the D2 receptor and the straightforward labeling procedure with high yields, make IBZM an excellent tracer for islet graft imaging.

After the discovery of D2 receptors on beta cells of pancreatic islets in 2005, (17) D2 receptor ligands were being applied to image pancreatic islets. $^{18}$F-L-3,4-dihydroxyphenylalanine positron emission tomography ($^{18}$F-DOPA PET) scans can be used to image congenital hyperinsulinism of infancy (CHI) (24, 25) and neuroendocrine tumors (NETs) such as insulinomas. (26) Due to their neuroendocrine nature, islets hold the ability to decarboxylate L-DOPA and accumulate the tracer according to the amine precursor uptake and decarboxylation (APUD) concept of Pearse (27). However, although the total pancreatic uptake of $[^{123}\text{I}]$IBZM is similar to the uptake of exendin, the significant background signal from the exocrine tissue precludes quantitative imaging of native islets in the pancreas by D2 receptor targeting. Another potential issue might be the expression of D2 receptors on delta cells (28). However, since delta cells only represent five percent of islet cells, it is very unlikely that tracer binding to the delta cells would significantly influence viable graft volume measurements by D2 receptor targeting.

Although islet imaging in the native pancreas is not feasible by targeting the D2 receptor due to the diffuse D2 receptor expression in this organ, it might be valuable in the transplantation setting. In 2011, Garcia \textit{et al.} showed that islets, transplanted in the spleen, could be visualized using $^{18}$F-fallypride PET (29). Furthermore, transplanted islets could be visualized using $^{18}$F-DOPA PET (30). In this study however, we preferred $[^{123}\text{I}]$IBZM in view of the favorable spatial resolution of preclinical SPECT systems as compared to PET (31). Furthermore, the spleen, kidney and the liver, wherein islets are most often transplanted both preclinically and clinically, show considerable $[^{123}\text{I}]$IBZM uptake (Figure 6), precluding islet graft detection using IBZM.
in these organs. Therefore, striated muscle appeared to be an excellent transplantation site to determine the value of D2 receptor targeting for islet imaging. In this model, islet grafts could clearly be delineated as areas of focal uptake, when compared to PET imaging of islets engrafted in the spleen or subcutaneously (29, 30), representing a substantial progress in molecular imaging of islet grafts.

Both for D2 receptor targeting and graft survival, striated muscle harbors advantages as a transplantation site when compared to liver and the kidney capsule. A common problem in both liver and kidney capsule transplantation is insufficient revascularization resulting in impaired islet oxygenation (32, 33). However, it has been shown that islets transplanted in the muscle show high vascular density and oxygenation (34). It was even stated that islets engrafted in the muscle create a vascular system which resembles the native pancreas (35) and patients were transplanted successfully in the muscle (36). Since the muscle is considered a promising novel site for islet transplantation, islet graft imaging via D2 receptor targeting might contribute to the further improvement of this promising therapy, despite its limited imaging capacities for liver grafts.

In view of the D2 receptor mediated binding to isolated islets in vitro and the distinct tracer uptake in the SPECT images, the present study clearly demonstrates that \[^{123}\text{I}]IBZM can be used to monitor the viability of islet grafts following transplantation in future studies. Nevertheless, in combination with other beta cell specific radiotracers such as \[^{111}\text{In}-\text{exendin}\], it might provide complementary information on the metabolic state of the islet graft. To summarize, IBZM imaging could provide vital complementary information on the viability and functionality of the islet graft. This may lead to early detection of aberrant processes in the transplant and eventually might improve the success of islet transplantation.

CONCLUSION

The labeling procedure was optimized to produce predominantly \[^{125}\text{I}]\text{-3-IBZM}\) at a high specific activity and radiochemical purity. The tracer showed D2 receptor-mediated binding to isolated islets and uptake within the transplant. Engrafted islets were clearly visualized on SPECT/CT images after injection of \[^{123}\text{I}]BZM\). In conclusion, \[^{123/125}\text{I}]\text{BZM}\) is a suitable tracer for islet graft imaging in vivo and its imaging capacities for transplanted islets will be further elucidated.
ACKNOWLEDGEMENTS
We thank Bianca Lemmers, Henk Arnts, Iris Lamers and Kitty Lemmens for their technical support with the animal experiments. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union’s Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n° 289932.

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Quantitative and longitudinal imaging of intramuscular transplanted islets of Langerhans with SPECT using $^{[123]}$IIBZM

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Adapted from: Diabetes, Obesity and Metabolism. 2017;19:604-608
ABSTRACT

Objective: A non-invasive imaging method to quantitatively and longitudinally monitor viable islet grafts could provide insight in the fate of islets after transplantation. Furthermore, it would grant the possibility to monitor effects of therapeutic interventions on viable graft volume and eventually, could help improve transplantation outcome. Here, we investigated the use of $^{123}$I-labeled dopamine 2 receptor antagonist Iodobenzamide ($[^{123}\text{I}]$IBZM) for viable graft volume quantification and longitudinal monitoring of islets grafts.

Methods: Six weeks after rats were transplanted intramuscularly with 1,000, 2,000, or 3,000 islets, they received 50 MBq $[^{123}\text{I}]$IBZM intravenously and one hour post injection SPECT images were acquired. Subsequently, animals were euthanized and the graft containing muscles were dissected for ex vivo SPECT and histological analysis. Viable graft volume was determined histologically by multiplying insulin-positive area by inter-slice distance. For longitudinal graft analysis, three rats were transplanted with 1,500 islets and monitored for ten weeks.

Results: Six weeks after transplantation, a clear signal of all grafts was observed by SPECT imaging. Moreover, the intensity of the SPECT signal correlated linearly with viable graft volume, as determined histologically. Longitudinal graft follow-up showed a clear SPECT signal of the transplant from three weeks until ten weeks after transplantation.

Conclusion: Here, we demonstrate for the first time the successful application of a radiotracer, $[^{123}\text{I}]$IBZM, for non-invasive, longitudinal and quantitative islet graft monitoring in vivo. Therefore, $[^{123}\text{I}]$IBZM SPECT might be a valuable tool to monitor and eventually further improve islet transplantation.

Key words: $[^{123}\text{I}]$IBZM, SPECT, islets of Langerhans, transplantation
INTRODUCTION

Besides insulin therapy, islet transplantation is considered one of the most promising therapies for type 1 diabetic patients with poor glycemic control. Despite improvements in the immunosuppressant regimen (1), islet transplantation still results in only short term insulin independence, while long term outcome remains poor (2). A reliable method to quantitatively and longitudinally monitor the islet graft would provide improved insight in islet loss after transplantation. Furthermore, it would grant the possibility to evaluate potential therapies and interventions that might improve long term transplantation outcome.

During the last decade, extensive efforts have been made to develop a non-invasive imaging method to monitor the islet graft, which resulted in a variety of methods for graft monitoring. Several groups used prelabeling methods to visualize the islet graft (3-8), while others targeted a receptor, specifically expressed on beta cells (9). One of these receptors is the glucagon-like peptide 1 (GLP-1) receptor, which can be successfully targeted using radiolabeled exendin to visualize the pancreatic islets (10, 11). Since a combination of different beta cell-specific radiotracers might reveal important complementary information on the state of the islet graft (for example in case of receptor down regulation under certain conditions), the search for alternative methods to monitor transplanted islets is ongoing.

Since the 1980’s, D2 receptors have been used as a target for brain imaging (12, 13). In view of the neuro-endocrine nature of pancreatic islets, the hypothesis arose that beta cells express D2 receptors. In 2005, this hypothesis was proven, when Rubi et al confirmed the expression of D2 receptors on both human and rodent islets (14). Furthermore, D2 receptors show excellent co-localization with insulin expression and mediate glucose-stimulated insulin section through a negative feedback mechanism (15-17). Ever since, the presence of these receptors has been exploited to image insulinomas (18), congenital hyperinsulism of infancy (CHI) (19, 20) and islets, both in the pancreas and after transplantation (21,23), using $^{18}$F-L-3,4-dihydroxyphenylalanine ($^{18}$F-DOPA) and $^{18}$F-fallypride Positron Emission Tomography (PET).

Another tracer displaying high affinity and specificity for the D2 receptor is iodobenzamide (IBZM), a strong receptor antagonist (24, 25). Labeled with I-123 or I-125, it is extensively used as a radiotracer for Single Photon Emission Computed Tomography (SPECT) in neurodegenerative diseases (26, 27). Recently, in a proof-of-concept study we showed specific binding of $^{125}$IIBZM to isolated islets in vitro and the feasibility to visualize islets, transplanted in the calf muscle of rats, using...
[\(^{123}\)I]IBZM SPECT/CT. In the present study, we aimed to characterize the potential of [\(^{123}\)I]IBZM for longitudinal and quantitative monitoring of the viable islet grafts \textit{in vivo} by determining the correlation between the SPECT signal of the graft and viable graft volume.

**MATERIALS AND METHODS**

**Animals**

Twelve weeks old female WAG/Rij rats (Charles River Laboratories, Erkrath, Sulzfield, Germany) were used both as islet donors as well as recipients. Animals had access to food and water ad libitum. The animal experiments were approved by the animal welfare committee of the Radboud University Nijmegen and carried out in accordance with their guidelines.

**Islet isolation**

Islets of Langerhans were isolated from WAG/Rij rats by collagenase digestion as described previously (28). Briefly, rats were suffocated using CO\(_2\)/O\(_2\), and 8 mL of ice cold RPMI 1640 (R0883, GIBCO, BRL Life Sciences Technologies, Bleiswijk, The Netherlands) containing 1 mg/mL collagenase type V (Sigma Aldrich, St Louis, MO, USA) was injected via the common bile duct. Pancreata were dissected and digested for 11 minutes and 30 seconds at 37 °C. Islets were purified on a discontinuous Ficoll gradient (densities 1,108; 1,096; 1,037 g/mL, Cellgro, Mediatech Inc., Manassas, VA, USA) by centrifugation at 625 × g for 16 minutes without brake. The islets were collected from the intersection of the second and third layer and cultured overnight at 37 °C with 5% CO\(_2\) in RPMI containing 10% fetal bovine serum (FCS) (HyClone, Celbio, Logan, UT, USA), 2 mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin (Sigma Aldrich) and 100 U/mL streptomycin (Sigma Aldrich). Islet purity and viability were assessed by microscopy.

**Islet transplantation**

Islets were transplanted intramuscularly as described previously (28). After overnight recovery, islets were collected, spun down, resuspended in serum free RPMI medium and counted. The recipient rats were anesthetized with isoflurane (induction 4-5%, maintenance 1.5-2% in air) (Abbott Laboratories, Toronto, ON, Canada). The right hind leg of the rats was shaved and disinfected with Betadine® (Meda Pharma B.V., Amstelveen, The Netherlands). Islets were aspirated into a catheter line and infused manually at low speed (injected volume 100 ± 10 µL) in the right calf muscle using a Hamilton syringe (Hamilton Company, Reno, NV, USA).
Experimental design for *in vivo* SPECT imaging of transplanted islets

To determine the correlation of SPECT signal with the viable graft volume, fifteen WAG/Rij rats (3 groups, n=5/group) were transplanted in the right calf muscle with 1,000, 2,000, or 3,000 islets as described above. SPECT/CT images were acquired six weeks after transplantation. For the longitudinal follow-up of the transplanted islets, three WAG/Rij rats were transplanted with 1,500 islets and SPECT/CT images were acquired seven times over a period of ten weeks (in week 2, 3, 4, 5, 6, 8 and 10). After the last SPECT/CT scan, rats were euthanized and muscles containing the transplant were collected for *ex vivo* SPECT and immunohistochemical analysis.

**SPECT acquisition**

One hour prior to SPECT acquisition, WAG/Rij rats (n=5 per group) were injected intravenously in the tail vein with 57.9 ± 4.8 MBq of $[^{123}]$IBZM (IBZM dose 1.7 ng in 500 µL injection fluid) (GE healthcare, Eindhoven, The Netherlands). SPECT/CT was acquired on a small animal SPECT/CT scanner (U-SPECT-II, MIlabs, Utrecht, Netherlands) with a 1.0 mm multipurpose rat and mouse collimator, using 24 bed positions and an acquisition time of 50 minutes. CT was acquired after SPECT acquisition for anatomical reference (65 kV, 615 µA, 1 bed position, spatial resolution 160 µm). Unfortunately, *in vivo* competition studies could not be performed due to the lethal effects of such high IBZM dose.

**Ex vivo** SPECT of muscles containing transplant

Immediately after dissection, the muscles containing the transplant were transferred to Eppendorf tubes with 4% formalin and SPECT/CT of the individual muscles were acquired using a 0.35 mm high-resolution mouse collimator (MILabs, Utrecht, The Netherlands), 42 bed positions and a SPECT acquisition time of 6 hours.

**SPECT reconstruction and quantitative SPECT analysis**

SPECT scans were reconstructed using U-SPECT-II reconstruction software (MILabs, Utrecht, The Netherlands) with the following settings: selection of the $^{123}$I photopeak (143-175 keV) corrected for two background regions (135-143 keV and 175-185.5 keV), pixel based OSEM, voxel size 0.75 mm$^3$, 1 iteration and 16 subsets. The uptake of $[^{123}]$IBZM was quantified using Inveon Reconstruction Workplace (IRW) (Siemens Healthcare, Den Haag, the Netherlands). A volume of interest was drawn around the graft. Total voxel intensity in the islet graft was corrected for background uptake in the contra-lateral control muscle. The activity calculated in the grafts was corrected for the injected activity of the animal.
Immunohistochemistry
The muscles containing the transplanted islets were fixed in 4% formalin, dehydrated and embedded in paraffin. Sections (4 µm) of the calf muscle were cut at levels 50 µm apart from each other and were stained with Haematoxylin and Eosin (HE) to localize the islet graft. Consecutive sections were stained for the presence of insulin and D2 receptor as follows. Antigen retrieval was performed using 10 mM sodium citrate buffer, pH 6.0 heated in a PT module (Thermo scientific, Waltham, MA, USA). For insulin staining, nonspecific binding was blocked by incubation for 30 minutes with PBS, 1% BSA (w/v) at RT while for D2 receptor staining blocking was performed using 5% normal donkey serum in PBS (w/v). Primary anti-insulin antibody (1:1,000) (A0564, DAKO, Copenhagen, Denmark) incubation for 60 minutes at RT in the dark was followed by incubation with Alexa fluor 488 goat-anti-guinea pig (1:500) (Life sciences technologies, Carlsbad, CA, USA) for 30 minutes at RT in the dark. For D2 receptor staining, primary anti-D2 receptor antibody (1:100) (Ab5084P, Millipore, Billerica, MA, USA) 16 h incubation at 4 °C in the dark was followed by incubation with Alexa fluor donkey-anti-rabbit (1:200) (Life sciences technologies) for 1 hour in the dark. Stained sections were mounted using fluoromount (DAKO).

Immunohistochemistry-based determination of viable graft volume
Sections stained for insulin, were analyzed using a DM5000 microscope (Leica, Solms, Germany) and images were obtained with a color camera (Evolution MP; Leica using Axio Vision 4.4 software). The insulin-positive area of the transplant was drawn on each section on all levels. Finally, the insulin-positive transplant area was multiplied with the 50 µm inter-slice distance to obtain the volume of the viable graft.

Statistical analysis
All values are expressed as mean values ± standard deviation (SD). All correlations stated in this paper were calculated using the Pearson correlation coefficient (Pearson r) in GraphPad Prism v. 5.03 (GraphPad Software, Inc., San Diego, CA, USA). The level of significance was set at p < 0.05.

RESULTS
Pancreatic islet isolation and transplantation
After overnight recovery in complete RPMI medium, the isolated islets showed normal islet morphology, as determined microscopically. The viability and purity of the isolated islets exceeded 90%. No unexpected or adverse effects were observed in the recipient rats after the transplantation procedure.
Immunohistochemical graft analysis and volume assessment
All grafts (n=15) could be detected in the calf muscle after HE staining. The location of the graft, determined by HE staining, co-localized with insulin staining and D2 receptor staining, as determined on consecutive sections (Figure 1). This indicates beta cell specific D2 receptor expression in the islet graft. The viable graft volume, calculated histologically from the insulin-positive graft area, increased with the number of transplanted islets. Viable graft volume ranged from $1.4 \times 10^6 \ \mu m^3$ to $1.2 \times 10^7 \ \mu m^3$. Two islet grafts were excluded from the volume assessment since no trustworthy viable graft volume could be calculated from the histological data due to technical errors during sectioning.

SPECT/CT imaging and correlation with graft volume
Six weeks after transplantation, all grafts (n=15) could be visualized by SPECT/CT, one hour after [$^{123}$I]IBZM injection. Figure 2 clearly shows differences in [$^{123}$I]IBZM uptake reflecting differences in viable graft volume. Furthermore, the measured SPECT signal in the islet grafts, with an average signal-to-noise ratio of $2.11 \pm 0.62$, showed a good
linear correlation (Pearson r = 0.73, p = 0.005; n=13) with the viable graft volume, as determined histologically as described above (Figure 3). For the image analysis, CT images were fused with the concomitant SPECT images for anatomical reference.

Figure 2: In vivo SPECT/CT images of a A) small, B) medium and C) large islet graft in the calf muscle, six weeks after transplantation, acquired 1 hour after injection of \[^{123}\text{I}]\text{IBZM}. The red arrows indicate the transplant.

Figure 3: Scatter plot of the correlation between the SPECT signal in the transplant expressed in arbitrary units (AU) (y-axis) and the viable graft volume (µm\(^3\)) as calculated from the histological analysis (x-axis) (Pearson r = 0.73, p = 0.005 ).
Ex vivo SPECT imaging
SPECT images of the dissected calf muscle samples showed a clear signal of the islet graft ex vivo. Furthermore, the SPECT signal of the islet graft observed ex vivo, corresponded nicely to the SPECT signal detected in the islet graft in vivo (Figure 4), which confirms that the signal observed in vivo originated from the islet graft in the muscle.

Longitudinal follow-up of transplanted islets
Figure 5 shows the longitudinal SPECT analysis of the intramuscular islet grafts. In all rats (n=3), islet grafts showed a clear SPECT signal from three weeks after transplantation until ten weeks (end of experiment) after transplantation (Figure 5). All transplants (n=3) were localized in the calf muscle by HE staining and co-localized with insulin and D2 receptor expression, as determined immunohistochemically.

Figure 4: A) In vivo SPECT/CT image and B) ex vivo SPECT image of a WAG/Rij calf muscle, transplanted with 3,000 islets. The red arrows indicate co-localization of the SPECT signal in the calf muscle detected in vivo and ex vivo.

Figure 5: In vivo SPECT/CT images of an islet graft in the calf muscle from three to ten weeks after transplantation. The red arrows indicate the transplant.
DISCUSSION
Recently, we have demonstrated the technical feasibility of non-invasive visualization of intramuscular islet grafts by D2 receptor targeting using $[^{123}\text{I}]$IBZM SPECT/CT (28). In this study, longitudinal and quantitative islet graft monitoring, using this technique, was explored. We demonstrated, for the first time, a high correlation (Pearson $r=0.73$) between the SPECT signal, obtained from a radiotracer, and the viable graft volume. Furthermore, we demonstrate the feasibility of islet graft monitoring from three until ten weeks after transplantation. These data go beyond previous work demonstrating the feasibility of nuclear islet graft imaging, since a linear correlation of viable graft volume and radiotracer uptake had never been shown in vivo.

Active targeting of peptide hormone receptors, expressed on beta cells, offers potential advantages over prelabeling methods. Active receptor targeting allows longitudinal in vivo monitoring of the islet graft by multiple radiotracer injections, providing real time information on the state of the islet graft. Currently, GLP-1R targeting is being extensively explored for beta cell imaging in the native pancreas and after islet transplantation (10, 11, 29, 30). However, the availability of an alternative tracer, targeting a different receptor, could provide complementary information about the islet graft and might therefore help to improve transplantation outcome.

Since D2 receptors are expressed on beta cells in the islets of Langerhans (14), they are considered a suitable target to monitor these insulin producing cells. Until now, D2 receptor targeting was mainly applied for CHI and insulinoma imaging (19, 31, 32). D2 receptor imaging is not suitable for imaging of the absolute islet mass in the native pancreas due to high diffuse tracer uptake in the exocrine pancreas. However, it has been demonstrated that islets transplanted into the spleen of rats could be visualized and semi-quantitatively analyzed by $[^{18}\text{F}]$-fallypride PET (21, 22). $[^{123}\text{I}]$IBZM is widely commercially available or can be produced using a facile labeling procedure (28) and therefore represents a highly interesting alternative for $[^{18}\text{F}]$-fallypride. In this study, we demonstrated for the first time, a high correlation (Pearson $r=0.73$) of viable graft volume and quantitative $[^{123}\text{I}]$IBZM SPECT signal, which indicates that tracer uptake is a representative measure for the amount of viable islets in the graft. Commonly used transplantation sites such as the liver, kidney or spleen show high background uptake of $[^{123}\text{I}]$IBZM, rendering these sites inferior to the muscle as a transplantation site for in vivo graft analysis using D2 receptor targeting. Furthermore, islet transplantation in the muscle is already applied for auto-transplantation in diabetes patients. Although skeletal muscle contains considerable dopamine levels, competition of tracer binding will be negligible since the potency of dopamine to displace IBZM binding is very low (24).
Longitudinal graft monitoring showed the possibility to image the islet grafts with \[^{123}\text{I}]\text{IBZM}\) for several weeks. A clear SPECT signal was observed in the islet grafts from three weeks until ten weeks (end of experiment) after transplantation. Furthermore, a gradual increase of the SPECT signal, followed by signal stabilization was observed. We have previously shown that the initial absence of detectable SPECT signal and the gradual signal increase can be explained by ongoing revascularization of the islet graft (33, 34). Islet vasculature is disrupted during the isolation process, which hampers tracer supply to the islet graft. However, in the first weeks after transplantation, functional blood supply in the islet graft is restored allowing reproducible \[^{123}\text{I}]\text{IBZM}\) targeting of the graft.

Taken together, \[^{123}\text{I}]\text{IBZM}\) SPECT provides an attractive tool to monitor survival of islet grafts. It allows longitudinal graft monitoring and holds the power to detect small differences in viable graft volume. The limited capacities of D2 receptor imaging for islets engrafted in the liver may no longer be a major drawback considering the increasing clinical interest in the muscle as a transplantation site (33, 35, 36). Therefore, \[^{123}\text{I}]\text{IBZM}\) SPECT might provide important information on the state of the islet graft and eventually, might help to predict and improve transplantation outcome.

**CONCLUSION**

In this study, intramuscular islet grafts could be visualized from three weeks after transplantation using \[^{123}\text{I}]\text{IBZM}\) as a tracer. The SPECT signal showed a high correlation with the calculated viable graft volume. In conclusion, \[^{123}\text{I}]\text{IBZM}\) can be successfully employed for non-invasive longitudinal and quantitative islet graft monitoring.

**ACKNOWLEDGEMENTS**

We thank Bianca Lemmers, Henk Arnts, Iris Lamers and Kitty Lemmens for their technical support with the animal experiments and the staff of the pathology department for tissue sectioning. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union’s Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n° 289932 and from the Institute of Genetic and Metabolic Disease, Radboud University Nijmegen.
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Improved quantification of the beta cell mass after pancreas visualization with $^{99m}$Tc-demobesin-4 and beta cell imaging with $^{111}$In-exendin-3 in rodents

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Adapted from: Molecular Pharmaceutics 2016;13:3478-3483.
**ABSTRACT**

**Objective:** Accurate assessment of the $^{111}$In-exendin-3 uptake within the pancreas requires exact delineation of the pancreas, which is highly challenging by MRI and CT in rodents. In this study the pancreatic tracer $^{99m}$Tc-demobesin-4 was evaluated for accurate delineation of the pancreas to be able to accurately quantify $^{111}$In-exendin-3 uptake within the pancreas.

**Methods:** Healthy and alloxan-induced diabetic Brown Norway rats were injected with the pancreatic tracer $^{99m}$Tc-demobesin-4 ($[^{99m}$Tc-N$_4$-Pro$^1$,Tyr$^4$,Nle$^{14}$]bombesin) and the beta cell tracer $^{111}$In-exendin-3 ($[^{111}$In-DTPA-Lys$_{40}$]exendin-3). After dual isotope acquisition of SPECT images, $^{99m}$Tc-demobesin-4 was used to define a volume of interest for the pancreas in SPECT images subsequently the $^{111}$In-exendin-3 uptake within this region was quantified. Furthermore, biodistribution and autoradiography were performed in order to gain insight in the distribution of both tracers in the animals.

**Results:** $^{99m}$Tc-demobesin-4 showed high accumulation in the pancreas. The uptake was highly homogeneous throughout the pancreas, independent of diabetic status, as demonstrated by autoradiography, whereas $^{111}$In-exendin-3 only accumulates in the islets of Langerhans. Quantification of both ex vivo and in vivo SPECT images resulted in an excellent linear correlation between the pancreatic uptake, determined with ex vivo counting and $^{111}$In-exendin-3 uptake, determined from the quantitative analysis of the SPECT images (Pearson r=0.97, Pearson r=0.92).

**Conclusion:** $^{99m}$Tc-demobesin-4 shows high accumulation in the pancreas of rats. It is a suitable tracer for accurate delineation of the pancreas and can be conveniently used for simultaneous acquisition with $^{111}$In-exendin-3. This method provides a straightforward, reliable and objective method for preclinical beta cell mass (BCM) quantification with $^{111}$In-exendin-3.

**Keywords:** exendin-3; demobesin-4; beta cell mass; pancreas imaging
INTRODUCTION

One of the key pathophysiological processes involved in the development of diabetes mellitus is the loss of beta cells. An accurate method to quantify the beta cell mass (BCM) during this process is essential to gain more insight in the pathophysiology of diabetes and for the development of new therapeutic strategies. Currently, mainly autopsy data on BCM are available (1, 2), that only give information about the BCM at one time point in the course of the disease and simultaneous functional data seem to be missing in most cases. In the last couple of years, there have been several attempts to quantify the BCM non-invasively by imaging (3), in order to provide real time information on the BCM during the development of diabetes.

A successful attempt to quantify the BCM was published by Brom et al (4). They correlated radiolabeled exendin-3 uptake, as determined with SPECT imaging, with the histologically determined BCM in rats. With this method exact delineation of the pancreas was challenging, especially in diabetic animals with low $^{111}$In-exendin-3 uptake as a result of low BCM, in combination with the high renal uptake of $^{111}$In-exendin-3 (>30% ID/g), so that a certain volume of interest within the pancreas was used as a surrogate marker for total beta cell mass. Besides the beta cells, the duodenum and stomach, organs located closely to the pancreas potentially overlapping with the pancreas on SPECT also express the glucagon-like peptide-1 receptor (GLP-1R) (5), and show accumulation of $^{111}$In-exendin-3, which can lead to inaccurate estimations of the actual BCM. Unfortunately, it is not easily possible to use CT or MRI scans to delineate the pancreatic tissue in these animals because the pancreas of rodents cannot reliably be distinguished from the surrounding tissues on these scans. To overcome this problem in SPECT imaging, a dual tracer imaging approach might be useful: $^{111}$In-exendin-3 to quantify the BCM, and another tracer to accurately delineate the pancreatic tissue. In addition to the dual tracer imaging approach a unilateral nephrectomy was performed to enable quantification of the BCM of a larger part of the pancreas. After exact delineation of the pancreas, the $^{111}$In-exendin-3 uptake within this region can be quantified. The use of two radionuclides for labeling, with different energy peaks, enables simultaneous acquisition of both images. Such a method would simplify the BCM quantification procedure and would be less vulnerable to inter-individual assessment differences due to manually drawn ROIs around the pancreas.

A potential tracer to accurately visualize the exocrine part of the pancreas is $^{99m}$Tc-demobesin-4. $^{99m}$Tc-Demobesin-4 binds to the gastrin releasing protein receptor (GRPR) physiologically expressed in the pancreas and is mainly used for visualization of GRPR positive tumors (6-8). Besides this high uptake in GRPR positive tumors it was...
also shown that several other bombesin analogs have high uptake in the exocrine part of the pancreas (6, 9). The GRPR agonists, like $^{99m}$Tc-demobesin-4 show higher pancreatic retention than the previously tested antagonist $^{99m}$Tc-demobesin-1 (6, 9). $^{99m}$Tc-demobesin-4 therefore has the potential to improve the visualization of the pancreas and thereby aid the quantification of $^{111}$In-exendin-3 signal within the pancreas.

In this study the feasibility to accurately determine the BCM by SPECT imaging in Brown Norway rats after co-injection of $^{99m}$Tc-demobesin-4 and $^{111}$In-exendin-3 was evaluated. To investigate the possibility to reliably detect even small amounts of beta cells one group of animals received alloxan to reduce the BCM in the animals.

**MATERIALS AND METHODS**

**Peptides and radiolabeling**

$^{111}$In-exendin-3

$[\text{Lys}^{40}](\text{DTPA})\text{exendin-3}$ (200 pmol) (Peptides Specialty Laboratories, Heidelberg, Germany) (referred to as exendin-3 in the remainder of the text) was incubated with $^{111}$InCl$_3$ (150 MBq) (Mallinckrodt Pharmaceuticals, ’s Hertogenbosch, The Netherlands) in 0.5 M MES buffer (Sigma Aldrich, St Louis, MO, USA), pH 5.5 at room temperature. After incubation for 20 min, 50 mM EDTA (Sigma Aldrich, St Louis, MO, USA) was added to reach a final concentration of 5 mM and 10% Tween80 (Sigma Aldrich, St Louis, MO, USA) to a final concentration of 0.1%. The labeling efficiency was determined on silica gel instant thin layer chromatography (ITLC) using 0.1 M EDTA in 0.1 M NH$_4$Ac pH 5.5 ($R_f=0$: $^{111}$In-exendin-3, $R_f=1$: $^{111}$In-EDTA). The reaction mixture was purified by solid-phase extraction using an HLB reversed-phase sorbent cartridge (30 mg, Water Oasis, Milford, MA, USA), as previously described (10). After purification the radiochemical purity was >99%, with an average radiochemical yield of ~60%.

$^{99m}$Tc-demobesin-4

Demobesin-4 was provided by the Molecular Radiopharmacy, NCSR Demokritos (Athens, Greece) and dissolved in 0.1% acetic acid in water and ethanol (8:2, v/v) at a final concentration of 1.8 mg/mL; aliquots thereof were stored at -20°C. Demobesin-4 (2.8 nmol) was labeled with $^{99m}$Tc pertechnetate (83 µL, 100-120 MBq) in 10 µL 0.5 M phosphate buffer (pH 11.5), 1 µL 0.1 M sodium citrate and 3 µL freshly prepared SnCl$_2$ (Sigma Aldrich) in pure ethanol (2 mg/mL). The mixture was incubated at room temperature for 30 min. After incubation, the pH of the solution was reduced to 7 by addition of 1 M HCl (4 µL), and 10% Tween80 was added to a final concentration of 0.1%. Radiochemical purity was determined by ITLC, using two mobile phases: 100%
aceton ($R_f = 0$; $^{99m}$Tc-demobesin-4, hydrolyzed $^{99m}$Tc, $R_f = 1$; $^{99m}$TcO$_4^-$) and 100% MeOH in 0.1 M NH$_4$Ac (1:1) ($R_f = 0$; hydrolyzed $^{99m}$Tc, $R_f = 1$; $^{99m}$Tc-demobesin-4, $^{99m}$Tc-citrate, $^{99m}$TcO$_4^-$) or reversed-phase high performance liquid chromatography (RP-HPLC) on a C-18 Alltech Alltima column, 4.6 x 250 mm, 5 µm (Fisher Scientific, USA). The column was eluted with 0.1% TFA in H$_2$O with a linear gradient from 10 to 40% acetonitrile in 30 min followed by a linear gradient from 40% to 100% in 10 min (flow rate 1 mL/min). Under these conditions $^{99m}$Tc-citrate elutes at 2.5 min, $^{99m}$TcO$_4^-$ at 3.4 min and $^{99m}$Tc-demobesin-4 at 29.1 min. The radiochemical purity of $^{99m}$Tc-demobesin-4 always exceeded 98%, and, because no purification was involved, a similar radiochemical yield was reached.

**Animal experiments**

Animal experiments were performed in six to eight week old Brown Norway rats (150 ± 7g), purchased from Charles River (Sulzfeldt, Germany). The animal experiments were approved by the animal welfare committee of the Radboud University Nijmegen. A unilateral nephrectomy of the left kidney was performed under inhalation anesthesia with isoflurane in O$_2$ and air (induction 4-5%, maintenance 1.5-2%) to improve visualization of the pancreas for quantification purposes. Prior to isoflurane anesthetics rats were given Carprofen (5 mg/kg s.c., Rimadyl®, Pfizer Animal Health B.V., Netherlands) for analgesia. Carprofen injections were repeated twice daily for two days after surgery.

**Alloxan treatment**

One week after surgery the rats were injected with 45 or 60 mg/kg body weight alloxan monohydrate (Sigma Chemical, St. Louis, MO, USA) (n= 4/group). Alloxan was dissolved in PBS (0.1 mg/µL), and protected from light and kept on ice before intravenous (i.v.) injection. Control rats were injected with PBS only.

**SPECT/CT acquisition**

One week after alloxan treatment blood glucose concentrations were measured and when hyperglycemia was confirmed in treated rats, diabetic (blood glucose: 32 ± 5.9 mmol/L) and control rats (blood glucose 6.5 ± 0.7 mmol/L) were i.v. injected with 14 ± 1.2 MBq $^{111}$In-exendin-3 (peptide dose 20 pmol, 200 µL) and 17 ± 2.8 MBq $^{99m}$Tc-demobesin-4 (peptide dose 0.56 nmol, 200 µL). Six healthy rats received a co-injection with either an excess of unlabeled exendin-3 (5 nmol) or an excess of unlabeled demobesin-4 (56 nmol) (n=3/group) to determine whether the uptake of the tracers is GLP-1R or GRPR mediated.
One hour after injection of the radiolabeled compounds SPECT/CT images were acquired (U-SPECT-II, MiLabs, Utrecht, The Netherlands). Rats were scanned under general anesthesia (isoflurane in air) for 50 min using a 1.0 mm multipurpose rat and mouse collimator.

**Biodistribution**

After the imaging procedure, rats were euthanized by CO$_2$/O$_2$ suffocation. Tissues were dissected, weighed and the uptake of the radiotracers in the tissues was determined using a gamma counter (Wallac 1480 wizard, Perkin Elmer, Waltham, MA, USA) with reference solutions of $^{99m}$Tc and $^{111}$In (200 µL samples containing 1% and 0.1% of the injected dose).

Counting was performed on the 140.5 keV for $^{99m}$Tc (120-140 keV) and the 171 and 245 keV photopeaks for $^{111}$In (150-541 keV). The counting windows were adjusted to minimize contamination of $^{111}$In activity in the $^{99m}$Tc window. Only when the amount of $^{111}$In in a tissue was several times higher than the amount of $^{99m}$Tc, the counts in the $^{99m}$Tc window were significantly contaminated with counts from the $^{111}$In-window.

The uptake of $^{111}$In-exendin-3 in the tissues was measured after decay of the $^{99m}$Tc. Uptake in the tissues was expressed as percentage of injected activity per gram of tissue (%ID/g). Immediately after dissection, all pancreata were transferred to 1.5-mL Eppendorf tubes containing 4% formalin, and subsequent embedding in paraffin.

**Ex vivo SPECT acquisition**

Pancreata were dissected and each pancreas was transferred to a 1.5-mL Eppendorf tube containing 4% formalin immediately after dissection. For each acquisition three or four pancreata (in total 11 pancreata (3 control, 4 treated with 45 mg/kg alloxan, 4 treated with 60 mg/kg alloxan)) in Eppendorf tubes were placed in a mouse animal bed and subsequently ex vivo SPECT images were acquired, using a 1 mm ultra-high sensitivity mouse collimator (MiLabs, Utrecht, The Netherlands) with an acquisition time of 6 h.

**SPECT/CT reconstruction and quantification**

SPECT images were reconstructed using the U-SPECT-Rec software (MiLabs, Utrecht, The Netherlands). The $^{111}$In and $^{99m}$Tc images were reconstructed separately. For $^{111}$In-exendin-3 images, only counts from the 245 keV photopeak (223-259 keV) of $^{111}$In were reconstructed. Since no counts from $^{99m}$Tc were measured in this window the quantification of the $^{111}$In images was only based on uptake of $^{111}$In in the beta cells. $^{99m}$Tc-demobesin-4 images were reconstructed with counts from the 140.5 keV
photopeak of $^{99m}$Tc. In this window, there are also counts from the 171 keV photopeak of $^{111}$In present, but the $^{99m}$Tc images were only used for delineation of the pancreatic tissue and not for quantification. The contribution of the counts from the 171 keV $^{111}$In photopeak in the $^{99m}$Tc window depends on the biodistribution of both tracers.

Images were analyzed using Inveon Research Workplace (Siemens Healthcare, Den Haag, The Netherlands). The $^{111}$In-exendin-3 images were used to draw a volume of interest (VOI) around the kidney, this VOI was dilated with a radius of 1 mm. Subsequently, the kidney VOI was copied to the $^{99m}$Tc-demobesin-4 image. The uptake in the kidney VOI was excluded. The pancreas was selected by drawing a cubical VOI around the pancreas in the $^{99m}$Tc-demobesin-4 window and subsequently the 50% hottest pixels within this VOI were selected.

This VOI of the pancreas in the $^{99m}$Tc-demobesin-4 image was copied to the $^{111}$In-exendin-3 image and the signal inside the VOI was measured. Standards with a known amount of $^{111}$In (0, 18.5, 37, 55.5 kBq) were scanned, quantified, and used to determine a calibration factor to convert the SPECT signal to uptake in kBq.

**Ex vivo autoradiography**

*Ex vivo* autoradiography was performed to visualize the distribution of the tracers in the pancreas. Sections (4 µm) were exposed to a phosphor imaging plate (Fuji Film BAS-SR 2025, Raytest, Straubenhardt, Germany) for 24 h at room temperature, to visualize uptake of $^{99m}$Tc-demobesin-4 and $^{111}$In-exendin-3. After 24 h, the imaging plate was developed using a radioluminography laser imager (Fuji Film BAS 1800 II system, Raytest, Straubenhardt, Germany). Subsequently, the same tissue sections were exposed for 6 days to a phosphor imaging plate to visualize only uptake of $^{111}$In-exendin-3. Images were analyzed with Aida image Analyzer software (Raytest, Straubenhardt, Germany).

**Statistical analysis**

All mean values are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism (version 5.0.3). Correlations were determined using the Pearson correlation coefficient ($r$). The level of significance was set at $p < 0.05$. 
RESULTS

Ex vivo autoradiography

Autoradiography of pancreatic sections of healthy and alloxan treated rats showed high tracer accumulation of $^{111}$In-exendin-3 restricted to the islets of Langerhans and a homogeneous uptake of $^{99m}$Tc-demobesin-4 within the pancreas (Figure 1). Uptake of both tracers was receptor mediated, because it could be blocked by an excess of unlabeled tracer (exendin-3 or demobesin-4) (Figure 1C and 1D).

<table>
<thead>
<tr>
<th>Healthy</th>
<th>Diabetic</th>
<th>Excess unlabeled demobesin-4</th>
<th>Excess unlabeled exendin-3</th>
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Figure 1: Digital autoradiography of pancreatic sections (4 µm) of healthy (A),(C),(D) and alloxan treated (60 mg/kg) (B) rats. All rats were injected with 14 ± 1.2 MBq $^{111}$In-exendin-3 and 17 ± 2.8 MBq $^{99m}$Tc-demobesin-4. In addition the rat in (C) received an excess of unlabeled demobesin-4, whereas the rat in (D) received an excess of unlabeled exendin-3. In (A),(B),(D) a homogeneous accumulation of $^{99m}$Tc-demobesin-4 was observed in the exocrine pancreas; this uptake could be blocked by an excess of unlabeled demobesin-4, although accumulation of the $^{111}$In-exendin-3 in the islets was still present (C). Injection of an excess of unlabeled exendin-3 blocked the uptake of $^{111}$In-exendin-3 in the islets, whereas the homogeneous uptake of $^{99m}$Tc-demobesin-4 was still present (D). The black bars represent 2 mm.

Biodistribution

$^{111}$In-exendin-3 showed a biodistribution pattern as previously described (4), with high specific uptake in the lungs (>10% ID/g) and high non-receptor mediated accumulation in the kidneys (>40% ID/g). Furthermore, specific uptake was observed in the pancreas of healthy rats, stomach and duodenum, since co-injection of an excess of unlabeled exendin-3 blocked uptake of the $^{111}$In-exendin-3 (Figure 2A).

The concentration of $^{99m}$Tc-demobesin-4 in the pancreas was 0.98 ± 0.27% ID/g, which could be blocked by co-injection of an excess of unlabeled demobesin-4 (0.13±0.03% ID/g), indicating GRPR mediated uptake. The uptake in all other organs was low (<0.5% ID/g), except in the lungs. The uptake of $^{99m}$Tc-demobesin-4 in the lungs was overestimated due to contamination of the $^{99m}$Tc-window with $^{111}$In. This
contamination is a result of the high uptake of $^{111}$In-exendin-3 in the lungs (confirmed in the excess exendin-3 group, where the $^{111}$In-exendin-3 uptake in the lungs was blocked (Figure 2B)). Unfortunately, it was not possible to accurately determine the $^{99m}$Tc activity in the kidneys, due to the extremely high kidney uptake of $^{111}$In-exendin-3. This leads to an underestimation of the %ID/g $^{99m}$Tc in the kidneys. On the basis of biodistribution data of animals injected with only $^{99m}$Tc, a kidney uptake of approximately 2.5% ID/g is expected (data not shown).

Ex vivo SPECT of the pancreas
The uptake of $^{99m}$Tc-demobesin-4 was clearly visualized in the pancreas of healthy and alloxan treated animals (Figure 3), whereas co-injection of an excess of unlabeled demobesin-4 blocked the receptor mediated pancreatic uptake of $^{99m}$Tc-demobesin-4. $^{111}$In-exendin-3 uptake co-localized with $^{99m}$Tc-demobesin-4 uptake, where hardly any $^{111}$In-exendin-3 uptake was observed in alloxan treated rats. Calculation of the correlation coefficient between the biodistribution data and $^{111}$In-exendin-3 uptake as determined by SPECT revealed a correlation coefficient (Pearson r) of 0.97 (p<0.0001) (Figure 3E).

Quantitative analysis of in vivo SPECT images
Specific accumulation of $^{99m}$Tc-demobesin-4 was observed in pancreatic tissue. In healthy animals, $^{111}$In-exendin-3 uptake in the pancreas co-localized with the $^{99m}$Tc-demobesin-4 uptake. In diabetic animals, negligible uptake of $^{111}$In-exendin-3 was observed within the VOI of the pancreas, based on $^{99m}$Tc-demobesin-4 uptake (Figure 4A and 4B). Quantification of the $^{111}$In-exendin-3 uptake within the VOI based on the $^{99m}$Tc-demobesin-4 images linearly correlated with the pancreatic uptake based on biodistribution data (Figure 4E, Pearson r =0.92). Co-injection of an excess of
unlabeled peptide-ligand, either exendin-3 or demobesin-4, blocked the specific tracer accumulation (Figure 4C and 4D).

Figure 3: Ex vivo SPECT images of a healthy (A), an alloxan treated pancreas (60 mg/kg) (B) and a rat co-injected with either an excess of unlabeled demobesin-4 or exendin-3 (C and D). $^{99m}$Tc-demobesin-4 is shown in gray, $^{111}$In-exendin-3 is shown in yellow. Correlation between uptake of $^{111}$In-exendin-3 determined by SPECT and biodistribution data is shown in (E), with a Pearson correlation coefficient of $r=0.97$, $p<0.0001$. The uptake on the y-axis is expressed in Bq/voxel, and the uptake determined with the gamma counter is expressed as %ID/g.

Figure 4: In vivo SPECT images of a healthy (A), an alloxan treated rat (60 mg/kg) (B) and rats co-injected with an excess of unlabeled demobesin-4 and exendin-3, respectively (C and D). $^{111}$In-exendin-3 uptake is shown in orange/yellow and $^{99m}$Tc-demobesin-4 uptake is shown in blue/white. Green arrow: pancreas, white arrow: kidney, red arrow: lungs. Correlation between the uptake of $^{111}$In-exendin-3 determined by SPECT and the biodistribution data are shown in (E) (Pearson $r=0.92$, $p<0.0001$). The uptake on the y-axis is expressed in kBq/VOI, whereas the uptake on the x-axis is expressed as %ID/g.
DISCUSSION
Radiolabeled exendin-3 is a promising tracer for BCM imaging in both the native pancreas (4, 11-15) and after islet transplantation (16, 17). However, exact delineation of the native pancreatic tissue is difficult and time consuming. Brom et al demonstrated the potential of radiolabeled exendin-3 not only for qualitative imaging of the BCM, but also for exact quantification of the BCM. In this study a linear correlation between BCM and $^{111}$In-exendin-3 uptake was observed, with a Pearson correlation coefficient of 0.89 (4). The quantification was based on the uptake in a small VOI in the pancreas, just above the kidney, which was used as a surrogate marker for total pancreatic BCM. In addition to the difficult delineation of the pancreas, the high kidney uptake of $^{111}$In-exendin-3 (>30% ID/g in rats) also hampers visualization of the $^{111}$In-exendin-3 uptake in the pancreas in rodents.

Because the pancreas is not clearly distinguishable from surrounding tissue on CT images in rodents and reduction of the high kidney uptake is difficult to achieve (18-20), quantification of the BCM in a larger VOI was not possible. To overcome these problems, one kidney was removed in our experiments and an additional tracer to visualize the whole pancreas was used. An additional tracer not only facilitates accurate delineation of pancreatic tissue and provides a straightforward, reliable, and objective method for BCM quantification, but it also allows quantification of the BCM in a larger VOI. The use of an exocrine tracer will not be necessary in a clinical setting because the pancreas morphology in humans is more defined than in rodents and therefore better visible on CT images. CT images could be used to delineate the pancreatic tissue and quantify the uptake of the BCM marker within this volume of interest.

In this study, the potential of $^{99m}$Tc-demobesin-4 as tracer for the exocrine pancreas, allowing exact delineation of the pancreas in beta cell imaging, was evaluated in a rat model. This tracer shows not only high uptake in rats with a high BCM, but also in rats with low BCM due to the expression of GRPR in the exocrine pancreas which is not influenced by loss of beta cell mass. As rats represent the preferred model for BCM imaging in vivo (21), quantification of the BCM in rats with low BCM is warranted in preclinical diabetes research, but this is also challenging and therefore, the need for an exocrine pancreas tracer is clear. Previously, Mikkola et al used $^{11}$C-methionine to verify the location of the pancreas, but in this study, the pancreatic tracer was not used for quantification purposes of a beta cell marker (11). More recently, Mathijs et al used the iodinated amino acid $^{123}$Iodo-L-phenylalanine (15) to determine the pancreatic VOI for quantification of $^{111}$In-exendin-3 in a dual tracer imaging approach. They showed a Pearson correlation coefficient of r=0.83 between pancreatic uptake
as determined in the gamma counter and SPECT quantification (15). In our study, we were able to achieve an excellent correlation between $^{111}$In-exendin-3 uptake quantified on *in vivo* SPECT images and *ex vivo* counting (Pearson r=0.92, p<0.0001). *Ex vivo* scanning of the pancreas and quantification resulted in an almost perfect correlation with *ex vivo* counting (Pearson r= 0.97). These results clearly demonstrate that $^{99m}$Tc-demobesin-4 in combination with surgical removal of the left kidney is indeed highly suitable for delineation of the pancreatic region for BCM quantification purposes. Besides this excellent correlation between the quantified *in vivo* $^{111}$In-exendin-3 uptake in SPECT images and *ex vivo* counting, $^{99m}$Tc-demobesin-4 offers two additional advantages over the $^{123}$I-labeled exocrine tracer. Deiodination of the $^{123}$Iodo-L-phenylalanine *in vivo* and consequent radioiodine release might increase radioactivity levels in the stomach, an organ close to the pancreas, and thereby hinder exact delineation of the pancreas. Another complicating factor for quantification purposes of the pancreas with $^{123}$Iodo-L-phenylalanine is the close proximity of the $^{123}$I peak to the low energy peak of $^{111}$In. $^{111}$In can contaminate the $^{123}$I window, and the high uptake of $^{111}$In in the stomach duodenum transition might be seen as $^{123}$I uptake.

In addition to using an exocrine pancreas tracer to improve BCM quantification, a unilateral nephrectomy (left kidney) was performed, to enable quantification of the BCM in a larger area of the pancreas. This is of importance because islets are heterogeneously distributed throughout the pancreas and therefore quantification of the uptake in a small VOI could lead to less accurate BCM estimations. The unilateral nephrectomy did not influence the biodistribution pattern of either $^{111}$In-exendin-3 or $^{99m}$Tc-demobesin-4. Both tracers are cleared via the kidneys, and because of the nephrectomy, all activity is cleared via one kidney, resulting in a higher radiation burden. Consequently, multiple injections might finally induce radiation damage to the kidneys, thereby potentially influencing the biodistribution of the tracer. Although this model might not prove optimal in longitudinal studies, the nephrectomy involved is necessary for accurate validation of the tracer in a preclinical setting. This advantage clearly outweighs the drawbacks.

In conclusion, the exocrine pancreatic marker $^{99m}$Tc-demobesin-4 showed high uptake in the pancreas, not only in animals with high BCM but also in animals with low BCM. The combination of using $^{99m}$Tc-demobesin-4 as an exocrine tracer (for delineation of the complete pancreas) and performing a unilateral nephrectomy allowed to precisely quantify the uptake in the total pancreas, resulting in a more robust determination of the uptake of $^{111}$In-exendin-3 in the pancreas of rodents. Therefore, we clearly suggest to use this model in future rodent studies with radiolabeled exendin-3 as a straightforward and objective quantification method.
ACKNOWLEDGEMENTS

The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programmes FP7/2007-2013/ under REA grant agreement n° 289932 and FP7/2007-2013 under grant agreement n° 222980 and from the Institute of Genetic and Metabolic Disease, Radboud University Nijmegen. No potential conflict of interest relevant to this article was reported.

We thank Erik de Blois from the Erasmus Medical Centre, Rotterdam for his advice on the $^{99m}$Tc labeling procedure.

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Whole organ and islet of Langerhans dosimetry for calculation of absorbed doses resulting from imaging with radiolabeled exendin

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*Adapted from: Scientific Reports 2017;7:39800*
ABSTRACT
Radiolabeled exendin is used for non-invasive quantification of beta cells in the islets of Langerhans in vivo. High accumulation of radiolabeled exendin in the islets raised concerns about possible radiation-induced damage to these islets in man. In this work, islet absorbed doses resulting from exendin-imaging were calculated by combining whole organ dosimetry with small scale dosimetry for the islets. Our model contains the tissues with high accumulation of radiolabeled exendin: kidneys, pancreas and islets. As input for the model, data from a clinical study (radiolabeled exendin distribution in the human body) and from a preclinical study with Biobreeding Diabetes Prone (BBDP) rats (islet-to-exocrine uptake ratio, beta cell mass) were used. We simulated $^{111}$In-exendin and $^{68}$Ga-exendin absorbed doses in patients with differences in gender, islet size, beta cell mass and radiopharmaceutical uptake in the kidneys. In all simulated cases the islet absorbed dose was small, maximum 1.38 mGy for $^{68}$Ga and 66.0 mGy for $^{111}$In. The two sources mainly contributing to the islet absorbed dose are the kidneys (33-61%) and the islet self-dose (7.5-57%). In conclusion, all islet absorbed doses are low (<70 mGy), so even repeated imaging will hardly increase the risk on diabetes.

Keywords: dosimetry; exendin; GLP-1R; islet imaging
INTRODUCTION

The beta cells in the islets of Langerhans in the pancreas produce insulin and play a key role in maintaining blood glucose levels within a normal range. In diabetes, the glucose homeostasis is disturbed by different underlying pathologies, resulting in insufficient beta cell function and/or mass. Until recently, the relation between beta cell mass (BCM) and beta cell function could not be investigated in vivo in humans due to the lack of a method to measure the BCM non-invasively.

Therefore, there is a major need for non-invasive determination of BCM in patients with diabetes (1), and people at risk of diabetes. Such a method could also provide more insight in the relation between BCM and function in healthy volunteers as compared to a population at risk or with diabetes. In the last couple of years various radiolabeled tracers have been developed for BCM quantification by PET and SPECT and evaluated in preclinical studies (2) with the first tracers now being tested in clinical trials (3-5). One promising tracer for beta cell quantification is radiolabeled exendin, which binds specifically to the beta cells (6) via the glucagon-like peptide-1 (GLP-1) receptor and is internalized upon receptor binding (3). This internalization and subsequent “metabolic trapping” of the tracer, labeled with a radiometal (3), leads to high target-to-background ratios.

The beta cells are the main cell type in the islets of Langerhans (~80% of the cells) (7), and are assumed to be homogeneously distributed within the islets in human (8). Besides beta cells, the islets of Langerhans consist of alpha, delta and pp cells. The islets of Langerhans are approximately spherical structures with diameters between 50 and 400 µm. The different cells types within the islets are of vital importance for maintaining glucose homeostasis and the accumulation of radioactivity leads to concerns about possible radiation-induced damage to the islets, especially after repeated radiotracer administration. To ensure imaging without damaging the islets, a method to calculate the islet absorbed dose is required.

Until now, no suitable model has been available to calculate absorbed doses in the islets. Some attempts have been made using whole organ-based dosimetry to calculate average whole body and pancreas absorbed doses when using radiolabeled exendin (9-12). Mikkola et al (9) used the rat biodistribution of NODAGA-exendin, labeled with ⁶⁴Cu or with ⁶⁸Ga to estimate human whole body absorbed doses with OLINDA/EXM (13). In a similar way, Wild et al (10) used the biodistribution in Rip1Tag2 mice for three different exendin radiopeptides (labeled with ⁶⁸Ga, ¹¹¹In or ⁹⁹mTc) to calculate the total body and pancreas dose. Recently, Selvaraju et al (12) calculated organ absorbed doses after administration of ⁶⁸Ga-exendin in different
animal species and humans. In all studies the pancreas absorbed dose was calculated assuming a homogeneous radioactivity distribution in the pancreas. However, only 1-2% of the pancreatic tissue consists of islets and the activity concentration in an islet is much higher than in exocrine tissue (3,9). Therefore, the absorbed dose in the islets is expected to be higher than in the exocrine tissue, which is not reflected by the models used so far, although acknowledged by Velikyan et al (11). Therefore, the islets should be included as a separate compartment in the islet absorbed dose calculation.

In this work, whole organ and small scale (µm-level) dosimetry was combined to calculate the islet absorbed dose after administration of radiolabeled exendin. In a previous clinical study, high accumulation of radiolabeled exendin in the pancreas and the kidneys was observed, while the radioactivity concentration in the remainder of the body was negligible (3). Therefore, the kidneys and pancreas were used as source organs in the organ level dosimetry part of the model and the remainder of the body was not included. To complete the model, small scale dosimetry of the islets was included.

The islet self-dose was calculated using Monte Carlo based small scale dosimetry using the MCNP code (14), whereas the absorbed doses due to kidneys and pancreas were calculated using S-values obtained from the OLINDA/EXM (11) software. OLINDA/EXM is a software program which can be used to obtain organ S-values or calculate organ absorbed doses after administration of radiopharmaceuticals. An S-value describes the contribution of the activity in one organ (source) to the dose in another organ (target). Accurate dose calculations require detailed information about the tracer distribution not only for different organs but also within specific organs. Therefore, not only organ based data on tracer distribution from a clinical study but also specific information on the tracer uptake in exocrine tissue and islets within the pancreas retrieved from preclinical data, were used as input for the model.

Finally, the model was applied to estimate islet absorbed doses and to investigate the effect of different radionuclides ($^{111}$In or $^{68}$Ga) and different biological factors (male or female, low or high kidney uptake, small or large islets, healthy or diabetic subjects) on the absorbed dose in the islets.
METHODS

Dosimetry model

The absorbed dose in one islet is the sum of absorbed doses originating from different sources. When source and target are the same, the resulting absorbed dose is called self-dose. Figure 1 shows the sources that contribute to the islet absorbed dose and the assumptions made in our model.

![Diagram of islet dosimetry model](image)

**Figure 1**: The different sources that contribute to the islet absorbed dose and the assumptions made in the model. Under the assumption of homogeneous distribution of the islets through the pancreas, the contribution of the surrounding islets and exocrine pancreas is combined in the self-dose of the pancreas (1). Based on previous observations the activity in the remainder of the body is neglected (2).

We assume that (I) all islets are homogeneously distributed through the pancreas and that (II) the surrounding islets and the exocrine tissue contribute to the islet absorbed dose as one source with a uniform distribution. This is defined as the self-dose of the pancreas.

Using these assumptions, the islet absorbed dose \( D_{islet} \) is given by:

\[
D_{islet} = A_0 \left( S_{pancreas} \epsilon_{kidneys} \tau_{kidneys} + S_{pancreas} \epsilon_{pancreas} \tau_{pancreas} + S_{islet} \epsilon_{islet} \tau_{islet} \right)
\]

Where S-values describe the contribution of activity of one tissue (source) to the absorbed dose in another tissue (target), \( \tau \) is the time integrated activity coefficient and \( A_0 \) the administered activity. For the use of S-values, it is assumed that activity is homogeneously distributed in the source and the absorbed dose is uniform in each target organ (12). This also holds for the calculation of the islet self-dose, where the islets are considered as spheres of homogeneously distributed activity. The total islet absorbed dose is slightly overestimated, since it is a combination of the islet self-dose and the pancreas self-dose, as the islet for which the islet absorbed dose is calculated is also part of the pancreas.
Model calculations were performed using an administered activity of 150 MBq for $^{111}$In, and 75 MBq for $^{68}$Ga (because of the higher sensitivity of PET imaging with $^{68}$Ga compared to SPECT imaging with $^{111}$In).

Each following section describes the contribution of one source (kidneys, pancreas and islet) to the islet absorbed dose. For each source we describe in which way S-values and time integrated activity coefficients were obtained and which assumptions were made.

**Kidneys**

The average absorbed dose in the islets originating from the kidneys is equal to the average dose to the pancreas originating from the kidneys under the assumption of equal absorption of photons, in both islets and exocrine tissue, the absorption of electrons is neglected due to their short range in tissue. Using this assumption, the islet absorbed dose due to the activity in the kidneys can be estimated using the S-values for the pancreas absorbed dose due to the kidneys ($S_{\text{pancreas} \to \text{kidneys}}$). S-values for an adult male and an adult female, for $^{111}$In and $^{68}$Ga (see supplementary material, Table 1) were taken from OLINDA/EXM, and are based on Monte Carlo simulations (15,16).

The time integrated activity coefficient of the kidneys was based on planar scintigraphic images, both anterior and posterior from a previous clinical study (clinical trial number: NCT01825148). The protocol was approved by the Institutional Review Board of the Radboud University Medical Center, written informed consent was obtained from all participants and the study was carried out in accordance with the approved guidelines. In the present dosimetry study, scintigrams of five healthy volunteers (2 male, 3 female, average age: 33.2 ± 13.6 years) acquired 0, 4, 24, 48, 96 and 168 h after injection of 150 MBq $^{111}$In-exendin-4 were used for calculation of the time integrated activity coefficient of the kidneys for each volunteer. The amount of activity in the kidneys at the different time points and the time integrated activity coefficients for $^{111}$In were calculated with SPRIND-software (17). For calculation of the $^{68}$Ga time integrated activity coefficients, also the $^{111}$In scintigrams were used, but the activity in the kidneys was corrected for the shorter half-life, assuming similar biological behavior for $^{111}$In-exendin and $^{68}$Ga-exendin.

In the islet dosimetry model the average time integrated activity coefficient of these five volunteers was used as input. For simulation of humans with “high” or “low” kidney uptake, the maximum and minimum time integrated activity coefficient of these five volunteers was used, respectively.
**Self-dose pancreas**

Within the pancreas, the activity in the exocrine part and in islets contributes to the islet absorbed dose. The S-values for the pancreas self-dose ($S_{\text{pancreas},\text{pancreas}}$) were also taken from OLINDA/EXM for an adult male and adult female, for $^{111}\text{In}$ and $^{68}\text{Ga}$ (see supplementary material, Table 1).

Since no human data is available on the specific accumulation of $^{111}\text{In}$-exendin in islets and in exocrine tissue an alternative approach using preclinical data was set up to calculate the contribution of the exocrine pancreas and the contribution of the islets to the islet absorbed dose. The animal experiments were approved by the animal welfare committee of the Radboud University Nijmegen and the study was carried out in accordance with the approved guidelines.

Nine female Biobreeding Diabetes Prone (BBDP) rats (5-7 weeks old) (Biomedical Research Models, Worcester, MA, USA), were euthanized two hours post injection (p.i.) of 18.5 ± 1.78 MBq $^{111}\text{In}$-exendin (peptide dose 20 pmol). Pancreata were dissected and activity in the pancreas for each rat was measured in a gamma counter (Wallac 1480 wizard, Perkin Elmer, Waltham, MA, USA), 2 weeks p.i.. The pancreata were fixed in formalin, embedded in paraffin and sliced into 4 µm sections for autoradiography and histological evaluation.

For autoradiography, a phosphor imaging plate was exposed to tissue sections of the pancreas (n=3 per rat)) for 7 days and developed using a radioluminography laser imager (Fuji Film BAS 1800 II system, Raytest, Straubenhardt, Germany), exported from AIDA software (Raytest) and further processed in MATLAB (version R2011a, The MathWorks, Inc., USA). The same sections were stained for the presence of insulin and the total pancreatic area and the area of the insulin positive islets were determined as described previously (3). Mask images of these delineated islets and of the total pancreatic area were created and saved. In MATLAB, autoradiography images and their corresponding histological sections were scaled and rotated to ensure optimal registration of the islets in both images. For each tissue section, the islet fraction was calculated by dividing the insulin positive area (islet mask) by the total pancreatic area (tissue mask). Furthermore the average activity outside the tissue, so called background (e.g. due to cosmic radiation) and the average activity in the exocrine tissue were calculated by selecting three regions outside the tissue (Figure 2A, B). For each tissue section the pixel values in the autoradiography images, representing the activity concentration in the exocrine tissue were corrected for the background.
Figure 2C and D show that in the autoradiography images, activity originating from the islets is also projected in the surrounding of the islets. This spill-over is caused by the distance between the tissue section and the phosphor imaging plate. Delineated islets were dilated (Figure 2E), to include spill-over originating from the islets in the calculation of the ratio between tracer uptake in the islets and in exocrine tissue. The activity in the dilated part (Figure 2E) was corrected for exocrine tissue activity, and included in the calculated islet pixel value. Finally, the average uptake ratio per pixel between islet and exocrine tissue (each section separately) can be calculated.

To calculate time integrate activity coefficients for each rat pancreas (exocrine tissue and islets), the fraction of injected activity in each rat pancreas was used. With this time integrated activity coefficient for each rat pancreas, the average uptake ratio per pixel and the islet fraction, the islet and exocrine time integrated activity coefficients can be calculated. Finally these time integrated activity coefficients were translated to
human time integrated activity coefficients using the method described by Kirschner et al (18). A more extensive description of the calculation of the pancreas self-dose is given in the supplementary material.

For calculation of the \(^{68}\text{Ga}\) time integrated activity coefficients, the activity in the pancreas was corrected for the shorter half-life, assuming similar biological behavior for \(^{111}\text{In}\)-exendin and \(^{68}\text{Ga}\)-exendin in the pancreas in rats, and the same translation to human.

**Self-dose islets**

Assuming islet sphericity, the sphere model (19) as used in OLINDA/EXM would be suitable for calculation of the islet self-dose, unfortunately the smallest sphere in OLINDA/EXM (diameter: 0.13 cm) (19) is much larger than the typical islet size (diameter: 50-400 µm). For calculation of S-values for \(^{111}\text{In}\) and \(^{68}\text{Ga}\) in spheres with sizes that cover the range of the islet sizes, new Monte Carlo simulations using the MCNP code (14) were performed. The methods for these Monte Carlo simulations were described previously (19), although for the present calculations only MCNP code was used and the comparison with results calculated with the EGS4 code was skipped. For the Auger electrons with energy <1 keV complete local absorption was assumed. For the electrons with energy >1 keV and for photons, Monte Carlo simulations were performed for spheres with diameters of 50, 100, 200 and 400 µm (see supplementary material, Table 2), which covers the typical range of the islet sizes. For each electron, positron and gamma spectrum from the MIRD-07 database for \(^{68}\text{Ga}\) and \(^{111}\text{In}\), 10 million initial particles were used as input, the energy cut-off was set at 1 keV and the ITS 3.0 electron energy loss straggling algorithm was set (DBCN 17j 2) (20). The density and elemental composition of the pancreas was according to ICRU report 44 (21), the beta cells were modelled according to soft tissue (male) in the same report.

Calculation of the time integrated activity coefficient for the islet was performed as described in the self-dose pancreas section.

**Use of the model**

To demonstrate the application of the model, several examples are described. Islet absorbed doses were calculated for healthy male (I), healthy female (II), male with a high or low kidney uptake (III), male with small or large islets (IV) and diabetic male (V). Islet absorbed doses were calculated for both \(^{111}\text{In}\) and \(^{68}\text{Ga}\) (details about the model input for the different examples are given in Table 1).
Table 1: Details and results of the examples.

<table>
<thead>
<tr>
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<th>Example II</th>
<th>Example III</th>
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<td>$^{111}$In $^{68}$Ga</td>
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<td><strong>Specific islet absorbed dose (mGy/MBq)</strong></td>
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</tbody>
</table>

**RESULTS**

Results for the examples specified above, are given in Table 1. Here, we describe the main results.

The use of $^{68}$Ga instead of $^{111}$In will result in a much lower islet absorbed dose

The use of $^{68}$Ga instead of $^{111}$In will result in an islet absorbed dose of only 0.99 mGy compared to 46.2 mGy (Table 1, example 1). The relative contribution of the islet self-dose to the total islet absorbed dose is smaller for $^{68}$Ga than for $^{111}$In (13% versus 39%
ISLET DOSIMETRY OF RADIOLABELED EXENDIN

of the total islet dose), whereas the relative contribution of the kidneys to the islet absorbed dose is in the same range for $^{111}\text{In}$ and $^{68}\text{Ga}$ (53% and 46%).

The islet absorbed dose is higher for females than for males
Calculation of the islet absorbed dose for males and females resulted in higher absorbed dose for females (57.6 versus 46.2 mGy for $^{111}\text{In}$ and 1.21 versus 0.99 mGy for $^{68}\text{Ga}$ (Table 1, example 1 and 2)). Although the absolute absorbed dose is higher for females, the relative contribution of all sources is similar.
The kidney uptake has a substantial effect on the islet absorbed dose
The islet absorbed dose in a subject with a high kidney uptake was approximately 15-30% higher than in a subject with low kidney uptake (40.0 mGy and 51.1 mGy for $^{111}$In and 0.92 mGy and 1.07 mGy for $^{68}$Ga (Table 1, example 3)). This difference in total islet absorbed dose is only a result of the difference in kidney time integrated activity.

Islet absorbed dose largely depends on islet size
The islet absorbed dose was calculated for both small (50 µm) and large (400 µm) islets. For $^{111}$In the islet self-dose was 15.4 mGy for islets with a diameter of 50 µm and 37.9 mGy for islets with a diameter of 400 µm (Table 1). The $^{68}$Ga islet self-dose was 0.07 mGy for 50 µm islets and 0.51 mGy for 400 µm diameter (Table 1, example 4). For the large islets this results in a high relative contribution of the islet self-dose to the total islet absorbed dose (57% for $^{111}$In and 37% for $^{68}$Ga). Compared to 50 µm diameter islets, the total islet absorbed dose in 400 µm islets is increased by 52% for $^{111}$In and by 48% for $^{68}$Ga.

The absorbed islet dose is slightly lower in diabetic subjects compared to healthy subjects
To simulate diabetic patients, meaning patients with a decreased BCM, the percentage islets in the pancreas was reduced from 2% to 0.2%. This strongly influenced the self-dose of the pancreas: for $^{111}$In in male the self-dose drops from 3.68 mGy to 1.51 mGy and for $^{68}$Ga from 0.40 mGy to 0.16 mGy (Table 1, example 1 and 5). The relative contribution of the whole pancreas to the total islet absorbed dose decreases from 8% in a healthy male to 3% in diabetic male for $^{111}$In and from 40% to 21% in $^{68}$Ga.

DISCUSSION
Up to now, the islet absorbed doses as reported in the literature were based on the mean absorbed dose in the whole pancreas and this could lead to an underestimation of potential islet damage resulting from the use of specific radiolabeled tracer molecules for beta cell imaging such as exendin. We have developed a model to calculate islet absorbed doses. The S-values as implemented in OLINDA/EXM were used to calculate the contribution of the kidneys and pancreas to the islet absorbed dose and small scale dosimetry was used to calculate the islet self-dose. The current model can simulate patients with differences in gender, islet size, percentage of islets in the pancreas and kidney uptake for tailoring the dosimetry to healthy subjects as well as patients with diabetes (thus with a lower percentage of islets).
In all our examples, substantial contribution of the activity in the islet itself to the total islet absorbed dose was found (with the maximum contribution in large islets, for $^{111}$In 57%, and for $^{68}$Ga 37%). Therefore, the specific accumulation of activity in the islets should be included in the islet absorbed dose calculation. The larger contribution of the islet self-dose for $^{111}$In compared to $^{68}$Ga (both absolute and in terms of percentage) to the total islet absorbed dose can be explained by the longer half-life of $^{111}$In, but is also a result of the short range of the conversion electrons of $^{111}$In (Table 2).

The islet self-dose for $^{111}$In is more than twice as high in 400 µm islets than in 50 µm islets. The increase in islet self-dose is even more pronounced for $^{68}$Ga, where the islet self-dose is more than 700% higher in large islets than in small islets. This higher islet self-dose in large islets, for both $^{111}$In and $^{68}$Ga, results in an increase in total islet absorbed dose of approximately 50%.
In the study of Wild et al (10) the islets were not included as a separate source and pancreas absorbed doses were calculated for $^{111}$In-exendin and $^{68}$Ga-exendin from mice data, resulting in a pancreas dose of 0.7 mGy/MBq for $^{111}$In-exendin and 0.2 mGy/MBq for $^{68}$Ga-exendin. Selvaraju et al (12) also calculated pancreas absorbed doses for $^{68}$Ga-exendin but in different species and found pancreas absorbed doses between 0.01 and 0.10 mGy/MBq. Inclusion of the islet self-dose in their models would have led to substantially higher islet absorbed doses than their reported average pancreas absorbed doses, because in our study the islet self-dose contributes up to 57% to the total islet absorbed dose.

The higher islet absorbed dose found in human female compared to male is a result of conversion of the rat time integrated activity coefficients to human (total body weight male 70.0 kg, female 56.9 kg) and the different $S$-values, due to a different distance between the organs in the standard male and female phantoms (15,16).

The five calculated examples showed that the contribution of the activity in the kidneys to the total islet absorbed dose is relevant, ranging from 33% for 400 µm islets in healthy males administered with $^{68}$Ga exendin, to even 60% in diabetic males administered with $^{68}$Ga-exendin (100 µm diameter islets). Therefore, reducing kidney uptake (e.g. with gelofusine (24)) is expected to not only increase image quality by increasing the pancreas to kidney activity concentration ratio, but would also lower the islet absorbed dose.

In all examples for which we calculated the islet absorbed dose, the islet absorbed dose was very low (maximum 66.0 mGy for $^{111}$In and 1.38 mGy for $^{68}$Ga), hardly increasing the risk on diabetes as a result of radiation (25). Our calculations also demonstrate that in order to minimize islet absorbed doses, the use of $^{68}$Ga-exendin is preferred over $^{111}$In-exendin.

In the future, the model could be refined by separating the pancreas into different compartments. In the current model an average islet absorbed dose is calculated, while the actual islet absorbed dose might for example be higher for islets in close proximity of the kidneys, compared to islets further away from the kidneys. By separating the pancreas into different compartments, islet absorbed doses for separate areas could be calculated. However, to obtain $S$-values for these compartments in relation to the kidneys and to each other new Monte Carlo simulations are required. Separation of the pancreas in different compartments would also enable simulation of inhomogeneous islet densities within the pancreas, as seen in humans (22).
In future research the sub-cellular distribution of the radiolabeled exendin should also be taken into account as radiolabeled exendin is internalized in the cells and this could have an effect on the potential cytotoxic effect especially for Auger emitting radionuclides. Several studies on sub-cellular distribution of Auger emitting radionuclides show a significant influence of this sub-cellular distribution on the nucleus absorbed dose (26-28). Especially if the Auger emitting radionuclide is taken up in the nucleus an increased nucleus absorbed dose is observed with potentially an increased cytotoxic effect on the cells. However, if the radionuclide is not taken up in the nucleus, but only in the cytoplasm and cell surface the average absorbed cell dose would overestimate the nucleus absorbed dose (27). It is currently not known if radiolabeled exendin is taken up in the cell nucleus. Uptake of the radiolabeled exendin in the nucleus would increase the nucleus absorbed dose for $^{111}$In-exendin, due to the short range (0.25-16 nm) of the most abundant Auger electrons (8.5 and 350 eV) and thereby the potential cytotoxic effect. However, when there is no uptake in the cell nucleus no enhanced nucleus absorbed dose and therefore cytotoxic effect is expected. To be able to accurately calculate this nucleus absorbed dose, more research on the sub-cellular radiolabeled exendin distribution is necessary.

Here, we have successfully combined human planar images, rat biodistribution data and ex vivo autoradiography to calculate the absorbed dose at islet level. The low maximum calculated islet dose (maximum 66 mGy) indicates that even repeated exendin imaging will hardly increase the risk on diabetes (25). Our data reveal that inclusion of the islet self-dose substantially contributes (up to 57%) to the islet absorbed dose, confirming that the islet self-dose should be included in the dose calculation.

**ACKNOWLEDGEMENTS**

The authors thank Desiree Bos and Hanneke Peeters for their excellent work in the animal experiments, staining and scanning of the slides and delineation of the islets.

This project has received funding from the European Community’s Seventh Framework Programme under grant agreement n° 222980 (Betalmage) and n° 602812 (BetaCure), the Juvenile Diabetes Research Foundation (JDRF), JDRF file number 37-2011-635, NIH grant 1R01 AG 030328-01 and The Institute of Genetic and Metabolic Disease, Radboud University Nijmegen.
AUTHOR CONTRIBUTIONS
I.K., W.W contributed in the design of the study, analyzed the data and wrote the manuscript. L.J. and C.F. performed the experiments and M.K. performed the Monte Carlo simulations. M.B., E.V. and M.G. contributed to the discussion and in the design of the study. All authors reviewed the manuscript.

COMPETING FINANCIAL INTERESTS
No potential conflict of interest relevant to this article was reported.

REFERENCES
Supplementary material

S-values

Supplementary tabel 1: S-values retrieved from OLINDA/EXM and used in the islet dosimetry model (M=male, F=female)

<table>
<thead>
<tr>
<th>S-values</th>
<th>$^{111}$In</th>
<th>$^{68}$Ga</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mGy/MBq.s)</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>$S_{\text{pancreas}}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$S_{\text{pancreas}}$</td>
<td>5.48E-5</td>
<td>4.63E-5</td>
</tr>
<tr>
<td>$S_{\text{pancreas}}$</td>
<td>0</td>
<td>1.47E-6</td>
</tr>
</tbody>
</table>

Calculation self-dose pancreas

In MATLAB, each autoradiography image was scaled and rotated to its corresponding histological section to ensure optimal registration of the islets in the autoradiography image with the islets in the pancreatic tissue (Supplementary Figure 1). The registration of the histological images and the masks of islets and pancreatic tissue with the autoradiography images was performed by two researchers (IK and WW). The registration of the histological and autoradiography images was scored from 1-5 (1 - very poor registration and 5 - excellent registration) by consensus. Only images with a score 3-5 (~80% of the images) were included for further analysis.

For each tissue section, the islet fraction($f_{ist, rat}$) was calculated by dividing the insulin positive area (islet mask) by the total pancreatic area (tissue mask). Subsequently, the average activity outside the tissue, so called background and the average activity in the exocrine tissue were calculated by selecting three regions outside the tissue and three regions in the exocrine tissue, respectively.
For each tissue section the pixel values in the autoradiography images, representing the activity concentration in the exocrine tissue were corrected for the background:

\[
A_{\text{exo rat/pix}} = A_{\text{exo,unc rat/pix}} - A_{\text{bg rat/pix}}
\]

(1)

Where \(A_{\text{bg rat/pix}}\) is the average background pixel value per section, \(A_{\text{exo,unc rat/pix}}\) the uncorrected average pixel value in the exocrine tissue and \(A_{\text{exo rat/pix}}\) the corrected average pixel value in the exocrine tissue.

In the autoradiography images the activity originating from the islets is also projected in the surrounding of the islets (spill-over). Delineated islets were dilated by 20 pixels on all sides, to include spill-over originating from the islets in the calculation of the ratio between tracer uptake in the islets and in exocrine tissue. The activity in the

Supplementary Figure 1: An example which shows the correlation of the position of the islets, immunohistochemically stained for insulin in brown (A and B), with the accumulation of \(^{111}\text{In-exendin}\) in islets visualized by autoradiography (C and D). B and D are magnifications of the areas indicated by the rectangles in A and C.
dilated part (rim) was corrected for exocrine tissue activity, and included in the calculated islet pixel value ($A_{isl\ rat/pix}$):

$$A_{isl\ rat/pix} = A_{isl,u\ rat/pix} + \frac{Area_{rim,lrat}(A_{rim,u\ rat/pix} - A_{exo,u\ rat/pix})}{Area_{isl,lrat}} - A_{bg\ rat/pix}$$

Where $A_{isl,u\ rat/pix}$ is the uncorrected average pixel value in the islet, $Area_{rim,lrat}$ is the number of pixels in the dilation area surrounding the islets, $A_{rim,u\ rat/pix}$ is the uncorrected average pixel value in the dilation area, $Area_{isl,lrat}$ is the number of pixels in the non-dilated islet area. Finally, the average uptake ratio per pixel between islet and exocrine tissue (each section separately) can be calculated:

$$R_{isl/exo, rat} = \frac{A_{isl\ rat/pix}}{A_{exo\ rat/pix}}$$

For calculation of the time integrated activity coefficients we assumed that immediately after injection the activity in the exocrine pancreatic tissue and in the islets is instantaneously distributed to its final position and from this moment decreases according to physical decay, with no biological clearance or redistribution between islets and exocrine tissue.

Time integrated activity coefficients for each rat pancreas (exocrine tissue and islets) were calculated. The fraction of the injected activity in each rat pancreas ($f_{ID,\ panc\ rat}$), determined by dividing the pancreatic uptake (extrapolated to t=0 h from the biodistribution data) by the administered activity, was used to calculate the time integrated activity coefficient for each rat pancreas:

$$\tau_{panc\ rat} = f_{ID,\ panc\ rat} \cdot \int_0^\infty e^{-\lambda t} dt = f_{ID,\ panc\ rat} \cdot \frac{1}{\lambda}$$

Where $\lambda$ is the physical decay constant.

The known pancreas weights (0.37-0.67 g) were converted to pancreas volumes ($V_{panc, rat}$) by assuming a pancreas tissue density of 1.079 g/cm$^3$ (1). In the autoradiography images the signal in one pixel originates from a tissue section of 4 µm thick leading to a voxel volume, $V_{vox}$ of 50x50x4 µm$^3$. The time integrated activity coefficient per voxel ($\tau_{panc\ rat/vox}$) for each rat is given by:

$$\tau_{panc\ rat/vox} = \frac{\tau_{panc\ rat}}{V_{panc, rat}} \frac{V_{panc, rat}}{V_{vox}}$$
\( \tau_{\text{panc rat/vox}} \) can be written as the weighted sum of the exocrine and islet time integrated activity coefficients per voxel (\( \tau_{\text{exo rat/vox}} \) and \( \tau_{\text{isl rat/vox}} \), respectively):

\[
\tau_{\text{panc rat/vox}} = (1 - f_{\text{isl rat}}) \cdot \tau_{\text{exo rat/vox}} + f_{\text{isl rat}} \cdot \tau_{\text{isl rat/vox}}
\]  

(6)

Under the earlier assumption of no biological clearance and no redistribution of activity between islets and exocrine tissue, equation (3) leads to:

\[
\tau_{\text{isl rat/vox}} = R_{\text{isl/exo rat}} \tau_{\text{exo rat/vox}}
\]  

(7)

Therefore, equation (6) can be rewritten as:

\[
\tau_{\text{panc rat/vox}} = (1 - f_{\text{isl rat}}) \cdot \tau_{\text{exo rat/vox}} + R_{\text{isl/exo rat}} \cdot f_{\text{isl rat}} \cdot \tau_{\text{exo rat/vox}}
\]  

(8)

\[
\tau_{\text{exo rat/vox}} = \frac{\tau_{\text{panc rat/vox}}}{f_{\text{isl rat}} \cdot R_{\text{isl/exo rat}} + (1 - f_{\text{isl rat}})}
\]  

(9)

To get the \( \tau_{\text{isl rat/vox}} \) equation (6) is rewritten as:

\[
\tau_{\text{isl rat/vox}} = \frac{\tau_{\text{panc rat/vox}} - \tau_{\text{exo rat/vox}} \cdot (1 - f_{\text{isl rat}})}{f_{\text{isl rat}}}
\]  

(10)

The time integrated activity coefficients per voxel for both exocrine tissue and islets for each rat were translated to humans with the following equation:

\[
\tau_{\text{exo human/vox}} = \tau_{\text{exo rat/vox}} \cdot \left( \frac{W_{TB, \text{rat}}}{W_{TB, \text{human}}} \right)
\]  

(11)

\[
\tau_{\text{isl human/vox}} = \tau_{\text{isl rat/vox}} \cdot \left( \frac{W_{TB, \text{rat}}}{W_{TB, \text{human}}} \right)
\]  

(12)

With for each rat \( W_{TB, \text{rat}} \), the total body weight of the rat (0.10 - 0.15 kg), and \( W_{TB, \text{human}} \), the total body weight of the human (male 70.0 kg, female 56.9 kg, as used for calculations in OLINDA/EXM).

Subsequently the time integrated activity coefficient per voxel for the human pancreas is calculated:

\[
\tau_{\text{panc human/vox}} = (1 - f_{\text{isl human}}) \cdot \tau_{\text{exo human/vox}} + f_{\text{isl human}} \cdot \tau_{\text{isl human/vox}}
\]  

(13)

Where \( f_{\text{isl human}} \) equals the volume fraction of islets in the human pancreas.
The time integrated activity coefficient per voxel for the human pancreas is multiplied by the number of voxels in the pancreas to get the time integrated activity coefficient of the human pancreas:

\[
\tau_{\text{panc, human}} = \tau_{\text{panc, human/vox}} \cdot \frac{V_{\text{panc, human}}}{V_{\text{vox}}} \tag{14}
\]

with \( V_{\text{panc, human}} \) the volume of the pancreas (which was calculated with the pancreas weight and with the same assumed tissue density of 1.079 g/cm\(^3\) (1) as in rat.

**Results Monte Carlo simulations for small spheres**

*Supplementary Table 2: Results of small sphere Monte Carlo simulations that were included in the model.*

<table>
<thead>
<tr>
<th>islet diameter (µm)</th>
<th>(^{111}\text{In} ) S-value (mGy/MBq.s)</th>
<th>(^{68}\text{Ga} ) S-value (mGy/MBq.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>beta</td>
<td>photon</td>
</tr>
<tr>
<td>50</td>
<td>16847</td>
<td>163</td>
</tr>
<tr>
<td>100</td>
<td>2476</td>
<td>38</td>
</tr>
<tr>
<td>200</td>
<td>413.9</td>
<td>8.5</td>
</tr>
<tr>
<td>400</td>
<td>80.1</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**REFERENCES**

General discussion
The aim of this thesis was to investigate the potential of several radiotracers to quantify the beta cell mass (BCM) in both the native beta cells and after islet transplantation. Furthermore, the potential damage to the islets as a result of radionuclide imaging was investigated. In this chapter we review and discuss the main findings and potential directions for future research.

Accurate quantification of the BCM would provide more insight in the role of the BCM in diabetes, but would also give more insight in the effect of certain therapies on BCM both in the pancreas and after transplantation. In the last couple of years several tracers were developed to non-invasively quantify the BCM, with tracers for PET and SPECT imaging holding the largest potential.

One of these tracers is radiolabeled exendin, which binds to the glucagon-like peptide-1 receptor expressed on the beta cells. The potential of $^{111}\text{In}$-exendin for quantification of the BCM in the native pancreas was already shown by Brom et al (1). Furthermore $^{18}\text{F}$-exendin and $^{111}\text{In}$-exendin were used to visualize transplanted islets (2-4). However, the full potential of radiolabeled exendin for quantification of transplanted islets was not yet investigated. In this thesis $^{111}\text{In}$-exendin was further characterized for quantification of islets transplanted in the muscle. In vitro experiments showed a linear correlation between the number of islets and tracer uptake. In vivo SPECT imaging showed a clear difference in SPECT signal between two groups of rats transplanted with different numbers of islets in the calf muscle. A follow up study showed a linear correlation between the transplant size, determined histologically, and quantitative analysis of the SPECT data. In this study it was possible to visualize as little as 50 transplanted islets.

Besides $^{111}\text{In}$-exendin, the potential of a different tracer, $^{123}\text{I}$-Iodobenzamide ($^{123}\text{IBZM}$) for BCM quantification was investigated. $^{123}\text{IBZM}$ binds to the dopamine D2 receptor (D2R), expressed in human and rodent islets. In contrast to $^{111}\text{In}$-exendin this tracer was not previously used for beta cell imaging and quantification. Unfortunately, $^{123}\text{IBZM}$ shows also uptake in the exocrine pancreas and is therefore not suitable for BCM quantification in the pancreas. However, with islet transplantation islets are separated from the exocrine tissue and therefore this tracer might be suitable for BCM quantification of transplanted islets. In vitro experiments showed specific tracer uptake in isolated islets. SPECT images showed clear uptake of transplanted islets and more importantly a linear correlation between transplant size and SPECT signal was observed. Furthermore, the transplanted islets could be followed successfully over time.
Next to quantification of the BCM in the muscle, these tracers can potentially be used for BCM quantification of islets transplanted at different sites. However, the tracers might not be suitable to use at all the different transplantation sites. Theoretically $^{111}\text{In}$-exendin could be used to quantify the BCM of islets transplanted in the liver, which is the current method for islet transplantation in the clinic, since the liver shows only low background uptake of $^{111}\text{In}$-exendin. However, due to dispersion of the islets throughout the whole organ, $^{111}\text{In}$-exendin uptake is spread over a larger area as compared to muscle transplantation which might complicate accurate BCM quantification. In contrast to $^{111}\text{In}$-exendin, BCM quantification in the liver with $^{123}\text{IBZM}$ is challenging, because of the relatively high liver uptake, due to its lipophilicity. High unspecific liver uptake doesn’t render BCM quantification impossible, but definitely complicates the quantification. Recently, a study was published using $^{11}\text{C}$-HTP, a precursor of serotonin, which also shows high liver uptake (5). In this study they applied a strategy to subtract the expected physiological background plus two standard deviations and analyzed only the areas with a signal above this threshold. With this method they were able to show a correlation between hepatic $^{11}\text{C}$-HTP uptake and transplanted islet mass.

Although $^{111}\text{In}$-exendin might be suitable to quantify the BCM of islets transplanted in the liver, this tracer cannot be used for quantification of islets transplanted under the kidney capsule, which is a frequently used transplantation site in rodent studies. The high non-receptor mediated kidney accumulation of $^{111}\text{In}$-exendin obscures the uptake in the islets transplanted under the kidney capsule and thereby hampers quantification of the BCM with $^{111}\text{In}$-exendin. Theoretically both $^{111}\text{In}$-exendin and $^{123}\text{IBZM}$ can be used to quantify the BCM at other transplantation sites; however, the data should be interpreted carefully, because the transplantation site could influence the reliability of the BCM quantification.

Translation of these tracers, for beta cell imaging and quantification, to the clinic should be relatively easy as both tracers are currently used in the clinic. However, in clinical practice a PET tracer is preferred over a SPECT tracer, because of the higher sensitivity and spatial resolution of clinical PET systems. Recently, the first imaging sessions of the native beta cells with the PET tracer $^{68}\text{Ga}$-exendin were performed in a clinical trial (data not published), but this tracer can also be used for imaging of transplanted islets. A PET tracer which could be used as alternative for $^{123}\text{IBZM}$ is the radiolabeled D2 receptor antagonist $[^{18}\text{F}]$allypride. This tracer is extensively used in clinical neuroimaging studies (6, 7) and is also used for preclinical imaging of beta cells (8, 9).
Before these tracers can be used in the clinic, the tracers should be extensively characterized in pathological conditions like inflammation, glucotoxicity, lipotoxicity, etcetera. These pathological conditions might change receptor expression and/or tracer uptake and thereby influence the measured BCM without an actual change in BCM. Potentially, both receptors and/or tracers would perform differently under these conditions and in this situation the combination of both tracers would provide information on the BCM but also on the metabolic state of the transplanted islets. Another factor that should be taken into account is the possibility that the tracer cannot reliably be used immediately after transplantation, which is the case for \(^{111}\text{In}\)-exendin in the muscle transplantation model. Due to disruption of the vasculature the radiotracer cannot reach the islets the first period after transplantation. A recent study by Eter et al (10) showed a steady increase in \(^{111}\text{In}\)-exendin signal in the first three weeks after islet transplantation. After this period the signal stabilized and the BCM could be measured reliably. The time after transplantation when a tracer could reliably be used for BCM quantification could be different for other transplantation sites, for example because of the closer vicinity to bloodvessels (e.g. intraportal infusion in liver). Pre-labeling of islets with iron oxide contrast agents (11, 12) would provide the possibility to follow islets immediately after transplantation with MRI. Since the contrast agent is already at the target site (in the islets), no intact vasculature is required to visualize the islets. However, it remains a matter of debate to which degree the presence of iron oxide contrast agents only shows viable and functional islets, or if it would also visualize the presence of dead islets.

In summary, both \(^{111}\text{In}\)-exendin and \(^{123}\text{IBZM}\) hold great potential to provide real time quantitative information on the BCM and allow long term follow-up after islet transplantation with a relatively easy translation to the clinic. Furthermore, they could be a useful tool to evaluate therapeutic interventions to improve transplantation outcome. However, the tracers should first be further characterized to acquire more knowledge on the expression of the targeted receptors during pathological conditions. Furthermore, BCM quantification and interpretation of the data should be done carefully as the transplantation site, revascularization after transplantation, and pathological conditions might influence the tracer uptake.

As mentioned before \(^{111}\text{In}\)-exendin was already successfully applied for BCM quantification in the pancreas (1). However, only a small part of the pancreas could be quantified, due to the high uptake of \(^{111}\text{In}\)-exendin in the kidneys. Furthermore, drawing a region of interest in the pancreas was complicated in animals with low BCM due to the low uptake of \(^{111}\text{In}\)-exendin in the pancreas and the pancreas of rodents is not clearly visible on CT images. The use of the exocrine tracer \(^{99m}\text{Tc}\)-demobesin-4 in
combination with a unilateral nephrectomy allowed quantification of the $^{111}$In-exendin uptake in a larger area of the pancreas. This makes the quantification less susceptible to inter-individual differences when drawing a region of interest in the pancreas and proved to be a good model for quantification of the BCM with $^{111}$In-exendin in rodents. This model should be used to further characterize $^{111}$In-exendin as a BCM marker in diabetic rodent models, but could also be used to investigate the effect of metabolic stress (e.g. glycotoxicity, lipotoxicity, cytokines) on GLP-1 receptor expression. For the most accurate and reliable results these studies should be performed in a rat model, because the uptake of $^{111}$In-exendin in rats resembles uptake in humans more closely as compared to mice. Mice with low BCM still have a considerable uptake of $^{111}$In-exendin in the exocrine tissue that can be blocked by an excess of cold exendin, although the GLP-1R expression is extremely low in the exocrine pancreas (13). In the future, the integration of MRI scanners in radionuclide imaging systems (PET/SPECT) (14) could replace the need for an exocrine tracer. The excellent soft tissue contrast in MRI would allow for accurate delineation of the pancreatic tissue. The combination of this high spatial resolution of the MRI system and the high sensitivity of the SPECT and PET systems could help to further elucidate the role of BCM during the development and after onset of diabetes. The use of an exocrine tracer will not be necessary in a clinical setting because the pancreas morphology in humans is more defined than in rodents and therefore better visible on CT images. In a clinical setting the CT images could be used to delineate the pancreatic tissue and quantify the uptake of the BCM marker within this volume of interest.

Already since the first studies with radiolabeled exendin questions were raised about the effect of ionizing radiation on the islets. The accumulation of radioactivity in the islets might lead to radiation induced damage which is potentially a large problem since islets cannot regenerate, and islet damage and death might result in diabetes. Previously some attempts were made to estimate the islet dose after administration of radiolabeled exendin, but in these studies the activity was assumed to be homogeneously distributed throughout the pancreas. However, only 1-2% of the pancreatic tissue consists of islets and the activity concentration in the islets is much higher than in the exocrine tissue. Therefore, these studies largely underestimate the absorbed dose in the islets. To be able to calculate more accurate islet absorbed doses due to the administration of radiolabeled exendin the islets were implemented as a separate compartment in the model and whole organ and small scale (μm-level) dosimetry was combined. As input for the model SPECT images of a clinical study, autoradiography and biodistribution data from a preclinical study were combined. With this model the islet doses were not only calculated for small and large islets, but
also kidney activity, percentage of islets in the pancreas, gender and radionuclide were varied to simulate various situations. In all simulated examples the islet absorbed dose was low (<70 mGy) and much lower than the dose shown to increase the risk in developing T1D, so even repeated imaging with radiolabeled exendin will hardly increase the risk on diabetes. The current model can be used to optimize the imaging protocol and investigate the effect of using a different radionuclide or a different administered activity and thereby minimizing the radiation exposure of the patient. In the future the dosimetric model can be refined by adding more patient data as input and replacing the preclinical input with clinical data, which will result in a more realistic dosimetric model. Replacement of the preclinical data requires information on the difference in exendin uptake between islets and exocrine tissue in humans. This information could be obtained from patients who are injected with radiolabeled exendin before they will undergo a pancreatectomy. This study is currently in preparation and will serve as a final validation for the use of radiolabeled exendin as BCM marker in a clinical setting. These data can also be used to provide crucial information on the endocrine/exocrine uptake ratio in humans which can be used to replace the preclinical data in the dosimetric model. The dosimetric model can also be adapted to calculate the absorbed dose as a result of a different beta cell tracer, for example $^{11}$C-HTP (15).

In this thesis we showed the great potential of $^{111}$In-exendin and $^{123}$IBZM, for BCM quantification of islets transplanted in the muscle and providing real-time information on the BCM. These tracers could provide crucial information on the relation between BCM and function over time, not only in a preclinical setting but potentially also in clinical trials. Although the results are promising, application of these tracers in different transplantation models should be done carefully and before translation to the clinic, both tracers should be further characterized to investigate the influence of pathological conditions on receptor expression. It might be possible that both tracers perform differently under certain pathological conditions and in this situation the use of both tracers could provide complementary information on the beta cells.

The exocrine tracer $^{99m}$Tc-demobesin in combination with a unilateral nephrectomy proved to be a robust model in rodents for BCM quantification with $^{111}$In-exendin which should be used to further characterize the this tracer in rodents. In the future the use of $^{99m}$Tc-demobesin in a preclinical setting might become expandable with the integration of MRI scanners in radionuclide imaging systems (PET/SPECT).

Based on the islet absorbed dose calculations, both $^{111}$In-exendin and $^{68}$Ga-exendin can safely be used for repeated imaging of patients. Furthermore, the dosimetric model can be used as a tool to select the optimal radionuclide for the procedure (i.e.
imaging or therapy) and could be adjusted to calculate the absorbed dose as a result of different beta cell tracers.

After further characterization the tracers described in this thesis could play an important role in further elucidating the changes in beta cell mass in diabetes and after transplantation. Additionally, the dosimetry model could help to select an optimal radionuclide and tracer for beta cell imaging, to minimize the absorbed dose to islets and kidneys.

REFERENCES

Summary

Diabetes mellitus is characterized by high glucose levels and the incapacity of the body to regulate this. In type 1 diabetes the insulin producing beta cells are destructed and therefore the body is not able to produce enough insulin. In type 2 diabetes the body does not respond properly to the produced insulin and loss of beta cell function often occurs. In the end stage of both types of diabetes significant amounts of beta cells are lost, however, the exact relation between the beta cell mass (BCM) and the onset and progression of diabetes is not known. Beta cell function can be measured with glucose, insulin and HbA\text{1c} measurements, but these tests do not provide information on the BCM.

Non-invasive imaging methods to monitor the BCM during the progression of the disease would not only provide crucial information about the role of BCM in diabetes but would also help to evaluate the effect of new therapies. A non-invasive imaging method could also be used to monitor islets after transplantation. Islet transplantation is a promising therapeutic option for type 1 diabetic patients with poor glycemic control. This method is initially successful; however, long-term transplantation outcome is poor. Metabolic stress and recurrence of autoimmunity cause a large number of the transplanted islets to die. The most promising approach to visualize the beta cells non-invasively is with the highly sensitive radionuclide imaging methods.

In this thesis two potential radiotracers to quantify the BCM both in the native pancreas and after islet transplantation are characterized. Furthermore, the potential of an exocrine pancreatic tracer to simplify quantification of beta cell specific radiotracers was investigated. Finally, the potential damage to the islets due to accumulation of one of the characterized radiotracers is investigated.
In **chapter 1** the specific requirements for a beta cell tracer are described and several potential tracers for beta cell imaging are discussed. Some of these tracers are already tested in a clinical setting. The first beta cell tracer to be tested in a clinical study was radiolabeled dihydrotetrabenazine (DTBZ). Although differences between healthy and diabetic subjects were observed with this tracer there is some discussion about the beta cell specificity of this tracer. More recently radiolabeled exendin and 5-HTP were tested in clinical studies. Both tracers show a difference in uptake between healthy and diabetic subjects and hold great potential for BCM quantification.

In the second part of chapter 1 the basics of dosimetry are explained. Dosimetry can be used to assess the risk of radiation induced damage to healthy tissues due to the use of ionizing radiation involved in radionuclide imaging.

In **chapter 2** the potential of $^{111}$In-exendin, one of the most promising tracers for BCM quantification, to visualize islets transplanted in the calf muscle was investigated. *In vitro*, a linear correlation between the number of islets and the $^{111}$In-exendin uptake was observed. SPECT/CT images acquired four weeks after transplantation showed a significant difference in SPECT signal between 400 and 800 transplanted islets in the calf muscle of Brown Norway rats. Histological analysis of the transplants showed excellent co-localization between $^{111}$In-exendin uptake, GLP-1R and insulin staining. The true potential of this tracer to quantify the BCM with *in vivo* SPECT imaging in islet transplants was investigated in a C3H mouse model in **chapter 3**. C3H mice were transplanted with 50, 100, 200, 400 or 800 islets. Four weeks after transplantation animals were injected with $^{111}$In-exendin and SPECT images were acquired. An excellent correlation was observed between SPECT quantification of $^{111}$In-exendin uptake and the graft volume, determined histologically (Pearson r=0.87). During this study it was possible to observe as little as 50 islets with SPECT imaging. These results show that $^{111}$In-exendin is a promising and sensitive tracer to quantify the BCM of transplanted islets.

The potential of an alternative tracer, radioiodinated iodobenzamide (IBZM), for visualization of transplanted islets was investigated in **chapter 4**. IBZM binds to the dopamine 2 (D2) receptor expressed on the beta cells. Radioiodinated IBZM showed specific binding to both INS-1 cells and isolated islets. Furthermore 1,000 islets transplanted in the calf muscle were visible with SPECT imaging 6 weeks after transplantation. Immunohistochemical analysis of the graft showed insulin and glucagon expression, indicating viable and functional islets.
In chapter 5 the potential of IBZM to quantify the BCM of transplanted islets was investigated. WAG/Rij rats were transplanted with 1,000, 2,000 or 3,000 islets and SPECT images were acquired 6 weeks after transplantation. Quantification of the SPECT signal in the transplant correlated linearly with the transplant volume (Pearson r=0.73). Furthermore, it was possible to follow the transplant over time. These results demonstrate that IBZM can be successfully applied for non-invasive, longitudinal and quantitative monitoring of transplanted islets. Unfortunately, this tracer is not suitable for the visualization of the islets in the native pancreas, due to a relatively high uptake in the exocrine pancreas.

As shown in chapter 2 and 3, $^{111}$In-exendin shows great potential for BCM quantification in a transplantation setting. However, BCM quantification in the native pancreas is more complicated due to dispersion of the signal over a larger area, especially in animals with low BCM. Since the pancreas of rodents is also not visible on CT images, it is difficult to delineate pancreatic tissue in these animals with low BCM. Therefore, a tracer accurately visualizing the entire pancreas would help to correctly delineate the pancreas and thereby improving BCM quantification. In chapter 6 the exocrine tracer $^{99m}$Tc-demobesin was evaluated. This tracer showed high accumulation in the pancreas of healthy animals and remained high after alloxan treatment, while the $^{111}$In-exendin uptake was reduced by more than 50%. Quantification of the $^{111}$In-exendin signal in a volume of interest based on the $^{99m}$Tc-demobesin uptake resulted in an excellent correlation (Pearson r=0.92) between $^{111}$In-exendin uptake determined in the gamma counter and $^{111}$In-exendin uptake determined from SPECT analysis. The use of $^{99m}$Tc-demobesin allowed quantification of $^{111}$In-exendin uptake in a larger region of the pancreas compared to studies were only $^{111}$In-exendin was used, resulting in a more robust quantification of the BCM.

The high accumulation of radiolabeled exendin in the islets of Langerhans might result in tissue damage. Since the uptake of exendin is much higher in the islets of Langerhans as in the rest of the pancreatic tissue the islet absorbed dose will be higher than the average pancreatic absorbed dose. Current models were not able to calculate the islet absorbed dose. In chapter 7 different dosimetry techniques were used to calculate the absorbed islet dose. Both SPECT scans from a clinical study and biodistribution and autoradiography data from a preclinical study were used to calculate the islet absorbed dose due to the use of radiolabeled exendin. Islet absorbed doses were not only calculated for small and large islets, but also kidney activity, percentage of islets in the pancreas, gender and radionuclide were varied to simulate different situations. The calculated islet absorbed dose was small in all cases.
(maximum 1.38 mGy for $^{68}$Ga and 66 mGy for $^{111}$In) and indicates that even repeated exendin imaging will hardly increase the risk on diabetes.

Both exendin and IBZM can be used for quantification of the BCM of transplanted islets. However, due to uptake of IBZM in the exocrine part of the pancreas, this tracer cannot be used for quantification of islets in the native pancreas. Quantification of the BCM in the native pancreas with $^{111}$In-exendin is possible, but difficult in animals with a low BCM. This quantification can be simplified by using the exocrine tracer $^{99m}$Tc-demobesin for delineation of the pancreatic tissue. The absorbed islet dose due to the use of radiolabeled exendin was also investigated. The calculated islet dose was much lower than the dose known to induce islet damage. Radiolabeled exendin can safely be used to calculate the BCM.
De insuline producerende cellen in het pancreas, de beta cellen, spelen een belangrijke rol in het reguleren van de glucose spiegels in het bloed. Na een maaltijd wordt glucose opgenomen in het bloed en geven de beta cellen insuline af om ervoor te zorgen dat de bloedglucose spiegel binnen de normale waarden blijft. Mensen met diabetes zijn niet in staat om hun bloedglucose spiegels te reguleren en hebben vaak hoge bloedglucose waarden. Er zijn twee types van diabetes: In type 1 diabetes worden de beta cellen vernietigd door een autoimmune respons, waardoor het lichaam niet meer in staat om genoeg insuline te produceren. In type 2 diabetes reageert het lichaam niet goed op de geproduceerde insuline en kunnen de beta cellen niet compenseren voor de grotere vraag aan insuline. De functie van deze beta cellen kunnen we bepalen door middel van metingen van insuline, glucose, HbA1c in het bloed of een glucose tolerantie test. Met deze methoden is het niet mogelijk om de hoeveelheid beta cellen (beta cel massa (BCM)) te bepalen en op dit moment zijn er geen methoden beschikbaar om de BCM te meten. De beschikbare informatie over de (veranderingen in) BCM is afkomstig van autopsie studies, maar in deze studies zijn niet de veranderingen in de hoeveelheid beta cellen in hetzelfde individu te meten. Hierdoor weten we niet de exacte relatie tussen de veranderingen in BCM en het ontstaan van diabetes. Daarom is er behoefte om een niet-invasieve methode te ontwikkelen om de BCM te meten. Zo’n methode kan ons niet alleen meer inzicht geven in de rol van de BCM in diabetes, maar kan ook gebruikt worden om te kijken naar het effect van nieuwe therapieën.

Eiland transplantatie is een veelbelovende therapie voor mensen met type 1 diabetes die hun bloed glucose spiegels slecht onder controle hebben. De eerste jaren na de transplantatie zijn de meeste patiënten onafhankelijk van insuline, maar enkele jaren na transplantatie moeten deze mensen toch weer insuline gaan gebruiken. Een methode om de BCM te meten zou meer inzicht kunnen geven in de processen na transplantatie die invloed kunnen hebben op de hoeveelheid beta cellen die nog
aanwezig zijn na transplantatie. Een potentiële methode om de beta cellen niet invasief in beeld te brengen zijn de zeer gevoelige beeldvormende technieken PET en SPECT na injectie van een beta cel specifieke radiogelabelde tracer.

In dit proefschrift worden radiotracers beschreven die gebruikt kunnen worden om de BCM te bepalen van zowel getransplanteerde eilandjes als in het pancreas. Verder hebben we ook bepaald of het gebruik van ioniserende straling voor beeldvormende doeleinden zorgt voor schade aan de eilandjes van Langerhans.

In hoofdstuk 1 is beschreven waar potentiele tracers voor bepaling van de BCM aan moeten voldoen. De beta cellen zitten in de eilandjes van Langerhans. Deze eilandjes van Langerhans hebben een diameter tussen de 50-400 µm en liggen verspreid in het pancreas. Om de BCM nauwkeurig te kunnen bepalen, moet een potentiele tracer specifiek zijn voor de beta cellen en dus niet binden aan ander pancreas weefsel. Verder moet de tracer geoptimaliseerd en gekarakteriseerd worden zodat een zo hoog mogelijk contrast in signaal tussen de beta cellen en het andere weefsel bereikt wordt. Factoren die geoptimaliseerd moeten worden zijn dosis van het toegediende peptide, tijdstip van scannen en de hoeveelheid toegediende radioactiviteit.

De afgelopen jaren zijn er al verschillende pogingen gedaan om de BCM te kwantificeren met radiotracers, de belangrijkste tracers zijn besproken in dit hoofdstuk. Van deze besproken tracers zijn er inmiddels drie gebruikt in klinische studies: radiogelabeld exendin, \( ^{11} \)C-HTP en radiogelabeled DTBZ.

In het tweede deel van dit hoofdstuk is de basis van dosimetrie uitgelegd. Dosimetrie kan gebruikt worden om een inschatting te maken van de kans op schade aan gezonde weefsels door de ioniserende straling van radiotracers. Om de stralingsdosis te berekenen is het noodzakelijk om te weten hoe de radiotracers zich door het lichaam verdelen.

In hoofdstuk 2 en hoofdstuk 3 is gekeken of \( ^{111} \)In-exendin gebruikt kan worden om getransplanteerde eilandjes in beeld te brengen en de ophoping van \( ^{111} \)In-exendin in de eilandjes te kwantificeren. \( ^{111} \)In-exendin bindt aan de glucagon-like-peptide-1 receptor (GLP-1R) die in het pancreas alleen tot expressie komt op de beta cellen. In vitro testen laten een lineaire relatie zien tussen het aantal eilandjes en de hoeveelheid gebonden \( ^{111} \)In-exendin. Deze binding aan de eilandjes is specifiek, omdat een overmaat van ongelabeled exendin de binding van \( ^{111} \)In-exendin aan de eilandjes blokkeert. Na deze succesvolle in vitro resultaten zijn WAG/Rij ratten getransplanteerd met 400 of 800 eilandjes en vier weken na transplantatie was er op
SAMENVATTING

SPECT/CT scans een significant verschil in $^{111}$In-exendin opname te zien in het transplantaat tussen deze twee groepen. Histologische analyse laat verder zien dat de opname van $^{111}$In-exendin overeenkomt met de hoeveelheid beta cellen bepaald door insuline kleuring en de receptor expressie aangetoond door GLP-1R kleuring. Vervolgens hebben we in hoofdstuk 3 bepaald of het mogelijk is om de BCM te kwantificeren met behulp van $^{111}$In-exendin SPECT. In deze studie zijn verschillende aantallen eilandjes getransplanteerd in de kuitspier van C3H muizen. Vier weken na transplantatie zijn SPECT/CT scans gemaakt en is de opname in het transplantaat gekwantificeerd. Verder is de grootte van het transplantaat bepaald op histologische coupes. Het uitzetten van de SPECT opname tegen de grootte van het transplantaat resulteerde in een sterke positieve correlatie (Pearson r=0.87). Tijdens deze studie was het mogelijk om slechts 50 eilandjes te visualiseren. Aan de hand van deze resultaten kunnen we concluderen dat $^{111}$In-exendin gebruikt kan worden om de BCM van getransplanteerde eilandjes te kwantificeren.

In hoofdstuk 4 en hoofdstuk 5 hebben we gekeken of een andere tracer, iodobenzamide (IBZM), gebruikt kan worden om getransplanteerde eilandjes in beeld te brengen en te kwantificeren. IBZM bindt aan de dopamine 2 (D2) receptor die tot expressie komt op de beta cellen. Met in vitro experimenten is aangetoond dat IBZM bindt aan de eilandjes. Ook waren 1.000 getransplanteerde eilandjes van Langerhans getransplanteerd in de kuitspier van WAG/Rij ratten zichtbaar op SPECT/CT beelden zes weken na transplantatie. In hoofdstuk 5 zijn WAG/Rij ratten getransplanteerd met verschillende hoeveelheden eilandjes. Kwantificatie van het SPECT signaal, zes weken na transplantatie liet een linear verband zien met de grootte van het transplantaat (bepaald op basis van histologie). Ook was het mogelijk om het transplantaat zichtbaar te maken op verschillende tijdstippen na transplantatie. Uit deze resultaten blijkt dat IBZM gebruikt kan worden om de BCM van getransplanteerde eilandjes te kwantificeren.

Zoals we in hoofdstuk 2 en hoofdstuk 3 bepaald hebben kan $^{111}$In-exendin gebruikt worden om de BCM van getransplanteerde eilandjes te kwantificeren. Deze tracer kan ook gebruikt worden om de eilandjes in het pancreas te kwantificeren. Het is alleen gebleken dat kwantificatie van het $^{111}$In-exendin signaal in het pancreas van ratten en muizen lastig is bij een lage BCM.

In hoofdstuk 6 hebben we onderzocht of $^{99m}$Tc-demobesin, een tracer die stapelt in het exocrine deel van het pancreas, het makkelijker maakt om de BCM in het pancreas te kwantificeren. $^{99m}$Tc-demobesin laat een hoge opname in het pancreas zien in zowel dieren met een hoge BCM als dieren die een lage BCM hebben. De opname van
$^{99m}$Tc-demobesin in het pancreas is gebruikt om de locatie van het pancreas op de SPECT beelden te bepalen. Vervolgens is het $^{111}$In-exendin signaal in dit gebied gekwantificeerd en uitgezet tegen $^{111}$In-exendin opname bepaald in de gamma counter, wat resulteerde in een lineaire correlatie (Pearson r=0.92). Doordat $^{99m}$Tc-demobesin een hoge opname in het pancreas heeft is het mogelijk om de $^{111}$In-exendin opname in een groter gebied te bepalen dan wanneer je alleen $^{111}$In-exendin gebruikt, wat zorgt voor een meer betrouwbare kwantificatie van de BCM. De exocrine tracer $^{99m}$Tc-demobesin zorgt ervoor dat de BCM makkelijk en objectief bepaald kan worden.

Radioactiviteit kan schade aan weefsel veroorzaken en de hoeveelheid radioactiviteit (dosis) is van invloed op de mate van schade aan de weefsels. In hoofdstuk 7 is gekeken of de accumulatie van radioactief gelabeld exendin schade aan de eilandjes van Langerhans kan veroorzaken. Doordat de accumulatie van exendin niet homogeen is in het pancreas, was het niet mogelijk om de geabsorbeerde dosis in de eilandjes met standaard modellen te berekenen, omdat deze modellen ontwikkeld zijn voor grote organen met een homogene verdeling van de radioactiviteit. Het gebruik van deze modellen voor het berekenen van de dosis op de eilandjes van Langerhans zou tot een onderschatting van de dosis leiden. In dit hoofdstuk hebben we SPECT beelden van een klinische studie en biodistributie en autoradiografie data van een preklinische studie gebruikt om de geabsorbeerde dosis in de eilandjes van Langerhans te berekenen. We hebben de eiland dosis berekend voor kleine en grote eilandjes, maar we hebben ook de opname in de nieren, het percentage eilandjes in het pancreas, het geslacht en het gebruikte radionuclide gevarieerd. In al deze situaties was de berekende eiland dosis veel lager dan de dosis die een verhoogd risico geeft op het ontwikkelen van diabetes type 1 door blootstelling aan ioniserende straling (>10 Gy). Deze studie suggereert dat meerdere scans met radioactief gelabeld exendin in hetzelfde individu gemaakt zouden kunnen worden.

In dit proefschrift zijn verschillende tracers gekarakteriseerd voor nauwkeurige bepaling van de BCM. Zowel $^{123}$IBZM als $^{111}$In-exendin zijn veelbelovende tracers om de BCM te bepalen na transplantatie van eilandjes van Langerhans. Door hoge opname van IBZM in het exocriene deel van het pancreas is het niet mogelijk om deze tracer te gebruiken voor BCM bepalingen in het pancreas. $^{111}$In-exendin heeft geen hoge opname in de exocrine cellen in het pancreas en kan daardoor ook gebruikt worden voor het kwantificeren van de eilandjes in het pancreas. De exocriene tracer, $^{99m}$Tc-demobesin, kan gebruikt worden om het pancreas duidelijk zichtbaar te maken in SPECT beelden, waardoor het makkelijker wordt om het $^{111}$In-exendin signaal te kwantificeren in dieren met een lage BCM. Verder hebben we de geabsorbeerde dosis
in de eilandjes bepaald wanneer gebruik wordt gemaakt van radiogelabeld exendin. De geabsorbeerde dosis in de eilandjes was veel lager dan de dosis die een verhoogde kans op het ontwikkelen van diabetes type 1 geeft. Radioactief gelabeld exendin kan dus veilig gebruikt worden om de BCM over tijd te bepalen.
List of publications


Willekens SMA, van der Kroon I*, Bos D, Joosten L, Nock BA, Maina T, Boerman OC, Brom M, Gotthardt M. Improved quantification of the beta cell mass after pancreas visualization with $^{99m}$Tc-demobesin-4 and beta cell imaging with $^{111}$In-exendin-3 in rodents. Mol Pharm. 2016;13:3478-3483


Dankwoord

Zoals bij wel meer mensen verliep ook mijn promotietraject alles behalve zonder problemen. Vooral in het begin liep het erg moeizaam en zonder de steun van een heleboel mensen, was dit proefschrift nooit tot stand gekomen.

Ten eerste wil ik mijn promotor Martin bedanken voor zijn creatieve en soms wilde ideeën, enthousiasme en zijn vertrouwen in mijn schrijfkunsten. De vrijheid die ik kreeg tijdens mijn promotie was erg fijn, al had ik in het begin van mijn promotie misschien af en toe wat extra sturing kunnen gebruiken. Martin, dankjewel voor de mogelijkheid om bij jou te promoveren en mezelf te ontwikkelen.

Waar Martin vaak met ideeën aan kwam zetten kon ik bij Maarten altijd terecht met alle mogelijke vragen die ik had. Maarten, ook al was je ontzettend druk, je maakte altijd tijd om te helpen. En tijdens de laatste fase van mijn promotie kon ik nog steeds met al mijn vragen bij jou terecht. Ik kan dan ook wel zeggen dat zonder jouw hulp de laatste twee papers zeker niet zo snel de deur uit gekomen waren.

Naast Martin en Maarten is er nog een persoon die een heel belangrijke rol tijdens mijn promotie heeft gespeeld. Stefanie, doordat onze promotie trajecten zo dicht bij elkaar lagen hebben wij heel wat uurtjes samen doorgebracht, in het lab, op PRIME voor onze talloze eiland isolaties, of gewoon achter onze computers. We hebben samen alle ups and downs van onze promoties meegemaakt. Doordat we precies van elkaar wisten waar we mee bezig waren konden we elkaar altijd om hulp vragen, ideeën bespreken, onze frustraties ventileren, maar was het bovenal gezellig. Helaas kon ik er op jouw promotie niet bij zijn, maar gelukkig ben jij er wel bij als paranimf op mijn promotie.
DANKWOORD

Daarnaast waren alle grote dierenexperimenten, vooral de enorme hoeveelheid eiland isolaties niet mogelijk geweest zonder de hulp van Lieke, Desiree, Cathelijne en Hanneke. In mijn laatste paar maanden in Nijmegen heb ik samen met Lieke in sneltrein vaart een project gedaan wat geresulteerd heeft in een mooie publicatie (hoofdstuk 6). Ik ben heel blij dat ook jij vandaag aan mijn zijde staat als paranimf.

Wietske, dankjewel dat je me bij het dosimetry project betrokken hebt. Ik heb met veel plezier aan dit project gewerkt. We wisten precies waar we naar toe wilden en we hadden dan ook binnen no time de eerste draft versie van de paper af, al liet de uiteindelijke publicatie wat langer op zich wachten. Gingen alle projecten maar zo!

Otto ook al was je niet heel veel betrokken bij mijn promotie, jouw inbreng in een aantal papers heeft ervoor gezorgd dat stukken enorm verbeterd zijn en daarnaast zijn jouw positiviteit en enthousiasme van groot belang voor de goede werksfeer op de afdeling.

Eric dankjewel voor je hulp en bijdrage aan de dosimetry paper. Als er een versie van de paper jouw kant op ging dan wist ik zeker dat alles grondig gelezen werd, en doordat je ook alles wilt begrijpen werden we gedwongen nog eens goed na te denken over bepaalde keuzes die we gemaakt hadden.

Mark, zonder jouw hulp was de dosimetry paper zeker niet zo volledig geworden als hij nu is, jij had de perfecte aanvulling op de kennis die in Nijmegen ontbrak.

Thea Maina and Berthold Nock, although you were only involved in the last project of my PhD project, I really appreciated your quick and useful replies on the paper in chapter 6.

Natuurlijk was er tijdens mijn promotie ook genoeg tijd voor gezelligheid met mijn kamergenootjes: Charlotte, Kristell, Maarten, Marlène, Peter, Rafke, Sandra, Selen, Stefanie, Stijn, Susanne, Tessa, Tom, Waël, Wietske en Willem, de andere mensen van beta image groep, en de nucleaire researchgroep. Samen lunchen, borrelen en natuurlijk de snoeppot leeg eten (banaantjes...).

Mijke, dankzij jou werden de tripjes tussen Eindhoven en Nijmegen in mijn laatste jaar een stuk leuker, plannen maken voor ons scaffold project, waar helaas niets concreets uit gekomen is, even wat dingen nabespreken over het werk of onze weekendplannen bespreken.
DANKWOORD

Tom, in mijn laatste maanden in Nijmegen kwam jij nog een project bij mij doen, qua timing was dit niet ideaal, omdat ik het grootste deel van jouw stage in India zat, maar je hebt geweldig zelfstandig gewerkt met een mooi verslag als resultaat. Inmiddels ben je zelf ook bezig met je eigen promotie onderzoek, heel veel succes!

Het was niet mogelijk geweest om de verschillende projecten af te ronden zonder de hulp van de medewerkers van de Urologie, Pathologie (dankjewel voor het snijden van de duizenden coupes!) en natuurlijk niet te vergeten de mensen op PRIME. Bianca, Iris, Kitty, Henk, Nicole dankjewel voor het meedenken als een project niet liep zoals verwacht, jullie flexibiliteit en het injecteren.

Naast de mensen op het werk zijn er natuurlijk ook nog een hoop andere mensen buiten het werk die de afgelopen jaren een belangrijke rol hebben gespeeld en met wie heel wat gezellige uurten zijn doorgebracht.

Ten eerste mijn studie vriendinnetjes Anne, Ariane, Rieneke, Sophie en Stephanie. Inmiddels hebben we elkaar al meer dan 10 jaar geleden leren kennen en in die tijd zijn er al heel wat promoties, bruiloften en baby’s voorbij gekomen. Ondanks dat we niet meer allemaal bij elkaar in de buurt wonen blijft het leuk om af te spreken en ik hoop dat dit nog vele jaren blijft lukken en dat er nog veel mooie herinneringen bij mogen komen.

Anniek en Renate, tijdens onze studententijd zagen we elkaar minimaal drie keer per week bij Hajrara, inmiddels zien we elkaar wat minder vaak, maar onze vriendschap is er zeker niet minder om geworden. Ook al woon ik niet meer om de hoek kan bij jullie altijd mijn ei kwijt en ik kijk altijd uit naar onze dates (met of zonder onze mannen) want die staan garant voor gezelligheid!

Chris, Miriam en Trystan, onder het genot van een lekker stuk limburgse vlaai en een kop thee wilden jullie altijd graag weten hoe het met mijn promotieonderzoek ging.

Lars en Es, liefste broer en beste schoonzus, ondanks dat onze levens zich op dit moment letterlijk aan de andere kant van de wereld afspelen is het fijn om te weten dat we altijd bij elkaar terechtkunnen. Hopelijk zien we elkaar snel weer in Curacao, Nederland of India!

Mama alles wat ik hier neerzet doet tekort aan hoe belangrijk jij bent voor mij. Je staat altijd voor me klaar, hebt een luisterend oor of opbeurend woord en geeft zoveel liefde. Ik weet dat het niet altijd makkelijk is zonder papa en met Lars en mij
aan de andere kant van wereld, maar ik ben super trots op hoe je het allemaal doet. Na vandaag hebben we er weer een mooie herinnering bij en ik weet zeker dat papa trots geweest zou zijn.

Lieve Eurion, jij bent mijn grootste steun en beste maatje. Alweer meer dan een jaar geleden zijn we begonnen aan ons grote avontuur in India en hebben we veel mooie dingen meegemaakt, met als meest bijzondere moment natuurlijk de geboorte van Tess. Ik kijk er naar uit wat de toekomst ons brengt.

Inge
Curriculum vitae

Inge van der Kroon was born on December 9, 1986 in Abcoude, the Netherlands. In 2005 she graduated from the Sint Ignatius Gymnasium in Amsterdam and moved to Eindhoven to start the bachelor Biomedical Engineering at the Eindhoven University of Technology. After obtaining her Bachelor’s degree in 2009, she started the Biomedical Engineering Master’s program, where she performed her final internship at the Biomedical NMR group resulting in her master thesis titled ‘Non-invasive measurement of mouse cardiac bioenergetics with $^{31}\text{P}$ Image Selected In Vivo Magnetic Resonance Spectroscopy (ISIS)’. During her master, she went to the Karolinska Institute in Stockholm, Sweden for a 3 month internship where she compared automatically segmented brain tissue fractions in both brain CT and MRI scans of patients from the Memory Clinic, Huddinge, Sweden. In 2011 she received her Master’s degree and in the same year she started her PhD project at the department of Radiology and Nuclear Medicine at the Radboud University Medical Center in Nijmegen, which resulted in the research presented in this thesis. During her PhD she evaluated different radiotracers for beta cell imaging.