Radiobiological effects of the thyroid gland

Transcriptomic and proteomic responses to ^{131}I and ^{211}At exposure

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Stay hungry.
Stay foolish.

Whole Earth Catalog (1974)

Abstract

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Transcriptomic and proteomic responses to ^{131}I and ^{211}At exposure

Nils Rudqvist
Department of Radiation Physics, Institute of Clinical Sciences, Sahlgrenska Academy at University of Gothenburg, Göteborg, Sweden, 2015

Radionuclides are widely used in medicine. ^{131}I is one of the most employed radionuclides and is administered to patients either bound to tumor targeting molecules or as halide to target the thyroid or thyroid cancer. ^{211}At is proposed for radionuclide therapy and preclinical and clinical research on ^{211}At-labeled tumor targeting molecules is on-going. The thyroid gland accumulates both ^{131}I and ^{211}At as halides and is an organ at risk. Additionally, ^{131}I exposure of thyroid may occur from radioactive fallout from e.g. nuclear accidents. There is a lack of knowledge of molecular mechanisms in thyroid cells after ^{131}I or ^{211}At exposure.

The overall aim of this work was to examine the transcriptomic and proteomic effects of ^{131}I and ^{211}At exposure on normal thyroid tissue in vivo. The influence of absorbed dose, dose-rate, time after administration, and radiation quality on gene expression regulation was studied. Another aim was to identify radiation-responsive genes in thyroid.

Mice and rats were i.v. injected with 0.064-42 kBq ^{211}At or 9-4700 kBq ^{131}I. Resulting absorbed dose to thyroid from ^{211}At and ^{131}I exposures were 0.023-32 and 0.0058-34 Gy, respectively. Transcriptomic and proteomic responses in thyroid and plasma were measured 1-168 h after administration using RNA microarray and liquid chromatography mass spectrometry, respectively. Fold-change and adjusted p-value cut-offs of 1.5 and 0.01 were used to determine statistically significantly regulated transcripts. Pathway analyses were performed using Gene Ontology and the Ingenuity Pathway Analysis tool (p-value < 0.05). Plasma T4 and TSH levels were measured in rats using ELISA.

The transcriptional response in thyroid tissue after ^{131}I and ^{211}At exposure varied with absorbed dose, dose-rate, time after administration, and radiation quality. In mice, 27 recurrently regulated genes were identified after ^{131}I or ^{211}At exposure and genes with similar function shared similar transcriptional regulation patterns. Additionally, regulation of several kallikrein genes was identified in mouse thyroid tissue after ^{131}I or ^{211}At administration. In rats, 2 recurrently genes were identified: *Dbp* and *Slc47a2*. Different biological functions were affected in response to different exposure conditions. For example, effects on immune response were found at 1, 6, and 168, but not 24 h after 1.7 kBq ^{211}At administration in mice. An impact on rat thyroid function with regulation of 13 genes crucial for thyroid hormone synthesis was identified. The proteomic response to 32 Gy suggests hypoxia in thyroid and decreased thyroid function.

Profound effects on gene expression regulation with distinct differences in response to different exposures were identified in mouse and rat thyroid tissue following ^{131}I or ^{211}At exposure. The transcriptional response likely depends to a varying degree on absorbed dose, dose-rate, time after administration, and radiation quality. Recurrently regulated genes were identified, and the biomarker applicability of these genes should be further assessed.

Keywords: astatine-211, iodine-131, radionuclide therapy, nuclear medicine, environmental exposure, thyroid, radiation biology, transcriptomics, proteomics, ionizing radiation, toxicity, normal tissue damage, microarray, LC-MS, biomarker

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Populärvetenskaplig sammanfattning

Jod-131 (^{131}I) är ett radioaktivt ämne som flitigt används inom sjukvården. ^{131}I ges för behandling av en överaktiv sköldkörtel eller av sköldkörtelcancer. ^{131}I kan också bindas till målsökande molekyler för behandling av cancer med annat ursprung än sköldkörteln. Ett exempel på ett sådant preparat är det radioaktiva läkemedlet ^{131}I-MIBG som används för att behandla vissa typer av hormonproducerande tumörer. Astat-211 (^{211}At) är ett annat radioaktivt ämne med potential för behandling av olika typer av cancersjukdomar.

När ^{131}I och ^{211}At används inom sjukvården kommer fritt ^{131}I eller ^{211}At finnas i blodet och ansamlas i frisk sköldkörtelvävnad. Anledningen till att fritt ^{131}I ansamlas i sköldkörteln är att sköldkörteln använder jod för att bilda de viktiga sköldkörtelhormonerna och att sköldkörteln inte kan skilja på radioaktivt och vanligt jod. Ansamling av ^{211}At i sköldkörteln beror på att ^{211}At delar vissa kemiska egenskaper med jod. Med andra ord, användning av ^{131}I och ^{211}At i sjukvården resulterar i oönskad bestrålning av frisk sköldkörtelvävnad, utöver bestrålning av den sjuka vävnaden som skall undersökas eller behandlas.

En annan källa till människors kontakt med ^{131}I är radioaktiva utsläpp vid kärnkraftsolyckor eller vid atombombsprängningar. Detta har hänt upprepande gånger under historien och senast i Fukushima i Japan, efter att en tsunami påverkade kärnkraftverk så kraftigt att ett radioaktivt utsläpp skedde.

Även om vi idag vet en hel del om strålningens effekter på sköldkörteln finns fortfarande stora kunskapsluckor. Det övergripande syftet med detta avhandlingsarbete var att öka kunskapen om biologiska effekter efter bestrålning av sköldkörteln med ^{131}I och ^{211}At och att försöka svara på följande frågor:

- Vilka biologiska funktioner påverkas vid bestrålning av sköldkörteln?
- Hur påverkar skillnader i bestrålningssättet effekterna på sköldkörteln?
- Går det att utforma test som gör det möjligt att veta om sköldkörteln har blivit bestrålad, och i så fall med vilken stråldos?

Våra studier visar att strålningens effekter på sköldkörteln beror mycket på bestrålningssättet. En viktig faktor som påverkar effekten på sköldkörteln är hur hög stråldos som ges per timme. Andra viktiga faktorer är mängden radioaktivitet vi ger och vid vilken tidpunkt efter behandling vi undersöker effekten. Vi har också visat att det är svårt att förutsäga vilka effekter ett bestrålningssätt har genom att studera ett annat. Detta beror på att effekterna varierar så mycket mellan olika bestrålningssituationer.. Resultaten visar att är det mycket viktigt att fortsätta studera effekter från strålning, framförallt vid låga stråldoser som hittills varit svåra att undersöka. Vi har även funnit en mängd olika möjliga biomarkörer för bestrålning av sköldkörteln. Dessa har potential att användas för att utforma ett test för att veta om sköldkörteln har blivit bestrålad, och i så fall ge ett mått på stråldos.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Nils Rudqvist**, Toshima Z Parris, Emil Schüler, Khalil Helou, Eva Forssell-Aronsson. *Transcriptional response of BALB/c mouse thyroids following in vivo astatine-211 exposure reveals distinct gene expression profiles*. EJNMMI Res, 2012, 2:32

II. **Nils Rudqvist**, Emil Schüler, Toshima Z. Parris, Britta Langen, Khalil Helou, Eva Forssell-Aronsson. *Dose-specific transcriptional responses in thyroid tissue in mice after ^{131}I administration.* Nucl Med Biol, 2015, 42(3)

III. **Nils Rudqvist**, Johan Spetz, Emil Schüler, Toshima Z. Parris, Britta Langen, Khalil Helou, Eva Forssell-Aronsson. *Transcriptional response in mouse thyroid tissue after ^{211}At administration: effects of absorbed dose, initial dose-rate and time after administration*. Submitted

IV. **Nils Rudqvist**, Johan Spetz, Emil Schüler, Toshima Z. Parris, Britta Langen, Khalil Helou, Eva Forssell-Aronsson. *Gene expression signature in mouse thyroid tissue after ^{131}I and ^{211}At exposure.* Submitted

V. **Nils Rudqvist**, Johan Spetz, Emil Schüler, Toshima Z. Parris, Britta Langen, Khalil Helou, Eva Forssell-Aronsson. *Transcriptional response to ^{131}I exposure of rat thyroid.* Submitted

VI. **Nils Rudqvist**, Johan Spetz, Britta Langen, Emil Schüler, Toshima Z. Parris, Carina Sihlbom, Khalil Helou, Eva Forssell-Aronsson. *Early proteomic response in thyroid gland after ^{131}I administration in mice*. Manuscript

Selection of related presentations

1. **Nils Rudqvist**, Toshima Parris, Szilard Nemes, Khalil Helou, and Eva Forssell-Aronsson. *Radiobiological effects on gene expression levels in normal mouse thyroid after ^{131}I irradiation*. 38th Annual Meeting of the European Radiation Research Society, Stockholm, Sweden, September 5-9, 2010

2. **Nils Rudqvist**, Toshima Parris, Szilard Nemes, Khalil Helou, and Eva Forssell-Aronsson. *Radiobiological effects (changes in gene expression) on mouse thyroid after irradiation with ^{211}At or ^{131}I*. 10th Congress of the World Federation of Nuclear Medicine and Biology, Cape Town, South Africa, September 18-23, 2010

3. **Nils Rudqvist**, Toshima Parris, Emil Schüler Khalil Helou, and Eva Forssell-Aronsson*. In vivo ^{211}At exposure reveals distinct absorbed dose-dependent gene expression profiles in mouse thyroid tissue.* 14th International Congress of Radiation Research, Warszawa, Poland, August 28 – September 1, 2011

4. **Nils Rudqvist**, Toshima Parris, Emil Schüler, Khalil Helou, and Eva Forssell-Aronsson. *In Vivo Iodine-^{131} Exposure Reveals Distinct Absorbed Dose-dependent Gene Expression Profiles in Mouse Thyroid Tissue*. From Dosimetry to Biological Effect: Radiobiology as Guide to Clinical Practice in Nuclear Medicine, Sorrento, Italy, November 5-9, 2011

5. **Nils Rudqvist**, Toshima Parris, Emil Schüler, Britta Langen, Khalil Helou, and Eva Forssell-Aronsson. *Astatine-211 exposure of Balb/c mice in vivo resulted in distinct effects on thyroid at 1, 6 hours and 7 days after injection*. 58th Annual Meeting of the Radiation Research Society, San Juan, Puerto Rico, September 30 - October 3, 2012

6. **Nils Rudqvist**, Toshima Parris, Emil Schüler, Britta Langen, Khalil Helou, and Eva Forssell-Aronsson*. Balb/c mice thyroids revealed distinct effects on gene expression at 1, 6 hours and 7 days after injection of exposure to ^{211}At*. 25th Annual Congress on European Association of Nuclear Medicine, Milano, Italy, October 27-31, 2012

7. **Nils Rudqvist**, Britta Langen, Toshima Parris, Emil Schüler, Khalil Helou, and Eva Forssell-Aronsson. *Transcriptional effects on normal tissues after administration of ^{211}At in mice*. Targeted alpha radionuclide therapy meeting, Oak Ridge, TE, United States, June, 2013

8. **Nils Rudqvist**, Britta Langen, Emil Schüler, Toshima Parris, Khalil Helou, and Eva Forssell-Aronsson. *Radiation-induced transcriptional response in kidneys, liver, lungs, spleen, and thyroid in mice exposed to ^{211}At*. 59th Annual Meeting of the Radiation Research Society, New Orleans, LA, USA, September 13-18, 2013

9. Eva Forssell-Aronsson, **Nils Rudqvist**, Britta Langen, Emil Schüler, Toshima Parris, and Khalil Helou. *Radiobiologiska effekter på thyroidea efter exponering för ^{211}At och ^{131}I*. Strålsäkerhetsmyndighetens forskningsdagar, Stockholm, Sweden, October 24-25, 2013

10. **Nils Rudqvist**, Britta Langen, Emil Schüler, Toshima Parris, Khalil Helou, and Eva Forssell-Aronsson. *Radiation-induced transcriptional response in thyroid tissue change with time in mice exposed to ^{211}At*. Cancerfondens planeringsgrupp för radionuklidterapi, Gothenburg, Sweden, November 14-15, 2013

11. **Nils Rudqvist**, Toshima Parris, Britta Langen, Emil Schüler, Johan Spetz, Khalil Helou, and Eva Forssell-Aronsson. *Genetic signatures detected in thyroid tissue in mice after ^{131}I and ^{211}At exposure*. 60th Annual Meeting of the Radiation Research Society, Las Vegas, NV, USA, September 20-24, 2014

12. **Nils Rudqvist**, Johan Spetz, Toshima Parris, Emil Schüler, Britta Langen, Khalil Helou, and Eva Forssell-Aronsson. *Transcriptional Relationships and Biological Functions of 27 Genes Commonly Regulated in Thyroid Tissue in Mice after ^{131}I and ^{211}At Exposure*. 27th Annual Congress on European Association of Nuclear Medicine, Gothenburg, Sweden, October 18-22, 2014

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Abbreviations

^{131}I	Iodine-131
^{211}At	Astatine-211
BASE	BioArray Software Environment
Bq	Becquerel
DNA	Deoxyribonucleic acid
DSB	Double strand break of DNA
EANM	European Association of Nuclear Medicine
ELISA	Enzyme-linked immunosorbent assays
eV	Electronvolt
GO	Gene Ontology
Gy	Gray
IA	Injected activity
i.v.	Intravenous
IPA	Ingenuity Pathway Analysis
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LET	Linear energy transfer
mRNA	Messenger RNA
NIS	Sodium-iodide symporter
RBE	Relative biological effectiveness
RIN	RNA integrity number
RNA	Ribonucleic acid
SSB	Single strand break of DNA
T3	Triiodithyronine
T4	Thyroxine
TH	Thyroid hormones
TG	Thyroglobulin
TPO	Thyroid peroxidase
tRNA	Transfer-RNA
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor

1 Background

1.1 Introduction

Radionuclides are widely used in medicine. Iodine-131 (^{131}I), a beta particle emitter, is one of the most employed radionuclides and is administered to patients either bound to tumor targeting molecules or as halide to target the thyroid [2-11]. Astatine-211 (^{211}At) is an alpha particle emitting radionuclide well suited for radionuclide therapy due to optimal physical properties [12]. ^{211}At is not yet fully utilized in clinics, but both preclinical and clinical research is on-going [13-18]. Medical use of ^{131}I and ^{211}At involves circulation of free radionuclide, and since the thyroid gland accumulates both ^{131}I and ^{211}At, the thyroid gland is an organ of risk [14, 19, 20]. Additionally, ^{131}I exposure of thyroid is associated with radioactive fallout, and about 1,760,000 and 100,000-200,000 TBq ^{131}I were released in conjunction with the nuclear power plant accidents in Chernobyl and Fukushima, respectively [21, 22].

Little is known about toxic effects from ^{211}At exposure on the normal thyroid. However, ^{131}I exposure may result in detrimental effects, e.g. hypothyroidism and thyroiditis [7, 20, 23]. The risk of carcinogenesis after medical use of ^{131}I is not clear, although an increased incidence of thyroid cancers were detected in individuals exposed to ^{131}I as children during the Chernobyl accident [21, 23].

Today we lack knowledge of molecular mechanisms that occur in thyroid cells after ^{131}I or ^{211}At exposure. This research project aimed to gain a deeper understanding of the biological effects of ^{131}I and ^{211}At exposure on normal thyroid tissue *in vivo*.

1.2 The thyroid gland

The thyroid gland is one of the largest endocrine gland that consists of one right and one left lobe connected by a small strip of tissue (i.e. the isthmus) and located in the neck across the trachea and below the thyroid cartilage. Thyroid tissue is composed mainly of follicles, three dimensional structures formed by a single layer of thyroid follicular epithelial cells lining a central lumen, i.e. the colloid. The microanatomy of mouse thyroid is shown in **Figure 1.1**. The thyroid follicle cells produce and secrete two thyroid gland hormones containing iodine (I); triiodothyronine (T_3) and thyroxine (T_4), both essential for e.g. regulation of normal tissue metabolism throughout the body. In this thesis, thyroid hormones (TH) will be used as a collective name for T_3 and T_4 hormones.

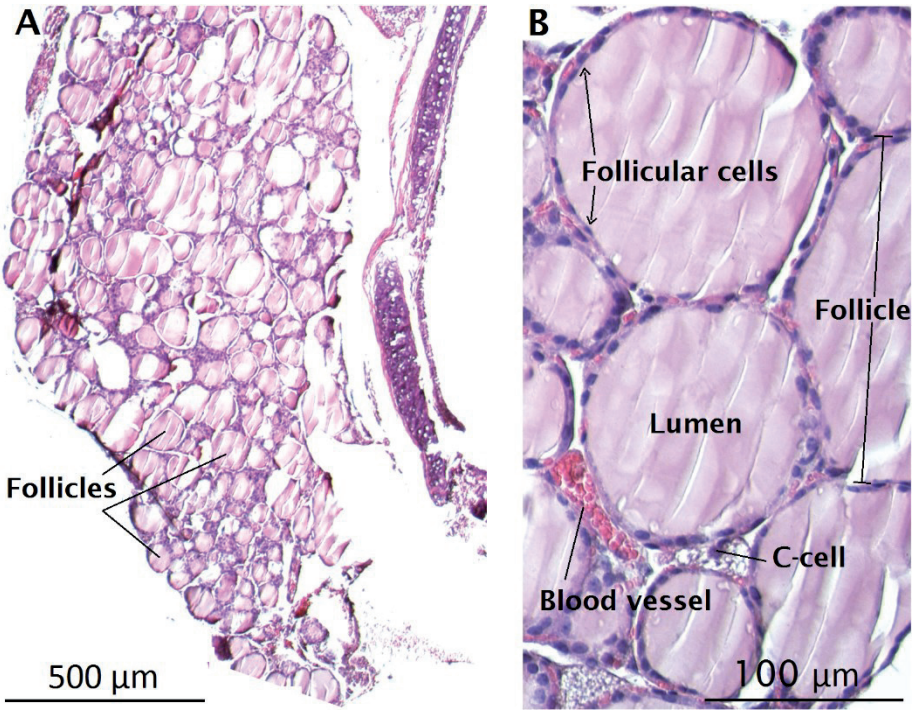

Figure 1.1 Microanatomy of mouse thyroid tissue. Both panel A and B show 4 µm hematoxylin-eosin stained microtome sections of female BALB/c nude mouse thyroid tissue at 4 and 20x magnification, respectively. The spherical structures are thyroid hormone producing follicles that consist of a single layer of thyroid follicular epithelial cells lining a central lumen, i.e. the colloid. Calcitonin producing parafollicular cells, i.e. the c-cells, are located on the periphery of the follicles and do not border the lumen. Blood vessels are located in close proximity to the follicles

1.2.1 Thyroid hormone synthesis and iodine transport

The thyroid hormone synthesis is illustrated in **Figure 1.2**. Iodide (I^-) is transported into the thyroid follicular cells across the basolateral membrane via the sodium-iodide symporter (NIS/SLC5A5), followed by transportation across the apical membrane into the colloid, partly via pendrin (SLC26A4) [24, 25]. Then, thyroid peroxidase (TPO) oxidizes I^- and iodinates tyrosine residues of thyroglobulin (TG), a storage molecule of thyroid hormones, produced by the thyroid follicular cells [26, 27]. Iodinated thyroglobulin binds to the megalin receptor (LRP2) and is transported into the follicular cell through endocytosis, after which proteolysis of thyroglobulin and secretion of released thyroid hormones occur [28, 29]. The rate of thyroid hormone production and secretion is controlled by a complex system with both negative and positive feedback loops. One crucial regulator is the thyroid stimulating hormone (TSH), that is secreted

by the anterior pituitary gland and binds to the thyroid stimulating hormone receptor (TSHR) and activates thyroid hormone synthesis and secretion [26].

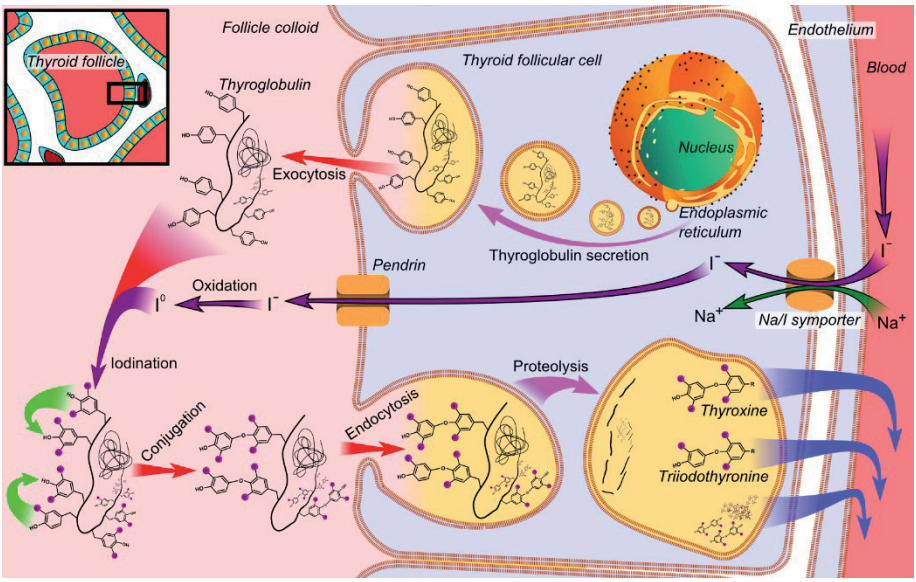

Figure 1.2 Synthesis of the thyroid hormones. The thyroid gland uses iodine to produce and secretes triiodothyronine (T_3) and thyroxine (T_4). Image in the public domain and retrieved from http://en.wikipedia.org/wiki/Thyroid_hormone on February 27, 2015, reprint from [1]

The thyroid gland mainly secretes thyroid hormones in the form of T_4, and serum T_4 levels are about 40 times higher compared to serum T_3 levels. The absolute majority of total serum T_3 (99.7 %) and T_4 (99.97 %) is bound to carrier proteins, e.g. thyroxine binding globulin and albumin [30]. Thyroid hormones are not active when attached to carrier molecules, but through deiodination of T_4, the more active thyroid hormone T_3 is produced [31, 32]. Then, T_3 is transported into the cells and binds to nuclear receptors for transcriptional regulation of target genes [30]. The thyroid hormones have a vital role in regulation of metabolism, and energy and oxygen consumption, although various other effects of thyroid hormones have been discovered. For example, thyroid hormones are critical for normal bone growth and development, and also play a role in the development and function of fat tissue [30].

The thyroid gland also contains parafollicular cells (C-cells), responsible for producing the hormone calcitonin that acts to decrease calcium concentration in blood (**Figure 1.1**).

1.2.2 Thyroid diseases

A dysfunctional thyroid may result in elevated or reduced serum thyroid hormone levels. These two conditions are called hypo- and hyperthyroidism, respectively.

Hypothyroidism is the condition when the thyroid gland produces insufficient amounts of thyroid hormones. Iodine deficiency is the most common cause of hypothyroidism, and 2007 the Lancet reported that approximately 2 billion individuals had insufficient iodine uptake, and that iodine deficiency was the single greatest cause of mental retardation [33]. Autoimmune thyroiditis is another cause of hypothyroidism, as well as side effects of external radiation therapy of the head and neck region or exposure to excessive amounts of iodide.

The opposite of hypothyroidism is called hyperthyroidism and is characterized of elevated serum thyroid hormone levels. Graves' disease is the most common cause of hyperthyroidism. It is an autoimmune disease when thyroid stimulating hormone receptor binding immunoglobulins (TSHR-Ab) binds to TSHR resulting in overproduction of thyroid hormones. Other reasons of elevated serum thyroid hormone levels are the presence of thyroid hormone overproducing (i.e. toxic) thyroid adenomas or multinodular goiter.

Several different types of thyroid cancer exist: papillary, follicular, medullary, and anaplastic thyroid carcinomas. Papillary and follicular thyroid carcinomas are most common and represent about 80 and 15 % of all thyroid cancer cases, respectively. Papillary and follicular thyroid carcinoma originate from the thyroid follicular cells, are usually well differentiated, have maintained iodide transport and are thus eligible for radioiodine treatment (see **Chapter 1.4.1**). Medullary thyroid carcinoma originates from C-cells and represent about 5-8 % of all thyroid cancer. Anaplastic thyroid cancer is the least common type of thyroid cancer, representing less than 5 % of all thyroid cancers. In this cancer form the thyroid follicle cells have a clearly reduced NIS expression and thus a lower iodide transport function.

1.3 Gene expression regulation

Proteins are biomolecules that perform a wide variety of tasks in a living organism. Examples of tasks are DNA repair or replication, or mediation of a response to stimuli (e.g. ionizing radiation or an extra-cellular signaling molecule). The blueprints of all proteins are stored as genes in DNA in the cell nucleus. When the cell demands a certain protein, e.g. in response to a stressor,

genes are activated or inactivated which results in initiation of protein synthesis (**Figure 1.3**).

The genetic information – the blueprints of proteins – is stored in the cell nucleus as DNA. This information is transferred to the site of protein production by mRNA, a single strand template of the DNA, created in a process called transcription. The proteins are then synthetized from mRNA templates in a process called translation.

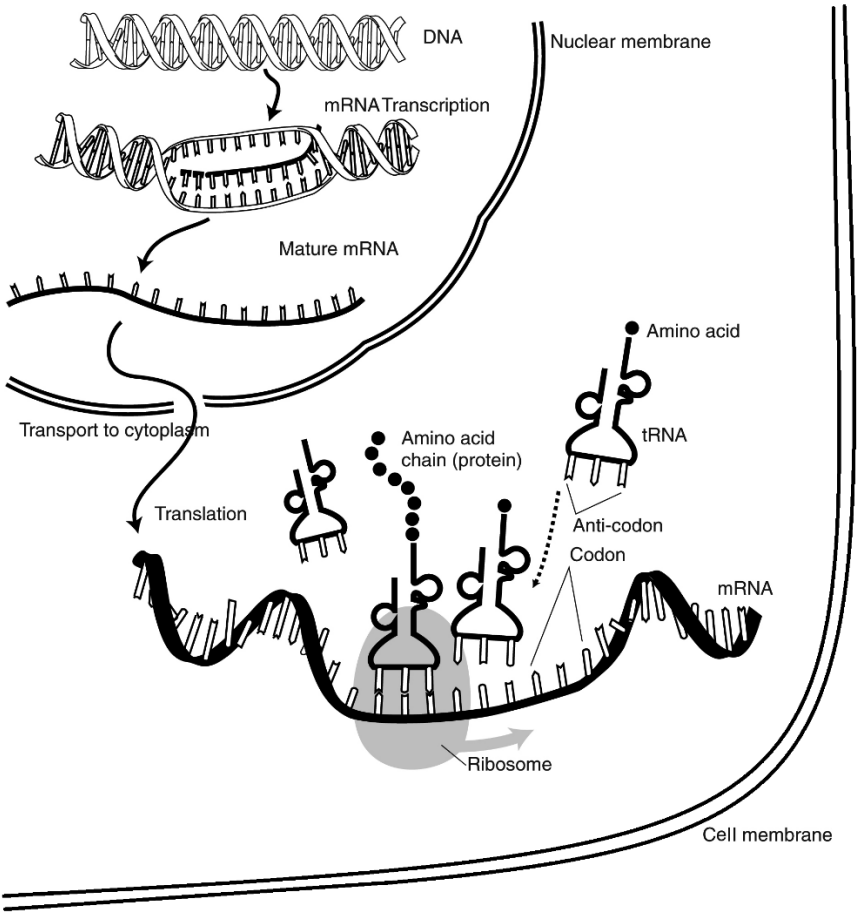

Figure 1.3 Protein synthesis simplified. Transcription of DNA is performed in the cell nucleus. The mRNA is matured, e.g. spliced, and transported to the ribosomes for translation. Picture was retrieved from Alan Benson, Summary of translation, http://www.ck12.org/user:YXJiZW5zb25AY2RzY2hvb2xzLm9yZw../section/Section-8.2:-Protein-Synthesis/ on March 25, 2015, Creative Commons Licence (CC BY-NC 3.0). Picture have been modified

1.3.1 Transcription of DNA

Transcription takes place in the cell nucleus where a complex of regulators together with the RNA polymerase binds to specific sites of the DNA and enables production of messenger RNA. The synthetized mRNA is thus a template of the DNA and carries the genetic information needed to assemble a certain protein. The initial mRNA molecule is further matured, e.g. by a process called splicing, in which the introns (non-coding RNA sequences) are excised and exons (protein coding sequence) are joined. The splicing process may result in different combinations of exons called transcripts, thus creating different proteins. In other words, one gene may encode for several transcripts leading to different proteins. The complete setup of all RNA transcripts within a cell is called the transcriptome, and the study of the transcriptome is called transcriptomics.

Several classical molecular techniques can be used to measure mRNA levels, e.g. reverse transcription quantitative polymerase chain reaction (RT-qPCR) and northern blotting. The development of modern techniques such as RNA microarrays and RNA sequencing has enabled researchers to assess whole-genome gene expression regulation in one single experiment. RNA microarrays are used to determine gene expression variations of all gene products at the same time, providing a snap-shot of what proteins are demanded at a certain time-point. RNA microarray technique is a method that offers relative quantification of changes in gene expression levels between two or more samples (usually between a test and control sample). Therefore, the choice of control is an important factor since it should represent a “normal” gene expression base-line.

1.3.2 Translation of mRNA to proteins

The mature mRNAs are transported out of the nucleus to ribosomes at the site of protein production in the cytoplasm where preproteins are produced followed by post-translational modifications to create the mature functional protein. Cellular functions requiring quick production or large amounts of a certain protein will store inactive preproteins within the cell. Thus, functions in the cell may be performed without *de novo* protein synthesis. Similarly to the transcriptome, the complete setup of all proteins within a cell is called the proteome.

Altered levels of predetermined single protein can be assessed using e.g. western blotting and enzyme-linked immunosorbent assays (ELISA). Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a technique available for analysis of the proteome. The method does not demand a pre-determined decision on what proteins are to be included in the measurements. This may be a good feature since it removes possible bias in selecting proteins to study. On the

other hand, proteins with high abundance are more likely to be detected in comparison with less frequent proteins. LC-MS/MS, together with chemical labeling, can be used to identify relative changes in protein levels between two or more samples.

1.3.3 Pathway analysis

Gene expression regulation is a dynamic process with fluctuation over time, and is affected by signaling pathways with up- and downstream regulation. To assess the effect of radiation on gene expression regulation, it is interest to investigate the effects on such networks of genes, or pathways, instead of assessing regulation of single genes. Also, the signaling pathways are linked to one or several biological functions, enabling prediction of changes in biological functions using transcriptomic and proteomic variations.

Several different methods can be used to assess effects on pathways or biological functions from altered transcriptomic and proteomic levels. One approach is by attaching biological information – Gene Ontology terms (GO) – to the genes. The Gene Ontology consortium is a large bioinformatics initiative in which over 100,000 scientific papers have been searched for gene functions [34]. The goal is to have a common vocabulary of biological functions to ease analysis of biological systems. The GO terms system is built like an ancestor chart, with specialized biological function in one end and more general biological function in the other. With this system design, all GO terms are connected, even though they can be far apart. For example, GO terms “single strand break repair” and “double strand break repair” share their parent GO term “DNA repair”. Another example is “single strand break” and “adaptation of immune response”, two GO terms far apart and first connected by their common ancestor GO term “response to stimulus. Another tool available for pathway analysis is the Ingenuity Pathway Analysis (IPA). IPA utilizes the Ingenuity Knowledge Base (IKB) to associate transcriptomic or proteomic changes with biological information [35, 36]. The canonical pathway analysis tool relates genes with pathways and enables biological interpretation of the data. The upstream regulator analysis tool identifies upstream regulators that could potentially explain changes in mRNA or protein levels, and the diseases and function analysis tool predicts disease or altered function from changes in mRNA or protein levels.

1.4 Thyroid exposure to ^{131}I and ^{211}At

The thyroid gland cannot distinguish between stable (^{127}I) iodine and ^{131}I. Consequently, the thyroid gland will accumulate circulating ^{131}I (which is

exploited when a thyroid disease can be treated by destroying thyroid tissue [19, 37]. Since ^{131}I and ^{211}At to some extent share chemical characteristics, selective uptake of ^{211}At will also occur in the thyroid, although not as high as the uptake of ^{131}I [19, 38-40]. While iodine is primarily transported into the thyroid follicle cells through NIS, astatine also has other transport mechanisms not dependent on NIS [41]. The uptake of ^{131}I and ^{211}At in thyroid can to a high extent be blocked by administration of, e.g., potassium iodide, although the effectiveness of blocking likely differs between ^{131}I and ^{211}At due to the additional uptake mechanisms of ^{211}At.

1.4.1 ^{131}I

1.4.1.1 Physical properties of ^{131}I

^{131}I undergo β^- decay with a half-life of 8.0 d to stable ^{131}Xe (**Figure 1.4**, **Table 1.1**). The β particles emitted by ^{131}I have a mean energy of 190 keV, a mean CSDA range of 400 μm (for ~190 keV beta particles), and a mean LET of 0.25 keV/μm [42, 43].

1.4.1.2 Environmental exposure to ^{131}I

There are several reasons why humans have been and likely will be exposed to ^{131}I from the environment. The most studied source of exposure originates from the breakdown of reactor 4 in Chernobyl 1986. Approximately $1.8 \cdot 10^{18}$ Bq ^{131}I was released and median thyroid absorbed doses of 356 and 39 mGy have been reported in Belarus and Russia, respectively (maximum thyroid absorbed dose was estimated to 9.5 and 5.3 Gy, respectively) [21, 44]. Only 6 years after the Chernobyl accident, an elevated thyroid cancer incidence was reported in Belarus children: from a few cases per year (2, 4, 5, and 6 cases in 1986, 1987, 1988, and 1989, respectively) to 29 and 55 cases in 1990 and 1991, respectively [45]. The highest increase in children thyroid cancer rate was found in regions that received fallout containing large amounts of ^{131}I and other short-lived iodine isotopes, e.g., Gomel, Brest, and Grodno [45]. A dose-response relationship has been suggested but with large uncertainties [44].

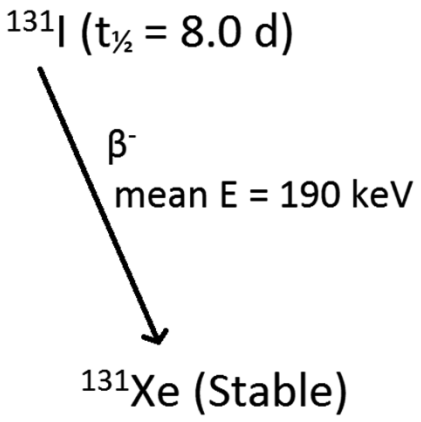

Figure 1.4 Simplified decay scheme of ^{131}I. ^{131}I decays in reality to metastable ^{131m}Xe that further decays to ^{131}Xe by emitting energy in

Table 1.1 Decay data for ^{131}I. Yields > 1% are included. * = mean energy [46]

Radiation	Yield (%)	Energy (keV)
^{131}I		
β^-	2.13	69.4*
β^-	7.36	96.6*
β^-	89.4	192*
γ	2.62	80.2
ce-K, γ	3.63	45.6
γ	6.06	284
γ	81.2	365
ce-K, γ	1.55	330
γ	7.27	637
γ	1.80	723
$K\alpha_1$ X-ray	2.59	29.8
$K\alpha_2$ X-ray	1.40	29.5
^{131m}Xe		
γ	1.99	233
ce-K, γ	63.5	199
ce-L_1, γ	11.9	227.7
ce-L_2, γ	2.56	228.1
ce-L_3, γ	6.29	228.4
ce-M, γ	4.57	232*
ce-N^+, γ	1.23	233*
$K\alpha_1$ X-ray	29.8	29.8
$K\alpha_2$ X-ray	16.1	29.5
$K\beta_1$ X-ray	5.72	33.6
$K\beta_2$ X-ray	1.91	34.4
$K\beta_3$ X-ray	2.94	33.6
Lα X-ray	3.22	4.11*
Lβ X-ray	3.07	4.49*
Auger-KLL	4.62	24.3*
Auger-KLX	2.14	28.5*
Auger-LMM	43.5	3.32*
Auger-LMX	23.9	4.18*
Auger-MXY	133	0.807*

Also, elevated anti-thyroperoxidase (TPO) antibody serum levels were found in individuals exposed to the Chernobyl ^{131}I release. Later it was reported that the elevated TPO antibody serum levels may have been a transient effect, and the authors suggested continuous monitoring of exposed individuals since thyroid dysfunctions may take decades to develop [47]. Other examples of environmental sources of ^{131}I include the Hanford nuclear fuel production site with release of $1.75 \cdot 10^{15}$ Bq ^{131}I, the Nevada nuclear bomb tests with release of $6 \cdot 10^{18}$ Bq ^{131}I, and the Fukushima accident with release of $100\text{-}200 \cdot 10^{15}$ Bq ^{131}I [22, 48, 49]. An increase of thyroid cancer was detected after the Nevada atomic bomb tests, but not as clearly as after the Chernobyl accident. It is not likely that the release of ^{131}I

in Fukushima will result in increased incidence of thyroid cancer since the population was exposed to a much smaller degree.

Additionally, handling and preparation of ^{131}I and ^{211}At labeled pharmaceuticals may result in accidental exposure of health care and research personnel.

1.4.1.3 Medical use of ^{131}I

^{131}I is routinely used in nuclear medicine; as halide or bound to targeting molecules. Already in 1946, treatment of hyperthyroidism with ^{131}I as halide was reported [2, 3], and today, this is one of the most common treatments in nuclear medicine. In Sweden in 2013, a total of 1735 hyperthyroidism treatments were performed, and the mean administered amount per treatment clinic was between 290 and 620 MBq ^{131}I, preceded by administration of ca 0.1-0.5 MBq ^{131}I for uptake measurement as basis for dose planning [50]. In ^{131}I treatment ca 50-300 Gy are given to hyperthyroid tissue [51, 52]. Hypothyroidism is the main side effect from ^{131}I therapy of hyperthyroidism. A transient elevation of thyroid hormones may also occur after ^{131}I treatment of hyperthyroidism, with potential heart failure as a result [51, 52]. Additionally, thyroid stunning, the phenomenon in which an administration of diagnostic amount of ^{131}I results in reduced uptake of the following therapeutic amount of ^{131}I, has been reported to occur clinically and pre-clinically over a wide range of absorbed dose [51, 53-55]. Immunological effects may occur after ^{131}I therapy of benign thyroid conditions. Elevated anti-TPO levels have been described as a marker of increased risk of side effects in patients following ^{131}I treatment of non-toxic goiter [56]. Additionally, transient increase of both pro- and anti-inflammatory cytokines has been reported after ^{131}I therapy of hyperthyroidism, although data is inconclusive [51, 57, 58]. It is not clear whether ^{131}I therapy of benign thyroid disorders may result in thyroid cancer, although such cases have been reported [51].

As previously mentioned, treatment of thyroid cancer using ^{131}I as halide was performed as early as in 1949 [5]. Today this procedure is usually only performed postoperatively for thyroid remnant ablation in patients with differentiated thyroid cancer [6].

^{131}I is also administered bound to different tumor targeting molecules. For example, ^{131}I-MIBG is used for diagnostic and therapeutic purposes of neuroendocrine tumors expressing the norepinephrine transporter, e.g. neuroblastoma, pheochromocytoma/paraganglioma, and gastrointestinal neuroendocrine tumors [7-10]. Many ^{131}I labeled antibodies have been developed

and clinically tested, e.g. ^{131}I-tositumomab (Bexxar ®, 2003-2014) for treatment of Non-Hodgkins lymphoma [59].

Preparations of ^{131}I-labeled pharmaceuticals will contain unbound ^{131}I as iodide [7, 60]. Furthermore, ^{131}I is released *in vivo* from metabolization or dehalogenation of ^{131}I-labeled pharmaceuticals [61]. Thus, administration of ^{131}I-labeled pharmaceuticals will most likely result in ^{131}I uptake in the thyroid gland. As previously mentioned, the thyroid gland may partially be blocked. The EANM procedure guidelines reports an absorbed dose to thyroid of 0.05 mGy/MBq administered ^{131}I-MIBG [9]. Others have reported higher values, e.g. 0.2-30 Gy to the blocked thyroid gland after 1.9-11 GBq ^{131}I-MIBG administration, and hypothyroidism developed in some patients [20]. Additionally, adverse effects such as hypothyroidism and cytopenia may occur in patients treated with Bexxar, and clinical evidence of hypothyroidism are evaluated annually.

1.4.2 ^{211}At

1.4.2.1 Physical properties of ^{211}At

^{211}At is an alpha particle emitting radionuclide with a half-life of 7.2 h. ^{211}At decays to either ^{211}Po (58 %) by electron capture (p + e -> n + ν) or to ^{207}Bi (42 %) by emission of 5.87 MeV alpha particles (**Figure 1.5**, **Table 1.2**) [46]. ^{211}Po then quickly ($t_{½}$: 0.52 s) decays to ^{207}Pb with emission of 7.45 MeV alpha particles, while ^{207}Bi decays much slower ($t_{½}$: 31.5 y) by electron capture to ^{207}Pb. Almost 99% of all energy emitted by ^{211}At is deposited from alpha particles (including decay chains). Emitted particles from ^{211}At have a mean linear energy transfer (LET) of 98.8 keV/µm, ^{211}At and the range of 5.87 and 7.45 MeV alpha particles in water is 48 and 70 µm, respectively [62].

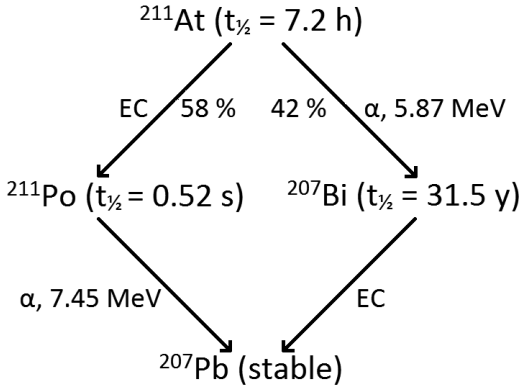

Figure 1.5 Decay scheme of ^{211}At

Table 1.2 Decay data for ^{211}At. Yields > 1 % are included, * = mean energy [46]

Radiation	Yield (%)	Energy (keV)
^{211}At		
$K\alpha_1$ X-ray	21.1	79.3
$K\alpha_2$ X-ray	12.6	76.9
$K\beta_1$ X-ray	4.80	89.8
$K\beta_2$ X-ray	2.27	92.5
$K\beta_3$ X-ray	2.51	89.3
Lα X-ray	7.54	11.1
Lβ X-ray	4.81	13.5
Lγ X-ray	1.90	13.3
Auger-L_2MM	4.15	10.2*
Auger-L_2MX	2.70	13.0*
Auger-L_2XY	4.46	15.2*
Auger-L_3MM	10.7	7.79*
Auger-L_3MX	7.11	10.6*
Auger-L_3XY	1.23	12.8*
Auger-MXY	66.4	3.16*
ΔE	16.7	0.576
A	41.7	5867
α recoil	41.7	113
^{211}Po		
A	98.9	7450
α recoil	98.9	144

1.4.2.2 Medical use of ^{211}At

Several treatment methods involving ^{211}At have been proposed and preclinical and clinical studies have been performed [13, 14], although no ^{211}At-labeled pharmaceutical has been approved for medical use yet. Similar to ^{131}I-labeled pharmaceuticals, incomplete labeling and *in vivo* dehalogenation of ^{211}At labeled pharmaceuticals will result in accumulation of ^{211}At in the thyroid gland. In a phase I trial of ovarian cancer using ^{211}At-labeled antibody fragments mean absorbed dose of 25 and 1.4 Gy/MBq/l to unblocked and blocked thyroid, respectively was reported [15]. In a preclinical study, ^{211}At-MABG (analogue to MIBG) was given to mice to treat neuroendocrine tumors expressing the norepinephrine transporter and similar relative thyroid uptake (%IA/g) of ^{211}At was reported as of ^{131}I after administration of ^{131}I-MIBG [17].

Free ^{211}At has been tried for therapy of NIS transfected cancer cells both *in vitro* and *in vivo* [18]. However, similar to administrations of ^{131}I as halide (discussed above), this resulted in much higher uptake in thyroid compared with tumor tissue [18].

As previously mentioned, NIS is not the only transporter of ^{211}At into thyroid follicular cells, therefore, administration of free ^{211}At for therapy of anaplastic thyroid cancer without sufficient NIS expression have been suggested [40]. These data also demonstrated too high concentration in normal tissues, such as thyroid, to be clinically useful.

Additionally, handling and preparation of ^{211}At and ^{211}At labeled pharmaceuticals may result in accidental exposure of health care and research personnel.

Detrimental effects on thyroid after administration of ^{211}At are not investigated.

1.4.3 Gaps in knowledge of biological effects

There are gaps in the knowledge of biological effects on thyroid tissue after ^{131}I and ^{211}At exposure. Detrimental effects from ^{131}I exposure on thyroid have been reported (and introduced in previous chapters), but the underlying molecular events are to a large extent unknown [44, 51]. Regarding ^{211}At exposure, knowledge of effects on thyroid is very limited.

The knowledge of underlying molecular mechanisms involved in radiation-induced response in vivo is very limited, especially at low and moderate absorbed doses [63]. There are several important issues to be addressed. How does variations in absorbed dose, dose-rate, time of exposure, time after

administration, and radiation quality impact radiation-induced biological effects in normal tissues? Is it possible to extrapolate biological effects between e.g. absorbed dose levels? Are there radiation-responsive biomarkers that can be utilized in triage following radioactive fallout or in clinics to predict normal tissue damage?

Lastly, improvements in early detection of tumors and higher survival rate after therapy increases the number of cancer survivors [64]. This results in an increasing number of patients in which late normal tissue damage may occur in [65]. Therefore, understanding of mechanisms of radiation-induced events in normal tissue becomes important.

1.5 Molecular radiation biology

There is a continuous rapid development of molecular techniques to be utilized within the field of radiation biology. Instead of main focus on DNA damage and repair, it has lately been recognized that the cellular response to radiation exposure is an orchestrated event in which complex intra- and extracellular signaling, pro- and anti-inflammatory cytokine cascades, and non-targeted effects are involved [66-69]. This orchestrated response involves gene expression regulation and thus protein synthesis.

Earlier, RNA microarrays did not cover the whole genome, and the first study utilizing RNA microarrays in the field of radiation biology profiled the expression of 1,344 genes in a myeloid cancer cell line 4 h after 20 Gy gamma exposure. [70]. In that study, the researchers identified 30 genes that previously were not related to radiation exposure. Today, there are many proposed radiation-responsive genes, of which a majority has been proposed based on *in vitro* or *ex vivo* experiments with acute high dose-rate photon irradiation [69, 71, 72]. Additionally, differences in radiation-induced gene expression were detected between e.g. the *in vitro* and *in vivo* setting, between different cell lines *in vitro* and different tissues *in vivo*, and between different individuals [68, 73-77]. Few *in vivo* studies of the transcriptional response after radionuclide administration have been performed, although lately this type of research has received some focus [78-83].

Since the transcriptional response is not always reflected at the protein level, it is also of interest to identify radiation-responsive proteins. In a review from 2006, over 300 scientific investigations of radiation-induced effects on protein level or post-translational modifications in vitro and in vivo were discussed, and 261 radiation-responsive proteins were suggested, of which a majority had been

identified using Western blotting after external exposure to photons from ^{137}Cs or ^{60}Co [84]. Although a majority of proteins were detected in human samples, studies were generally performed in an *in vitro* setting, which may exclude potential effects from systemic regulation. Additionally, data from literature were used to construct a panel of 20 protein biomarkers for a variety of absorbed dose levels and time points after exposure [84]. In a more recent paper, about 300 more proteins were added to the list of radiation-responsive proteins [85]. Radiation-responsive proteins could be valuable as radiation biomarkers in a triage situation, e.g. after accidental exposure to ionizing radiation. Studies of radiation-induced effects on the proteome are few, especially after radionuclide administration.

2 Aims

The overall aim of this work was to study the transcriptomic and proteomic effects of a variety of ^{131}I and ^{211}At exposure situations on normal thyroid tissue *in vivo*..

The specific aims were:

- To investigate effects of absorbed dose (and dose rate) on the transcriptional response in mouse thyroid early after ^{211}At administration (**Paper I**)

- To investigate effects of absorbed dose (and dose rate) on the transcriptional response in mouse thyroid early after ^{131}I administration (**Paper II**)

- To investigate effects of dose-rate and time after administration on the transcriptional response in mouse thyroid early after ^{131}I and ^{211}At administration (**Paper III**)

- To re-evaluate the transcriptional data from **Papers I**, **II**, and **III** together to gain a better understanding of variations in transcriptional regulation due to absorbed dose, dose-rate, time after administration, and radiation quality (**Paper IV)**

- To investigate effects of absorbed dose (and dose rate) on the transcriptional response in rat thyroid and thyroid function early after ^{131}I administration (**Paper V**)

- To identify effects on the proteome in mouse thyroid tissue and plasma early after ^{131}I administration (**Paper VI**).

- To identify novel and validate previously proposed genes related to radiation exposure in thyroid tissue from mice and rats early after ^{131}I or ^{211}At administration (**Papers I-VI**)

3 Material and methods

3.1 Radiopharmaceuticals

^{131}I was obtained as Na^{131}I from GE Healthcare (Braunschweig, Germany) and ^{211}At was produced at the Cyclotron and PET Unit at Rigshospitalet in Copenhagen, Denmark, using the ^{209}Bi(α,2n)^{211}At reaction. The complete method of acquiring ^{211}At is described in [86].

Measurement of ^{131}I and ^{211}At activity concentrations in stock solutions was performed using a gamma counter (Wallac 1480 Wizard® 3", Wallac Oy, Turku, Finland).

3.2 Dosimetry

The MIRD formalism was used to calculate the mean absorbed dose to the thyroid, $\overline{D}_{thyroid}$:

$$\overline{D}_{thyroid} = \tilde{A}_{thyroid} \sum n_i \, E_i \phi_i / m_{thyroid} \, .$$

The time-integrated (cumulated) activity, $\tilde{A}_{thyroid}$ for mouse and rat was based on previously reported data on relative activity concentration data (given as %IA) after i.v. administration of ^{131}I and ^{211}At using linear interpolation (**Papers I-IV**, **VI**) [19, 39]. For $\tilde{A}_{thyroid}$ of ^{211}At in mouse between 24 and 168 h the ^{211}At activity in thyroid was assumed to change only by physical decay. The average energy released per decay, $\sum n_i \, E_i$, where n_i is the yield of radiation *i* with energy E_i was determined using data from ENSDF, National Nuclear Data Center, Brookhaven National Laboratory, Upton, NY, USA. In mouse thyroid, the absorbed fraction, ϕ_i, was set to 0.742 and 1 for radiation emitted ^{131}I and ^{211}At, respectively [87]. A standard mouse thyroid mass, $m_{thyroid}$, of 3 mg was used. In rats, the absorbed dose was estimated using previously reported biokinetic and dosimetry data from Sprague Dawley rats on iodide deficient chow (0.05 ppm) for 5 days prior i.v. injection (6.1 Gy/MBq IA). Unpublished results indicate that a standard laboratory chow (2 ppm) resulted in approximately 9 times lower thyroid ^{131}I activity concentration than with iodide-deficient chow. By assuming similar biokinetics for both animal models, the mean absorbed dose per injected activity was estimated to 0.68 Gy/MBq IA in rat thyroid tissue. Dosimetric calculations only included particles emitted in the thyroid, and additionally, for ^{211}At

calculations, calculations only included energy deposited from alpha particles emitted by ^{211}At and ^{211}Po.

3.3 Study designs

The details of different experiments included in this thesis are shown in **Table 3.1**.

In four papers, the Illumina RNA microarray technique was used to assess genome-wide transcriptional variations in mouse thyroid tissue after ^{131}I (**Paper II**) and ^{211}At (**Papers I**, **III**, and **IV**) administrations. For rat thyroid tissue after ^{131}I exposure the transcriptional response was studied using the Agilent RNA microarray technique (**Paper V**). Additionally, LC-MS/MS was used to identify proteins with altered protein levels in mouse thyroid tissue and blood plasma (**Paper VI**).

Animals in **Papers I-VI** were i.v. injected with radionuclides via tail vein. Animals had access to water and standard laboratory chow *ad libitum*. Animals were killed by cardiac puncture under anesthesia with pentobarbital sodium and tissue samples were quickly excised and snap-frozen using liquid nitrogen. Additionally, plasma was collected in **Papers V-VI**. All samples were stored at -80°C awaiting analysis.

All studies were approved by the Ethical Committee on Animal Experiments in Gothenburg, Sweden.

3.3.1 Paper I

The Illumina RNA microarray technique was used to investigate the ^{211}At-induced transcriptional response in mouse thyroid tissue. Twenty-one female mice (BALB/c nude) were divided into groups of three. Animals in five groups were administered with 0.064-42 kBq ^{211}At (absorbed dose to thyroid: 0.05-32 Gy). Mice in the sixth group were mock-treated with an empty syringe and used as controls. All mice were killed 24 h after treatment.

Animals with thyroids exposed to 0.05 and 0.5 Gy were in reality killed together with animals in **Paper III**. When included in **Paper I**, regulation profiles of the 0.05 and 0.5 Gy groups were determined using control animals of **Paper I** and not **III**.

Table 3.1 Study design of all experiments included in the thesis

Species	Radio-nuclide	Methods	Paper	Exposure time (h)	Injected activity (kBq)	$\bar{D}_{thyroid}$ (Gy)
Mouse	^{211}At	Illumina RNA Microarray	III, IV	1	1.7	0.023
					100	1.4
				6	1.7	0.32
					7.5	1.4
			I, IV	24	0.064	0.050
					0.64	0.50
					1.7	1.4
					14	11
					42	32
			III, IV	168	1.7	1.8
	^{131}I	Illumina RNA Microarray	II, IV	24	13	0.85
					130	8.5
					260	17
		LC-MS/MS	VI	24	490	32
Rat	^{131}I	Agilent RNA Microarray, ELISA	V	24	9.0	0.0058
					88	0.057
					170	0.11
					260	0.17
					340	0.22
					760	0.50
					1300	0.84
					4700	3.0

Absorbed doses are thyroid mean absorbed doses estimated using the MIRD formalism. In **Paper IV**, a new transcriptional dataset that included re-evaluated normalized raw intensity values from **Papers I**, **II**, and **III** was generated

3.3.2 Paper II

The Illumina RNA microarray technique was used to investigate the ^{131}I-induced transcriptional response in mouse thyroid tissue. Eight female mice (BALB/c nude) were divided into groups of two. Animals in three groups were administered with 13-260 kBq ^{131}I (thyroid mean absorbed doses: 0.85-17 Gy,

respectively). Mice in the remaining fourth group were mock-treated with an empty syringe and used as controls. All mice were killed 24 h after treatment.

3.3.3 Paper III

The Illumina RNA microarray technique was used to further investigate the ^{211}At-induced transcriptional response in mouse thyroid tissue, but at different time-points compared with **Paper I**. Eighteen female mice (BALB/c nude) were divided into six groups with three animals per group. Mice in three groups were administered 1.7 kBq ^{211}At and killed 1, 6, and 168 h after administration (thyroid mean absorbed doses 0.023-1.8 Gy). Mice in two groups were injected with 100 and 7.5 kBq ^{211}At and killed at 1 and 6 after administration, respectively (thyroid mean absorbed dose: 1.4 Gy). Mice in the remaining sixth group were mock-treated with an empty syringe in the tail vein and used as controls (killed 24 h after mock-treatment).

3.3.4 Paper IV

A new transcriptional dataset that included re-evaluated raw intensity values from **Papers I-III** was generated to further investigate the ^{131}I- and ^{211}At-induced transcriptional response in mouse thyroid tissue. There was one difference in **Paper IV** compared with previous papers: data from mice injected with 0.064 and 0.64 kBq ^{211}At and killed 24 after administration were compared with data from control animals of **Paper III**.

3.3.5 Paper V

The Agilent RNA microarrays technique was used to investigate the transcriptional response in rat thyroid tissue 24 h after ^{131}I administration. Thirty six male Sprague Dawley rats were divided into 9 groups. Eight groups received 9.0-4700 kBq ^{131}I (estimated thyroid mean absorbed doses: 0.0058-3.0 Gy). Rats in the he ninth group were mock-treated with i.v. injection of NaCl in the tail vein. All rats were killed 24 h after administration.

3.3.6 Paper VI

Liquid chromatography tandem-mass spectrometry (LC-MS/MS), together with chemical labeling, was used to investigate the proteomic response to ^{131}I exposure in mouse thyroid tissue and plasma. Eleven female (BALB/c nude) mice were divided into two groups and i.v. injected with 150 µl NaCl containing 0 kBq (n=6) or 490 kBq (n=5) ^{131}I. Mice were killed 24 h after administration and mean absorbed dose to thyroid was estimated to 32 Gy in exposed mice.

3.4 RNA microarray (Papers I–V)

Briefly, RNA was extracted from homogenized thyroid tissue samples. RNA microarray was then used to determine genome-wide mRNA levels. Differential expression of transcripts was determined by comparing mRNA levels between irradiated and non-irradiated thyroid tissue samples.

3.4.1 Extraction of RNA

Collected thyroid tissue samples were homogenized using the Mikro-Dismembrator S ball mill (Sartorius Stedim Biotech, Aubagne Cedex, France) or the TissueLyser LT (Qiagen, Hilden, Germany). Total RNA was extracted from homogenized samples using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer´s instructions. RNA was extracted from pooled mice and individual rat thyroid samples in **Papers I-IV** and **V**, respectively.

RNA quality control using the NanoDrop ND-1000 and the RNA 6000 Nano LabChip Kit with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA concentrations, purity and Integrity Number (RIN) values were determined. A RIN cut-off value of 6 was used.

3.4.2 Hybridization and data preprocessing

3.4.2.1 Mouse studies (Papers I–IV)

Hybridization using MouseRef-8 Whole-Genome Expression Beadchips (Illumina, San Diego, CA, USA) containing 25,435 probes was performed at the Swegene Center for Integrative Biology at Lund University (SCIBLU, Lund, Sweden). Illumina BeadArray Reader scanner and BeadScan 3.5.31.17122 image analysis software were used for image acquisition and subsequent image analysis, respectively. Then, data were preprocessed (e.g. quantile normalization of raw signal intensities) using the web-based BioArray Software Environment (BASE) system.

3.4.2.2 Rat study (Paper V)

Hybridization using Agilent SurePrint G3 Rat GE 8x60K (Agilent, Santa Clara, CA, USA) containing 30,003 probes was performed at the Bioinformatics and Expression Analysis core facility at Karolinska Institute (BEA, Stockholm, Sweden). Slides were scanned using Agilent DNA microarray scanner G2505C at 3 μm resolution (Agilent, Santa Clara, CA, USA). The fluorescent intensities of the scanned images were extracted and preprocessed with the Agilent Feature

Extraction Software (version 10.7.3.1). Data were quantile normalized using Partek software (Partek Incorporated, St Louis, MO, USA).

3.4.3 Identification of regulated transcripts

In **Papers I-V**, statistically significant differentially expressed (regulated) transcripts between test and control samples were determined using Nexus Expression software (BioDiscovery, El Segundo, CA, USA) with |fold change| and Benjamini-Hochberg adjusted p-value cut-offs of 1.5 and 0.01, respectively

In **Papers III-V**, The R statistical computing environment was used to perform hierarchical clustering and to create heat maps (version 0.97.551, http://www.r-project.org) [88]. Hierarchical clustering was accomplished using the `hclust` function (`stats` package, version 3.1.1) with the complete linkage algorithm and Lance-Williams dissimilarity update formula and heat maps were created with the `heatmap.2` function within the `gplots` package (`gplots` package, version 2.14.2).

3.5 Liquid Chromatography Tandem Mass Spectrometry (Paper VI)

Briefly, proteins were extracted from tissue samples. LC-MS/MS, together with chemical labeling, was then used to determine relative changes in protein levels between two or more tissue samples.

Protein digestion and chemical labeling

Totally, 100 µg of total protein from each sample tissue or plasma sample was denatured and digested with trypsin after reduction and alkylation of disulfide bridges. For tissue samples a reference pool was generated from an aliquot of each sample, representing the same total protein amount. For the plasma analysis, one sample was selected as reference and repeatedly analyzed. Peptides were subjected to isobaric mass tagging reagent TMT® according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). In a set, each sample and a reference were labeled with a unique tag from a TMT 6plex or 10plex isobaric mass tag labeling kit. After TMT labeling, the samples in a set were pooled and fractionated by strong cation exchange chromatography.

LC–MS Analysis and search for protein TMT Quantification

The 6- or 10-plexed TMT-labeled samples were analyzed on an Orbitrap Fusion Tribrid mass spectrometer interfaced to an Easy-nLC II (Thermo Fisher Scientific,

Waltham, MA, USA). Peptides were separated using reversed-phase nanoLC separation and injected into the mass spectrometer in positive ion mode. MS scans and multiple MS analysis were performed in a data-dependent multinotch mode. Ions were selected for fragmentation (MS2) by collision induced dissociation (CID) for identification followed by multinotch (simultaneous) isolation of the top ten MS2 fragment ions selected for fragmentation (MS3) by high energy collision dissociation (HCD) and detection in the Orbitrap for quantification.

A database matching per set was performed using software with the Mascot search engine (Matrix Science, Boston, MA, USA) towards the Mus Musculus Swissprot (Swiss Institute of Bioinformatics, Switzerland) using relevant mass tolerances and modifications. The detected peptide threshold was set to 1 % false discovery rate (FDR). The TMT reporter ion intensities were used to calculate protein ratios with peptides unique for a given protein and normalized using the protein median for relative quantitation

3.6 Pathway analysis (Paper I–VI)

In the present work, gene ontology, canonical pathway, upstream regulator, and disease and function analyses was used to connect genes with biological information.

3.6.1 Gene Ontology (Papers I–VI)

In **Papers I-IV**, Nexus Expression software was used for enrichment of regulated transcripts and affected Gene Ontology (GO) terms were identified with a p-value cut-off of 0.05. Identified GO terms were classified according to biological function using an in-house developed categorization model (created using parental GO terms, http://geneontology.org) [79]. Main and subcategories are shown in **Table 3.2**.

In **Paper V**, Nexus Expression out-put data was searched for genes with thyroid related GO terms.

In **Paper VI**, functional annotation of proteins using GO terms were performed with the DAVID bioinformatics resource tool (http://david.abcc.ncifcrf.gov/) [89, 90] with a p-value cut-off of 0.05.

Nexus Expression and DAVID use a similar approach to identify affected GO terms. Statistical significance is determined by comparing the fraction of

regulated genes associated with a certain GO term with the fraction of all genes associated with the same GO term. Fisher´s exact test was used to determine if these levels are statistically significantly different. For example, if 50 % regulated genes are associated with metabolism, but metabolism is only represented by 5 % of the total genes in the genome, then it is likely that metabolism is an important biological function under that specific condition.

3.6.2 Ingenuity Pathway Analysis (Papers IV–VI)

QIAGEN´s Ingenuity Pathway Analysis software (IPA; Ingenuity Systems, Redwood City, CA, USA) was used to generate upstream regulation (**Papers IV-V**), canonical pathway (**Papers IV-V**), and disease and function analyses (**Papers IV-VI**).

Statistical analyses in IPA are made using Fisher´s exact test by comparing measured transcriptomic or proteomic changes with a reference set (i.e. the Ingenuity Knowledge Base, IKB) [35, 36]. Additionally, IPA uses previously reported experimental data (in IKB) to predict activation or inhibition of upstream regulators and downstream diseases and functions. For all IPA analyses, a p-value cut-off of 0.05 was used, and when predicting activation or inhibition, a |z-score| ≥ 2 was considered statistically significant.

3.7 ELISA (Paper V)

Enzyme-linked immunosorbent assay (ELISA) was used to determine thyroid stimulating hormone (TSH) and total thyroxine (T4) plasma concentration levels in rats.

3.7.1 T4 plasma levels (Paper V)

Total thyroxine (T4) plasma concentrations in rats were determined using the Thyroxine (T4) ELISA (mouse/rat) kit (Genway Biotech, Inc., San Diego, CA, USA), according to the manufacturer's instructions. Statistical significance was determined using Student's t-test ($p \leq 0.05$).

3.7.2 TSH plasma levels (Paper V)

The Rat Thyroid Stimulating Hormone (TSH) ELISA kit (Cusabio, Wuhan, Hubei Province 430206, China) was used to measure plasma concentrations of TSH in rats. This was performed according to the manufacturer's instructions, with one exception: a sample and standard volume of 50 µl instead of 100 µl was used due to insufficient amount of plasma. Additionally, due to limited amount plasma, TSH measurements were not performed in duplicate for one and two animals in

each/most group/s, and one animal administered 260 kBq was excluded from the analysis. Student's t-test was used to determine statistical significance ($p \leq 0.05$).

Table 3.2. Classification of Gene Ontology terms

Main and subcategories	Biological processes that...
1. 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
2. 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
3. Cellular integrity	
Physico-chemical environ.	…are related to e.g. regulation of ion homeostasis or transport
Cytoskeleton & motility	…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 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
4. 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 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
5. Cell communication	
Intercellular signaling	…facilitate communication between cells
Signal transduction	…regulate or effect signal transduction
6. Metabolism	
Proteins, amino acids	…regulate or facilitate metabolic processes for proteins/amino acids
Lipids, fatty acids	…regulate or facilitate metabolic processes for lipids or fatty acids
Carbohydrates	…regulate or facilitate metabolic processes for carbohydrates
Signaling molecules	…regulate or facilitate metabolic processes for signaling molecules
Nucleotide/nucleic acid-related	…regulate or facilitate metabolic processes for nucleic acid-related
Other	…are part of metabolism but not related to other subcategories
General	…are valid for any of the specific subcategories
7. Stress response	
Oxidative stress response	…respond to e.g. superoxide, hydrogen peroxide, or ROS
Inflammatory response	…regulate or facilitate pro- 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 related to other specific subcategories
8. 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

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4 Results and Discussion

4.1 Mouse studies (Papers I–IV, VI)

4.1.1 Transcriptional profiles (Papers I–IV)

The number of regulated transcripts in mouse and rat thyroid after ^{131}I and ^{211}At exposure is shown in **Figure 4.1**. In thyroids of mice, the number of regulated transcripts varied from 60 (24 h, 17 Gy, ^{131}I) to 1636 (24 h, 0.050 Gy, ^{211}At). An increased absorbed dose/dose-rate correlated with a higher number of regulated transcripts at early time points (1 and 6 h) after ^{211}At administration. At a somewhat later time point (24 h after administration), lower absorbed dose/dose-rate resulted in the highest number of regulated transcripts both for ^{211}At and ^{131}I (**Papers I-II**).

Hierarchical clustering revealed clear differences between regulation profiles of both similar and different exposures (**Figure 4.2**). For example, regulation profiles for 1.4 Gy at 1, 6, and 24 h after ^{211}At administration were clustered far apart although the absorbed dose was similar. The difference in dose-rate is probably partly responsible for this, and dose-rate effects on the gene expression regulation have previously been described *in vivo* following radionuclide administration [80, 83].

There are several differences in the properties of ^{131}I and ^{211}At exposure that may explain differences in the response between ^{131}I and ^{211}At. For example, to deposit an average absorbed dose of 1 Gy to the follicular cell nucleus, 800 beta particle tracks are needed from ^{131}I exposure, but only 3 alpha particle tracks from ^{211}At exposure [92]. Also, the mean range of alpha and beta-particles emitted from ^{211}At and ^{131}I is about 60 and 400 μm, respectively [43, 62]. Additionally, alpha particles emitted from ^{211}At have a much higher LET compared with beta particles and other electrons emitted by ^{131}I, and more deposited energy per particle track length can result in more complex DNA lesions with more remaining DNA damage after repair [93]. Altogether, a much more heterogeneous dose-distribution will be created for ^{211}At exposure compared with ^{131}I exposure, and when the mean absorbed dose decreases below 1-2 Gy, it is the number of irradiated cells that decrease rather than the energy deposited in each hit cell. Additionally, when fewer cells are hit, the measured response is more likely to originate from non-targeted effects, i.e. bystander cells [94].

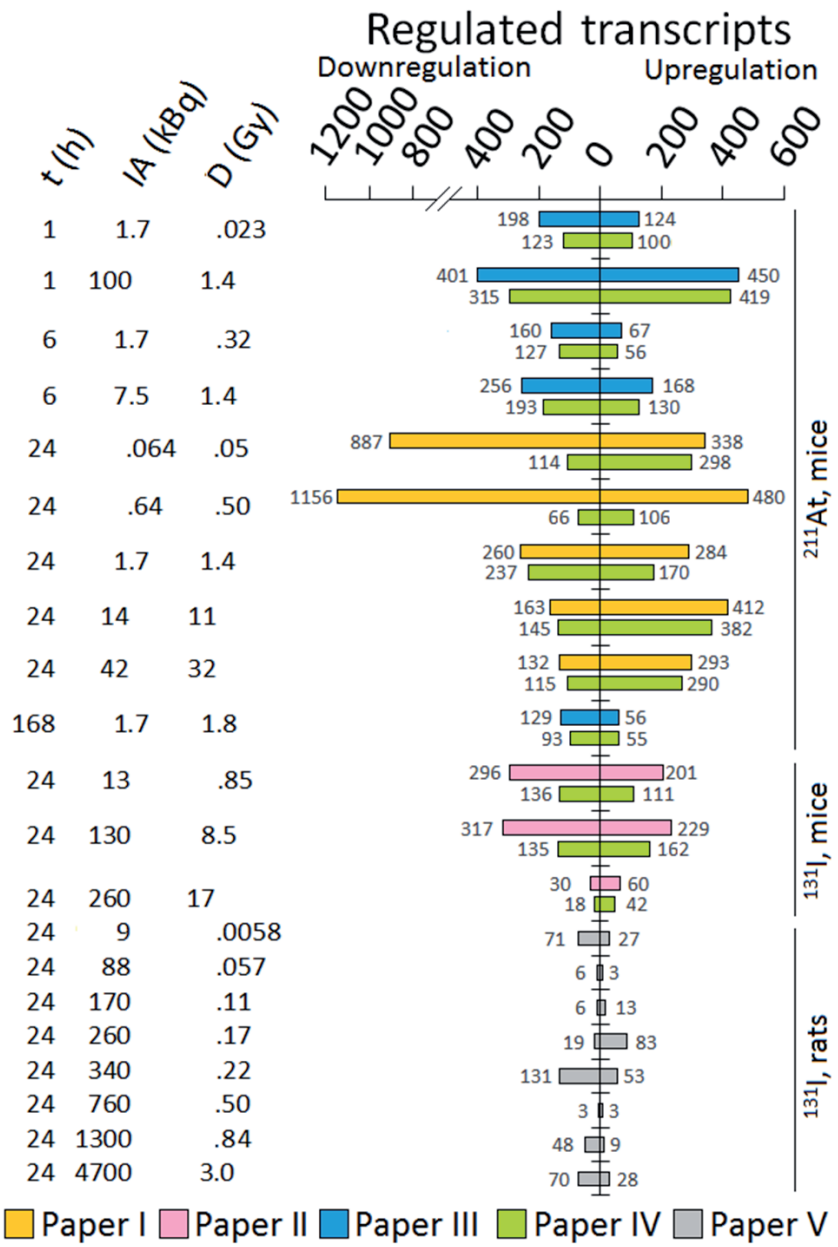


Figure 4.1 Number of regulated transcripts in thyroid tissue after ^{131}I or ^{211}At exposure. t: time after injection, IA: injected activity, and D: absorbed dose

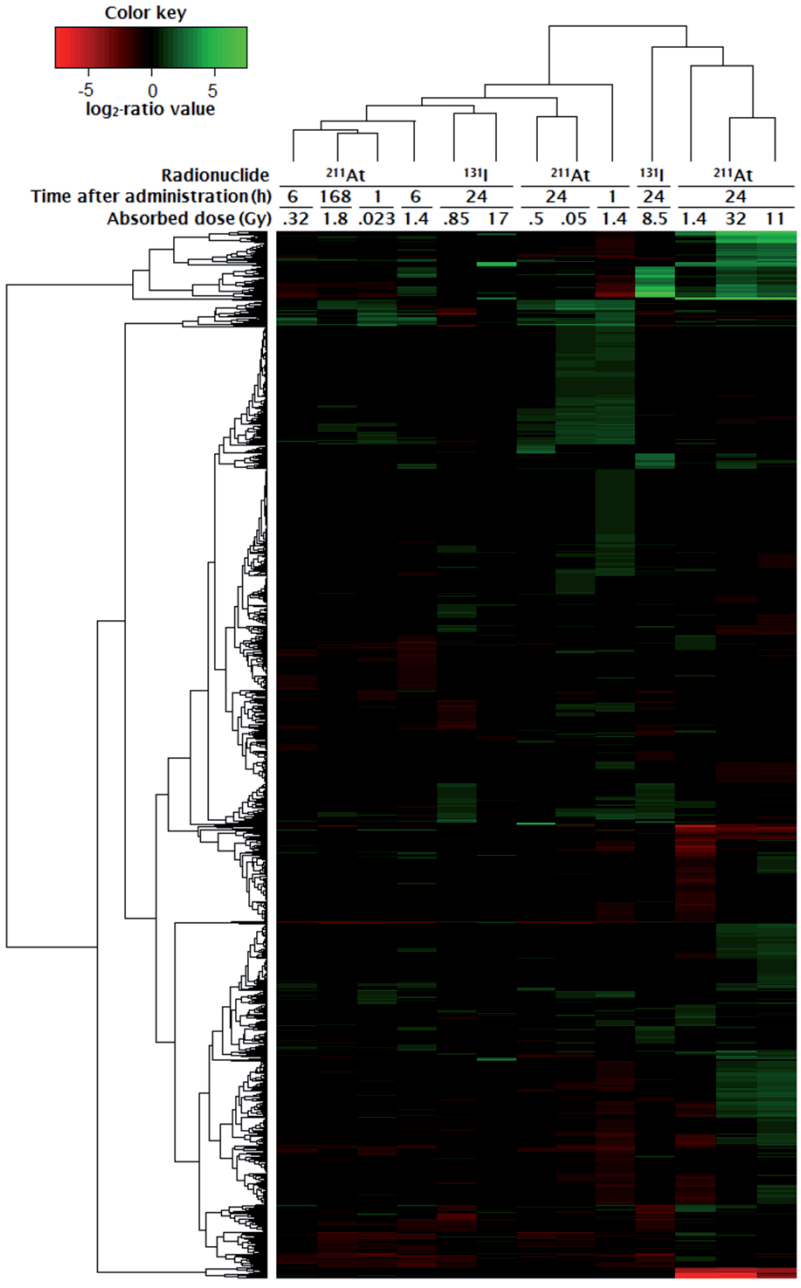


Figure 4.2 Hierarchical clustering of regulation profiles. Transcripts were hierarchically clustered using the gplots package in R/Bioconductor. The rows represent transcripts and columns represent exposure

Another difference between ^{131}I and ^{211}At exposure is the shorter half-life of ^{211}At compared with ^{131}I (7.2 h and 8 d, respectively). Therefore, a similar mean absorbed dose from ^{211}At exposure will be delivered over a shorter period of time compared with that from ^{131}I exposure. Taking into account that cells are dynamic systems consisting of complex regulatory networks acting with a large variation in response depending on the stressor, the rate of which absorbed dose is deposited, will most probably affect the type and strength of the radiation-induced response.

Altogether, these findings show variations in the radiation-induced response with exposure condition by the number of regulated genes, and the specific genes regulated. It is likely that changes in transcriptional patterns depend with varying degree on, but not excluded to, the following parameters: exposure time, injected activity, absorbed dose, dose-rate, dose distribution, and radiation quality. Each exposure condition, i.e. combination of these parameters, may then yield a specific response in the target tissue. The results from this work suggest that dose-rate is the most influential parameter on early response when comparing absorbed dose, dose-rate, and time after administration.

Relative biological effectiveness (RBE) is the ratio between biological effect of one radiation type and that of a standard radiation quality (usually 250 keV X-rays), given the same absorbed dose. The biological end-point may vary but is usually cell survival fraction, and RBE is valuable in radiation therapy. It is hard to consider potential effects of RBE when studying whole genome transcriptional regulation, since RBE demands a quantitatively measurable end-point for two different radiation qualities/sources. Although such end-points may exist for e.g. one specific gene or signaling pathway, the present work shows that different exposure conditions results in different type or pattern and not in strength of response, as mentioned above. This results in difficulties in defining a suitable end-point for determining RBE. Therefore, the RBE concept may have limited use within radiation transcriptomics (and radiation proteomics).

4.1.1.1 Differences in regulation profiles between Papers I and IV

In **Paper I**, 0.05 and 0.5 Gy from ^{211}At exposure resulted in 1225 and 1636 regulated transcripts, respectively, in contrast to 412 and 172 regulated transcripts in the 0.05 and 0.5 Gy groups in **Paper IV**, where data from **Papers I-III** were re-evaluated. The difference between **Paper I** and **IV** is that two different control groups were used to determine regulation profiles of the 0.05 and 0.5 Gy groups.

All ^{211}At mouse thyroid data were run in two different sessions. The first contained the 1.4, 11, 32 Gy groups at 24 h, and one non-irradiated control group (control A). The second contained data of all other time-points (1, 6, and 168 h), but also the 0.05 and 0.5 Gy groups at 24 h, and another control group (control B). In **Paper I**, regulation profiles of 0.05 and 0.5 Gy were determined using control A, and in **Paper IV**, control B.

Differences in results between two microarray sessions may be due to batch effects when performing microarray assay or to biological differences between analyzed samples [95]. To analyze the impact of batch effects, we combined the results from the sessions and performed hierarchical clustering of the raw quantile normalized intensity values from the two different projects with and without using a batch correction [95]. We found that batch correction did not have a large impact on the hierarchical clustering of raw data. This suggests that the discrepancies in regulation profiles for the 0.05 and 0.5 Gy groups between **Papers I** and **IV** most probably are due to biological variation between control animals. Differences in gene expression regulation between the control groups of **Papers I** and **III** are surprising since animals were of similar age and treated in similar ways, e.g. free access to food and water, and similar mock-treatment. It is also worth mentioning that a large majority of regulated transcripts in the 0.05 and 0.5 Gy groups in **Paper I** show relatively low fold changes, which may suggest that changes between control groups exist, but are generally small in terms of magnitude of expression.

Gene expression variations with a ≤ 31% difference in $\log_2$ ratio between **Papers I** and **IV** (for the 0.05 and 0.5 Gy groups) were detected independently of the control group used are shown in **Figure 4.3**. Radiation-induced changes in regulation of these genes may be regarded as robust since they remain regulated with small differences even when changing controls. Twelve of these genes (*Ccl9*, *Cpa3*, *Ctgf*, *Dpt*, *Eno3*, *Fstl1*, *Ltf*, *Lum*, *Ogn*, *S100a8*, *S100a9*, *Scara3*) were among the 27 recurrently regulated genes found when including all data on the transcriptional response in mouse thyroid after ^{131}I or ^{211}At exposure (**Paper IV**). Therefore, these genes are of special interest, but further research should be focused on validating their potential role as specific biomarkers in thyroid of ^{131}I and ^{211}At exposure and if they have a more general radiation-induced response.

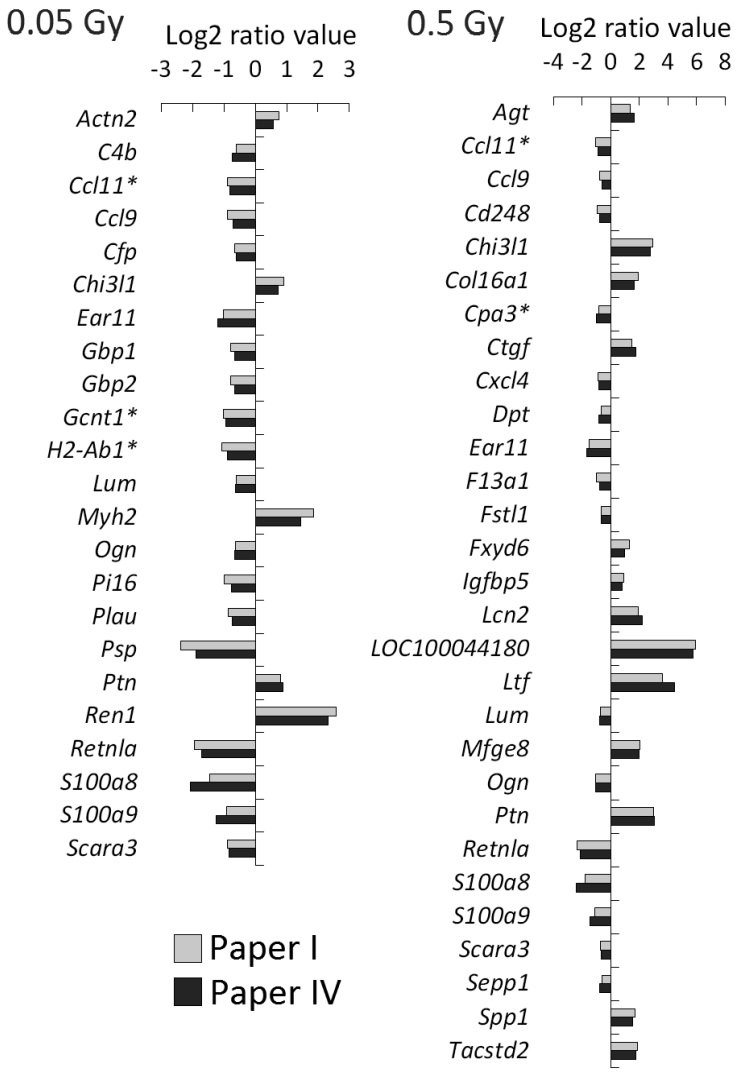
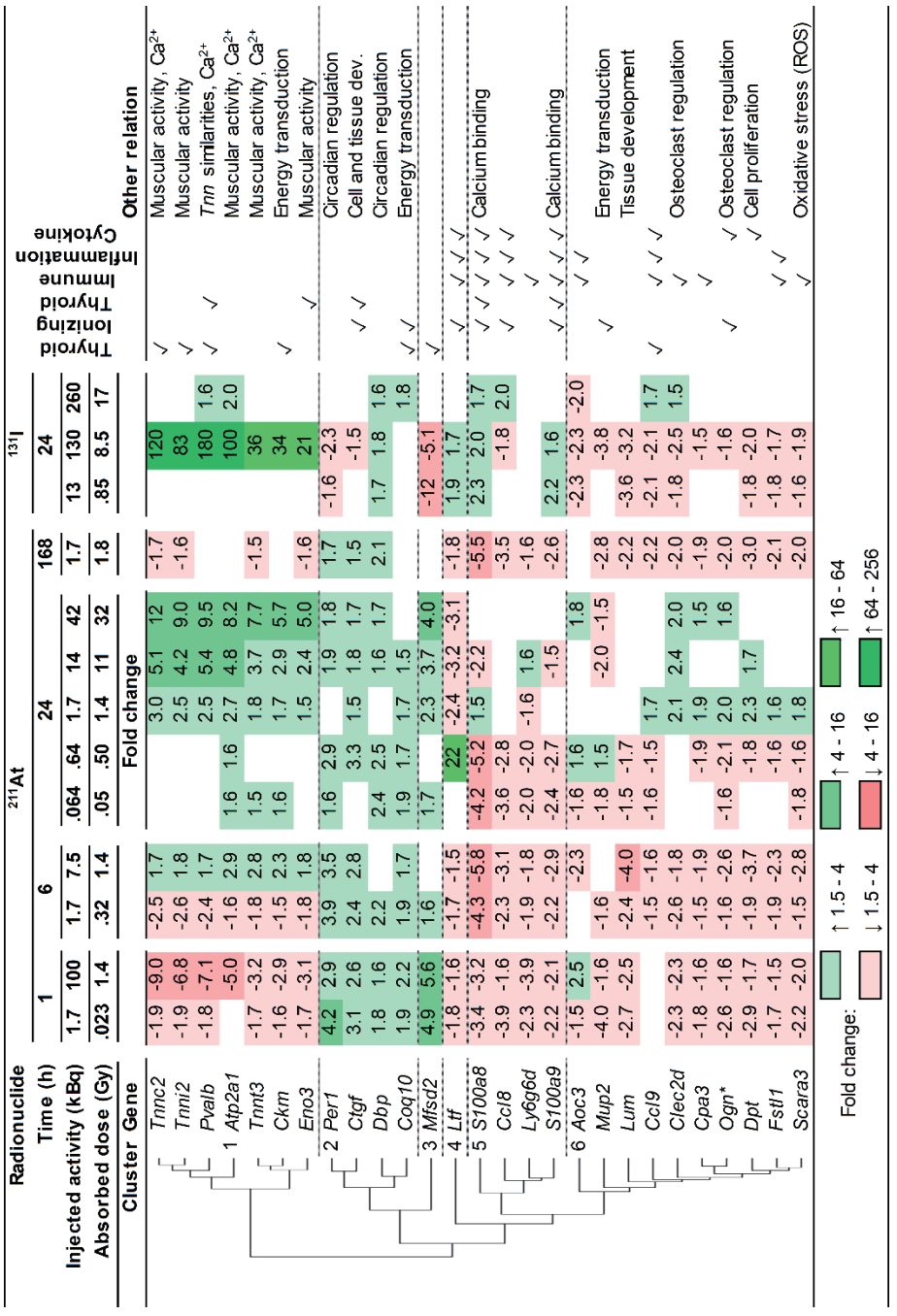


Figure 4.3. Genes with robust regulation 24 h after ^{211}At administration (0.05 and 0.5 Gy). The genes that had ≤31 % difference in $\log_2$-ratio when comparing regulation profiles after exposure with two different controls

4.1.2 Recurrently regulated genes (Paper IV)

Twenty-seven recurrently regulated genes were identified after ^{131}I or ^{211}At exposure (**Table 4.1**). These genes can be divided into 6 clusters using hierarchical clustering.

Table 4.1 Recurrently regulated genes in mouse thyroid after ^{131}I or ^{211}At exposure



The regulation of genes in **cluster 1** (*Atp2a1*, *Ckm*, *Eno3*, *Pvalb*, *Tnnc2*, *Tnni2*, and *Tnnt3*) varied with both absorbed dose and time after ^{211}At administration. Notably, all genes in cluster 1 had a monotonous increase in upregulation with absorbed dose at 24 h after ^{211}At administration. Also, at 24 h after ^{131}I administration, 8.5 Gy resulted in very high upregulation of these genes (fold change 21-180). Genes in cluster 1 were related to muscular activity and/or calcium activity (*Atp2a1*, *Eno3*, *Pvalb*, *Tnnc2*, *Tnni2*, and *Tnnt3*). Genes in **cluster 2** (*Coq10b*, *Ctgf*, *Dbp*, and *Per1*) were upregulated after all ^{211}At exposures, whereas *Per1* and *Ctgf*, and *Dbp* and *Coq10b* were down- and upregulated, respectively, after ^{131}I exposure. The genes in cluster 2 were related to different biological functions, e.g. energy transduction (*Coq10b*), cellular & tissue development (*Ctgf*), and circadian rhythm (*Dbp* and *Per1*) [96-98]. *Dbp* was also recurrently regulated after ^{131}I exposure of rat thyroid (**Paper V**), see next chapter. **Cluster 3** consisted only of one gene, *Mfsd2*, which was up and downregulated after ^{211}At and ^{131}I exposure, respectively, indicating a difference between exposure with radiation of different quality. *Mfsd2* seems to have various biological roles e.g. in metabolism, body growth and development, and motor function [99]. The only gene in **cluster 4** (*Ltf*) was generally downregulated after ^{211}At exposure, but upregulated after ^{131}I exposure, also suggesting different effects after exposure with radiation of different quality. The four genes in **cluster 5** (*Ccl8*, *Ly6g6d*, *S100a8*, *S100a9*) showed similar pattern as cluster 4 and were generally down- and upregulated after ^{211}At and ^{131}I exposure, respectively. Genes in **cluster 6** (*Aoc3*, *Ccl9*, *Clec2d*, *Cpa3*, *Dpt*, *Fstl1*, *Lum*, *Mup2*, *Ogn*, and *Scara3*) were downregulated at low absorbed doses and upregulated at high absorbed dose levels although the change from down- to upregulation occurred at a lower absorbed dose level for ^{211}At compared with ^{131}I. The *Ltf* gene in cluster 4, the *Ccl8*, *Ly6g6d*, *S100a8*, *S100a9* genes in cluster 5, and *Aoc3*, *Ccl9*, *Clec2d*, *Cpa3*, *Fstl1*, and *Scara3* genes in cluster 6 are all related to the immune system in various ways, and many related to both inflammation and the cytokine network [100-110]. The 27 genes with recurring regulation could potentially be used to discriminate between different exposures with variation in e.g. radiation quality, absorbed dose, and time after administration, and might be considered as potential general biomarkers for ^{131}I and ^{211}At exposure of thyroid. Additionally, the results suggest that genes with similar function share similar regulation patterns (e.g. especially genes in clusters 1, and 4-6). More research is needed to determine the role, and the sensitivity and specificity, of these 27 genes after ^{131}I and/or ^{211}At exposure.

4.1.3 Regulation of previously proposed biomarkers (Paper I–IV)

Of about 100 radiation-responsive genes from literature fifteen were regulated in mouse thyroid tissue after ^{131}I or ^{211}At exposure (*Amy2*, *Ccnd1*, *Ccnd1*, *Cdkn1a*, *Cxcl12*, *Ephx2*, *Fos*, *Gadd45g*, *Gadd45g*, *Gja1*, *Gjb2*, *Gjb2*, *Hspe1*, *Lep*, and *Tgfbr2*) (**Table 4.2**) [71, 72, 111, 112]. ^{131}I and ^{211}At exposure generally resulted in different direction of regulation of genes responding to both radionuclides. Regulation of these radiation-responsive genes was both sensitive to changes in absorbed dose and time after administration. For example, *Amy1* was downregulated with high fold changes at 1.4-32 Gy, 24 h after ^{211}At administrations but not after any other exposure, and was thus both sensitive to absorbed dose, time after administration, and radiation quality.

Table 4.2 Regulation of previously proposed biomarkers of radiation exposure

Radionuclide	^{211}At										^{131}I		
Time (h)	1		6		24					168	24		
Injected activity (kBq)	1.7	100	1.7	7.5	.064	.64	1.7	14	42	1.7	13	130	260
Absorbed dose (Gy)	.023	1.4	.32	1.4	.05	.50	1.4	11	32	1.8	.85	8.5	17
Gene	Fold change												
Amy2							-28	-21	-47				
Ccnd1		-1.5					-2.0					1.7	1.5
Ccnd1							-2.0					1.7	
Cdkn1a	2.3	3.0		1.7	1.6						-1.7	-2.0	
Cxcl12				-2.7			1.5			-1.6	-1.6		
Ephx2					1.5						-1.6	-1.6	
Fos							2.1				4.0	6.2	
Gadd45g	2.3	2.3	2.2	2.0	3.0	1.8	2.0				-3.1		
Gadd45g	1.9	2.0	1.8	1.6	2.7		2.2				-3.7		
Gja1		1.9		-1.5	2.2								
Gjb2	1.5			1.5			-1.6	1.5					
Gjb2	1.6			1.7			-2.3	1.9					
Hspe1		1.6										-1.6	
Lep		1.7	-1.8	-2.1			1.6	-2.6					
Tgfbr2	-1.6			-2.2									

Fold change: ↑ 1.5 - 4 ↑ 4 - 16 ↑ 16 - 64

↓ 1.5 - 4 ↓ 4 - 16 ↓ 16 - 64

Ccnd1, *Gadd45g*, and *Gjb2* were regulated with two transcripts each

The *Cdkn1a* and *Gadd45g* genes are regulated by p53 and commonly regulated after radiation exposure. Both *Cdkn1a* and *Gadd45g* were up- and downregulated after ^{211}At exposure ^{131}I exposure, respectively, but not at the higher absorbed dose levels and longer time periods (*Gadd45g*). In previous studies recurring regulation of *Cdkn1a* was identified after higher absorbed doses in kidneys at 24 h, and 4, 8 and 12 months after ^{177}Lu-octreotate administrations [82, 113]. Additionally, of administration of 8 MBq ^{137}Cs resulted in transiently elevated

expression levels of *Cdkn1a* in blood in mice [78]. Altogether, the *Ckn1a* gene may be an indicator of exposure to different radionuclides in several tissues in mice and knowledge of how different exposure conditions affect *Cdkn1a* expression is warranted.

4.1.4 Regulation of kallikrein genes (Papers IV, VI)

In the investigations, kallikrein 1 (*Klk1*) and 12 kallikrein 1-related (*Klk1b*) genes were frequently regulated with fold change between -3.8 and 110 after ^{131}I and ^{211}At exposure (**Table 4.3**). Downregulation was prominent at 1 h (with highest dose-rate), while upregulation was prominent at 6, 24, and 168 h after ^{211}At exposure with large fold changes especially after 11 and 32 Gy exposure (24 h, ^{211}At). Following ^{211}At exposure, the *Klk1* and *Klk1b*-related genes were rarely regulated below 1.4 Gy, suggesting a cut-off in absorbed dose. ^{131}I exposure did not have the same impact as ^{211}At on regulation of kallikrein and kallikrein 1-related genes; only *Klk1* and three *Klk1b*-related genes were regulated at 17 Gy, 24 h after ^{131}I administration.

Table 4.3 Regulation of kallikrein-related genes

Radionuclide	^{211}At										^{131}I		
Time (h)	1		6		24					168	24		
Injected activity (kBq)	1.7	100	1.7	7.5	.064	.64	1.7	14	42	1.7	13	130	260
Absorbed dose (Gy)	.023	1.4	.32	1.4	.05	.50	1.4	11	32	1.8	.85	8.5	17
Gene	Fold change												
Klk1								1.9	1.8				6.5
Klk1b5		-1.8			-1.6		2.2	22	16				
Klk1b5								2.9	2.7				3.7
Klk1b4		-1.6					3.7	51	33				
Klk1b4								2.9	3.1				2.8
Klk1b24		-1.7		1.9	-1.6			19	12	1.8			
Klk1b16	1.6	-1.6		1.6			1.7	18	12	1.6			
Klk1b22		-1.5					4.3	71	46				
Klk1b21		-1.9	1.6	1.8			1.5	19	14	1.6			
Klk1b21		-1.9		1.9			1.7	23	15	1.7			
Klk1b27		-1.9					1.6	25	17				
Klk1b27								3.0	3.0				3.1
Klk1b26		-1.5					8.7	110	82				
Klk1b11		-2.3		1.5			1.9	31	20				
Klk1b11		-2.0					3.6	67	45				
Klk1b9		-2.2					2.1	39	24	1.5			
Klk1b9		-1.9					2.6	33	22				
Klk1b1	-1.8	-3.8	-1.7	-1.5	2.0	-1.9		4.7	2.9				
Klk1b8		-1.9						19	15				

Fold change: ↑ 1.5 - 4 ↑ 4 - 16 ↑ 16 - 64 ↓ 1.5 - 4 ↓ 4 - 16 ↑ 64 - 256

Kallikreins are a subgroup of serine peptidases with various physiological functions. KLK1 (tissue kallikrein) is found in several species including humans,

rats, and mice, and is a common ancestral protein to many species-specific kallikrein-related peptidases [114]. The human and mouse genomic regions containing the kallikrein genes (located on chromosomes 19 and 7, respectively) are highly syntenic with the exception of a 290 kb long genomic region between *Klk1* and *Klk15* in the mouse genome that contains 13 *Klk1*-related genes with high *Klk1* homology (70-90 %) [115]. The *Klk1* and *Klk1b*-related genes are homologous to KLK3 in human, although their function is not fully understood in mouse. For example, the prostate-specific antigen, a biomarker for prostate cancer, is encoded by KLK3 in humans but not by the *Klk1* and *Klk1b*-related genes in mice.

A relation between radiation and kallikrein expression has previously been proposed. Elevated levels of a protein identified as kallikrein 1-related peptidase b24 precursor (mouse) was found in rat urine 24 h after 10 Gy total body irradiation together with decreased levels of the serine protease inhibitor A3K precursor protein, known to inhibit kallikrein [116]. In another study, plasma kallikrein levels decreased with absorbed dose between 0 and 19 Gy, 2-24 h after local irradiation of hind legs of rats [117]. As a result of this, and because our previous studies showed upregulation of *Klk1* and *Klk1b*-related genes, we used LC-MS/MS to identify proteins with altered levels in the mouse plasma (**Paper VI**). Although some animals showed elevated levels of kallikreins both in thyroid tissue and plasma, no statistically significant difference in kallikrein levels were detected in either thyroids or plasma of mice exposed to 32 Gy and killed 24 h after ^{131}I administrations. Kallikrein has also been associated with radiation-induced death of various species. Administration of soy bean trypsin inhibitors (SBTI) reduced the mortality rate in mice exposed to 6.7 Gy from 100% to 50% and chickens exposed to 8 Gy from 96% to 4% [118]. The authors suggest that the decrease in mortality originated from a radioprotective effect on the vascular system due to inhibition of tissue pre-kallikrein.

Genes involved in the kallikrein network may be potential biomarkers of radiation exposure in general, and of ^{131}I and ^{211}At exposure in particular. Further research is needed to elucidate the relationship between radiation exposure, and kallikrein levels. It is also suggested that the radioprotective role of SBTI should be further investigated.

4.1.5 Gene Ontology pathway analysis (Papers I–IV)

Enrichment of the transcriptional response in mouse thyroid after ^{131}I or ^{211}At exposure using Gene Ontology (GO) terms is shown in **Table 4.4**. Data was gathered from respective papers, except for the 24 h data after 0.064 and 0.64

kBq ^{211}At administrations (0.05 and 0.5 Gy). Enrichment data for these two groups were re-evaluated using transcriptional regulation data from **Paper IV**.

Altogether, few GO terms related to DNA or gene expression integrity were identified, and when such terms were identified, it was generally for the lowest absorbed dose levels used in the experiments. Additionally, no GO terms related to DNA repair and few GO terms related to cell cycle regulation and cell death were identified in the present investigations. These results were not expected, especially not for the highest absorbed doses and dose rates, and shortest time points studied. One explanation could be that abundant of DNA damages at high absorbed doses might give rise a transient transcriptional activation of DNA repair that is not present after 24 h. Another explanation is that DNA damage caused in the present studies is repaired without *de novo* protein synthesis. A further explanation of the lack of DNA repair GO terms may be that many genes are associated with DNA repair in the GO database. This may result in difficulties in achieving a statistically significant overrepresentation of DNA repair related genes in an experiment.

Many GO terms related to metabolism and organismic regulation were identified in the present work. Metabolism was especially affected at the highest dose-rate (1.4 Gy, 1 h, ^{211}At). Interestingly, almost no nucleic acid related GO terms were identified in the present investigations, supporting the lack of DNA damage response discussed above. Systemic regulation was similarly affected at all exposures studied. Regulation of muscle contraction is categorized under systemic regulation and was the most recurrently regulated GO term in the present investigations. Additionally, the GO term circadian rhythm was recurrently affected by ^{211}At exposure but not by ^{131}I exposure.

Several identified GO terms were categorized under inflammation and immune response. No GO term related to inflammation was identified at any absorbed dose level, 1, 6 and 24 h after ^{211}At administrations, although at 168 h after administration of 1.7 kBq ^{211}At, 4 GO terms related to inflammation were identified. At 24 h after 130 and 260 kBq ^{131}I administrations (8.5 and 17 Gy), 1 GO term related to inflammation was identified. This result may seem surprising since it is thought that radiation exposure results in regulation of both pro- and anti-inflammatory cytokines, shortly after exposure [66, 67]. The lack of inflammatory GO terms could possibly be due to the time-points used; too late for detection of early regulation of e.g. cytokines, but too early for late inflammatory effects. These results are in agreement with a previous study on transcriptional

Table 4.4 Categorized Gene Ontology terms identified in thyroid tissue in mice administered with ^{131}I or ^{211}At

Radionuclide	^{211}At										^{131}I		
Time after administration (h)	1		6		24					168	24		
Injected activity (kBq)	1.7	100	1.7	7.5	.064	.64	1.7	14	42	1.7	13	130	260
Mean absorbed dose (Gy)	.023	1.4	.32	1.4	.05	.50	1.4	11	32	1.8	.85	8.5	17
Cell communication	***2***	***2***	***4***	***3***			***2***	***2***		***1***	***4***	***3***	***1***
Intercellular signaling			2	1									
Signal transduction	2	2	2	2			2	2		1	4	3	1
Cell cycle and differentiation	***1***	***3***	***3***	***2***	***2***	***2***	***2***	***3***	***1***	***5***	***5***	***4***	***4***
Apoptotic cell death		1	1			1					2	1	
Cell cycle regulation				1	1					1	1	1	1
Cell death	1				1	1				1			
Differentiation & aging		2	2	1			2	3	1	3	2	2	3
Cellular integrity	***3***	***5***	***8***	***7***		***4***	***9***	***4***	***5***	***4***	***4***	***6***	***11***
Cytoskeleton & motility	1		4	1		1	2	2	2	2	2	1	3
EM & CM			1	4		2				1	2	3	2
General		1					1						
Physico-chemical environ.	1	1	2	2		1	3	2	3	1		2	2
Supramolecular maint.	1	3	1				3						4
DNA integrity	***2***	***2***	***2***		***2***	***2***					***1***		
Chromatin organization	2	2	2		2	2					1		
Gene expression integrity	***1***		***1***				***1***				***4***		
General											3		
Nucleus-specific											1		
RNA processing							1						
Transcription	1		1										
Metabolism	***7***	***19***	***8***	***7***	***7***	***4***	***5***	***4***	***3***	***9***	***8***	***5***	***7***
Carbohydrates	1	2		1	1				1	1			
General	1	3	1		2						4		2
Lipids, fatty acids	2	5	1	2	4	3		2		3		1	1
Nucleic acid related			2										
Other	2	5	2	1		1	3	1	2	4	3	3	2
Proteins, amino acids	1	2	2	3			1			1	1	1	2
Signaling molecules		2					1	1					
Organismic regulation	***5***	***5***	***6***	***11***	***2***	***5***	***4***	***6***	***3***	***8***	***8***	***7***	***6***
Ontogenesis	2	1	1	8			1		1	4	4	2	1
Reproduction								1			1		
Systemic regulation	3	4	5	3	2	5	3	5	2	4	3	5	5
Stress responses	***8***	***2***	***7***	***2***	***2***	***2***	***2***	***2***	***1***	***13***	***2***	***6***	***12***
General													1
Immune response	7	1	5	2	2	1		2	1	6			9
Inflammatory response										4		1	1
Other	1	1	2			1	2			3	2	5	1

Regulated transcripts were associated with biological functions using Gene Ontology (GO) terms ($p \leq 0.05$). The data given is number of identified GO terms for each category and subcategory. The grey scale indicate absolute number of identified GO terms, i.e. white equals no identified GO terms = white and darkest gray indicate 8 (max) GO terms. Abbreviations: EM=extracellular matrix and CM=cellular membrane

regulation (microarray, 7,680 human cDNA clones) in kidney and brain tissue, 8 and 24 h after 10 Gy exposure, where very few genes related to inflammation was identified [74]. Additionally, the protracted exposure employed in the present studies may possibly affect the presence of an inflammatory response. Several GO

terms related to an immune response were identified. For ^{211}At exposure, 1.7 kBq resulted in many GO terms related to immune response at 1, 6, and 168 h (0.023, 0.32, and 1.8 Gy) but not at 24 h (1.4 Gy) after administration. Also, at 1 and 6 h after ^{211}At administration, a smaller amount of administered ^{211}At resulted in a higher number of immune response related GO terms. For ^{131}I exposure, 0.85 and 8.5 Gy did not result in any immunological processes, while 17 Gy exposure yielded the highest number of immune response related GO terms in any of the mouse studies. Together these results suggest that 1) after a similar administration of 1.7 kBq ^{211}At, the immune response may involve different phases at different time periods, 2) a low absorbed dose results in a larger immune response compared with high absorbed doses, at least at 1 and 6 h after ^{211}At administration, and 3) the immune system may respond differently to high absorbed doses from ^{131}I and ^{211}At.

In **Paper IV**, 27 genes were identified with recurring regulation after ^{131}I or ^{211}At exposure (**Table 4.1**). Based on the literature, several genes with similar transcriptional regulation profiles were associated with immune response [100-110]. This is discussed further in **Chapter 4.1.2**. Additionally, five proteins (ANK1, HBA, HBB1, HBB2, and PMGE) with reduced levels in thyroid tissue (detected by LC-MS/MS) were linked to the GO term "immune system development (**Paper VI**).

4.1.6 Ingenuity Pathway Analyses (Paper IV)

The transcriptional response to ^{131}I and ^{211}At exposure was related to thyroid cancer signaling and various cancers according to the IPA canonical pathway and diseases and function tools, respectively. Cancer induction is a multi-step process and e.g. thyroid cancer resulting from ^{131}I exposure from Chernobyl took several years to develop [45]. It is uncertain whether indications of thyroid cancer can be observed at the transcriptional level at these early times after start of radiation exposure, or at all [45]. Unfortunately, all thyroid tissue from mice killed in the present study were used for microarray analysis, and no study of genomic rearrangements (a known feature of thyroid cancer after ^{131}I exposure) was possible [119].

An impact on calcium and integrin-linked kinase signaling was identified after ^{131}I and ^{211}At administrations in mouse thyroid using IPA. Several of the genes associated with calcium signaling were among the 27 recurrently regulated genes (cluster 1) after ^{131}I and ^{211}At exposure (*Tnni2*, *Tnnc2*, *Tnnt3*, and *Atp2a1*) (**Table 4.1**). Additionally, several other recurrently regulated genes are also related to calcium signaling in the literature, but not in the IPA canonical pathway analysis,

e.g. gene products of *Clec2d* and *Ogn* both inhibit osteoclasts that can release Ca^{2+} into the blood [120, 121]. Integrin-linked kinase signaling has previously been identified in the cellular response to radiation and shown to mediate pro-survival and anti-apoptotic signaling after radiation exposure, and integrin expression has been proposed as an indicator of short time radiation exposure [122, 123]. Additionally, in blood in mice administered with ^{137}Cs, integrin signaling was the top canonical pathway (IPA) and genes related with integrin-signaling were upregulated at 2 and 3 days and downregulated at 20 and 30 days after administration [78]. In the present work, no clear temporal effect on integrin-signaling was seen during the somewhat shorter time range used. However, we demonstrate that genes related to integrin-linked kinase signaling were generally up- and downregulated at higher and lower absorbed dose levels, respectively, suggesting different involvement of this signaling pathway at different absorbed dose levels.

Several peroxisomal proliferator-activated receptors (PPARs) and PPAR ligands were suggested as upstream regulators using the IPA upstream regulator analysis. A relation between PPARs and radiation has previously been recognized. Administration of a PPARα agonist prevented some cognitive function impairments in rats 26 weeks after fractionated whole brain irradiation (40 Gy) [124]. Additionally, PPARα knock-out inhibited radiation-induced apoptosis in mouse kidney tissue via regulation of *Nfkb* and anti-apoptosis factors within hours to days after 10 Gy whole body irradiation [125].

4.1.7 Proteomic changes after ^{131}I exposure (Paper VI)

Seventeen proteins with altered levels were identified in thyroid tissue after 32 Gy at 24 h after ^{131}I administration (**Table 4.5**). The majority of these 17 proteins had decreased concentrations in thyroid tissue: A2M, ACADL, ANK1, ARL3, B3AT, CAH1, DHB8, DHDH, HBA, HBB1, HBB2, PMGE, and SUCA, while ABRAL, HYOU1, RL24, and RL5 had increased concentrations. Twenty proteins were identified with altered concentrations in mouse plasma after 32 Gy at 24 h after ^{131}I administration (**Table 4.6**). Proteins with altered levels in plasma were generally increased (increased levels: AATC, C1TC, CYC, FABPH, FAS, GSTM1, K6PF, KAD1, KCRM, LDHB, MAOX, MYG, NCAM1, PGAM2, PGM1, RANG, and S1PBP; decreased levels: CHIA, 5NT3A, and VNN3). No proteins were identified with changed level in both thyroid and plasma.

Table 4.5 The 17 proteins identified with altered levels in mouse thyroid tissue 24 h after ^{131}I administration, together with transcriptional regulation in mouse thyroid exposed to ^{131}I and ^{211}At (Paper IV)

	Radionuclide	^{131}I	^{211}At										^{131}I		
	Time (h)	24	1		6		24					168	24		
	Injected activity (kBq)	490	1.7	100	1.7	7.5	.064	.64	1.7	14	42	1.7	13	130	260
	Absorbed dose (Gy)	32	.023	1.4	.32	1.4	.05	.50	1.4	11	32	1.8	.85	8.5	17
Protein	Gene symbol	Relative protein level (fold change)	Transcriptional regulation (fold change)												
A2M	*A2m*	-1.3													
ABRAL	*Abracl*	1.5													
ACADL	*Acadl*	-1.3													
ANK1	*Ank1*	-1.3													
ARL3	*Arl3*	-1.7													
PMGE	*Bpgm*	-1.5				-3.4	2.9						-2.9		
CAH1	*Ca1*	-1.7	3.6				3.0						2.9	2.9	
DHDH	*Dhdh*	-1.2													
HBA	*Hba*	-1.5	3.8												
HBB1	*Hbb-b1*	-1.7	3.2				3.5								
HBB2	*Hbb-b2*	-1.6													
DHB8	*Hsd17b8*	-1.5						-3.8						3.2	
HYOU1	*Hyou1*	1.5													
RL24	*Rpl24*	1.7													
RL5	*Rpl5*	1.2						-3.0						3.4	
B3AT	*Slc4a1*	-1.5													
SUCA	*Suclg1*	-1.4													

Fold change: ↑ 1.5 - 4, ↑ 4 - 16, ↑ 16 - 64; ↓ 1.5 - 4, ↓ 4 - 16, ↓ 16 - 64, ↓ ≥ 64

Altered protein levels were identified by comparing the protein content in thyroids from mice administered 490 kBq ^{131}I with thyroids from mock-treated controls. Thyroid absorbed dose was 32 Gy in the exposed group. Transcriptional data is from Paper IV

Fewer proteins with altered thyroid or plasma levels were identified compared with the number of regulated transcripts in thyroid tissue after ^{131}I or ^{211}At exposure. This may be due to a change in absorbed dose, and the transcriptional response to 32 Gy at 24 h after ^{131}I administration was not measured. Another reason is that thyroid tissue was pooled in the transcriptional studies, but individual samples were used in the proteomic study. Data from pooled samples (technical replicates) are likely more similar compared with data from individual samples (biological replicates). This results in less statistical variation in pooled samples and a higher number of statistically significant "hits". Additionally, RNA microarray determines transcriptional changes of a genome-wide predetermined set of genes while LC-MS/MS determines change in protein levels based on the total protein content of samples. Thus, proteins with low abundance may remain undetected using LC-MS/MS but are detected at the transcriptional level using RNA microarray. It is also shown that protein and transcriptional data not always correlate [126].

Table 4.6 The 20 proteins identified with altered levels in mouse plasma 24 h after ^{131}I administration, together with transcriptional regulation in mouse thyroid exposed to ^{131}I and ^{211}At (Paper IV)

	Radionuclide	^{131}I	^{211}At										^{131}I		
	Time (h)	24	1		6		24					168	24		
	Injected activity (kBq)	490	1.7	100	1.7	7.5	.064	.64	1.7	14	42	1.7	13	130	260
	Absorbed dose (Gy)	32	.023	1.4	.32	1.4	.05	.50	1.4	11	32	1.8	.85	8.5	17
Protein	Gene symbol	Relative protein level (fold change)	Transcriptional regulation (fold change)												
KAD1	*Ak1*	1.8													
CHIA	*Chia*	-7.0							-210	-11	-200				
KCRM	*Ckm*	1.6	-1.6	-2.9	-1.5	2.3	1.6		1.7	2.9	5.7			34	
CYC	*Cycs*	1.6											1.7	1.8	
FABPH	*Fabp3*	2.1	3.1	6.0			9.1	4.0				2.4	-2.4		
FAS	*Fasn*	2.0		2.0			1.7								
AATC	*Got1*	1.5													
GSTM1	*Gstm1*	1.9													
LDHB	*Ldhb*	1.9													
MYG	*Mb*	2.3		-2.3	-2.4		1.7			2.1	8.1	-1.6	-1.8	4.3	
MYG	*Mb*	2.3			-1.5		1.6			1.5	6.4			2.0	
MAOX	*Me1*	1.3													
C1TC	*Mthfd1*	1.3													
NCAM1	*Ncam1*	2.1													
5NT3A	*Nt5c3a*	-1.1													
K6PF	*Pfkm*	2.8				1.9								2.7	
PGAM2	*Pgam2*	1.6	-1.5	-2.8	-1.6	2.3			1.9	3.5	7.0			29	
PGM1	*Pgm1*	1.5													
RANG	*Ranbp1*	3.6													
S1PBP	*S100pbp*	1.2													
VNN3	*Vnn3*	-1.3													

Fold change: ↑ 1.5 - 4; ↑ 4 - 16; ↑ 16 - 64; ↓ 1.5 - 4; ↓ 4 - 16; ↓ 16 - 64; ↓ ≥ 64

Altered protein levels were identified by comparing the protein content in plasma from mice administered 490 kBq ^{131}I with plasma from mock-treated controls. Thyroid absorbed dose was 32 Gy in the exposed group. Transcriptional data is from Paper IV and shows transcriptional regulation in thyroid tissue exposed to ^{131}I and ^{211}At

Since ^{131}I was given i.v. other tissues than the thyroid will also contain ^{131}I. For example, it is estimated that when the thyroid receive 32 Gy, the blood, liver, lungs, kidneys, spleen and stomach, will receive absorbed doses of 20, 7.6, 4.0, 11, 12, and 54 mGy [39, 87]. We have previously described the transcriptional response in liver, lungs, kidneys (medulla and cortex), and spleen excised from the same animals used in **Papers I-IV** [79-81, 127]. We then demonstrated that these tissues responded differently after radiation exposure, with varying degree of regulation of thyroid hormone dependent genes and ionizing radiation responsive genes. Having this in mind, altered protein levels, especially in plasma, may not be exclusively derived from the irradiated thyroid gland, but may instead be a result of systemic regulation after ^{131}I administration. Additionally, of the 20 proteins with altered levels in plasma, 8 were regulated in thyroid after various exposures from ^{131}I or ^{211}At irradiation in previous studies (**Papers I-IV**), indicating an origin of protein synthesis in the thyroid gland.

4.1.7.1 Effect on thyroid function (Paper VI)

Lower levels of KCRM, LDHB, and MYG (creatine kinase M-type, L-lactate dehydrogenase B chain, and myoglobin, respectively) were identified in plasma after administration of 490 kBq ^{131}I (thyroid absorbed dose 32 Gy). Previously, decreased and increased levels of KCRM, LDHB, and MYG have been associated with hypo- and hyperthyroidism, respectively, and it has been suggested that altered levels of KCRM, LDHB, and MYG are mainly derived from skeletal muscle [128]. Furthermore, transcriptional levels of *Ckm* and *Mb* (genes encoding KCRM and MYG) changed after ^{131}I and ^{211}At exposure in thyroid gland, and transcriptional regulation of *Ckm* and *Mb* varied with both absorbed dose and time after administration (**Paper IVFel! Hittar inte referenskälla.**). For example, at 1 h after ^{211}At administration, a higher absorbed dose resulted in stronger transcriptional downregulation of both *Ckm* and *Mb*, but at 24 h, an increased absorbed dose resulted in stronger upregulation. Interestingly, at 6 h after ^{211}At administration, transcriptional regulation shifts from down- to upregulation with increased absorbed dose. Time-dependent variations in iodide transport and NIS mRNA expression have been demonstrated previously in cultured thyrocytes, with different patterns after ^{131}I and ^{211}At exposure [53]. It should be further studied if plasma KCRM and MYG levels show similar changes as transcriptional regulation of *Ckm* and *Mb*, respectively, after ^{131}I and ^{211}At exposure respectively, and if KCRM and MYG may be suitable biomarkers of effect on thyroid function (after ^{131}I or ^{211}At exposure).

4.1.7.2 ^{131}I induced effect on CHIA levels (Paper I and VI)

Plasma levels of CHIA decreased 7 fold at 24 h after administration of 490 kBq ^{131}I (thyroid absorbed dose 32 Gy). Strong transcriptional downregulation was identified in thyroid after exposures of 1.4-32 Gy, 24 h after ^{211}At administration (**Table 4.6**). No transcriptional regulation was identified after ^{131}I administrations, but this may partly be explained by a higher thyroid absorbed dose from ^{131}I exposure in the proteomic study (32 Gy) compared with thyroid absorbed doses in the transcriptional study (0.85-17 Gy). We have not found any previous relation between radiation and *Chia* gene expression in literature, and the physiological function of CHIA is not clear, The physiological function of CHIA is not clear, although it have been suggested to play a role in various biological processes, e.g. defense, immune response, and inflammation [129, 130]. Conclusively, *Chia*/CHIA is a potential biomarker of exposures of high absorbed doses of ^{131}I and ^{211}At, and understanding the biological mechanism of radiation-induced regulation of *Chia*/CHIA is warranted.

4.1.7.3 GO terms related to oxygen supply in thyroid after ^{131}I exposure (Paper VI)

GO pathway and IPA analysis of proteomic changes in thyroid tissue revealed an impact on hematopoiesis in general, and on erythrocyte homeostasis in particular. Proteins involved in this response were ANK1, HBA, HBB1, HBB2, and PMGE, all showing decreased levels. ANK1 (AnkyrinR) is a protein that couples e.g. the Cl^-/HCO_3^- anion exchanger (alias: B3AT, encoded by the SLC4A1 gene, also reduced in thyroid tissue in the present investigation) to the cytoskeleton of erythrocytes [131]. HBA, HBB1, and HBB2 are all different hemoglobin subunits, and hemoglobin release oxygen in the presence of CO_2. This process is mediated by carbonic anhydrase enzyme (CAH1), a protein also found reduced in thyroid tissue in the present investigation [132]. Additionally, PMGE (bisphosphoglycerate mutase) may promote the release of oxygen from hemoglobin [133]. Reduced levels of ANK1, HBA, HBB1, HBB2, and PMGE suggest a hypoxic condition in mouse thyroid 24 h after 490 kBq ^{131}I administration (32 Gy). This hypothesis is further supported by increased levels of HYOU1 (hypoxia upregulated protein 1), a protein induced during hypoxic conditions [134]. Further studies are needed to fully elucidate the effect on hematopoiesis, and oxygen level in thyroid after ^{131}I exposure.

4.2 Rat studies (Paper V)

The rat thyroid gland has a mass of about 20 mg, which allows RNA extraction and analysis on individual samples, in contrast to mouse (~3 mg), where pooled tissue samples were used to ensure enough amounts of RNA.

4.2.1 Transcriptional regulation (Paper V)

The numbers of regulated transcripts in rat thyroids 24 h after ^{131}I administration are shown in **Figure 4.1**. Totally, 429 regulated transcripts were identified in normal rat thyroid 24 h after administration of 9-4700 kBq ^{131}I. The number of regulated transcripts after each exposure varied from 6 (760 kBq) to 184 (340 kBq). The number of regulated transcripts showed no clear dependence on absorbed dose or dose-rate, similar to that in mouse thyroid tissue at 24 h after administration (**Paper V, Figure 4.1**). Few similarities were seen between different exposure levels (F**igure 4.4**). Fewer transcripts were identified as regulated in rat thyroid compared with mouse thyroid, which to some extent can be explained by RNA extraction from individual and pooled thyroids in the rat and mouse study, respectively (analysis of individual tissue samples gives higher

statistical variation between samples, and fewer statistically significant transcripts can be expected).

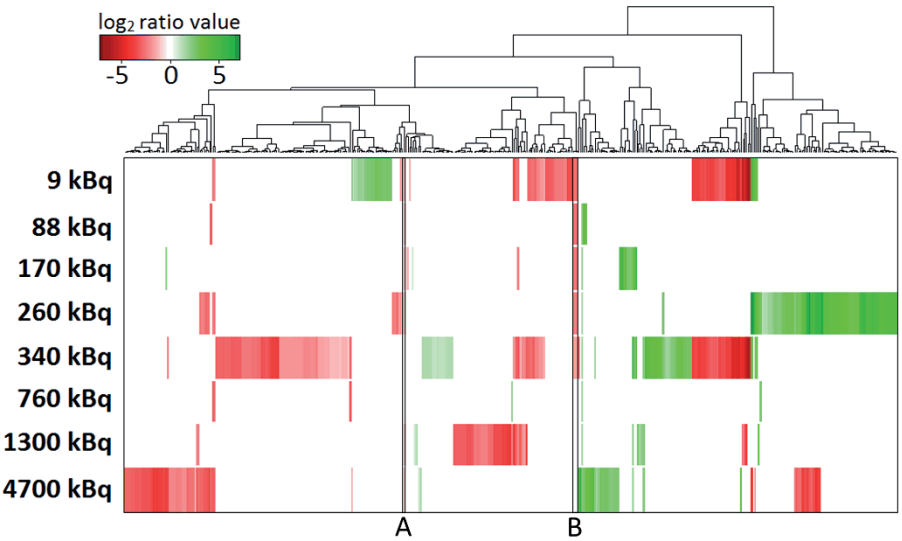


Figure 4.4 Hierarchical clustering of the 429 regulated transcripts in thyroids in rats injected with 9-4700 kBq ^{131}I. Regulated transcripts with a fold change ≥ 1.5 (log2 ratio ≥ 0.58) and an adjusted p-value ≤ 0.01 were hierarchically clustered according to log2 ratios using the hclust and heatmaps.2 functions in the R statistical environment. Statistically not significant transcripts were given the log2 ratio 0. The transcript above "A", downregulated after 9-260 and 1300-4700 kBq administrations, is the *Slc47a2* transcript. The transcripts above B are three *Dbp* transcripts, downregulated after 9-340 kBq administrations

4.2.2 Recurrently regulated genes (Paper V)

Three transcripts associated with the *Dbp* gene and one with the *Slc47a2* gene were recurrently regulated in rat thyroids after ^{131}I exposure (**Figure 4.5**). The three *Dbp* transcripts encode the transcription factor D site of albumin promoter binding isoform 1 protein, and had similar levels of expression within each group. When comparing regulation levels between groups, however, administrations of 9-170 kBq resulted in similar levels of downregulation while the strength of downregulation decreased monotonously between 170 and 340 kBq. The pattern of monotonous decrease in downregulation with increasing absorbed dose have previously been identified in mouse kidney tissue 8 and 12 months after ^{177}Lu-octreotate administration, although the absorbed doses were higher (19-45 Gy) [113]. In rats, a small and not statistically significant increase of *Dbp* expression was identified at 4700 kBq (**Figure 4.5**). Recurring upregulation of *Dbp* were also seen after ^{131}I and ^{211}At exposure of mice thyroid (**Table 4.1**). Additionally,

upregulation of *Dbp* was identified in mouse kidney 24 h after ^{177}Lu-octreotate administration (0.13-13 Gy to kidneys) [113]. Altogether, this data suggest that *Dbp* is a potential biomarker of ^{131}I and ^{211}At exposure of thyroid tissue, and also to ^{177}Lu-octreotate in kidneys. Further evaluations of variations in *Dbp* gene expression with dose-rate, absorbed dose, and time after administration, and the effects of different radiation qualities in different tissues should be performed.

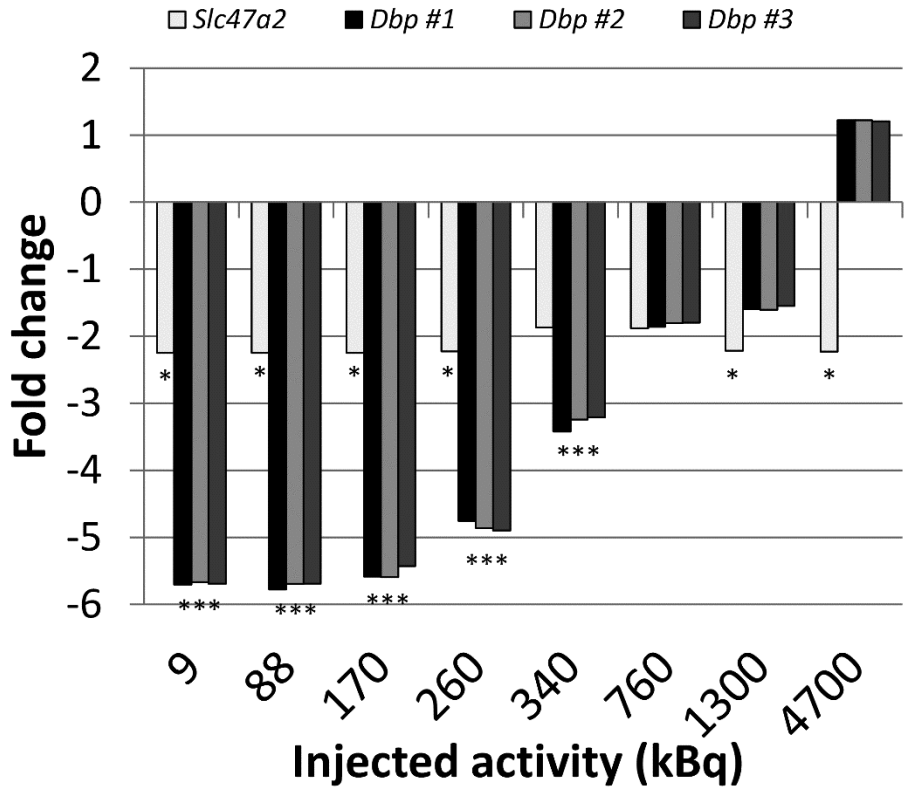


Figure 4.5 Effect on fold change of the 4 recurrently regulated transcripts in rat thyroid 24 h after injection with 9-4700 kBq ^{131}I. In total, four transcripts were regulated in ≥ 5 of 8 groups. * indicates regulation, i.e. fold change and false discovery rate adjusted (Benjamini Hochberg) p-value cutoffs of 1.5 and 0.01, respectively. Fold change cannot take a value between -1 and 1

The *Slc47a2* encode the solute carrier family 47 member 2 transporter protein, and was regulated with similar levels in groups receiving 9-260, 1300, and 4700 kBq. To our knowledge, a relationship between Slc47a2 expression and ^{131}I exposure has not been previously reported. This protein is normally expressed in

kidneys in rodents and seems to be involved in the excretion of toxics and drugs, but the action of this protein is probably not fully understood in rodents. Further research is warranted to determine the biomarker applicability of Slc47a2 to ^{131}I exposure.

4.2.3 Effect on thyroid function (Paper V)

From the transcriptional response, an effect on thyroid function was predicted using GO terms and IPA, GO term annotation revealed 12 genes associated with thyroid function, e.g. *Tg*, *Tpo*, *Slc5a5*/*Nis*, and *Tshr*. Generally, regulation of these genes was statistically significant in groups administered 9 and 340 kBq ^{131}I (**Table 4.7**). IPA canonical pathway and diseases and functions analysis associated the transcriptional response to 9 and 340 kBq with an impact on thyroid hormone biosynthesis and with a change in thyroid function, respectively. IPA was not able to predict activation or inhibition of diseases and functions or canonical pathways, but downregulation of *Tg*, *Tpo*, *Slc5a5*/*Nis* and *Tshr* indicate decreased thyroid hormone biosynthesis. For 340 kBq ^{131}I administrations, an impact on thyroid function was supported by decreased T4 plasma, although no changes were detected after administration of 9 kBq ^{131}I (**Figure 4.6**). Additionally, an IPA upstream regulator analysis predicted inhibited upstream regulation of TSH in response to 9 and 340 kBq. No statistical significant changes in TSH levels were detected in any group, although some animals showed elevated TSH plasma levels.

Downregulation of *Slc5a5*/*NIS* 24 h after administration of 9 and 340 kBq ^{131}I supports the concept of thyroid stunning in which a diagnostic amount of ^{131}I diminishes the uptake of subsequent therapeutic administrations of ^{131}I through downregulation of NIS [135].

Table 4.7 Regulation of genes related to thyroid function. Genes with statistically significant (fold change ≥ 1.5, adjusted p-value ≤ 0.01) changes in gene expression are shown in bold

Gene	Biological process (Gene Ontology)	Fold change (adjusted p-value)								
		9 kBq	88 kBq	170 kBq	260 kBq	340 kBq	760 kBq	1300 kBq	4700 kBq
Hoxd3	Thyroid gland development	**3.5 (0.0096)**	1.1 (1)	3.5 (0.065)	1.7 (1)	1.3 (1)	1.1 (1)	1 (1)	1.5 (1)
Tbx1	Thyroid gland development	1.5 (1)	-1.1 (1)	1.9 (0.72)	1.1 (1)	**2.8 (0.0002)**	-1.2 (1)	1.4 (1)	-1.4 (1)
Cpq	Thyroid hormone generation	**-3.5 (0.0046)**	-1.6 (1)	-2 (0.61)	-1.6 (1)	**-3.7 (0.0001)**	-1.3 (1)	-2.1 (0.28)	1.1 (1)
Dio1	Thyroid hormone generation	**-9.8 (0.0038)**	-2.3 (1)	-2.8 (1)	-2.5 (1)	**-13 (0.0001)**	-1.7 (1)	-4.3 (0.23)	-1.2 (1)
Hhex	Thyroid gland development	**-6.1 (0.0006)**	-2.1 (1)	**-4.6 (0.0064)**	-2.5 (0.6)	**-9.2 (0.0001)**	-2.8 (0.2)	**-4.9 (0.0022)**	-1.1 (1)
Iyd	Thyroid hormone generation	**-13 (0.0024)**	-2 (1)	-2.9 (1)	-1.5 (1)	**-16 (0.0003)**	-1.4 (1)	-2.8 (1)	-1.2 (1)
Nkx2-1	Thyroid gland development	**-18 (0.001)**	-2.8 (1)	-4 (0.84)	-2 (1)	**-12 (0.0002)**	-1.9 (1)	-3.2 (0.81)	1.2 (1)
Nkx2-1	Thyroid gland development	**-12 (0.0075)**	-2.3 (1)	-3.7 (1)	-2 (1)	**-11 (0.0034)**	-1.5 (1)	-3.2 (1)	1 (1)
Pax8	Positive regulation of thyroid hormone generation, thyroid gland development	**-21 (0.0001)**	-2.8 (1)	-4.3 (0.24)	-2.6 (1)	**-30 (0.0001)**	-1.9 (1)	-6.5 (0.036)	1 (1)
Slc5a5 /NIS	Iodide transport, sodium ion transmembrane transport, sodium ion transport, thyroid hormone generation	**-21 (0.0001)**	-2.3 (1)	-4.3 (0.51)	-2.1 (1)	**-13 (0.0001)**	-2 (1)	-5.7 (0.15)	1.1 (1)
Tg	Iodide transport, thyroid gland development, thyroid hormone generation, thyroid hormone metabolic process	**-48 (0.0001)**	2.8 (0.75	-4.6 (0.027)	-1.2 (1)	**-24 (0.0001)**	-2 (1)	**-5.3 (0.0074)**	1.1 (1)
Tpo	Thyroid hormone generation	**-69 (0.0001)**	3.5 (0.87	-6.1 (0.06)	-2.1 (1)	**-52 (0.0001)**	-2.6 (1)	-6.1 (0.033)	1.3 (1)
Tshr	Thyroid-stimulating hormone signaling pathway	**-7.5 (0.0022)**	-2.6 (1)	-2.5 (1)	-1.6 (1)	**-4.9 (0.0096)**	-1.7 (1)	-2.3 (0.9)	1.1 (1)

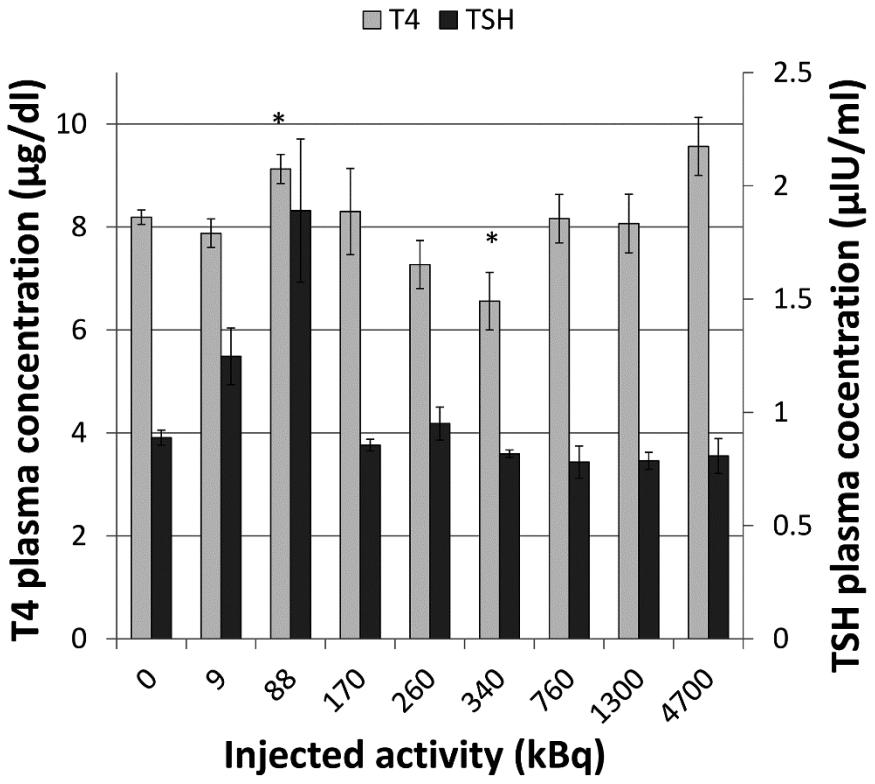


Figure 4.6 Plasma T4 and TSH levels in rats 24 h after ^{131}I administration. Plasma concentrations of T4 and TSH were measured using ELISA. Bars represent mean concentration values ± SEM. * indicates statistically significant difference between irradiated and control groups (Student's t-test, $p \leq 0.05$)

4.3 Methodological considerations

It would have been valuable to have data from more animals per group in this work. This was not the case for several reasons. One is the high cost per analyzed sample when using RNA microarray and LC-MS/MS. Another reason is to reduce the number of animals due to ethical aspects. Additionally, similar genotype in inbred mice and working under controlled conditions help reducing the number of animals/samples needed to achieve the same statistical power as in experiments in which e.g. human samples are analyzed [136].

The studies were performed on animals and not on humans, of practical and ethical reasons. The protein coding DNA of humans, mouse, and rats are similar, and e.g. 99% of the genes in mouse have counterparts in humans [137]. This

makes the use of animal models most valuable in radiation transcriptomics and proteomics, since all types of exposure and tissue sampling is not possible on humans. We believe that the results obtained in this work are representative for humans, but cannot be directly translated to humans.

Both rats and mice were used in the present work. Although rats have larger thyroid glands, which enables, e.g., histological sampling and immuno-histochemistry, long-term studies using mice should also be performed since most data available on ^{131}I and ^{211}At effects on thyroid tissue *in vivo* are from experiments on mice. Other advantages with mice for this type of work is that the mouse genetics is more studied, and the mouse is more commonly used within scienc. Thus more techniques are available for the mouse, and, e.g., Ilumina does not offer microarray assays for the rat any longer.

The thyroid gland consists of several different cell tissue types, e.g. thyroid follicular cells, endothelial cells, connective tissue, and parafollicular cells, and it is estimated that 70-80% of the cells in thyroid tissue are thyroid follicular cells [138]. Additionally, parathyroid tissue might also have been present in analyzed samples, although parathyroid cells are not supposed to accumulate ^{131}I or ^{211}At but are exposed from primarily ^{131}I in thyroid. The identified transcriptional response in the thyroid samples is most probably generated from all these different cell types (i.e. the data is convoluted). Due to high abundance of thyroid follicular cells in thyroid, the majority of isolated RNA is likely to originate from these cells. Still, in the present work, presented effects are on thyroid (with all cell types) and not only on thyroid follicular cells. In a study not included in this thesis, we performed deconvolution of convoluted transcriptional thyroid data [139]. Deconvolution (performed using a bioinformatics method within the R statistical computing environment) increased the detection rate of statistically significant transcripts and may be useful when searching for biomarkers of a specific cell type.

When injecting ^{131}I and ^{211}At in the blood, other tissues than thyroid are also irradiated. We have performed studies on systemic effects after ^{131}I and ^{211}At administration, and proposed that extra-thyroidal tissues such as liver, lungs, kidneys and spleen respond with different magnitude in transcriptional regulation of thyroid hormone and ionizing radiation responsive genes [79, 127]. Therefore, the measured radiation-induced response in thyroid tissue and plasma could be influenced by systemic effects, with interaction of other tissues and, e.g., the immune system.

We have also excised kidney cortex and medulla, liver, lung, and spleen tissue samples from the animals of which thyroid tissue was removed (data published in [79-81]). RNA from all different tissues were extracted simultaneously and run in the same microarray session. Hierarchical clustering of raw quantile normalized intensity values of data from these tissue samples reveal a high degree of similarity in gene expression for each tissue type, regardless if that tissue was from animals administered radionuclides or not). These data show that test and control tissue samples should be of the same type.

In the present work, the expression of several genes together with related signaling pathways have been suggested as potential biomarkers of ^{131}I and/or ^{211}At exposure. There are different definitions of a biomarker [140]. In the present work, the WHO definition, which is rather inclusive than exclusive, is used and includes "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction" [141]. In the present work most effects of ^{131}I and/or ^{211}At exposure were identified in thyroid in animals, and we believe that these results have increased our understanding of biological effects of radiation exposure *in vivo*. To collect a thyroid biopsy from humans for biodosimetric purposes in a triage situation will likely not be performed due to ethical reasons. Still, knowledge of radiation-induced effects on a target organ (in this case thyroid) will likely prove necessary in order to determine good biomarkers of this effect in more easily available samples, such as plasma or urine. One example of this is transcriptional regulation of *Chia* in thyroid tissue together with altered plasma CHIA levels. Additionally, we recognize the need to properly validate a biomarker to determine its usefulness for e.g. biodosimetric purposes and risk assessments.

5 Conclusions

In this work, effects of ^{131}I and ^{211}At exposure on gene expression regulation in thyroid in mice (**Papers I-IV**) and rats (**Paper V**) were investigated. The effects of absorbed dose, dose-rate, time after administration, and radiation quality were assessed, and large differences in the transcriptional regulation profiles and associated biological functions were found between different exposures. These results suggest that it is difficult to extrapolate effects from one exposure type to another. The effect of ^{131}I administration on the thyroid and plasma proteome was also assessed, and several proteins were found with altered levels.

The conclusions related to the specific aims presented in **Chapter 2** are presented below.

Distinct differences in transcriptional regulation between different absorbed dose levels (0.05-32 Gy to thyroid) were found in thyroid in mice 24 h after ^{211}At administration (**Paper I**). The proportion of dose-specific gene expression increased with decreased absorbed dose. Additionally, substantial differences in associated biological functions were found at the different absorbed dose levels.

Distinct differences in transcriptional regulation and affected biological functions between different absorbed dose levels (0.85-17 Gy to thyroid) were identified in thyroid in mice 24 h after ^{131}I administration (**Paper II**). The number of regulated transcripts decreased with increased absorbed dose, and the proportion of dose specific gene expression were similar at the different absorbed dose levels.

In **Paper III**, we showed that the amount of genes involved in the response to ^{211}At exposure increased with dose-rate in thyroid in mice at 1 and 6 h. Also, the number of regulated genes decreased with time after administration of 1.7 kBq ^{211}At. Regulation profiles of groups administered 1.7 kBq showed a higher degree of similarity compared with the other exposures. Thus, the regulation profiles were dependent on both absorbed dose and time after exposure, but initial dose-rate was the most influential parameter. Functional annotation of regulated genes revealed exposure-specific effects on biological processes.

In **Paper IV**, the transcriptional data from **Papers I**, **II**, and **III** were re-evaluated together to gain a better understanding of variations in transcriptional regulation due to absorbed dose, dose-rate, time after administration, and radiation quality. A profound effect on gene expression in mouse thyroid tissue after ^{131}I and ^{211}At exposure was detected. Hierarchical clustering revealed distinct differences

between transcriptional profiles of both similar and different exposures, demonstrating the necessity for better understanding of radiation-induced changes in cellular activity. It is likely that variations in transcriptional regulation profiles will depend to varying degree on, but not excluded to, the following parameters: exposure time, injected activity, absorbed dose, dose-rate, and radiation quality. Each unique setup of these parameters may then yield a specific response in the target tissue.

Different amounts of injected ^{131}I demonstrated distinct transcriptional responses to ^{131}I exposure in thyroid in rats (0.0058-3.0 Gy to thyroid) (**Paper V**). A transcriptional response related to thyroid function was found already at very low absorbed doses to thyroid (0.0058 and 0.22 Gy). ^{131}I induced changes in T4 plasma levels were also detected, when thyroids were exposed to absorbed doses as low as 0.057 and 0.22 Gy.

Relatively few proteins were identified with altered levels in thyroid and plasma 24 h after administration of 490 kBq 131I in mice (**Paper VI**). Additionally, functional annotation of the proteomic response indicates hypoxia in thyroid tissue. Functional annotation of identified proteins suggests an impact on hematopoiesis, reduced oxygen levels, and decreased thyroid function.

Twenty-seven genes were recurrently regulated in mouse thyroid tissue after ^{131}I and ^{211}At administration (*Aoc3*, *Atp2a1*, *Ccl8*, *Ccl9*, *Ckm*, *Clec2d*, *Coq10b*, *Cpa3*, *Ctgf*, *Dbp*, *Dpt*, *Eno3*, *Fstl1*, *Ltf*, *Lum*, *Ly6g6d*, *Mfsd2*, *Mup2*, *Ogn*, *Per1*, *Pvalb*, *S100a8*, *S100a9*, *Scara3*, *Tnnc2*, *Tnni2*, and *Tnnt3*) (**Papers I-IV**). These genes are potential biomarkers of ^{131}I and ^{211}At exposure of thyroid. Clustering of the 27 recurrently genes revealed a connection between specific transcriptional profiles and biological functions. This was seen especially for genes related to immunological response and inflammation, including the cytokine network. Effects on kallikrein regulation (at high absorbed doses from ^{131}I and ^{211}At exposure) and integrin-linked kinase and calcium signaling were identified in thyroid and are potential biomarkers of ^{131}I and ^{211}At exposure of thyroid.

In rats, few recurrently genes were regulated, but *Dbp* showed a decrease in downregulation with absorbed dose, and should be further studied as a potential biomarker of radiation exposure. Studies of the mouse plasma proteome revealed several potential biomarkers of ^{131}I exposure. One protein of special interest is CHIA with a clearly lower plasma level in mice after 24 h (32 Gy to thyroid). Strong transcriptional downregulation of *Chia* were also identified after 1.4-32 Gy ^{211}At exposure in mouse thyroid tissue.

Generally, few previously proposed biomarker genes of radiation exposure were found regulated in this work. The reason is likely that many of the suggested radiation-responsive genes were identified *in vitro* and our experiments were performed *in vivo*. Two p53 responsive genes of interest, *Gadd45g* and *Cdkn1a*, were recurrently regulated after ^{131}I and ^{211}At exposure in thyroid.

Altogether, we consider transcriptomic and proteomic techniques, especially in the *in vivo* setting, important tools to gain further insight on the very complex biological response to radiation exposure, and suitable for biomarker discovery.

6 Future perspectives

The overall aim of this research project was to increase the knowledge of biological effects of ^{131}I and ^{211}At exposure on normal thyroid tissue *in vivo*. This research project have given rise to several issues which should be examined further.

The transcriptional response to ^{131}I and ^{211}At exposure should be further assessed in both mouse and rat thyroids to gain better understanding of variations in radiation-induced transcriptional regulation due to absorbed dose, dose-rate, time after administration, and radiation quality. These studies should be performed at a wider range of time-points after administration, together with a large variety of absorbed doses and dose-rates.

One important aspect is the translation of data from the present work to humans. For this reason, it would be very valuable to sample thyroid tissue and blood from patients administered with ^{131}I. Thyroid tissue (both from healthy individuals and patients) is likely hard to obtain, but blood is likely more accessible. Assessment of radiation-induced transcriptomic and proteomic changes in blood from mice and humans exposed to various amounts of ^{131}I and ^{211}At could aid in finding biomarkers usuable in triage situations.

The transcriptional data in the present work were used to predict effects on biological functions. These effects should be further investigated using functional measurements. It is interesting to gain further insight in effects of changed exposure condition on iodine uptake, thyroid hormone production, integrin signaling, oxygen supply, immune response, DNA repair, and cancer induction, to mention a few. The present work may help guiding the direction of future functional investigations.

The twenty-seven recurrently regulated genes in mouse thyroid should be further assessed in thyroid tissue after exposure. It is of interest to study their role in radiation-induced response, their cellular origin of transcription, and also whether these changes persist to the protein level. It would also be valuable to investigate the plasma proteome after ^{131}I and ^{211}At exposure under similar exposure conditions presented in this thesis. Additionally, the specificity of regulation of these 27 recurrently regulated genes should be assessed, i.e. if they are regulated in response to other stressors or specifically after radiation exposure.

The focus of the present work was the acute and early transcriptional response to ^{131}I and ^{211}At exposure. Since thyroid function disorders may develop long time after irradiation, there is a need for experimental data on long-term effects of ^{131}I and ^{211}At exposure and to assess differences due to age of the animals. For this purpose, we have recently completed a long-term experiment in which young and adult rats were given different amounts of ^{131}I and killed after up to 9 months.. Thyroid and blood were sampled from these animals, as well as extrathyroidal tissues such as liver, lung, kidneys and spleen. Experimental data from this long-term study is valuable and may help in identifying biomarkers of ^{131}I and ^{211}At exposure and to relate these biomarkers to the impact on thyroid function. Additionally, to study the response also in young rats is interesting since e.g. thyroid cancer after ^{131}I exposure is known to mainly develop in humans exposed as children [44]. Together with the data on the acute response presented in this thesis, knowledge of long-term effects could potentially help in developing a predictive test of affected thyroid function.

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