Biomarker discovery and assessment for prediction of kidney response after ^{177}Lu-octreotate therapy

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Cover illustration by Nils Rudqvist, Emil Schüler, and Johan Spetz.

"One of the symptoms of an approaching nervous breakdown is the belief that one´s work is terribly important."

Bertrand Russell

Abstract

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Patients suffering from neuroendocrine tumors are oftentimes presented with spread disease at the time of diagnosis. Therapy using somatostatin analogs is today the only potentially curative treatment option for these patients. However, the kidneys are the dose-limiting organs in this type of therapy and the biological impact from radiopharmaceutical treatment is not fully understood. Furthermore, considering the large inter-individual variations in renal absorbed dose and toxicity, biomarkers for radiation damage would be of great significance in this type of therapy.

The aims of this project were to study the normal kidney tissue response *in vivo* in mice following ^{177}Lu and ^{177}Lu-octreotate administration, to identify potential biomarkers following ^{177}Lu exposure and evaluate their dependencies of absorbed dose, dose-rate, and time after injection, and to correlate these results with functional and morphological effects.

The injected activity ranged between 0.3 and 150 MBq following ^{177}Lu/^{177}Lu-octreotate administration and the biological effect was investigated between 15 minutes and one year after administration. Transcriptional and miRNA variations were studied using microarray analysis and protein expression was investigated using mass spectrometry. Correlations between the transcriptional and protein variations were performed with functional parameters, as determined by ^{99m}Tc-DTPA/^{99m}Tc-DMSA scintigraphy, and with the morphological effects following ^{177}Lu-octreotate administration.

The number of differentially regulated transcripts following ^{177}Lu/^{177}Lu-octreoate administration was dependent on absorbed dose, dose-rate, time after injection, and tissue (kidney cortex or medulla) investigated. No transcript was found to be differentially regulated at all exposure conditions. The most recurrently regulated genes were the *Serpina10* gene in kidney cortex, and the *Egr1*, *Pck1*, and *Hmgcs2* genes in kidney medulla. Substantial differences in response were found between ^{177}Lu-octreotate and [177]LuCl$_3$. Concerning the miRNA and protein data, a high absorbed dose-specificity was found, with few miRNAs/proteins found recurrently regulated at most exposure conditions.

The transcriptional analyses showed a strong and diverse transcriptional response and the functional analyses revealed clear negative effects on renal function, with enhanced negative effects with absorbed dose and time after administration. Several potentially useful biomarkers were detected at the transcriptional level, markers with potential applicability in early prediction of late renal injury after ^{177}Lu/^{177}Lu-octreotate exposure.

Populärvetenskaplig sammanfattning

De tre vanligaste behandlingsformerna för cancer är idag kirurgi, kemoterapi och extern strålbehandling. Trots att dessa metoder kontinuerligt har förbättrats så är behandlingen av spridd sjukdom (flera metastaser i annan del av kroppen än ursprungstumören) fortfarande den största utmaningen inom cancerterapi. Kirurgi och extern strålbehandling lämpar sig inte för behandling av spridd tumörsjukdom samtidigt som det idag är svårt att bota spridd sjukdom med kemoterapi.

Radionuklidterapi är en metod som har använts sedan 1940-talet. Denna metod går ut på att man administrerar ett radioaktivt ämne som tas upp i kroppen och på så vis behandlar tumören inifrån. Den mest använda radionukliden inom radionuklidterapi är radioaktivt jod. Principen för behandlingen är att man utnyttjar sköldkörtelns förmåga att ta upp och lagra jod. Genom denna behandling kan man mycket effektivt behandla tumörer i sköldkörteln, men kan även användas vid andra sjukdomstillstånd såsom hypertyreos (överaktiv sköldkörtel) och struma (förstorad sköldkörtel).

Behandling av tumörer med radionuklider är dock begränsad då där oftast inte finns specifika upptagsvägar i tumören för rena radioaktiva nuklider. En nyare metod inom radionuklidterapi är istället användningen av tumörsökande molekyler märkta med radionuklider, även kallade radioaktiva läkemedel. Fördelen med radioaktiva läkemedel är att de kan söka upp alla tumörer i kroppen och på så vis mycket effektivt behandla metastaserad sjukdom. I detta arbete har radionukliden ^{177}Lu använts ibland tillsammans med den tumörsökande substansen octreotate. Octreotate binder till receptorer på tumörcellernas yta och lämpar sig bra för behandling av neuroendokrina tumörer då dessa ofta har flera sådana receptorer jämfört med andra vävnader och organ. ^{177}Lu bundet till octreotate (^{177}Lu-octreotate) har visat bra resultat i behandlingen av patienter med neuroendokrina tumörer.

Nackdelen med denna metod är dock att det finns risk för att njurarna tar skada av behandlingen och det är ofta njurarna som begränsar hur mycket av det radioaktiva läkemedlet man kan ge. Vid denna typ av behandling vill man att tumören tar upp en stor andel av läkemedlet, och den del som tumören inte tar upp ska lämna kroppen snabbt för att inte orsaka biverkningar. Det mesta an det radioaktiva läkemedelet lämnar kroppen via njurarna och en del stannar kvar i njurarna och kan orsaka biverkningar. Ett stort problem är också att man inte vet den totala mängd av det radioaktiva läkemedlet (stråldos) som njurarna tål innan skada uppstår. Troligen tål njurarna mer än den mängd läkemedel man ger idag. Om man kan ge patienterna större mängd av det radioaktivta läkemedlet så bör fler patienter botas. Dessutom finns stora skillnader i känslighet för strålning mellan patienter, vilket innebär att deras njurar tål olika mycket av det radioaktiva läkemedlet.

Målet med denna avhandling var att undersöka njurarnas effekter efter injektion av ^{177}Lu-octreotate. Resultaten visar att de biologiska effekterna på njurarna är mycket beroende av mängden radioaktivt läkemedlet som vi ger, men även vid vilken tidpunkt efter injektion som vi undersöker. Biverkningarna på njurfunktionen efter denna typ av behandling kommer ofta sent, vilket betyder att det kan ta lång tid innan någon uppenbar skada kan urskiljas. Resultaten visar också att biverkningarna vid högre administrerad mängd både blir kraftigare och att de uppkommer tidigare efter behandlingen. Vi har även letat efter biologiska markörer, som vi hoppas kunna använda för att i ett tidigt skede förutsäga risken för att njurarna tar skada, för att på så vis kunna optimera behandlingen individuellt för varje patient. Ett antal markörer har undersökts och studierna visade att vissa av dessa eventuellt kan förutsäga njurens svar på behandlingen.

List of papers

This doctoral thesis is based on the following five papers, which will be referred to in the text by Roman numerals:

I.   **Schüler E**., Rudqvist N., Parris T.Z., Langen B., Helou K., Forssell-Aronsson E. *Transcriptional response of kidney tissue after* ^{177}*Lu-octreotate administration in mice*. Nucl Med Biol, 41(3):238-247, 2014

II. **Schüler E**., Rudqvist N., Parris T.Z., Langen B., Spetz J., Helou K., Forssell-Aronsson E. *Time- and dose rate-related effects of internal* ^{177}*Lu exposure on gene expression in mouse kidney tissue*. Nucl Med Biol, 31(10):825-832, 2014

III. **Schüler E**., Larsson M., Parris T.Z., Johansson M.E., Helou K., Forssell-Aronsson E. *Potential biomarkers for radiation-induced renal toxicity following* ^{177}*Lu-octreotate administration in mice* (submitted)

IV. **Schüler E**., Dalmo J., Larsson M., Parris T.Z., Helou K., Forssell-Aronsson E. *Proteomic and functional analysis for the assessment of radiation induced kidney response after* ^{177}*Lu-octreotate administration in mice* (manuscript)

V.   **Schüler E**., Parris T.Z., Helou K., Forssell-Aronsson E. *Distinct microRNA expression profiles in mouse renal cortical tissue after* ^{177}*Lu-octreotate administration*. PLoS ONE 9(11):e112645, 2014

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Related presentations

1. **Schüler E**., Parris T.Z., Rudqvist N., Helou K., Forssell-Aronsson E. *Global transcriptional response on mouse kidney following internal irradiation with ^{177}Lu-octreotate.* Conference: From dosimetry to biological effects: radiobiology as guide to clinical practice in nuclear medicine. Sorrento, Italy, Nov 2011

2. **Schüler E**., Rudqvist N., Parris T.Z., Helou K., Forssell-Aronsson E. *Injection with ^{177}Lu-octreotate reveals distinct gene expression response in kidney tissue*. Society of Nuclear Medicine and Molecular Imaging Congress, Miami, USA, June 2012

3. **Schüler E**., Rudqvist N., Parris T.Z., Langen B., Helou K., Forssell-Aronsson E. *Biological effects of ^{177}Lu-octreotate therapy in mouse: in vivo normal kidney tissue response evaluated with gene expression microarray*. Radiation Research Society (RRS) Meeting, San Juan, Puerto Rico, October 2012

4. **Schüler E**., Rudqvist N., Parris T.Z., Langen B., Helou K., Forssell-Aronsson E. *Dose-rate related effects on gene expression in kidney tissue after intravenous injection of ^{177}LuCl$_3$ in mouse*. European Association of Nuclear Medicine Congress, Milan, Italy, October 2012

5. **Schüler E**., Rudqvist N., Parris T.Z., Langen B., Helou K., Forssell-Aronsson E. *Dose-rate effects in radionuclide therapy: global transcriptional regulation in kidney tissue in vivo after ^{177}LuCl$_3$ administration.* SWE-RAYS Meeting, Uppsala, Sweden, August 2013

6. **Schüler E**., Rudqvist N., Parris T.Z., Langen B., Helou K., Forssell-Aronsson E. *Dose-rate effects in radionuclide therapy: global transcriptional regulation in kidney tissue in vivo after ^{177}LuCl$_3$ administration*. Radiation Research Society (RRS) Meeting, New Orleans, USA, September 2013

7. **Schüler E**., Parris T.Z., Helou K., Forssell-Aronsson E. *Micro-RNA signature of ^{177}Lu-octreotate treatment effects in renal cortical tissue. Cancerfondens planeringsgrupp för onkologisk nuklidterapi*, Gothenburg, Sweden, November 2013

8.   **Schüler E**., Larsson M., Parris T.Z., Johansson M., Helou K., Forssell-Aronsson E. *Kidney toxicity profiles after ^{177}Lu-octreotate administration.* SWE-RAYS Meeting, Malmö, Sweden, August 2014

9.   **Schüler E**., Larsson M., Parris T.Z., Helou K., Forssell-Aronsson E. *Radiation-induced renal toxicity from ^{177}Lu-octreotate: long term effects and biomarker development.* Radiation Research Society (RRS) Meeting, Las Vegas, USA, September 2014

10. **Schüler E**., Larsson M., Parris T.Z., Helou K., Forssell-Aronsson E. *^{177}Lu-octreotate therapy and renal toxicity: novel biomarkers predicting absorbed dose.* European Association of Nuclear Medicine Congress, Gothenburg, Sweden, October 2014

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Abbreviations

^{111}In	Indium-111
^{177}Lu	Lutetium-177
^{90}Y	Yttrium-90
BASE	BioArray Software Environment
cDNA	Complementary DNA
Da	Dalton
DNA	Deoxyribonucleic acid
DOTA	Dodecanetetraacetic acid
DSB	Double strand break
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GFR	Glomerular Filtration Rate
GO	Gene Ontology
Gy	Gray
H	Hydrogen
i.v.	Intravenous
IgG	Immunoglobulin G
K	Potassium
keV	Kiloelectron volts
mRNA	Messenger-RNA
miRNA	Micro-RNA
Na	Sodium
NE	Neuroendocrine
PRRT	Peptide Receptor Radionuclide Therapy
qPCR	Quantitative polymerase chain reaction
RIN	RNA Integrity Number
RNA	Ribonucleic acid
ROI	Region-Of-Interest
SSB	Single strand break
SST	Somatostatin
SSTR	Somatostatin receptor
tRNA	Transfer-RNA

x

Background

Peptide receptor radionuclide therapy

Radiolabeled somatostatin (SST) analog-based therapy has become a novel approach for the treatment of somatostatin receptor (SSTR)-overexpressing neuroendocrine (NE) tumors. There are five different somatostatin receptor subtypes, SSTR1-5, all belonging to the G protein-coupled receptor family [1, 2]. Patients who are considered for peptide receptor radionuclide therapy (PRRT) with radiolabeled SST analogs include those with inoperable metastasized tumors, with high expression of SSTR. Since the introduction of somatostatin receptor scintigraphy using ^{111}In-DTPA-octreotide (Octreoscan®, Mallinckrodt Medical, Petten, The Netherlands) in the late 1980´s, Ocreoscan has become the golden standard in staging of SSTR positive NE tumor disease [3-6]. Thanks to promising results, including high tumor-to-normal-tissue activity and dose ratios, clinical therapy studies using ^{111}In-octreotide was later attempted with encouraging results with regard to clinical benefits and biochemical responses [3-5]. However, tumor shrinkage was rarely observed, due to the far from optimal therapeutic properties of [111]In (high photon contribution and very low electron energies) [7, 8].

Through the development of somatostatin analogs with higher receptor affinity (DOTA-octreotide and DOTA-octreotate) labeled with therapeutic radionuclides ([90]Y: high energy electron emitter, and [177]Lu: medium energy electron emitter), increased therapeutic efficacy was achieved [9]. With higher electron energies, the dependence of homogeneous SSTR expression, internalization, and proximity to the DNA was reduced, compared with [111]In (Table 1).

Successful results related to tumor regression, increased overall survival, and improved quality of life have been reported through the use of ^{177}Lu-octreotate and ^{90}Y-octreotide for patients with different types of NE tumors, with response rates of about 50% [10-14]. These results are superior compared with chemotherapy, which is often the only other treatment modality available for these patients, where response rates seldom reaches 20% [15-17].

The side effects associated with PRRT can be categorized as direct and delayed effects of radiotoxicity. The direct effects include nausea, vomiting, abdominal pain, and mild hair loss [18, 19]. These reactions are often normalized after the end of therapy and are easily treated. Hematologic effects observed after treatment include decreased blood cell count, which are also regarded as acute effects, However, this

Table 1. Physical data for ^{111}In, ^{177}Lu, and [90]Y [20]. Values in parenthesis represent yield.

Radio-nuclide	Half-life	Beta [keV]*	Conversion electron [keV]	Auger electron [keV]	Gamma [keV]	Characteristic X-ray [keV]
[111]In	2.8 d		145 (8%)	0.51 (191%)	171 (90%)	23 (24%)
			219 (5%)	2.6 (67%)	245 (94%)	23.2 (44%)
				3.2 (31%)		26.1 (4%)
				3.6 (4%)		26.1 (8%)
				19.2 (11%)		26.6 (2%)
						22.3 (5%)
[177]Lu	6.7 d	47 (12%)	48 (5%)	1.9 (19%)	113 (6%)	7.9 (1%)
		111 (9%)	102 (3%)	6.3 (5%)	208 (11%)	9 (1%)
		149 (79%)	103 (103)	8.1 (3%)		54.6 (2%)
			111 (111%)			55.8 (3%)
[90]Y	2.7 d	934 (100%)				

* Average energy

condition is usually mild and transient, and blood transfusion is seldom needed [21, 22]. The delayed side effects include toxicity of liver and kidneys. Liver toxicity is most often presented in patients with liver metastases, and the distinction between treatment effects and metastasis progression is challenging [13]. The major risk organ is instead the kidneys where primary clearance of the radiolabeled SST analogs occurs. Also, accumulation and retention occurs, which in a therapeutic setting can be dose limiting due to the relatively long effective half-life of the radiopharmaceutical in the renal tissues [23]. To provide optimal therapy and to minimize normal tissue toxicity, we need to have a better understanding of biological responses following exposure.

Radiopharmaceuticals

Several parameters, such as type of radiation emitted, range of the particle *vs.* tumor size, half-life, biokinetics, and the effect on SSTR expression are to be considered in the choice of radionuclide [7, 24]. The most used radionuclides in PRRT with somatostatin analogs are [177]Lu ($\bar{E}(e^-)$=147 keV) and [90]Y ($\bar{E}(e^-)$=934 keV) (Table 1). The effect of particle range can have significant consequences on treatment efficiency. Depending on the distribution of the radiopharmaceutical in the tumor, smaller tumors might be more efficiently treated with radionuclides emitting lower electron energies compared with higher electron energies, due to the resulting higher absorbed fraction [25, 26]. Furthermore, the particle range will probably also have

an effect on normal kidney tissue response, where higher electron energies will allow for direct irradiation of the glomerulus, the most radiosensitive subunit of the kidney [27]. In addition, the choice of somatostatin analog and the type of radionuclide bound will also affect treatment efficiency. Studies on patients have shown that the tumor/kidney absorbed dose ratio is higher with ^{111}In-DOTATOC compared to ^{111}In-DOTATATE [28], while the opposite is observed for ^{177}Lu-DOTATOC *vs.* ^{177}Lu-DOTATATE [29].

Biodistribution / dosimetry
The biodistribution of the radiopharmaceutical within the total body is of vital importance for treatment planning. The biodistribution of ^{177}Lu-octreotate has to some extent been studied in patients undergoing treatment [30, 31]. However, the most detailed knowledge comes from animal studies.

Uptake of radiolabeled somatostatin analogs in normal tissues is generally lower than in tumor tissue [30, 32-34]. The highest normal tissue uptake in patients takes place in the kidneys with a maximal kidney uptake of between 1 and 4 %IA [31, 34-38]. Furthermore, large individual variation in uptake has been observed in the kidneys after ^{177}Lu-octreotate administration, with absorbed dose per administered activity between 0.33 and 2.4 Gy/GBq (mean value 0.8 Gy/GBq) after infusion of basic amino acids [31]. In animal models, kidney uptake values of 2.2-7.7 %IA/g have been reported in neuroendocrine tumor bearing Balb/c nude mice 24h after injection of ^{177}Lu-octreotate [39-42]. In C57BL/6N mice, uptake values of 3.8-9.1 %IA/g have been reported with large variations with the amount of injected activity [43]. The generally higher uptake values in e.g. the kidneys could be due to several factors, including strain differences (where Balb/c mice are immunodeficient with lack of T-cell production) or other physiological effects that the tumor tissue might have on the systemic properties of the organism.

The biodistribution after [177]LuCl$_3$ administration has not been as extensively studied. At 24h and 7 days after injection, uptake values of 3.7 and 1.8 %IA/g have been reported (3.4 MBq) [41].

Dose limiting organs
Normal tissues set the limit for the amount of activity that can be safely administered to a patient. In most types of therapy using radiopharmaceuticals, the bone marrow is regarded as the limiting organ, which has a tolerance dose of 2.5 Gy (TD$_{5/5}$ − 5% injury probability within 5 years) and 4.5 Gy (TD$_{50/5}$ − 50% injury probability within 5 years), defined for fractionated external radiation exposure (2Gy/fraction given 5d/w) [44]. In more recently developed therapies using ^{177}Lu-octreotate and ^{90}Y-octreotide, the dose limiting organ beside the bone marrow is the kidneys, where excretion of the major part of the radionuclide occurs [37, 38]. The

renal tolerance dose according to external radiation therapy is 23 Gy ($TD_{5/5}$) and 28 Gy ($TD_{50/5}$) [45]. However, due to lower and continuous exponentially decreasing dose rates, inhomogeneous dose distribution within the organs and body, and particles of varying ionization density in radionuclide therapy compared with external irradiation, significant differences in radiobiological effects will most probably arise [24, 27], and studies on patients and animals demonstrate possibly higher tolerance doses for kidneys when ^{177}Lu-octreotate and ^{90}Y-octreotide is administered [46, 47]. However, since there is still limited knowledge of the effects of radionuclide therapy on normal tissues, tolerance doses are difficult to define.

Clinically, bone marrow toxicity (reduced leukocyte count) is often observed after ^{177}Lu-octreotate administration, with subsequent recovery within two months [48]. In early ^{90}Y-DOTATOC studies nephrotoxicity has been shown with renal function loss and end-stage renal disease [21, 27, 49, 50].

Radiation biology

While it is assumed that the genetic background of an organ or tissue has a major role in the response to radiation, the radiation effects on cells at the molecular level are still largely unknown. The genetic information for all developmental and functional processes in the cell is located in the DNA. The functional unit of the genetic information is the gene which is built up of nucleotides. Genes, i.e. the genetic information, are thereby transcribed to RNAs and then translated into proteins, the functional unit of all processes in an organism (Figure 1).

The process of transcription occurs in the nucleus and is carried out by replicating DNA into complementary RNA sequences using transcription factors and the enzyme RNA polymerase II. The initiation and stop of the gene expression is regulated by specific sequences known as promoter and terminator regions. The resulting primary mRNA then consists of coding (exons) as well as non-coding (introns) sequences. Mature messenger RNA (mRNA) is produced through RNA capping, polyadenylation, and RNA splicing.

The mRNA carries the genetic code from the DNA to the ribosomes for translation of the RNA into a protein. At the ribosomes, the mRNA molecule is read in groups of three nucleotides which correspond to a specific amino acid. The transfer RNA (tRNA) carries the different amino acids to the ribosomes in the protein synthesis process, and neighboring amino acids bind to each other by a peptide bond between the carboxyl group and the amino group of the two amino acids. The protein will then undergo modifications before the active protein is produced.
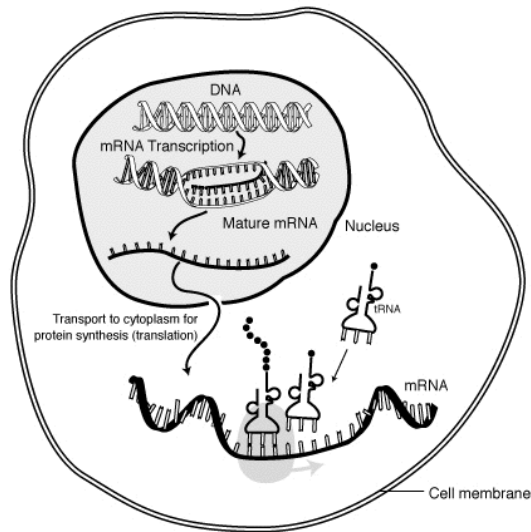
Figure 1. Schematic illustration of the central dogma of the cell. The DNA is transcribed, the pre-mRNA is spliced, and mature mRNA is produced. The mature mRNA is then transported into the cytoplasm where translation occurs in the ribosomes. Image is in the public domain and was retrieved from http://en.wikipedia.org/wiki/Messenger_RNA on Nov 9, 2014.

Epigenetics

The human genome consists of 20,000-25,000 protein coding genes. However, only a small fraction of these genes are actively translated into proteins in a specific tissue. Furthermore, protein expression differs between different organs and tissues due to both qualitative and quantitative effects in gene expression. This variation in gene expression without altering the DNA nucleotide sequence is called epigenetics and includes DNA methylation, chromatin remodeling, and various RNA mediated processes.

The process of DNA methylation (addition of a methyl group to cytosine or adenine in the DNA) and repression of gene expression occurs by allowing normal hydrogen binding. This in turn may cause the DNA molecule to be inaccessible to transcription factors and thereby gene silencing. Effects on gene expression can also be affected by cromatin remodeling through histone deacetylation, which causes changes in the histone structure and results in an increased compaction of the DNA, blocking the possiblity for RNA polymerase to bind to the DNA, thereby inducing transcriptional silencing. The process of deacetylation of lysine in the histones will increase the positive charge of the side chains of the histones, thereby increasing the strength of binding to the negatively charged DNA.

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Epigenetic regulation can also occur through RNA based mechanisms [51]. It is now well known that not all of the RNA is translated into proteins [52, 53]. Micro-RNAs (miRNA) is the most studied non-coding RNA, and their function include RNA silencing and post-transcriptional regulation of gene expression [54]. Similar to mRNA, miRNA is transcribed by RNA polymerase II and folds to a double stranded hairpin loop structure. Precursor-miRNA (pre-miRNA) is then produced via interaction with the protein DGCR8 and the enzyme Drosha and exported out of the nucleus in to the cytoplasm where the loop is cleaved by the enzyme Dicer, yielding a mature miRNA of about 22 nucleotides in length. The mature miRNA is then a part of the RNA-induced silencing complex (RISC) containing Dicer, the protein argonaute and many other associated proteins. The miRNA binds to mRNA, and upon imperfect binding, translation will be inhibited, while after perfect binding, the mRNA will be degraded through the RISC complex [51].

Single miRNA may target several mRNAs, and several miRNAs may be specific for the same mRNA. The majority of human genes are regulated, either directly or indirectly, by miRNAs [55, 56]. It has also been found that miRNAs play an essential role in fundamental cellular processes including cell metabolism, cell differentiation, apoptosis, and cell signaling as well as in cancer differentiation [55-58]. Recently, the role of miRNA in the response to ionizing radiation has been investigated, and miRNAs has been found to be important in the DNA damage response [59-62].

Cellular responses

Traditionally the DNA has been considered to be the principal target for biological effects of radiation, where bioloical effects might be induced either by direct actions of the radiation, with direct ionization of a target atom in DNA, or by indirect actions, where the radiation interacts with e.g. water to produce free radicals that react with DNA. A variety of DNA lesions may be produced, e.g. single-strand breaks (SSB), double-strand breaks (DSB), base damage, and DNA-DNA and DNA-protein cross-links [24, 63]. Following radiation-induced DNA damage, complex cellular responses including DNA repair, cell cycle arrest, mitotic catastrophe, necrosis, and apoptosis are potentially induced [64, 65].

However, the radiation biology paradigm should include more than the direct action of radiation on the DNA. The so called non-target effect, which includes effects that are not directly DNA damage-related, should also be considered, e.g. membrane-mediated signaling, abscopal effects, bystander effects, genomic instability, adaptive response, hypersensitivity, and inverse dose-rate effects [63]. The observation and acceptance of these responses has fundamentally changed the view of radiation-

induced responses, and that the observed effects are not only due to direct ionization or free radicals.

The non-target effect can be described as biological responses similar to those of directly exposed cells, which occur in non-irradiated cells [24, 63]. Observed responses include DNA damage, epigenetic changes, carcinogenesis, cytotoxicity, and more [63, 66, 67]. The mechanisms underlying non-targeted effects are not fully understood, but emerging evidence suggest the involvement of cytokines, including TNFα and IL-8, as well as reactive oxygen species [68-70]. Also smaller molecules, such as nucleotides and peptides, may be involved through gap junctions. This direct cell-to-cell communication is hypothesized to be involved in the non-targeted effect of normal tissue, and studies of alpha-irradiated human fibroblasts with inhibited intercellular communication showed reduced induction of both p53 and CDKN1A levels, as well as reduced cell killing [71, 72]. However, in tumor tissue, gap junctions are generally down-regulated [63].

In vivo evidence of the non-targeted effect includes studies in mice, where a reduction in tumor growth rates have been observed after high dose exposures of non-cancerous tissues [73] and partial irradiation of rat lung also resulted in damage in non-irradiated lung tissue [74].

Kidney toxicity

Normal kidney function
The primary function of the kidneys is to remove metabolic waste products from the body through filtration of the blood and excretion, as well as to regulate blood volume and composition, electrolytes, blood pH, and blood pressure [75, 76]. The kidneys receive blood from the renal arteries which branch directly from the abdominal aorta, allowing about 20% of the cardiac output to be transported directly to the kidneys.

The functional unit of the kidneys is the nephron, which is responsible for the urine production by filtration, reabsorption, and tubular secretion (Figure 2). The nephron consists of the glomerulus, proximal tubule, loop of Henle, distal tubule, and the collecting ducts. Filtration of the blood takes place in the glomerulus where the plasma is filtered through the glomerular barrier, which consists of capillary endothelium (negatively charged surface, glycocalyx), basement membrane (net negative charge), and epithelium of the Bowman's capsule. The epithelium of the Bowman´s capsule will only allow passage of molecules smaller than ~60 kDa, and together with the charge selectivity of the capillary endothelium and the basement

membrane, the passage through the glomerulus will depend on the size, charge, and shape of the molecule [77, 78].

The filtrate (primary urine) then drains into the Bowman´s space. Reabsorption and secretion takes place in the proximal tubule, loop of Henle, and the distal tubule. The reabsorption process can be divided into active and passive transport, where active transportation takes place through Na^+/K^+ pumps, whereas passive transportation takes place through Na^+ symporters, ion channels, and osmosis. Furthermore, different transporter proteins are located in the tubular cell membrane, responsible for reabsorption of e.g. peptides. In total, 99% of H_2O, 100% of glucose, 99.5% of Na^+, and 50% of urea is reabsorbed. Furthermore, all proteins and peptides are reabsorbed by the proximal tubules and transferred into lysosomes and digested by proteolytic enzymes. The catabolic products are then either transferred back into the circulation or excreted. The primary secretion of urea, bile salts, metabolites,
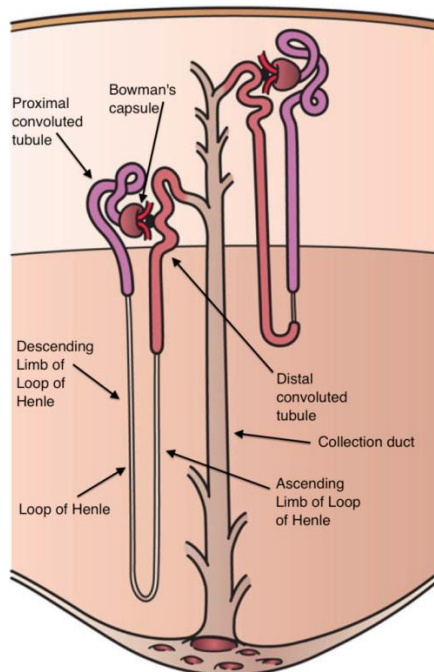
Figure 2. Schematic illustration of nephron in the cortex (lighter part) and medulla (darker part). Image is in the public domain and were retrieved from http://en.wikipedia.org/wiki/Nephron on Nov 9, 2014.

drugs, and creatinine takes place in the proximal tubule, whereas organic acids, K^+, and H^+ are primarily secreted in the distal tubule.

Further functions of the kidneys include production and secretion of hormones, renin, erythropoietin, and vitamin D, important for cardiovascular, hematologic, and skeletal muscle homeostasis [76]. Renin is released during low blood flow or low Na^+ concentration. This will lead to the production of angiotensin II, which will result in increased retention of salt and water. In cases of low blood oxygen, the kidneys will secrete erythropoietin. This hormone will stimulate red blood cell development in bone marrow. The conversion of vitamin D to its active form also takes place in the kidneys, and this hormone is among others included in calcium homeostasis [75].

Renal handling of ^{177}Lu-octreotate
After ^{177}Lu-octreotate administration, the majority of the radiolabeled peptide will go through glomerular filtration and be excreted into the primary urine. Then, a small fraction will be retained in the kidneys through reabsorption in the tubules from the primary urine [79]. Uptake of ^{177}Lu in the kidneys is highly heterogeneous, where the highest accumulation in rats takes place in the cortex, whereas the outer medulla has a concentration at 24h of 50-60% of the cortical activity, with no uptake detected in the inner medulla or the renal pelvis. However, a study on mice showed differences in ^{111}In uptake in the kidneys after ^{111}In-octreotide of female and male mice , where male mice were comparable to rats while the highest uptake in female mice was found in the outer medulla with less uptake in the cortex [80]. In addition, uptake in the cortex is highly heterogeneous, where it has been found that the majority of the uptake takes place in the proximal tubules in both rodents and humans, while only very low amounts are detected in the distal tubules and the glomeruli [79, 81].

The uptake mechanisms of radiolabeled somatostatin analogs in the kidneys are not fully understood. However, animal model studies have indicated that receptor-mediated endocytosis via the megalin-cubilin complex and SSTR are involved, as well as amino acid/oligopeptide transporters, pinocytosis, and passive diffusion [37, 46, 79, 82]. The megalin-cubilin complex is a scavenger protein receptor with high expression in the proximal tubule and is involved in the reabsorption of hormones, drugs, toxins, enzymes, and other proteins [37, 83-85]. It is today considered the most important receptor involved in protein uptake in the proximal tubule. The megalin receptor has a negative charge, and thus high affinity for the positively charged somatostatin analogs in the primary urine [79]; this was confirmed by both *in vivo* and *in vitro* studies with ^{111}In-octreotide/octreotate [80, 86]. In the case of

uptake in the lysosomes in the proximal tubular cells, the radionuclide can be retained due to intracellular binding to metal binding proteins [27, 37].

Animals as model

The effects of radiation exposure in mammals are influenced by both individual genetic predisposition and radiation dose. It is clear that the genetic background underlying different effects and reactions is both polygenic (multiple genes interact) and heterogeneous (the effect of different genes can sometimes give the same end result). Consequently, it is particularly difficult to analyze and identify the optimal absorbed dose response underlying the development of human side effects and reactions related to cancer treatment, because people from a genetic point of view, are very diverse group in which each individual is also subjected to its own specific set of complex environmental factors. One way to reduce the influence of such factors that complicate the optimal absorbed dose response is the use of an animal model system. If isogenic animals are used under controlled conditions, influence from factors such as genetic heterogeneity and variable environmental factors can greatly be reduced. Results obtained in the study of the models can be easily translated to humans using the available information from comparative gene mapping between humans and the model.

In the present thesis, mice were used as a model to investigate the temporal and absorbed dose related response of the kidneys after ^{177}Lu-octreotate and ^{177}LuCl$_3$ administration. Mouse is a suitable model to improve our understanding of the effects of radiation exposure in mammals. However, there are both discrepancies and similarities between man and mice and an understanding of these differences are important in the evaluation of these trials [38]. In the kidneys, the expression of all five SSTR subtypes has been found in both man and mice, while expression in rat kidney is limited to SSTR3 & 5 [87-91]. However, the importance of somatostatin receptor expression in the kidneys concerning renal uptake of radiolabeled somatostatin analogs is in question. In rats, co-infusion of unlabeled octreotide did not effectively block renal uptake [79], and in SSTR-2 knockout mice, no difference in biodistribution was found between these and normal mice [92]. Also in man, SSTR expression in the kidneys seems to be less important [93].

In both rodents and humans, inhomogeneous activity distributions have been described in the kidneys [80, 81], as well as a similarity in uptake mechanisms, including the megalin/cubilin complexes in the proximal tubule cells [86, 94]. However, factors that differ substantially between the species is kidney size and the size of the nephrons [78].

Kidney injury after radionuclide therapy
Data on kidney toxicity after radionuclide therapy are limited. In external beam radiation therapy, observed radiation-induced nephropathy includes azotemina, hypertension, and anemia [95]. Furthermore, mesangiolysis, atrophy and tubulointerstitial scarring may be induced. Some of these effects can be reversible or progression can occur to chronic nephropathy. Chronic nephropathy is related to loss of kidney mass and function, which can develop up to many years after therapy [37, 95].

In rats and nude mice, dose-dependent late damage of the proximal tubules has been observed after ^{177}Lu-octreotate administration [47, 96, 97]. Furthermore, selective and dose-dependent morphological changes have been observed in the renal cortex after 35 to 58 Gy of ^{177}Lu-octreotate. In patients, both acute and chronic nephropathy has been observed after administration of ^{90}Y-labeled somatostatin analogs [21, 98]. However, more data are needed to fully understand the nature of the induced kidney injury and to predict tolerance doses for this type of therapy.

Biomarkers
The general definition of a biomarker is "a measurable indication of a specific biologic state that is relevant for a specific process" [99]. The ideal biomarker should originate from the damaged cells and display organ specificity. The dose-response of the ideal biomarker should be directly dependent on the extent of damage, and the change in expression should be expressed early after insult. The turn-over rate should be quick in order to be able to follow the disease process and the measurement technique of choice should be quick and reliable [76, 100].

Advances in basic biological understanding are vital for the development of biomarkers for clinical practice, and biomarker development concerning the renal effects after ^{177}Lu-octreotate administration requires a detailed knowledge of the organ under investigation [101]. It is important to remember that after exposure to stressors, induced kidney injury is a result of the relationship between cell dysfunction, cell death, proliferation, inflammation, and recovery and biomarker dependency on these relationships needs to be understood [102].

Renal function
The glomerular filtration rate (GFR), which describes the quantity of glomerular filtrate formed in the nephrons of both kidneys per unit of time, is the most examined parameter in the assessment of renal function [75]. GFR, adjusted for body surface area, is 100-130 ml/min/1.73m^2 in men and women, and after age 40, GFR decreases with age. Chronic renal dysfunction demonstrated by decreased GFR

is generally followed by altered electrolyte and volume balance, decreased red blood cell production, hypertension, and altered bone mineralization [75, 103].

The routinely used endogenous marker for renal function is serum creatinine. Creatinine is produced through metabolic processes in the muscles and is freely filtered by the glomerulus without renal tubular reabsorption [75], and increased levels in serum is an indicator of reduced GFR. However, several drawbacks exist when using serum creatinine and studies have shown a lack of power for the identification of early renal injury and dysfunction [102]. Furthermore, GFR needs to be reduced up to 50% before a change in serum creatinine levels is detected [103-105]. Serum creatinine also highly depends on muscle mass, age, sex, medications, and hydration status. Significant injury can thus exist with no change in serum creatinine levels due to renal reserve, enhanced tubular section, and other factors [102, 106, 107].

A more robust endogenous marker of GFR may instead be serum cystatin C, which is independent of muscle mass, sex, and age [108]. The production of cystatin C takes place in all nucleated cells at a constant rate and is freely filtered by the glomeruli and reabsorbed and catabolized by the tubules. Increased level in serum is an indicator of reduction in GFR [108-110]. Cystatin C may be a more reliable marker of GFR than creatinine, especially in cases of mild reductions of GFR [111]. The lack of sensitive functional assessment methods of the kidney may, however, limit the possibility of early and effective prevention of functional loss. Therefore, GFR should not be considered as the sole method for assessment of kidney response following ^{177}Lu-octreotate administration. Markers for renal injury and damage, specifically for tubules due to the high uptake of ^{177}Lu-octreotate in this part of the kidneys, could potentially prove invaluable in the characterization and investigation of renal damage.

Measurement of kidney injury
A number of biomarkers have been proposed for kidney injury. Most notably are N-acetyl-beta-(D)-glucosaminidase (NAG), neutrophil gelatinase associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), interleukin-18 (IL-18), and liver-type fatty acid binding protein (FABP) [75]. Furthermore, increased levels of β_2-macroglobulin (B2M), α_1-microglobulin (A1M), and retinol-binding protein (RBP) in urine are indicative of tubular damage [75, 112]. However, the use of single markers may not be sufficient given the complex response found after radiation exposure and the heterogeneity of response of various dysfunction conditions in the kidneys. Furthermore, many patients have systemic diseases, which complicate the development of biomarkers [113]. The use of multiple markers may be needed to monitor interplay between different potential mechanisms [102, 114].

Morphological changes

Upon whole kidney irradiation, morphological changes can induce a reduction in renal function which will be both time- and absorbed dose-dependent. Both tubular and glomerular alterations will be induced in the early stages of radiation nephropathy. Concerning the effects on the glomeruli, segmentalization (injury to the glomerular capillaries and mesangium), as well as nuclear enlargement have been observed, while the tubular effects include tubulolysis and tubular atrophy [95].

Following radiation exposure, an increase in cellular proliferation in both glomerulus and tubules has been observed, likely as a response to radiation induced cell death. Also pronounced changes in cell phenotype have been observed in irradiated kidney cells, which in part may be due to radiation-induced chronic and persistent oxidative stress [95].

Aims

The overall aim of this thesis was to study the normal kidney tissue response *in vivo* after ^{177}Lu/^{177}Lu-octreotate administration. The main objective was to investigate the biological response at the gene expression level and to identify and evaluate potential biomarkers.

The specific aims of the separate studies included in this thesis were:

- To investigate the effects of absorbed dose on the transcriptional response in kidney tissue in mice after ^{177}Lu-octreotate administration (Paper I)

- To investigate the effects of dose rate and time after injection on the transcriptional response in kidney tissue in mice after ^{177}LuCl$_3$ administration (Paper II)

- To investigate the effects of absorbed dose at late time points on the transcriptional response and function of kidney tissue in mice after ^{177}Lu-octreotate administration (Paper III)

- To investigate the effects of absorbed dose at late time points on the proteomic and functional response in renal cortical tissue after ^{177}Lu-octreotate administration (Paper IV)

- To investigate the effects of absorbed dose on the miRNA response in renal cortical tissue after ^{177}Lu-octreotate administration, and the influence of miRNA on the expression of target genes (Paper V)

Methodological aspects

Radiopharmaceuticals

^{177}Lu, in the form ^{177}LuCl$_3$, and the somatostatin analog DOTA-Tyr^3-octreotate were acquired from I.D.B. Holland (I.D.B. Holland BV, Baarle-Nassau, Netherlands). Preparation and radiolabeling were conducted according to the manufacturer´s instructions. The fraction of peptide bound ^{177}Lu was determined by instant thin layer chromatography with 0.1 M sodium citrate as the mobile phase, where a fraction > 99% was considered satisfactory. The ^{177}Lu-octreotate stock solution was diluted with saline solution to the final activity concentrations.

Preparation of ^{99m}Tc-DTPA (diethylene-triaminepenta-acetate) and ^{99m}Tc-DMSA (dimercaptosuccinic acid) was performed according to the instructions by the manufacturer (Covidien, Millington, Dublin, Ireland).

Before and after administration, the activity in the syringes was measured with a well-type ionization chamber (CRC-15R; Capintec, IA, USA) to determine the injected activity in each animal.

Animals

The animal strains BALB/c nude mice and C57BL/6N mice were used in the present investigations. Both strains are inbred, and the BALB/c nude mice are immunodeficient and lack T-cell production. During the animal trials, the animals were kept under normal nutritional conditions with extra care not to induce unwanted stress to the animals. All injections were performed through the tail vein and the animals were killed through cardiac puncture under anesthesia. The kidneys were surgically removed and one kidney was flash frozen in liquid nitrogen and stored at -80°C until analysis. The other kidney was harvested for either activity concentration measurement (Paper I and II) or stored in formalin followed by histological analysis (Paper II, and III). All studies were approved by the Ethical Committee on Animal Experiments in Gothenburg, Sweden.

Dosimetry

The absorbed dose estimation to the kidneys was based on the Medical Internal Radiation Dose (MIRD) pamphlet 21 formalism [115]:

$$\bar{D}(r_S, T_D) = \frac{\tilde{A}(r_S, T_D) \sum_i E_i Y_i \phi(r_T \leftarrow r_S, E_i, T_D)}{M(r_T, T_D)},$$

where $\tilde{A}(r_S, T_D)$ is the time integrated activity in the source organ, r_S, during the time of interest, T_D. E_i is the energy and Y_i is the yield of the radiation i. $\phi(r_T \leftarrow r_S, E_i, T_D)$ is the absorbed fraction in the target organ, r_T, and $M(r_T, T_D)$ is the mass of the target tissue. In the calculations, r_S and r_T were considered the same. $\sum_i E_i Y_i$ was approximated to 147 keV, only including emitted electrons. The value of the absorbed fraction was taken from the literature ($\phi(r_T \leftarrow r_S, E_i, T_D)$=0.93 [116]). $\tilde{A}(r_S, T_D)$ was calculated using previously published biodistribution data [40, 41, 43] and activity concentration measurements of the kidneys in the individual studies. The absorbed dose was calculated to whole kidney.

RNA extraction, analysis, and data processing (I, II, III, V)

The flash-frozen kidneys were dissected into cortical and medullary tissues and homogenized using the Mikro-Dismembrator S ball mill (Sartorius Stedim Biotech, Aubagne Cedex, France). Total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), or the miRNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer´s instructions. RNA Integrity Number (RIN) values were retrieved using RNA 6000 Nano LabChip Kit with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and samples with RIN values above 6.0 were accepted for further analysis.

In Papers I-III, the extracted RNAs were processed at the Swegene Center for Integrative Biology at Lund University (SCIBLU). MouseRef-8 Whole-Genome Expression Beadchips (Illumina, San Diego, USA), containing 25,435 probes were used for hybridization. Image acquisition and subsequent analysis were performed with Illumina BeadArray Reader scanner and BeadScan 3.5.31.17122 image analysis software, respectively. Data preprocessing and quantile normalization of raw signal intensities were conducted through the use of the web-based BioArray Software Environment (BASE) system. Nexus Expression 2.0/3.0 (BioDiscovery, Hawthorne, USA) software was used for further analysis with $\log_2$-transformed, normalized expression values and a variance filter.

In Paper V, the RNA samples were processed at the TATAA Biocenter (Gothenburg, Sweden) and hybridized on to the Mouse miRNA oligo chip 4plex, containing approximately 1,300 miRNAs (Toray Industries, Tokyo, Japan). The chips were scanned with the Gene Scanner 3000 (Toray Industries, Tokyo, Japan) using standard settings for mouse miRNA v.19. Further analysis was conducted through the use of GenEx (Multid Analyses AB, Gothenburg, Sweden).

Table 2. Categories of biological processes. Reprint from [117], with kind permission from Britta Langen.

Category	Biological processes that...
DNA integrity	
Damage and repair	...recognize damage or initiate or facilitate repair pathways
Chromatin organization	...maintain the structural integrity of DNA on the chromatin level
Gene expression integrity	
Transcription	...are involved in transcription or its regulation
RNA processing	...are involved in processing immature or mature RNA or its regulation
Translation	...are involved in translation or its regulation
Cellular integrity	
Physico-chemical environment	...are associated with e.g. regulation of ion homeostasis or transport
Cytoskeleton & motility	... establish or regulate cytoskeleton integrity, chemotaxis or cellular motility
Extracellular matrix & CM	...regulate biogenesis of the cellular membrane (CM), maintain the extracellular matrix, regulate cell adhesion, etc.
Supramolecular maintenance	...are involved in or regulate e.g. protein (re)folding, protein oligomerization or modification, general transport of molecules or vesicles, etc.
General	...are valid for any of the above subcategories
Cell cycle and differentiation	
Cell cycle regulation	...are involved in e.g. cell growth, regulation of growth arrest, etc.
Differentiation & aging	...regulate e.g. cellular development, proliferation, or aging
Apoptotic cell death	...are involved in regulating pro-apoptotic or anti-apoptotic pathways
Cell death	...in non-apoptotic cell death, e.g. cytolysis
General	...are valid for any of the above subcategories
Cell communication	
Intercellular signaling	...facilitate communication between cells, e.g. synaptic or hormone signaling
Signal transduction	...regulate or effect signal transduction, e.g. signal processing within a cell
Metabolism	
Proteins, amino acids	...regulate or facilitate anabolic or catabolic processes for proteins or amino acids
Lipids, fatty acids	...regulate or facilitate anabolic or catabolic processes for lipids or fatty acids
Carbohydrates	...regulate or facilitate anabolic or catabolic processes for carbohydrates
Signaling molecules	...regulate or facilitate anabolic or catabolic processes for signaling molecules
Nucleic acid-related	...regulate or facilitate anabolic or catabolic processes for nucleic acid-related
Other	...are part of metabolism but not associated with other specific subcategories
General	...are valid for any of the specific subcategories
Stress responses	
Oxidative stress response	...respond to e.g. superoxide, hydrogen peroxide, or other reactive oxygen species
Inflammatory response	...regulate or facilitate pro-inflammatory or anti-inflammatory responses
Immune response	...regulate or facilitate e.g. the acute-phase response, responses to pathogens, phagocytosis, or concern immune-specific biosynthesis
Other	...are part of stress responses but not associated with other specific subcategories
Organismic regulation	
Behavior	...regulate behavioral responses of the organism
Ontogenesis	...regulate or facilitate developmental processes on the organ or organism level
Systemic regulation	...are involved in organismic regulations with systemic relevance
Reproduction	...regulate or facilitate e.g. germ cell development, parturition, or pregnancy

To determine differentially expressed transcripts and to control the false discovery rate, the Benjamini-Hochberg method was used in paper I-III (*26*). A p-adjusted value of <0.01 and a fold change of at least 1.5 (up- or down-regulation) were considered statistically significant.

Gene Ontology (GO) terms were used to determine affected biological processes from the differentially regulated gene sets. The biological processes were categorized based on the GO terms employing a *p* value cutoff of <0.05 and ancestor charts. Eight main categories with more than 30 subcategories were generated to account for the higher level cellular functions (Table 2).

Further analysis, including pathway analysis, up-stream regulator analysis, and target gene analysis, was conducted using the IPA software (Ingenuity Systems, Redwood City, CA, USA).

Protein extraction, analysis, and data processing (IV)

The fresh frozen kidneys were dissected and the kidney cortex was homogenized using a FastPrep®-24 instrument (MP Biomedicals, OH, USA). Total protein concentration was determined with Pierce™ BCA Protein Assay (Thermo Fisher Scientific, Waltham, USA). Total protein (100 µg/sample) was diluted and trypsin digested. After centrifugation the filtrates were subjected to isobaric mass tagging reagent TMT® according to the manufacturer's instructions (Thermo Fisher Scientific). The peptides were further purified by Strong Ion Exchange Spin columns (Thermo Fisher Scientific) according to the manufacturer's guidelines. The samples were desalted using PepClean C18 spin columns (Thermo Fisher Scientific) according to the manufacturer's guidelines.

The samples were then analyzed on an Orbitrap Fusion Tribrid mass spectrometer interfaced to an Easy-nLC II (Thermo Fisher Scientific). Ions were injected into the mass spectrometer under a spray voltage of 1.6 kV in positive ion mode. MS scans was performed at 120 000 resolution and m/z range of 400-1600. MS raw data files for each TMT (Tandem Mass Tag Reagent) set were merged for relative quantification and identification using Proteome Discoverer version 1.4 (Thermo Fisher Scientific).

Quantitative real-time PCR (qPCR) (I,II,V)

In order to validate gene expression data identified by microarray analysis, several genes with significant differential expression were selected for qPCR analysis in Papers I and II. Predesigned TaqMan assays (Applied Biosystems, Carlsbad, CA,

USA) were used on cDNA synthesized from the same RNA extraction as the microarray experiments (SuperScript^{TM} III First-Strand Synthesis SuperMix, Invitrogen, Carlsbad, CA, USA). The standard curve method was used for quantification and the geometric mean of three endogenous controls was used for normalization of the samples. In Paper V, the samples were reversely transcribed using the Universal cDNA Synthesis Kit II (Exiqon, Vedbaek, Denmark) according to the manufacturer´s protocol. The Pearson correlation coefficient was used in all studies to calculate the correlation between the microarray and the QPCR methods.

Western blotting (II)

To investigate the effect of transcriptional regulation of *Havcr1* and *Lcn2* at the protein level, Western blotting was carried out. Fresh frozen tissue samples (renal cortical and medullary tissue) were suspended in Mammalian Cell Lysis Buffer including Benzonase® Nuclease and Protease Inhibitor Solution and homogenized using the Mikro-Dismembrator S ball mill (Sartorius Stedim Biotech, Aubagne Cedex, France). The samples were centrifuged and the supernatant was transferred into microcentrifuge tubes and immediately frozen and stored at -20°C.

Extracts (100 µg) were resolved by SDS-PAGE on 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA), and later transferred to nitrocellulose membranes. The HAVCR1 protein was detected using anti-TIM-1 (MAB 1817, R&D Systems, Minneapolis, MN, USA) and Rat IgG Horseradish Peroxidase-conjugated antibody (HAF005, R&D systems, Minneapolis, MN, USA). LCN2 was detected using anti-LCN2 (PAB 9542, Abnova, Taipei city, Taiwan) and Rabbit IgG Horseradish Peroxidase-conjugated antibody (NA934V, Amersham, Piscataway, USA). Anti-ACTB and Mouse IgG Horseradish Peroxidase-conjugated antibody was used for detection of beta-actin as loading control (NA931V, Amersham, Piscataway, USA). SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA) was used for detection and digitalized images were acquired using Fujifilm Luminescent Image Analyzer LAS-1000 (Fujifilm, Tokyo, Japan).

Scintigraphy (III,IV)

To investigate the kidney function after ^{177}Lu-octreotate, dynamic and static scintigraphic images were obtained using a single headed gamma camera (ADACT 210, ADAC Laboratories A/S, Aalborg, Denmark). The gamma camera was equipped with a medium energy parallel-hole collimator (256x256 image matrix), and images were acquired with a 20% energy window over the 140 keV photon peak of ^{99m}Tc. Mice were i.v. injected with ^{99m}Tc-DTPA followed by dynamic

scintigraphic acquisition. Two to three days after ^{99m}Tc-DTPA scintigraphy, static images with ^{99m}Tc-DMSA were obtained. Three hours after ^{99m}Tc-DMSA injection, a static 3 min image was acquired. In all scintigraphic images, a syringe with known ^{99m}Tc activity was also imaged as calibration.

The ImageJ software was used for image processing [118]. A region-of-interest (ROI) was outlined for each kidney at 10% level of maximal pixel count in the ROI and total number of counts was collected from the ROI. In addition, the number of counts in whole body and in a ROI around the calibration syringe was collected from each image. In the case of accidental subcutaneous injection, the number of counts in a ROI around the position of injection was subtracted from the number of counts in whole body.

Histology (II,III)

To study morphological changes in the kidneys, tissue slices (2 µm thick) were acquired from the formalin fixed kidneys and stained with hematoxylin-eosin.

Results & Discussion

During radionuclide therapy, normal tissue exposure is inevitable, and will limit the amount of activity that can safely be administered. Risk of normal tissue toxicity will thus limit treatment efficiency. In ^{177}Lu-octreotate therapy, the kidneys are the main limiting organ for late toxicity, where active reabsorption and retention takes place. However, there are substantial differences in absorbed dose to the kidneys among individual patients who have undergone this therapy. Furthermore, the biological response will not be strictly dose-dependent, but tolerance and subsequent toxicity will also vary strongly between individuals [119]. A deeper knowledge about the biological effects following ^{177}Lu-octreotate administration is of vital importance in order to optimize this type of therapy. In addition, biomarkers for kidney function and damage need to be defined in order to easily and effectively predict and evaluate kidney toxicity after ^{177}Lu-octreotate administration.

Effects on transcriptional level (I,II,III)

The number of differentially regulated transcripts following ^{177}Lu administration was found to be dependent on absorbed dose, dose-rate, time after injection, and kidney tissue type (Figure 3). At 24h after injection, the total number of uniquely regulated transcripts at all dose levels was 281 and 480 in kidney cortex and medulla, respectively (Paper I). Initially, an increase in the number of differentially regulated transcripts was found with absorbed dose. The highest number of regulated transcripts was found at 4.3 Gy (kidney cortex) and 1.3 Gy (medulla), with a subsequent decrease with absorbed dose. The number of regulated transcripts was also found to be dose-rate (Paper II) and time (Paper II and III) dependent. In general, higher dose-rates (0.23-16 mGy/min) induced more transcriptional regulation compared with lower dose-rates (0.028-0.11 mGy/min) in both kidney cortex and medulla. A general increase in the number of regulated transcripts was also found with time where higher numbers of regulated transcripts were found at later time points (12 *vs.* 4 months).

The increased number of differentially regulated transcripts with dose-rate is hypothesized to depend on the radiation insult on the tissues. With lower dose-rate, the damage induced per time interval will be lower, potentially allowing the cell to repair while at the same time continue with its normal function. At higher dose-rate, the higher insult per time interval will instead induce a stronger cellular response with increased repair and protection and decreased normal metabolic function.
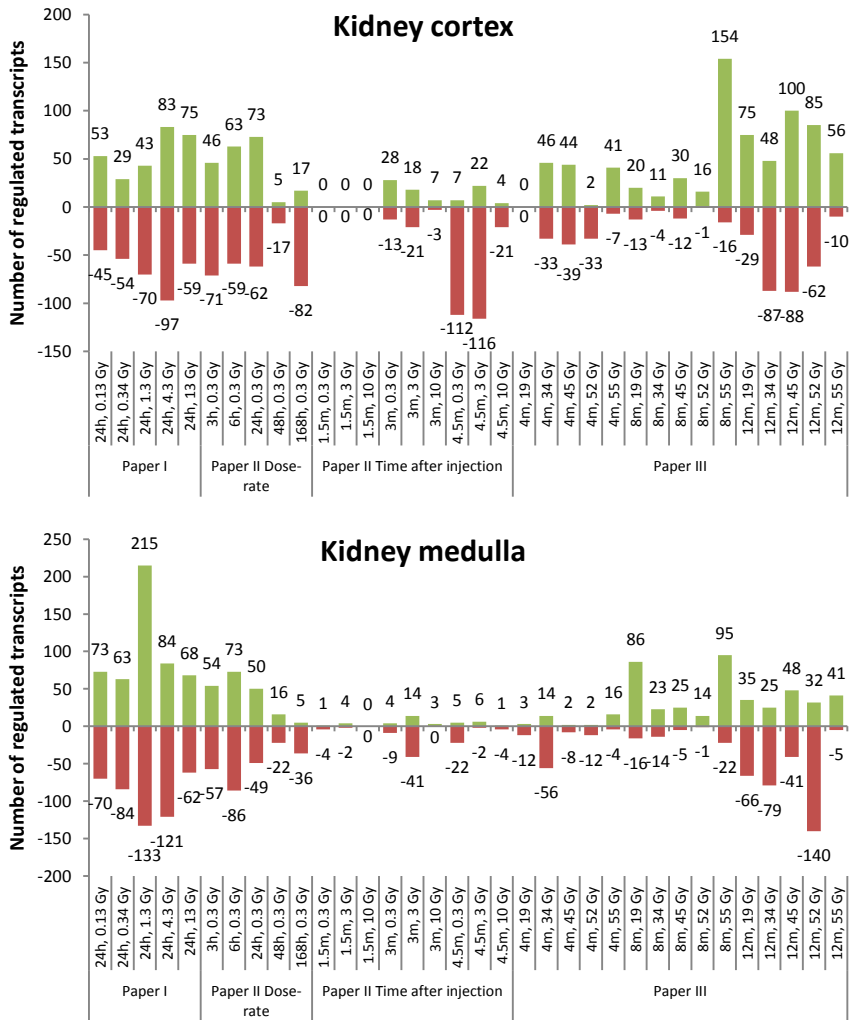
Figure 3. Total number up- (positive numbers, green bars) and down-regulated (negative numbers, red bars) transcripts in Paper I, II, and III.

Similar arguments can also be applied to the observed absorbed dose dependence, where the number of differentially regulated transcripts generally increased with absorbed dose (Figure 3). It may be suggested that the strongest response is observed when the absorbed dose is high enough to impose severe cellular damage but low enough to allow repair. It has previously been shown *in vitro* that low dose/low dose-rate will display markedly different effects compared with the same dose delivered acutely [120]. It has also been suggested that low dose-rate exposure

leads to a decrease in activation of key molecules in the cell, e.g. ATM (ataxia-telangiectasia mutated). ATM is a kinase that plays an important role in the detection of DNA damage and initiation of cell cycle arrest, which could potentially result in decreased activation of early DNA damage responses of exposed cells [121, 122] The increased number of differentially regulated transcripts with time could instead be due to the physiological conditions within the kidney. It is known that radiation will affect the physiology of the exposed tissue, and the increase in number of differentially regulated transcripts with time may be due to cellular adaptations that occur to this new environment.

In total, Papers I, II, and III represent 34 specific exposure conditions of kidney tissue. When comparing the differentially regulated transcripts between the studies, no transcripts were found which were recurrently regulated at all exposure conditions. The most recurrently regulated transcripts are presented in Table 3, where the *Serpina10* gene in kidney cortex, and the *Egr1*, *Pck1*, and *Hmgcs2* genes in kidney medulla were the most commonly observed (16 different exposure conditions). Three of the recurrently regulated transcripts were differentially regulated in both kidney cortex and medulla: *Cdkn1a*, *Dbp*, and *Hmgcs2*, which are involved in cell cycle progression, regulation of transcription, and metabolic processes, respectively.

A frequently observed tendency among the recurrently regulated transcripts was the dependence of the type of injected substance and time after injection. For example, the *Cdkn1a* gene was differentially regulated at 1.3-13 Gy in Paper I and also recurrently at 4-12 months after ^{177}Lu-octreotate administration in kidney cortex and medulla, while after ^{177}LuCl$_3$ administration the gene was only differentially regulated in kidney cortex after 10 Gy at 140 days after injection, and not at all in kidney medulla. Considering the expression of *Serpina10* and *Lcn2*, these genes were recurrently differentially regulated at the different exposure times 3-168h for both ^{177}LuCl$_3$ and ^{177}Lu-octreotate, while they were rarely differentially regulated at later time points. This shows the importance of studying the transcriptional response at multiple time points and also the difficulties in translating the results between absorbed doses, time points, and injected substance.

Table 3. Recurrently regulated genes in Papers I, II, and III found in more than 12 exposure conditions. Data for human homolog expression was retrieved from www.proteinatlas.org.

Kidney cortex

| Gene symbol | Full name | Expression of human homolog in kidney | | |
| | | RNA expression (FPKM) | Protein localization (score) | |
			glomeruli	tubules
Serpina10	Serpin peptidase inhibitor A10	0	ND	medium
Hmgcs2	3-hydroxy-3-methylglutaryl-CoA synthase 2	66	ND	medium
Napsa	napsin A aspartic peptidase	67	ND	high
Ccrn4l	CCR4 carbon catabolite repression 4-like	4	N/A	N/A
Dbp	D site of albumin promoter binding protein	13	ND	low
Cdkn1a	cyclin-dependent kinase inhibitor 1A	18	ND	ND
Spink6	serine peptidase inhibitor, Kazal type 6	0	low	low
Havcr1	hepatitis A virus cellular receptor 1	3	N/A	N/A
Mep1b	meprin A, beta	0	ND	ND

Kidney medulla

| Gene symbol | Full name | Expression of human homolog in kidney | | |
| | | RNA expression (FPKM) | Protein localization (score) | |
			glomeruli	tubules
Egr1	early growth response 1	165	medium	medium
Pck1	phosphoenolpyruvate carboxykinase 1	490	ND	high
Hmgcs2	3-hydroxy-3-methylglutaryl-CoA synthase 2	66	ND	medium
Cdkn1a	cyclin-dependent kinase inhibitor 1A	18	ND	ND
Cml3	camello-like 3	*human homolog not available*		
Dbp	D site of albumin promoter binding protein	13	ND	low
Lcn2	lipocalin 2	10	ND	ND
Angptl4	angiopoietin-like 4	5	ND	medium
Socs2	suppressor of cytokine signaling 2	18	medium	medium
Cfd	complement factor D	2	ND	high
Igf2	insulin-like growth factor 2	24	medium	medium

FPKM=Fragments per kilobase of exon per million fragments mapped

N/A=Not available

ND=Not detected

Affected biological processes

From the differentially regulated gene sets, GO terms were used to determine affected biological processes (the biochemical reactions known to be regulated by the expression-affected genes). Generally, an increased association with stress response and a reduced association with metabolic processes were seen with increasing absorbed dose (Paper I). Concerning the dose-rate effect, the highest overall number of differentially regulated transcripts was associated to stress response, with the strongest association at the lowest dose-rate investigated (0.028 mGy/min) (Paper II). This result could also indicate that radiation-induced activation of immune response may be time dependent and not a dose-rate effect. The group exposed to the lowest dose rate in this study was killed one week after irradiation, and it can be hypothesized that with higher absorbed doses, an earlier induction of immune response may occur.

At longer time points, the association with stress response was reduced in a time dependent manner after exposure to 10 Gy ^{177}Lu. However, the study of the long term effects on kidney tissue after ^{177}Lu-octreotate administration revealed that the recurrently differentially regulated transcripts were primarily found to be down-stream targets of different cytokines and transcription factors (Paper III). The most strongly associated up-stream regulators included *Ifng* and *Tnf* in kidney cortex, and *Ifng*, *Il1B*, and *Nr3c1* in kidney medulla. The *Ifng*, *Tnfa* and *Il1* genes have been shown to be involved in "danger" signaling following irradiation, with the purpose of orchestration of tissue repair [123]. However, these genes are also involved in cell cycle arrest, cell survival, DNA repair, and senescence, and may thus indicate high level integration between cell and tissue responses [123]. Also the *Nfkb* complex was found to be an up-stream regulator, one of the most frequently activated transcription factors by radiation exposure [123-125]. The transcription factor *Egr1* was recurrently regulated in both tissues in this work, and has previously been found to be frequently regulated after irradiation [123-125]. Taken together, radionuclide exposure of the kidneys induces a stress response which is persistent and measurable up to 12 months after initial insult.

Differences in exposure conditions

In this work, the transcriptional variations were studied using both ^{177}LuCl$_3$ and ^{177}Lu-octreotate. In Paper I and II, the same absorbed dose was delivered during 24h, but with ^{177}Lu-octreotate and ^{177}LuCl$_3$ respectively. The observed response was markedly different. The number of regulated transcripts in kidney cortex was 83 and 135 after ^{177}Lu-octreotate and ^{177}LuCl$_3$ administration, respectively, while in kidney medulla the corresponding numbers were 147 and 99. Of the differentially regulated transcripts, 11 and 37 transcripts were differentially regulated in kidney cortex and medulla, respectively, after both ^{177}Lu-octreotate and ^{177}LuCl$_3$ administration.

Although the same radionuclide has been used, the biodistribution within the body and organs are markedly different between the two molecules, which might explain some of these observed differences. In a comparison of the biodistribution between ^{177}LuCl$_3$ and ^{177}Lu-octreotate in Balb/c nude mice the activity concentration in most organs was highest for ^{177}LuCl$_3$ [41]. These results may be due to the rapid and non-specific accumulation of ^{177}LuCl$_3$ in the extra-cellular fluid in the tissues, together with differences in handling of the kidneys. Specifically for the kidneys, a higher accumulation of ^{177}LuCl$_3$ was found at 24h compared with ^{177}Lu-octreotate (3.7 *vs*. 2.2 %IA/g). Furthermore, a more homogenous absorbed dose can be assumed to be delivered after ^{177}LuCl$_3$ due to non-specific distribution. However, the difference in response is not assumed to be due to the somatostatin analog itself. We have previously found that only minor transcriptional variations are induced in the kidneys 24h after administration of octreotate (data not shown). Therefore, the difference in response is assumed to be caused by differences in the irradiation parameters, and not by the somatostatin analog. However, a combination of effects in the biological response to the conjugate cannot be excluded.

Protein expression (IV)

The number of differentially regulated proteins in renal cortical tissue varied with time after injection and absorbed dose (Paper IV). No proteins were found to be recurrently regulated at all exposure conditions, and only three proteins (LRPPRC, PCK1, and RANBP1) were found differentially regulated at three exposure conditions, indicating that the affected proteins were primarily specific for the injected amount and time studied. Highest association among the affected proteins was found for RAN signaling, including oxidative phosphorylation, mitochondrial dysfunction, and valine degradation.

A comprehensive review of radiation responsive proteins have previously been performed by Marchetti et al [126], where 261 genes with altered protein expression were listed. Of these, only 29 have been observed *in vivo*, indicating the low correlation between *in vivo* and *in vitro* studies. In Paper IV, out of the 128 uniquely regulated proteins, 24 have previously been identified as responsive to ionizing radiation. In addition to the differences in response between *in vivo* and *in vitro* settings, this low correlation could also be due to tissue specific regulation as well as divergent responses between radiopharmaceutical and pure radiation exposure.

The correlation between the transcriptional data (Paper I, II, and III) and the protein data (Paper IV) was in general low. Regulated transcripts corresponding to the MEP1B, PCK1, SLC12A1, HSPA8, MIOX, DCN, ETHE1, FGG, AASS, BDH1, EHHADH, MYH11, S100G, ACY1, CYCS, DMGDH, HNRNPA2B1, IDH2,

LGMN, SLC4A1, ST13, and TGM1 proteins were, however, detected when comparing the entire data sets. No altered protein expression levels were found for the *Cdk1a*, *Dbp*, *Lcn2*, and *Per2* genes that were previously found recurrently regulated at the transcriptional level. The reason for this large deviation in results is surprising but it is known that the transcriptional and the protein expression do not always correlate [127]. Translation of an mRNA molecule to a functional protein takes place in several steps, including post-transcriptional and post-translational events causing deviations between the transcript and protein levels. Other potential reasons for the lack of protein expression of our previously proposed candidate biomarkers for ^{177}Lu-octreotate exposure include potential rapid degradation of the produced protein and/or fast excretion from the kidneys to urine or reabsorption to the blood. Therefore, further studies using plasma and urine samples need to be conducted in order to identify novel biomarkers and evaluate the role of the previously reported candidate biomarkers.
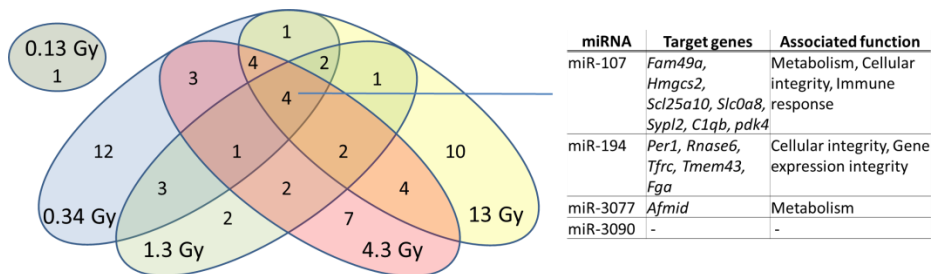
miRNA regulation (V)

The role of miRNA in the response to ionizing radiation has recently been explored [61, 128], and several miRNAs have been identified as recurrently regulated following ionizing radiation exposure [59, 60, 62]. In Paper V, the miRNA response following different amounts of injected ^{177}Lu-octreotate 24h after administration was investigated. In total, 57 unique miRNAs were identified as differentially regulated, with up-regulation as the primary mode of regulation. The most recurrently regulated miRNAs (regulation at four of five exposure conditions) were miR-194-5p, miR-107-3p, miR-3090-5p, and miR-3077-5p (Figure 4).

The impact of miRNA regulation on mRNA transcriptional patterns was studied through a target gene analysis, where the miRNA data was analyzed in conjunction with the mRNA data obtained in Paper I. The target prediction analysis revealed a strong association with immune response pathways, with highest association with antigen presentation pathway and interferon signaling. The predicted upstream regulators corresponded well with the pathways affected, with IFNG inhibition and TP53 and TP73 activation.

The majority of the miRNAs that were differentially regulated in Paper V have also previously been observed after radiation exposure. For example, the let-7 miRNA family were regulated at 0.05-19 Gy in a variety of cell lines and time points (0.5-168h) after photon radiation, and is involved in cell proliferation, differentiation, and inhibition of the RAS pathway [61, 129-132]. The miR-10 family is involved in DNA damage response [133] and radioprotective/radiosensitizing effects [62, 134]

and the miR-15 family is involved in apoptosis and cell cycle control through targeting of the BCL2 and E2F1 proteins [135, 136].

miRNA	Target genes	Associated function
miR-107	*Fam49a, Hmgcs2, Scl25a10, Slc0a8, Sypl2, C1qb, pdk4*	Metabolism, Cellular integrity, Immune response
miR-194	*Per1, Rnase6, Tfrc, Tmem43, Fga*	Cellular integrity, Gene expression integrity
miR-3077	*Afmid*	Metabolism
miR-3090	-	-

Figure 4. The distribution of differentially regulated miRNAs in mouse kidney cortex 24h after ^{177}Lu-octreotate administration. The 0.13 Gy dose level did not share any regulated miRNAs with the other dose levels. The target genes and associated biological function of the four miRNAs that were differentially regulated at four dose levels are shown to the right.

Systemic response

The radiation response within specific organs is complex due to the heterogeneity of cell types, where each cell type is subjected to its own specific and complex gene expression. Furthermore, a consequence of radionuclide administration is that all organs and tissues in the body are exposed to various amounts of ionizing radiation emitted by the radionuclide with resulting whole body systemic effects, which complicates the analysis of specific organs. Therefore, the detected responses in kidney tissue may be understood as not only a single consequence of kidney irradiation, but also an effect of communication between different cells, tissues and organs. Thus, systemic effects must also be considered when evaluating the biological response [137]. In addition, the biological effect will also be dependent on spatial, temporal, and tissue factors, which further complicates the observed response [138]. It is clear that low-dose/low dose-rate irradiation will display markedly different effects compared with the same dose delivered acutely. Previous studies have shown an increase in effectiveness of cell killing when low dose-rate irradiation was employed, compared with high dose-rate exposure, under certain conditions [120]. Also, induction of radio-resistance and radio-adaptation has been reported in normal cells [120].

Potential biomarkers

The biological response observed after radiation exposure is a relationship between cell dysfunction, cell death, proliferation, inflammation, and recovery. In the pursuit of novel biomarkers, these relationships need to be considered and a basic biological understanding and a detailed knowledge of the disease/injury under investigation is vital. The ideal biomarker should originate from the damaged cells and display organ specificity. The dose-response relationship of the biomarker should be directly dependent on the extent of functional damage, and the change in expression should be expressed early after exposure and measurable in easily available tissue/fluid sample. The turn-over rate of the biomarker molecule should be fast in order to be able to follow the disease process and the measurement technique of choice should be easy and reliable [100].

The search for biomarkers after radiation exposure has primarily been studied *in vitro*. However, systemic responses, heterogeneous and varying absorbed dose distribution in radionuclide therapy, and heterogeneity in cell types within tissues and organs make it hard to translate *in vitro* response to *in vivo* situation. Indeed, preclinical work on cell lines often fails to translate to the clinic [139]. Molecular features may be lost in the adaptation to culture conditions [140]. Gene expression profiles have been suggested as potential biomarkers for biodosimetry, primarily *in vitro* and *ex vivo*, and 97 genes have been proposed as markers for radiation [141-143]. Recurrently observed genes include *Gadd45*, *Cdkn1a*, *Mdm2*, *Mapk*, *Pcna*, and *Ddb2*. Also potential protein biomarkers for radiation exposure has been studied, and the top candidates include ATM, H2AX, CDKN1A, and TP53 [126]. However, with the exception of *Cdkn1a*, these genes are rarely regulated in our data sets. Considering kidney damage, few studies have investigated the kidney response following exposure to ionizing radiation. However, extensive studies have been performed following chemically induced kidney injury in the search for biomarkers. The most prominent findings include *Lcn2* (NGAL), *Havcr1* (KIM-1), and *Il-18* [144-147]. These potential markers are all expressed in the proximal tubules, and also other cell types, and proposed as early indicators of acute kidney injury. However, their applicability as markers for chronic kidney damage is still to be determined.

In the transcriptional data presented in this thesis, expression levels for the *Havcr1*, *Lcn2*, and *Cdkn1a* genes were generally the most prominent after ^{177}Lu-octreotate administration (Figure 5). The *Havcr1* gene, also known as KIM-1 (kidney injury molecule 1), has previously been used in preclinical studies as a predictor of kidney tubular damage in a variety of animals, and in humans [144, 145]. These studies have shown that *Kim-1* mRNA levels are elevated after tubular damage, as well as at the protein level. In the present studies, the *Havcr1* gene was significantly regulated
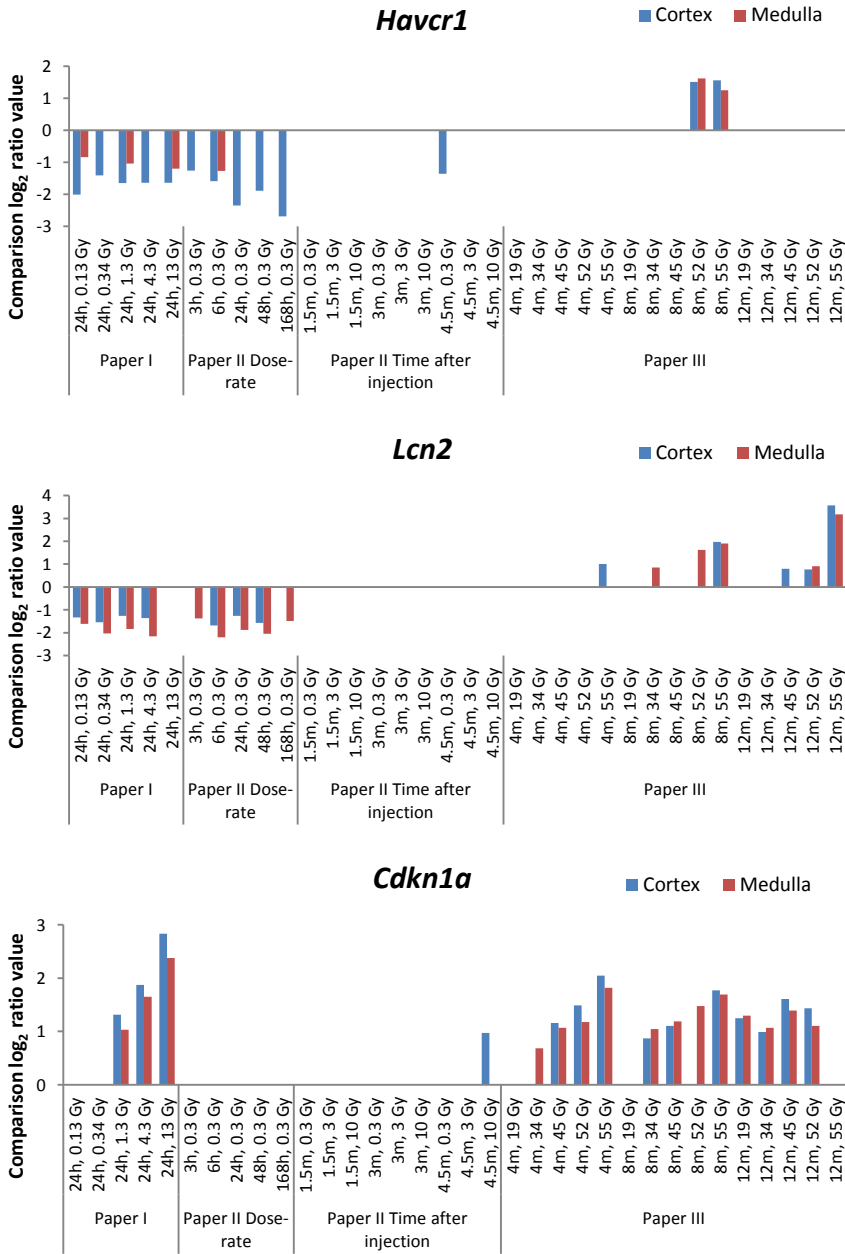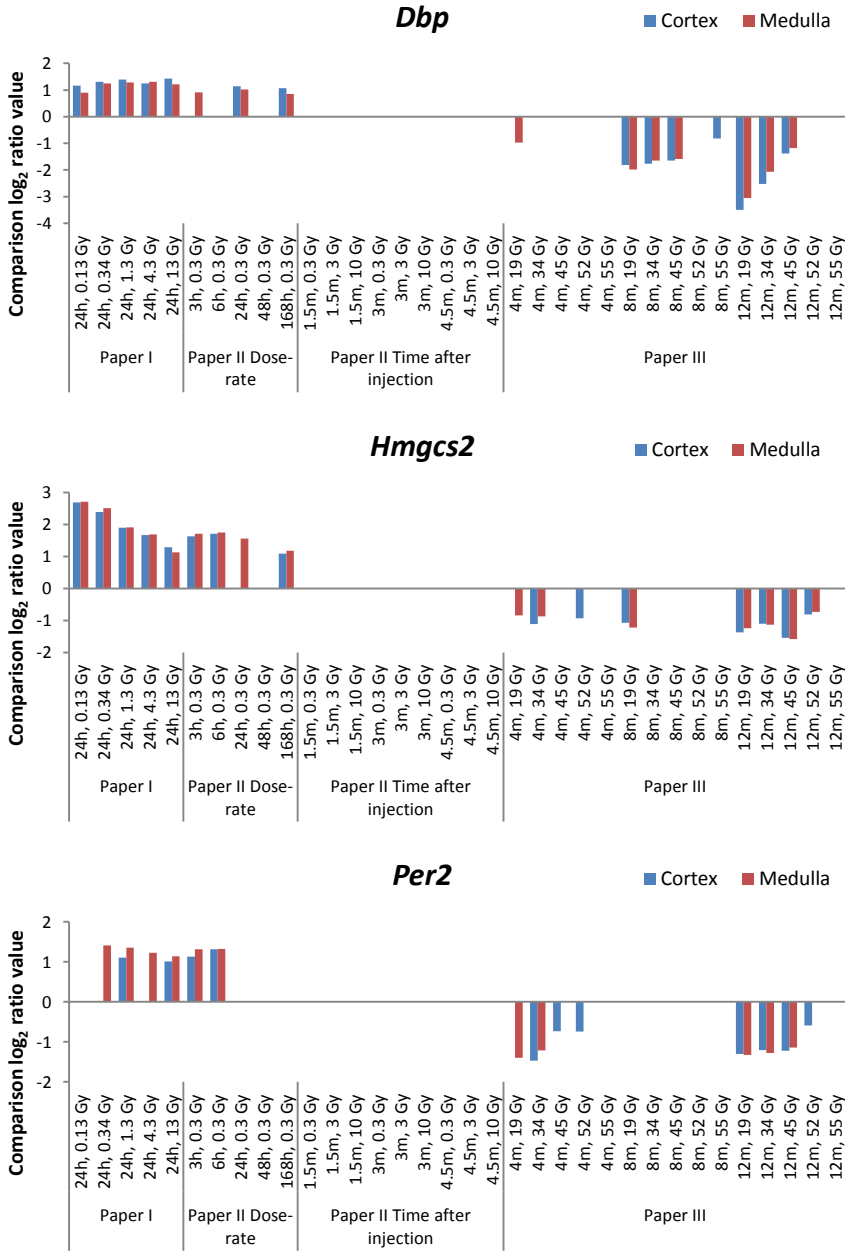
Figure 5. The transcriptional variations of *Havcr1*, *Lcn2*, and *Cdkn1a* as observed in Paper I, II, and III. Only statistically significant regulation is shown.

at all absorbed doses in Paper I and at all dose-rates in Paper II in kidney cortex while at longer time points this gene was rarely regulated (Paper II and III). Also the expression of *Lcn2* gene (NGAL) has been suggested as a predictor of tubular injury and other studies have shown that the NGAL gene expression was up-regulated within a few hours of harmful injury and was among the earliest genes induced after injury where the NGAL protein was detected in both blood and urine [147, 148]. The *Lcn2* gene was significantly regulated at all absorbed doses in Paper I and at all dose-rates in Paper II in kidney medulla. No regulation was observed at longer time points after ^{177}LuCl$_3$ exposure. However, after ^{177}Lu-octreotate, this gene was recurrently regulated, especially at higher absorbed doses at 8 and 12 months after injection. Commonly for both these genes are that they have primarily been studied after acute kidney injury, and that their expression has been found to be strongly up-regulated [144, 145, 147, 148]. The last finding is in contrast to the results presented in this thesis, where these genes were found down-regulated at the early time points investigated. Only at longer time points were these genes found to be up-regulated. The reason for this deviation is unknown, but could be due to that in the previous studies, the kidney injury was chemically induced and significant damage was present before measurements, while in the present studies, the gene expression levels were measured as early as 3h after injection, when probably no kidney damage was yet induced.

The *Cdkn1a* gene is a cyclin dependent kinase inhibitor highly involved in cell cycle progression and has previously been seen to be consistently regulated in response to ionizing radiation [141-143]. The *Cdkn1a* gene has also been found to be up-regulated shortly after acute renal failure [148]. While the gene was only statistically significantly regulated after 10 Gy at 140d after injection of ^{177}LuCl$_3$, it was recurrently regulated after ^{177}Lu-octreotate administration in a dose-dependent manner independent of time point investigated (Figure 5) [149, 150].

Identifying biomarkers for kidney toxicity and the ability to observe this change in expression at early time points is of highest concern to optimize individualized patient treatment. Other genes were also identified which showed significant de-regulation at both early and late time points. With the exception of *Cdkn1a*, the *Car3*, *Dbp*, *Hmgcs2*, *Mup2*, *Per2*, *S100a6*, and *Upk1b* genes in kidney cortex and *Actb*, *Adipoq*, *Car3*, *Cfd*, *Ckb*, *Dbp*, *Hmgcs2*, *Nupr1*, *Per2*, and *Upk1b* genes in kidney medulla were found to be differentially regulated at both 24h and either at 4, 8, or 12 months after ^{177}Lu-octreotate administration (Paper I and III). The *Dbp* and *Hmgcs2* genes were differentially regulated at all the studied dose levels (0.13-13 Gy) in both tissues in Paper I, and the *Per2* gene was differentially regulated at absorbed doses between 0.34 and 13 Gy (Figure 6). In Paper III, the *Dbp* and *Per2* showed clear dose-response in both tissues, while *Hmgcs2* was recurrently regulated

Figure 6. The transcriptional variations of *Dbp*, *Hmgcs2*, and *Per2* as observed in Paper I, II, and III. Only statistically significant regulation is shown.

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in both tissues and displayed clear dose response at 24h after injection. The expression of these genes at both early and late time points may warrant their use to evaluate the response to effects on the renal tissues.

The use of single markers may, however, not be sufficient given the complex radiation response and kidney heterogeneity of various dysfunction conditions. Furthermore, patients often have systemic diseases, which may complicate biomarker definition [113]. The use of multiple markers may be needed and may be more useful, since interplay between different pathways could be monitored [102]. Furthermore, the ability to measure the biomarker levels in easily obtained samples, such as plasma/serum or urine, would be advantageous from a clinical point-of-view.

Renal scintigraphy

99mTc-DTPA scintigraphy
The study of the relationship between biomarker expression and functional and morphological effects is important in order to identify an association between early indicators and late toxicity. 99mTc-DTPA is used for the external measurement of glomerular filtration rate (GFR) in the clinic, exclusively cleared by glomerular filtration in the kidneys, without secretion or reabsorption [108, 151, 152]. Normally following the clearance of 99mTc-DTPA there is a rapid uptake phase of 1-3 minutes followed by a slow excretion phase when the tracer passes to the bladder [151]. The slope of the uptake phase correlates well with the glomerular filtration rate but due to practical issues this (uptake) time-phase could only be measured in Paper IV, and the data in Paper III illustrate the excretion phase of 99mTc-DTPA. The functional effects of the kidneys following 177Lu-octreotate administration, as measured through 99mTc-DTPA scintigraphy, revealed a clear reduction in excretion of 99mTc-DTPA to the bladder. While no difference in filtration or excretion rate could be observed at 4 months after injection at any absorbed dose, at 8 months, an altered time-activity curve was observed for the group injected with 90 MBq, while the 150 MBq group displayed accumulation of the radio-tracer with no emptying into the bladder. A further alteration of the time-activity curve was observed at 12 months for the 90 MBq group. The accumulation of the radio-tracer for the 90 MBq group at 12 months is comparable to that observed for the 150 MBq group at 8 months, indicating a prolonged time until renal function loss with reduced absorbed dose. However, no effects on the time activity curves were observed for the group injected with 30 MBq at any time point. Furthermore, measurements of the 150 MBq group at 12 months were not possible due to the poor physical condition of these mice. In Paper IV, altered 99mTc-DTPA time-activity curves after 177Lu-octreotate

administration were obtained, with a generally increased ^{99m}Tc activity in the kidneys after ^{177}Lu administration. An absorbed dose-dependent delay in time until maximum intensity was found. However, no obvious dose-dependent alteration in the overall time-activity curve pattern was observed. The clear accumulation of ^{99m}Tc-DTPA in the kidneys following high absorbed doses was only found for the fractionation group (4x15 MBq at 12 months) in Paper IV.

A previous study on rats injected with 460 MBq ^{177}Lu-octreotate ^{111}In-DTPA scintigraphy at 100-120 days after administration showed reduced maximal uptake compared with controls, with almost total excretion at 20 min and stabilization at 40 min after injection. This finding is in line with the results observed in the present study at 4 months, where the activity concentration in the kidneys was stabilized at time points > 20 min. To our knowledge, a rise in renal time-activity-curve has not previously been observed after ^{177}Lu-octreotate administration. However, similar results have previously been shown with ^{99m}Tc-DTPA scintigraphy in dogs with nephrolithiasis [153]. Nevertheless, there were no indications that the mice developed calculi in the kidneys or ureters after irradiation, and the effect observed in the present study is most likely due to obstruction due to cell death.

^{99m}Tc-DMSA scintigraphy
^{99m}Tc-DMSA scintigraphic images are used for morphological studies in the clinic, demonstrating anatomy, tumors, renal trauma and inflammation, and to study relative differences in uptake between left and right kidney to estimate the renal functional mass [151]. ^{99m}Tc-DMSA scintigraphy has also successfully been used after ^{177}Lu-octreotate administration to study renal function in rats [96, 154]. A dose-dependent decrease in ^{99m}Tc-DMSA concentration in kidneys was observed at 109-146 d after administration of ^{177}Lu-octreotate (23, 9.9, and 1.4 %IA/g for 0, 278, and 555 MBq, respectively), indicating severe tubular damage for the highest injected activity [96]. Furthermore, a shift in distribution of ^{99m}Tc-DMSA was observed with increased accumulation in the outer medulla with increased injected activity of ^{177}Lu-octreotate. Similar results were observed 90 d after therapy using 460 MBq ^{177}Lu-octreotate, with a significant reduction in renal ^{99m}Tc-DMSA uptake. However, in paper III, no clear reduction could be observed at any time point or absorbed dose investigated, despite the functional aberration observed for ^{99m}Tc-DTPA. Nevertheless, in Paper IV, a reduction in ^{99m}Tc-DMSA uptake was observed in most groups but without a clear absorbed dose-response relationship.

Histological evaluation
Signs of kidney toxicity were observed through histological evaluation at the highest activity level investigated (150 MBq, 55 Gy) at 8 and 12 months after

administration. The observed effects were located to the glomeruli with observed sclerosing and segmentalization of the glomerular tuft. Signs of cell death were observed in the glomeruli at 12 months after administration in the form of nuclear fragmentation and segmental necrosis. Furthermore, indications of inflammation were seen. The results are in contrast to previously published morphological effects of ^{177}Lu-octreotate in rats (46-92 Gy, 4.5 months after injection) and nude mice (35-58 Gy, 6 months after injection) [47, 96], where the primary changes were located in the proximal and distal tubules. Those changes included inhomogeneous nuclei, apoptosis, and necrotic cells in the proximal and distal tubules in rat kidney and flattened epithelium, loss of brush border, dilation, and empty lumina in mouse kidney. No significant changes of the glomeruli were observed in these studies. Explanations for these differences in response are not obvious. Speculations may include divergences between animal strains, and differences in absorbed dose distribution within kidneys of different size, time after administration, and repair processes in kidney tissue.

Concluding remarks

Radionuclide therapy using radiolabeled peptides has recently become clinically available for treatment of various tumor types. However, kidneys and bone marrow are the dose limiting tissues. For the kidneys, the exposure limits are based on the tolerance doses established from external beam radiation therapy, despite significant differences between the two modalities. It is hypothesized that the exposure limit for the kidneys in radionuclide therapy can be increased without exceeding tolerable nephrotoxicity, leading to higher patient cure rates. However, we lack knowledge in the underlying mechanisms of radiation-induced effects on kidney tissue, which would give us a better understanding of nephrotoxicity after exposure to radiopharmaceuticals.

In this work, genome-wide transcriptional responses were integrated with functional and morphological data from kidney tissue after administration of ^{177}Lu-octreotate and [177]LuCl$_3$ to better understand the radiobiological response, and to identify and evaluate potential novel biomarkers for radiation-induced kidney injury. Furthermore, the effects of ^{177}Lu-octreotate on the renal cortical response were evaluated by protein and miRNA analysis.

The main conclusions for the individual papers included in this thesis can be summarized as follows:

Distinct differences in the transcriptional response were found 24h after administration of various amounts of ^{177}Lu-octreotate (Paper I). Significant differences in affected biological processes was observed between higher and lower absorbed doses, with lower association with metabolism and higher association with stress response with increasing absorbed dose.

The transcriptional response was found to be predominantly uniquely regulated at specific dose-rates following ^{177}Lu administration, and generally more transcripts were regulated at higher dose-rates (Paper II). Concerning time after injection, the number of differentially regulated transcripts increased with time, but decreased with absorbed dose, and the strongest affected associated biological processes included metabolism and stress-response.

At longer time points after ^{177}Lu-octreotate administration, clear negative effects was observed on the kidneys after high absorbed dose exposure, as determined by functional analysis (^{99m}Tc-DTPA) and histological evaluation (Paper III). In

addition, a strong transcriptional response was observed, with recurrent regulation of e.g. *Cdkn1a*, *C3*, *Dbp*, *Lcn2*, and *Per2*.

The evaluation of the proteomic response at longer time points after ^{177}Lu-octreotate administration on renal cortical tissue revealed a low number of proteins which were differentially regulated at multiple exposure conditions (Paper IV). The functional evaluation revealed altered time-activity curves following administration of ^{99m}Tc-DTPA, with increased time until maximum intensity with increasing absorbed dose, and a generally decreased accumulation of ^{99m}Tc-DMSA following ^{177}Lu-octreotate administration, but no clear dose-response relationship was observed.

The miRNA response was in general dose-specific 24 h after ^{177}Lu-octreotate administration (Paper V). Stress-response related pathways were strongly associated and recurrent regulation of miR-194-5p, miR-107-3p, miR-3090-5p, and miR-3077-5p was observed.

Altogether, the results from these studies demonstrate distinct differences in the biological response after different absorbed doses, dose rates, and time after injection. The number of differentially regulated transcripts was generally higher at higher dose rates as well as at later time points. Furthermore, a reduced number of regulated transcripts were observed with increasing absorbed dose after [177]LuCl$_3$ administration; this effect was not observed after ^{177}Lu-octreotate administration. When comparing the regulated transcripts between the studies, no transcripts were recurrently regulated at all exposure conditions. The most recurrently regulated transcripts were related to the *Serpina10* gene in kidney cortex, and the *Egr1*, *Pck1*, and *Hmgcs2* genes in kidney medulla. Three of these transcripts were regulated in both kidney cortex and medulla, i.e. *Cdkn1a*, *Dbp*, and *Hmgcs2*. Considering only ^{177}Lu-octreotate exposure, the most recurrently regulated transcripts were connected to *Cdkn1a*, *Dbp*, *C3*, *Lcn2* and *Per2* genes. A frequently observed tendency among the recurrently regulated transcripts was the dependence of the type of injected substance and time after injection. Furthermore, a strong association with stress response-related transcripts was found, especially for higher absorbed doses. Interestingly, ^{177}Lu-octreotate administration induced a stress response of the kidneys which is persistent and measurable at least up to 12 months.

Future perspectives

The primary focus of this work was to increase our understanding of the kidney response to ^{177}Lu-octreotate and to identify potential novel biomarkers for radiation induced injury. In future work, several aspects of the current work should be explored further.

The biomarkers which are proposed in this work ought to be further tested in animals at the protein level in, e.g., plasma and urine in order to evaluate their potential for clinical usage. The protein biomarker levels need to be correlated with the functional and morphological effects of the kidneys.

Clinical verification should then be performed, where the proposed biomarkers should be tested in patients receiving ^{177}Lu-octreotate treatment. Measurements should be performed both on plasma and urine samples collected at various time-points after treatment, and careful long term follow-up of these patients should be undertaken in order to correlate kidney function/injury with the biomarker levels in the collected samples. A better understanding of the functional parameters studied today is also needed.

Studies must be conducted in order to more fully understand the different dependencies of dose (both concerning absorbed dose and amount of peptide administered), dose-rate, and time after administration. Also the influence of other factors, e.g. tumor burden and inflammation processes, should be investigated in relation to biomarker levels.

A clear difference between ^{177}Lu and ^{177}Lu-octreotate exposure to the kidneys were found in the present investigation, and this effect should be further explored in animals in order to obtain a better understanding of the biological response. It is desirable to be able to separate the part of the response that is due to the exposure of ionizing radiation, and the part that is due to the hormone analog, but synergistic effects might also be present.

There are still great uncertainties in the tolerance dose of normal tissues following PRRT, and the limits used today are defined for external radiation therapy. There are indications that the tolerance doses after PRRT are higher than for external radiation therapy, and studies should be designed to specifically test the tolerance doses following ^{177}Lu-octreotate administration.

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Paper I

Transcriptional response of kidney tissue after ^{177}Lu-octreotate administration in mice

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Paper II

Time- and dose rate-related effects of internal ^{177}Lu exposure on gene expression in mouse kidney tissue

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Paper III

Potential biomarkers for radiation-induced renal toxicity following ^{177}Lu-octreotate administration in mice

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Paper IV

Proteomic and functional analysis for the assessment of radiation induced kidney response after ^{177}Lu-octreotate administration in mice

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Paper V

Distinct microRNA expression profiles in mouse renal cortical tissue after ^{177}Lu-octreotate administration

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