Long-term radiobiological effects of ^{131}I exposure

– dose, age and time related transcriptomic and proteomic response in rats

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I was taught that the way of progress is neither swift nor easy
– Marie Curie

Abstract

^{131}I is commonly used in the clinic for treating thyroid diseases, using the physiological uptake of iodine in thyroid, but also for other target tissues. ^{131}I is also commonly released during nuclear accidents. Children are in general more radiation sensitive, and an increased number of thyroid cancers was seen in children but not in adults after the Chernobyl accident. There us a lack of knowledge about long-term radiobiological mechanisms and response in vivo.

The aim of this thesis was to study the ^{131}I induced long-term effects in rat thyroid tissue and plasma by investigating the transcriptional and translational expression, and to propose potential biomarkers related to age at exposure, time after exposure, and dose.

The radiation induced transcriptomic and proteomic response was studied in thyroid tissue and plasma from young and adult rats, 3-12 months after ^{131}I injection, using mRNA microarray technique and mass spectrometry. The number of significant transcripts and proteins was in general highest for low doses (5-50 kBq) and for young rats, but showed no general time-related trend. From these transcripts and proteins, biomarker candidates were identified. Biological functions associated to the significant transcripts and proteins were identified, and metabolic and hormonal effects were in common in most studies. Young rats demonstrated more affected canonical signaling pathways than adults one year after exposure.

In conclusion, radiobiological effects were detected late after exposure (3-12 months), and biomarker candidates (single markers and panels) were proposed for ^{131}I exposure, dose, age, and time after exposure, some connected to thyroid function and cancer. The results increases the knowledge in radiobiology, and may be valuable for improvement of radiation therapy and radiation protection.

Key words: radiation, thyroid, plasma late effects, biomarkers, transcript, protein

Sammanfattning

Jod tas naturligt upp i sköldkörteln, liksom dess radioaktiva isotop ^{131}I, som är vanligt förekommande i sjukvården. ^{131}I används för behandling av överfunktion hos sköldkörteln (hyperthyreos) och vid sköldkörtelcancer. ^{131}I kan också bindas till olika typer av bärar-molekyler som binder till andra vävnader i kroppen, till exempel ^{131}I-MIBG som används för behandling av neuroblastom hos barn. ^{131}I är också en av de vanligaste radionukliderna som släpps ut vid kärnvapensprängningar och kärnkraftsolyckor. Efter Tjernobylolyckan ökade antalet sköldkörtelcancrar hos barn men inte hos vuxna. Kunskapen om de bakomliggande biologiska effekterna efter ^{131}I bestrålning och cancerinduktion är liten, speciellt vad gäller låga doser och lång tid efter bestrålning.

Målet med denna avhandling var att undersöka bakomliggande biologiska effekter av ^{131}I bestrålning i sköldkörtelvävnad och blod hos unga och vuxna råttor genom att studera gen- och proteinuttryck lång tid efter bestrålning. Särskilt studeras skillnaderna mellan unga och vuxna individer, då barn generellt anses mer strålkänsliga.

Totalt sett visade försöken att många gener och proteiner hade ändrade uttryck (ökade och/eller minskade) även lång tid efter bestrålning när man jämförde vävnadsprover från bestrålade och obestrålade råttor. Utifrån dessa resultat hittades olika samband och vissa av dessa gener och proteiner föreslås som tänkbara biomarkörer kopplade till bestrålning. Speciellt föreslås tänkbara biomarkörer kopplade till ^{131}I-exponering, stråldos, ålder vid bestrålning, och tidpunkt efter bestrålning, liksom biomarkörer med känd koppling till sköldkörtelfunktion och cancer. Många av dessa biomarkörer är involverade i ämnesomsättning och hormonproduktion.

Resultaten av detta arbete ökar förståelsen för biologiska effekter av strålning och kan bidra till förbättrad strålbehandling och strålskydd.
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List of papers

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

I. **Larsson, M**., Rudqvist, N., Spetz, J., Shubbar, E., Langen, B., Parris, TZ., Helou, K., Forssell-Aronsson, E.

Long- term transcriptomic and proteomic effects in Sprague Dawley rat thyroid and plasma after internal low dose ^{131}I exposure

PloS One 2020;15(12):e0244098.

II. **Larsson, M**., Rudqvist, N., Spetz, J., Langen, B., Parris, TZ., Helou, K., Forssell-Aronsson, E.

Age related long-term response in rat thyroid tissue and plasma after internal low dose exposure to ^{131}I

Submitted

III. **Larsson, M**., Shubbar, E., Spetz, J., Parris, TZ., Langen, B., Berger, E., Helou, K., Forssell-Aronsson, E

Late age- and dose-related effects on the proteome of thyroid tissue in rats after ^{131}I exposure

Submitted

Selection of related presentations

1. **Larsson M**, Rudqvist N, Spetz J , Parris T, Langen B, Helou K, Forssell-Aronsson E. Transcriptome and proteome analysis for potential biomarker discovery of long-term effects in rat thyroid and blood tissue after ^{131}I exposure. Swerays, Stockholm, Sweden. Aug 25-26, 2016
2. **Larsson M**, Rudqvist N, Spetz J , Parris T, Langen B, Helou K, Forssell-Aronsson E. Potential biomarkers for long-term effects in thyroid tissue after ^{131}I exposure in rats. Radiation Research Society, Hawaii, USA, Oct 16-19, 2016
3. **Larsson M**, Rudqvist N, Spetz J, Parris T, Langen B, Helou K, Forssell-Aronsson E. Exposure of rats to ^{131}I - potential biomarkers of long-term effects and cancer induction in the thyroid. Höstmöte Onkologisk Radionuklidterapi, Uppsala, Sweden, Nov 24-23, 2016
4. **Larsson M**, Rudqvist N, Spetz J , Parris T, Langen B, Helou K, Forssell-Aronsson E.^{131}I exposure in rats potential biomarkers and functional analysis for long-term effects in thyroid. European Association of Nuclear Medicine congress 2017, Wien, Austria, Oct 22-26, 2017
5. **Larsson M**, Rudqvist N, Spetz J, Parris T, Langen B, Helou K, Forssell-Aronsson E. Long-term effects in thyroid and plasma after internal low dose exposure with ^{131}I in rat.Gothenburg Cancer Meeting, Göteborg, Sweden, May 6-7, 2019
6. **Larsson M**, Rudqvist N, Spetz J, Parris T, Langen B, Helou K, Forssell-Aronsson E. Low-dose exposure of ^{131}I in young rat thyroid tissue and plasma. European Radiation Protection Week, Stockholm, Sweden, Oct 14-18, 2019
7. **Larsson M**, Shubbar E, Spetz J , Parris T, Langen B, Helou K, Forssell-Aronsson E. Proteomic expression analysis of rat thyroid tissue 12 months after low-intermediate ^{131}I exposure. European Radiation Research Society, Lund, Sweden, Sep 13-17, 2020

Content

Abbreviations

A	Adenine
Bq	Becquerel
C	Cytosine
cDNA	Complementary DNA
CNA	Copy number alteration
DNA	Deoxy ribonucleic acid
ELISA	Enzyme linked immunosorbent assay
G	Guanine
GO	Gene ontology
Gy	Gray
H&E	Haematoxylin and eosin
HGF	Hepatocyte growth factor
HPLC	High-performance liquid chromatography
I	Iodine
IAEA	International atomic energy agency
ICRP	International commission on radiological protection
IPA	Ingenuity pathway analysis
KI	Potassium iodine
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LNT	Linear-no-threshold
mRNA	Messenger RNA
MIBG	Meta iodo benzylguanidine
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PTC	Papillary thyroid cancer
RNA	Ribonucleic acid
RT-qPCR	Real time quantitative polymerase chain reaction
SNP	Single nucleotide polymorphism
Sv	Sievert
T	Thymine
Tf-RETs	Transferrin receptor positive reticulocytes
TG	Thyroglobulin
TPO	Thyroid peroxidase
U	Uracil
Xe	Xenon

1. Background

1.1 Iodine isotopes, especially ^{131}I

Iodine has several different isotopes, where some of them are unstable. One of the most commonly used radioactive iodine isotopes in the clinic is ^{131}I. It decays by beta emission (mean energy 190 keV, half-life of 8 days) to stable ^{131}Xe, via excited states of ^{131}Xe or to a small extent (0.39%) via ^{131m}Xe (half-life of 11.9 d) (Table 1.1). ^{131}I is mainly used for therapeutic purposes, due to the high abundance of emitted electrons and a suitable half-life. ^{131}I also emits gamma radiation that can be used for external detection and imaging. ^{123}I is used for diagnostic purposes and has a half-life of about 13 hours.^{125}I is preferred in most laboratory use, due to its long half-life (60 d) and emission of low energy photons and electrons. ^{124}I is a positron emitter that is used in PET imaging (half-life of 4.2 d). The most important iodine isotopes released during nuclear accidents is ^{131}I, but also ^{132}I (half-life 2.3 h), ^{133}I (half-life 21 h) and ^{135}I (half-life 6.6 h) are released (1).

1.2 The thyroid

The human thyroid gland weighs about 20 g, consists of two main lobes and is located below the larynx (Figure 1.1). The organ is mainly built up by follicles containing follicular cells, which produce the iodine containing hormones thyroxine (T4) and triiodothyronine (T3). The follicular cells take up iodine (as iodide) from the blood by the sodium-iodide symporter, NIS, and the iodine is used to produce T4 and T3 hormones. The thyroid hormones are involved in vital body functions such as metabolism, temperature regulation, normal growth and development of the nervous system in children (2). Thyroid C-cells are located in the connective tissue between the thyroid follicles. The C-cells produce the hormone calcitonin that regulates the calcium concentration in the extracellular fluid. Most of the calcium in the body is found in bones but it is also a necessary component for some of the enzymes in the coagulation system.

Table 1.1. *- ^{131}I decay data. The table includes emissions with yields >1 % (3). When ^{131}I decays 0.39 % of transitions are via metastable xenon, ^{131m}Xe, before decay to stable ^{131}Xe. The radiation types emitted consist of β^- (electrons), γ (photons), conversion electrons (ce), characteristic X-rays and Auger electrons. * denotes mean energy*

^{131}I			^{131m}Xe		
Radiation	**Yield (%)**	**Energy (keV)**	**Radiation**	**Yield (%)**	**Energy (keV)**
β^-	2.13	69.4*	γ	1.99	233
β^-	7.36	96.9*	ce-K, γ	63.5	199
β^-	89.4	192*	ce-L_1, γ	11.9	227.7
γ	2.62	80.2	ce-L_1, γ	2.56	228.1
ce-K, γ	3.63	45.6	ce-L_1, γ	6.29	228.4
γ	6.06	284	ce-M, γ	4.57	232*
γ	81.2	365	ce-N^+, γ	1.23	233*
ce-K, γ	1.55	330	$K\alpha_1$ x-ray	29.8	29.8
γ	7.27	637	$K\alpha_2$ x-ray	16.1	29.5
γ	1.80	723	$K\beta_1$ x-ray	5.72	33.6
$K\alpha_1$ x-ray	2.59	29.8	$K\beta_2$ x-ray	1.91	34.4
$K\alpha_2$ x-ray	1.40	29.5	$K\beta_3$ 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*

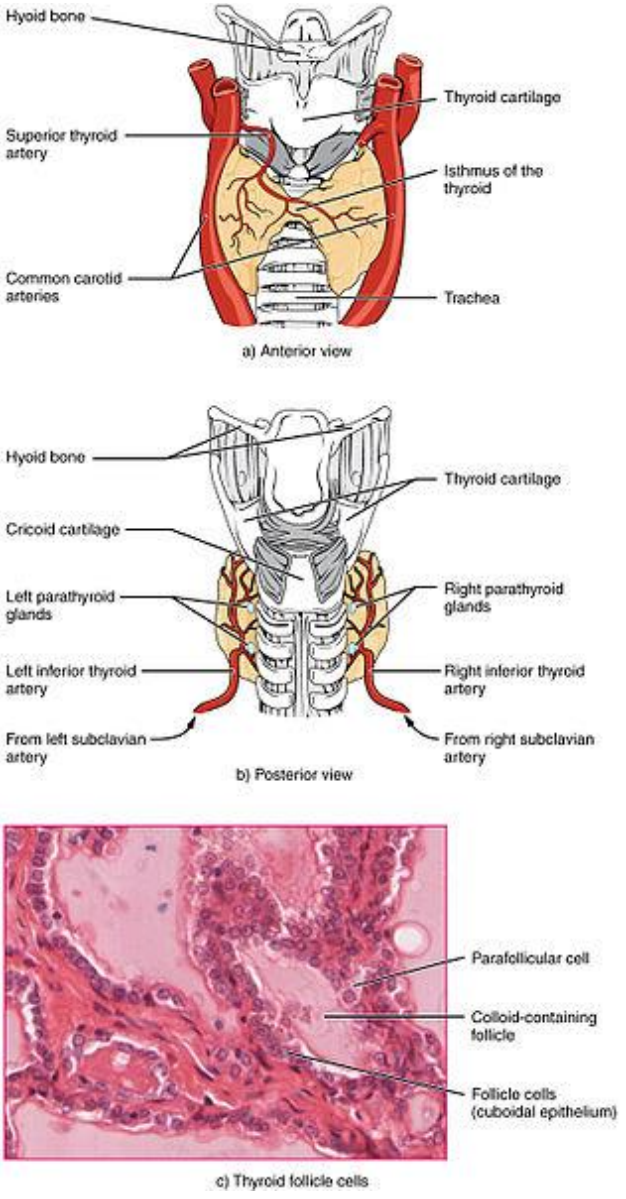

Figure 1.1. The thyroid gland. *The thyroid seen from a) anterior view, b) posterior view and c) using microscopy. The figure was retrieved from Wikipedia, The thyroid gland, https://upload.wikimedia.org/wikipedia/commons/d/d2/1811_The_Thyroid_Gland.jpg*

1.3 Medical use of ^{131}I

During the 1940s, iodine (I), ^{131}I was the first radionuclide introduced for therapeutic purposes, and was initially clinically used for thyroid cancer treatment. ^{131}I is still used for treatment of thyroid tumour remnants and metastases after total thyroidectomy (4, 5). During the last decades, efforts to develop new radiopharmaceuticals are ongoing, and more specific tumour markers (targets) for targeted therapy have been defined, such as hormone receptors at the surface of the cell. The main advantages of targeted radionuclide therapy is that the radiopharmaceutical binds to specific targets on the tumour cell, and is often given systemically. Thus, radionuclide therapy has the possibility to treat metastatic cancer disease.

The choice of radionuclide is important for therapeutic use. The radionuclide needs to deposit a relatively high energy over a rather short pathway, by for example electrons or alpha particles, to locally damage and hopefully kill the tumour cells. The half-life of the radionuclide should be sufficiently long to enable accumulation in the tumour, and still give a high absorbed dose to the tumour. The uptake in the tumour should be higher and/or the retention time longer compared with those for normal tissue (6). The optimal radionuclide should be produced with a high specific activity, and the amount of emitted photons should be low to spare normal tissue, especially considering risk organs in patients such as bone marrow (7). Radionuclide treatment may spare more normal tissues compared with external irradiation, where the radiation source is outside the body and the radiation needs to pass normal tissues to reach the tumour, unless the tumour is superficial.

After exposure to ^{131}I, either orally or by i.v. injection, ^{131}I is transported by blood in the form of iodide, and then 20-40 % is accumulated in the thyroid, small amounts are accumulated in e.g. the salivary glands, breast and stomach, while most of the remainder is excreted in the urine by the kidneys (8). The thyroid is primarily locally irradiated from the emitted beta particles with a mean range of up to 400 μm in soft tissue (9). The contribution from photons from ^{131}I located in the thyroid or the remainder of the body is low, less than 5 % (10).

The physiological uptake of ^{131}I is used to treat different thyroid diseases, such as hyperthyroidism and thyroid cancer. The natural uptake can also be a disadvantage if the thyroid tissue is exposed to ^{131}I from, for example, nuclear power plant accidents (e.g., Chernobyl, Fukushima). The thyroid can also be exposed to ^{131}I as iodide from radionuclide therapy using different ^{131}I-based radiopharmaceuticals, when free ^{131}I is present in the administered solution or is released in the body after administration. Potassium iodide, KI, can be administered to reduce the ^{131}I uptake and radiation dose to the thyroid, but up to 64 % of the treated children with neuroblastoma received significant absorbed doses to thyroid, leading to hypothyroidism (too low production of thyroid hormones) after treatment with ^{131}I-labelled MIBG (11). Irradiation of the thyroid can also cause hypothyroidism (underactive thyroid) leading to the development of autoimmune thyroid disease. Hypothyroidism is generally more severe at a young age, especially in foetus since thyroid hormones are essential for normal development of the nervous system, and IQ can be negatively affected with insufficiently thyroid hormone levels (12, 13). Other common symptoms that occur in both children and adults are fatigue, resistance to cold, weight gain, constipation, muscle weakness and memory difficulties (12, 13). However, thyroid hormone substitutes are readily available.

1.4 Radiation induced cancer risks

Cancer is one of the most common causes of death in the developed countries today. There is a large variation between different types of tumours and each type has specific features. Hanahan *et al.* initially distinguished six hallmarks of cancer, including “sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis” and later included ” reprogramming of energy metabolism and evading immune destruction”. Two processes are involved in these hallmarks: genomic instability and inflammation (14, 15).

It is well known that ionising radiation increases the risk of cancer development, and some of the known cancer forms are leukemias and various

solid cancers like breast, lung, ovarian and thyroid cancers. The main contribution to cancer is genetic and epigenetic DNA damages, such as base changes, methylation, deletions and chromosomal rearrangements. Ionising radiation affects the DNA structure and function either directly or indirectly (targeted and non-targeted effects). Furthermore, radiation affects DNA molecule either via direct or indirect (e.g. via free radicals) action.

The linear no-threshold (LNT) model is a general model to estimate the excessive cancer risk from radiation exposure, mainly developed for radiation protection purposes. The model is based on epidemiological data from moderate-high doses, and then the risk is linearly extrapolated to lower doses (< 50-100 mGy) to become zero for non-exposed persons (16).

There are two different ways of describing cancer induction, short- and long-term effects. The short term effects include repopulation, initiation and the inactivation of normal and pre-malignant stem cells (recovery time about a month). The long term effects consider spontaneous cancer induction (17, 18).

1.4.1. ^{131}I and thyroid cancer risks

The thyroid is sensitive to radiation and thyroid cancer incidence normally increases linearly with dose, and an effect is seen already at doses below 100 mGy (low dose), and at doses above 5 Gy (high dose) the increase in effect is weakened. The Chernobyl data indicates that thyroid cancer can be induced at doses below 100 mGy (19).

Radiation-induced effects have been seen even when the thyroid was not in the radiation field *e.g.* during treatment of neuroblastoma and lymphoma (20). One interesting finding was that radiation induced cancer seems to have more severe chromosomal changes from DNA double stand breaks (amplification and gene fusion) compared to sporadic cancers (point mutations) (21).

The majority of radiation induced thyroid cancers are papillary thyroid cancers (PTC). The latency time of PTC is often more than 5-10 years and the radiation induced cancers behave in a similar way as those in non-ex-

posed patients and are usually not aggressive (20, 22). The risk of developing thyroid cancer is increased up till 40 years after exposure (23). The thyroid cancer incidence is about three times higher for women than men.

Children are normally more radiosensitive than adults and the difference in incidence of thyroid cancers was first noted when treating children with benign diseases during the 1920-1960th (20). Compared with adults, children are more sensitive to radiation probably because they have a more active cell proliferation. Other factors that contributes to a higher sensitivity is largely unknown, but may include higher metabolism and a difference in hormone profile compared to adults (24).

Furthermore, children have a longer expected life-time than adults and therefore higher incidence of radiation induced cancers might be expected (25). Since the age at exposure is an important factor, children under three years of age are not recommended radiation treatment (20). Patients younger than 10 years that receive radiation therapy are more sensitive to radiation even at low doses compared to older children and, thus has a larger risk of developing thyroid cancer. The risk for developing secondary cancer is increased from 10 to at least 20 years after the first primary tumour (26). The correlation with radiation exposure at a young age and thyroid cancer incidence has been established, and the number of cancer patients was increased with a factor of 2-4 times compared to a non-exposed population.

1.5 Nuclear power plant accidents

There are a number of accidents and nuclear test weapon detonations that involves ^{131}I release. Some examples with corresponding estimated ^{131}I release are Hanford reservation nuclear production complex (27 PBq ^{131}I 1944-1972), Marshall islands nuclear testing site (233,100 PBq ^{131}I between the years of 1946-1958), Nevada nuclear test site (5,550 PBq ^{131}I 1952-1970), Chernobyl (1,850 PBq ^{131}I 1986), and Fukushima (511 PBq ^{131}I 2011) (27).

1.5.1 Chernobyl

Chernobyl has been called "the greatest nuclear catastrophe in human history" by the IAEA (28). The estimated amount of released ^{131}I was $1.8*10^{18}$ Bq. Totally 200 000 km^2 area was contaminated and of this 71 % of the area is located in Ukraine, Belarus and Russia (28). The iodine exposure during and after the accident primarily came from milk and dairy consumption and leafy vegetables, as well as inhalation (24, 28-30). Administration of KI protects the thyroid from ^{131}I accumulation, and the best effect for a one time exposure is seen when the administration is done up to 24 h before the exposure until 8 h after exposure (27, 31). For continuous exposure the blockage of the thyroid can be kept at over 90 % for a daily intake of 15 mg KI (32). In general, few or no efforts were made early after the Chernobyl accident to protect the population in the most contaminated areas from ^{131}I intake (33).

The estimated absorbed dose to the thyroid from ^{131}I depends on several factors, including the population (age, gender, thyroid mass, living area, outdoor activity, and diet), the method used for calculation of dose (detector measurements, interviews, simulations), and which factors that were accounted for (radionuclides, potassium iodine, milk and local food consumption) (28, 34-39). The estimated thyroid doses was higher for the Belarussian populations compared to the Ukrainian cohort, where children in Bryansk had the highest estimated dose (40-42). The majority of the absorbed doses to thyroid are in the interval of 0.001-10 Gy (only about 0.7 % had a dose that exceeded 10 Gy) (29, 30, 36, 43-46). However, the dose has recently been re-evaluated using Monte Carlo based simulations. The mean dose to the entire population was 0.43 Gy. More than half of the population received a dose of less than 0.2 Gy, and only 0.5 % had a thyroid dose above 5 Gy, and the highest dose seen was 8.7 Gy (47). Moreover, the correlation between thyroid dose and cancer risk was estimated, and the relationship was linear for doses below 2 Gy and linear quadratic for doses in the range of 2-5 Gy. An additional factor that affected the cancer risk was stable iodine intake, where a higher intake seemed to decrease the cancer risk (48). The cancer incidence after the Chernobyl accident in children started to increase about 4-5 years after the accident (49, 50).

The effects of irradiated thyroid tissue were increased thyroid size, increased proliferation rate, and difference in tissue structure compared to non-exposed individuals. However, the T4, TSH and serum TG levels were normal, even though some studies report increased TSH levels (51-53). Furthermore, a small increase in hypothyroidism, hyperthyroidism and autoimmune thyroiditis were reported (53). The radiation induced thyroid cancers in children were most common in the Gomel and Bryansk regions and the vast majority was of papillary origin (43, 54-56). In a large screening study of almost 12000 individuals that were under 18 years old at the time of the Chernobyl accident, and tested 10-18 years after the accident, about 8 % had developed thyroid nodules. The risk of developing thyroid nodules increased with absorbed dose to the thyroid and a lower age at the exposure (24). For children (younger than 18 years at the accident) living in the four most contaminated areas in Ukraine, Belarus and Russia, 6000 thyroid cancers were discovered (19). A contributing factor to the high number of thyroid cancers detected after the Chernobyl accident is due to extensive screening (23).

1.5.2 Fukushima

Ten years ago (March 2011) a nuclear power plant accident occurred in Fukushima, Japan, when 160 PBq ^{131}I was released (57). The absorbed dose to the thyroid for the persons living in close proximity to the power plant were around a few mGy, and in total 116 and 71 thyroid cancers were found after the first and second screening of children exposed to ^{131}I. However, these cancers are assumed to be sporadic tumours (randomly induced for other reasons than irradiation) identified due to the extensive screening. The PTC cases obtained from the Fukushima accident had more similarities with adult PTC cancer than with the Chernobyl children cancers (58). Recently, a histological evaluation of PTCs related to the Chernobyl and the Fukushima accidents, respectively and including corresponding controls was performed. The correspondence between the Japanese tissues from persons living in the Fukushima area and unexposed controls were large. However, no such correspondence was found for the PTCs from the Chernobyl accident with any of the other tissue materials. The authors concluded that no correlation with radiation exposure could be seen for the

Fukushima related PTCs, but for the Chernobyl related PTCs it was apparent (59).

1.6 Biomarkers for ^{131}I exposure in normal thyroid and for thyroid cancer

To be able to predict biological effects and to give the best treatment to radiation-exposed individuals, radiation related biomarkers could be an option. "The ideal radiation biomarker should provide information about dose and time and should be independent of environmental and confounding factors such as smoking, drug therapy, age, etc" (60). In biological dosimetry "a biodosimeter can be characterized as a physiological molecule with an expression pattern that is quantitatively altered when exposed to ionizing radiation" (61). There are several difficulties for estimating dose in an exposed individual, due to the radiation field, heterogenic exposure, and the unsureness of which parts of the body that have been exposed, both for external and internal exposure (61). In recent years, microRNA has been proposed as interesting molecules for radiation biodosimetry, but further evaluation of the suggested candidates are needed (60). However, the most widely used method today is dicentric chromosome analysis, but it is time consuming and user-dependant, and faster omics based methods would be of interest (60). Gene expression analysis can be used for evaluation of radiation exposure and dose, *e.g.* for peripheral blood lymphocytes (60). Protein expression analysis can be used and there are several markers proposed for radiation exposure, and some early examples are C-reactive protein and amylase (61, 62). Metabolomics is another interesting method, since the response to radiation is related to changes in metabolites, and can be studied in urine, blood, saliva, and faeces, but also in soft tissue (including thyroid) (60, 63). However, the paradigm of finding one optimal biomarker candidate has recently shifted, especially for lower absorbed doses towards a panel of different transcript and/or protein biomarkers, eventually using different omics techniques (60).

1.6.1 The transcriptional and translational processes

During DNA transcription, the information in the DNA is copied (transcribed) into a single stranded ribo-nucleic acid (RNA) molecule (Figure 1.2) (64). During and after the transcription RNA is spliced, meaning that non-coding RNA (introns) are removed and only coding RNA (exons) are

ligated together. The final RNA molecule is called messenger RNA (mRNA). The mRNA is then transported to ribosomes in the cytoplasm, and its genetic information read and translated into the protein using transfer RNA (tRNA), adding amino acids in the right order. In the human genome there are approximately 20,000 genes, 80,000 variants of transcripts, resulting in 250,000-1,000,000 different proteins (65).

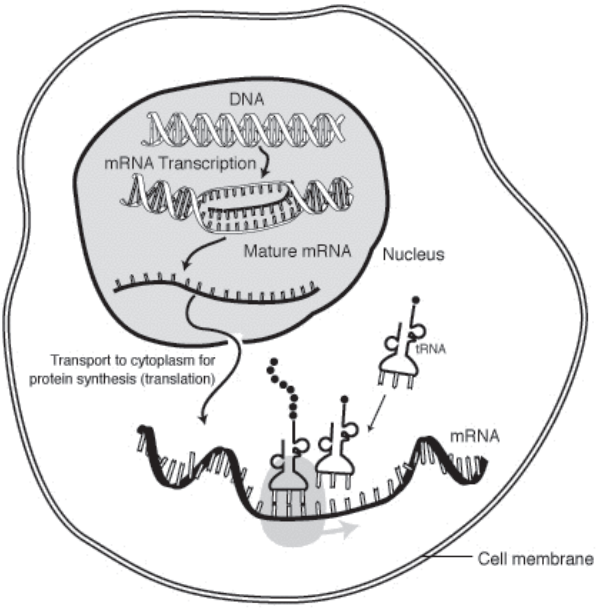

Figure 1.2. The synthesis of proteins in a simplified way.
DNA is transcribed to mRNA in the nucleus and transported to the ribosome in cytoplasm, where the mRNA code is translated to protein using tRNA. The figure was retrieved from Wikipedia, Messenger RNA, ***https://en.wikipedia.org/wiki/Messenger_RNA#/media/File:MRNA-interaction.png*****.**

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1.6.2 Previously proposed biomarkers for ^{131}I exposure of normal thyroid tissue

Our research group has previously identified and partly validated potential biomarkers (transcripts and proteins) in thyroid tissue from rats and mice exposed to low-intermediate absorbed doses (0.0058 Gy-1 Gy), and intermediate-high doses (1-32 Gy) from ^{131}I (66-69). These studies were focussed on acute and short-term effects up to 24 h after ^{131}I injection. The number of regulated transcripts decreased with increased absorbed dose from ^{131}I. In summary, the suggested biomarkers from gene identification included *Agpat9, Klk1,* the *Klk1b* family, *Plau, Prf1 and S100a8* (67, 68). Furthermore, the *Dbp* gene was down-regulated in rats exposed to low or moderate absorbed doses (68). Four proteins were also suggested as biomarkers related to absorbed dose: PGAM2, CHIA A2M and CAH1. In a proteomic analysis in mice, relatively few regulated proteins were identified with altered levels in thyroid and plasma 24 h after administration of ^{131}I, and functional analysis indicated hypoxia, effects on hematopoiesis and decreased thyroid function (69). Furthermore, the circadian rhythm also affected radiation induced gene expression in the thyroid, where highest number of significantly regulated transcripts was found after exposure in the morning, and many of the regulated transcripts belong to the kalikrein family (66).

1.6.3 Biomarker candidates related to PTC induced after the Chernobyl accident

Different biomarker candidates, genetic aberrations and signalling pathways have been identified from PTC tissue from children irradiated during the Chernobyl accident (Tables 1.2-1.3). There are large differences in the results, since the analytical method varied, but also the reference groups used, together with individual differences. However, some results were obtained in several studies, including rearrangement of *RET/PTC1* and *RET/PTC3* genes, an increased number of copy number alterations (CNAs), single nucleotide polymorphisms (SNPs) related to *FOXE1* gene, and the protein CLIP2.

Table 1.2. *Suggested biomarkers for PTC induced due to the Chernobyl accident***.**

Biomarker candidates	Ref.
ABCC3, C1orf9, C6orf62, FGFR1OP2, HEY2, NDOR1, STAT3, UCP3, ANKRD46, CD47, HNRNPH1, NDOR1, SCEL, SERPINA1	(70)
DIRC3, NRG1, PTCSC3, MBIPI	(58)
TG, TSH	(71, 72)
TPO, SLC5A5	(72)
BLC2	(21, 73)
CLIP2	(21, 74-76)
BAG2, CHST3, GLOM1, KIF3C, NEURL1, PPIL3, RGS4	(75, 76)
SFRP1, MMP1, ESM1, KRTAP2-1, COL13A1, BAALC, PAGE1	(77)
CA12, BID, CCND2, TFF3, LRP1, DUSP1, TSP1	(78)
FOXE1	(81)
ATMIVS22-77, TP53arg72Pro	(79)
DIRC3, NRG1, PTCSC2/FOXE1, PTC5C3, MBIPI	(58)
ATMIVS22-77 TP53Arg72Pro ATMG5557A, XRCC1Arg399Gln	(80)
PPARG, NTRK1, NTRK3	(49)
PAX8, KIT, CYR61, PAPSS2, FHL1, PIP3-E, ELMO1, CTGF, ZFP36L2, NCAM1, SORD, FBLN1, GLUL, ANK2, CHRDL1, DEPDC6, PDLIM3, ZMAT4, NUAK2, LRRN3, SCL43A3, ODZ1, KCNJ2, CAMK2N1, TRA, CYP1B1, PDZRN4, GABBR2, CA12, GALNT7, MPZL2, TIAM1, PDZK1P1, SCG5, DMD, LPL, AMIGO2, PLXNC1, SPOCK1, C8orf4, ABCC3, TNIK, ETV1, CDH6, TMEM100, NT5E, HEY2, PLAG1, LMO3, ZMAT3, HPN, AUTS2, ADAMTS9, ST3GAL5, MTUS1, TSC22D1, AK1, PPP1R7, KLHDC8A, C10orf72	(81)
SERPINE1, DUSP1, TRIB1, S100A10, RDH12, ANXA1, GNAL	(78)
DNA gain chromosome 7 (7p14.1-q11.23)	(82)

Table 1.3. *Genetic aberrations and affected signalling pathways in PTC induced by exposure due to the Chernobyl accident.*

Genetic aberrations	**Ref.**
RET rearrangements	(76, 80, 83, 84)
RET/PTC	(74)
RAS rearrangements	(58, 74)
RET/PTC1	(21, 58, 73, 83, 85)
RET/PTC2, RET/PTC4, RET/PTC5, RET/PTC6, RET/PTC7, RET/PTC8, RET/PTC9, AKAP9/BRAF, AGK/BRAF, TPR/NTRK1, ETV6/NTRK3, PAX8/PPARG	(21, 49)
RET/PTC3	(1, 21, 49, 58, 73, 78, 85)
CCDC6/PTEN	(21)
CREB3C2/PPARG	(21, 49, 85)
RET/NTRK	(73)
BRAF rearrangements	(21, 58, 73, 74, 78, 84)
NTRK rearrangements, TPRIN/TRK1	(49)
Signalling pathways	
MAP-kinase	(21, 58, 74, 76)
RAS-RAF-MAP kinase signalling pathway	(78)

1.6.4 Biomarkers used or proposed for thyroid tissue, function and cancer

There are some biomarkers that are clinically used for characterisation of thyroid tissue function: the thyroglobulin (TG) glycoprotein, and the thyroid peroxidase protein (TPO) (86). TG is produced only in the follicular cells of the thyroid. This feature is conserved for this type of cells even in neoplasms, and therefore serum TG can be used as a marker for residual thyroid tissue after intended total thyroidectomy, as well as recurrent and metastatic thyroid cancer (87, 88). Also, the TPO protein and *TG* gene can be used as biomarkers for thyroid tissue, unless the TSH levels are suppressed (89).

The number of peripheral blood micronuclei transferrin receptor positive reticulocytes (Tf-RETs) increases within the first day of ^{131}I treatment for thyroid cancer and decreases the following 2-5 days. This method may be used as a bio-dosimeter, since it can detect doses as low as 100 mSv and has been suggested as a marker for chromosomal damage (90).

1.7 Methods for transcriptomic analysis of tissue samples

There are two methods for global transcriptomic analysis, the RNA-seq and the RNA expression microarray (91). In this thesis, Agilent rat microarray expression chips were used to identify differentially expressed transcripts (Figure 1.3). The Agilent microarray chip consists of probes (holes) that contains short oligonucleotides 60-100mers to analyse each mRNA transcript separately. The test samples are labelled with florescent dye (commonly Cy3) (92). The test samples are purified, replicated, coupled and hybridised onto the glass slide. A scanner is used for reading the signal intensity and the data obtained is then processed and normalised.

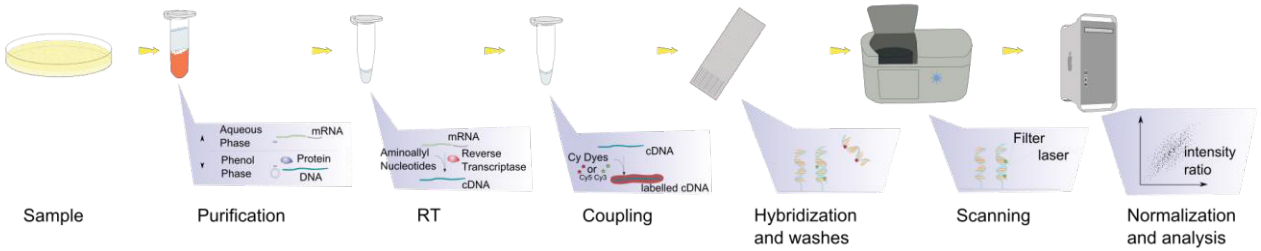

Figure 1.3. A schematic overview of the mRNA microarray analysis process.
The figure was retrieved from Wikipedia, The steps required in a microarray experiment, ***https://upload.wikimedia.org/wikipedia/commons/thumb/e/e8/Microarray_exp_horizontal.svg/1920px-Microarray_exp_horizontal.svg.png***

To verify the results from the microarray analysis, real-time quantitative polymerase chain reaction (RT-qPCR) was used to measure the expression of a certain gene (Figure 1.4). The sample RNA is converted to cDNA. The cDNA solution is incubated with nucleotides, primer, DNA polymerase and a detector probe (Taq-man). The detector probe consists of complementary bases to the gene of interest, a fluorescent dye and a quencher that absorbs all the fluorescence from the dye. The primer binds to the cDNA, and then DNA polymerase binds to the 3´end of the primer and starts copying the DNA (elongation process). When passing the primer the quencher becomes inactivated and the fluorescent dye is released. This process is repeated in cycles by controlling the heat and making the DNA molecule divide into two chains (denaturate).The amount of fluorescence is measured in real time by a spectrometer coupled to the thermocycler, and is a measure of the amount of transcripts of the selected gene in the original sample (93).

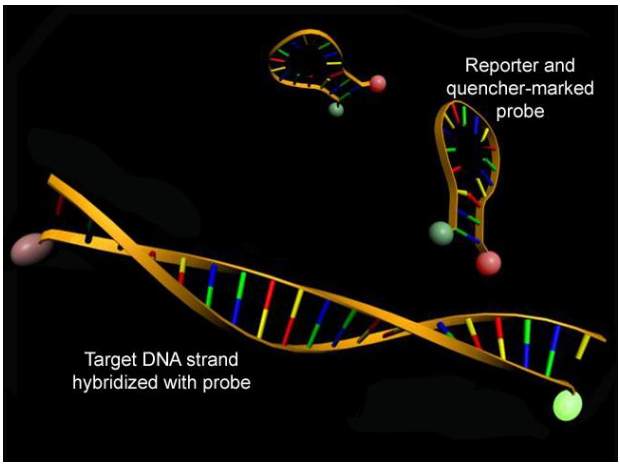

Figure 1.4. A schematic overview of the RT-qPCR process.
The figure was retrieved from Wikipedia, Real time PCR uses fluorophores in order to detect levels of gene expression

https://upload.wikimedia.org/wikipedia/commons/0/05/Molecular_Beacons.jpg.

1.8 Methods for proteomic analysis of tissue samples

There are several methods to detect the amount of a single protein, and the methods can be divided into two main groups: spectrometry methods and antibody-based methods. The spectrometry method scan be divided into two subgroups: high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). The antibody-based methods include enzyme-linked immunosorbent assays (ELISA), 2D gel-electrophoreses, Western blot techniques, immunohistochemistry and immunofluorescence methods (94). In this thesis, LC-MS-MS and ELISA methods were used, since they are sensitive and can detect proteins with concentrations down to pg/mL.

When using LC-MS-MS the proteins are first separated by LC, usually HPLC. First tissue samples are homogenised *e.g.* using a lysis buffer, then each protein is resolved into peptides, commonly using the enzyme trypsin and labelled with mass tags. A column using an acetonitrile gradient separates the peptides. The peptide ions are then sprayed into the MS system that separates them by mass, charge and time of flight (Figure 1.5). This makes it possible to obtain a spectrum of peptides and by combining these data the proteins can be identified together with their concentrations (95).

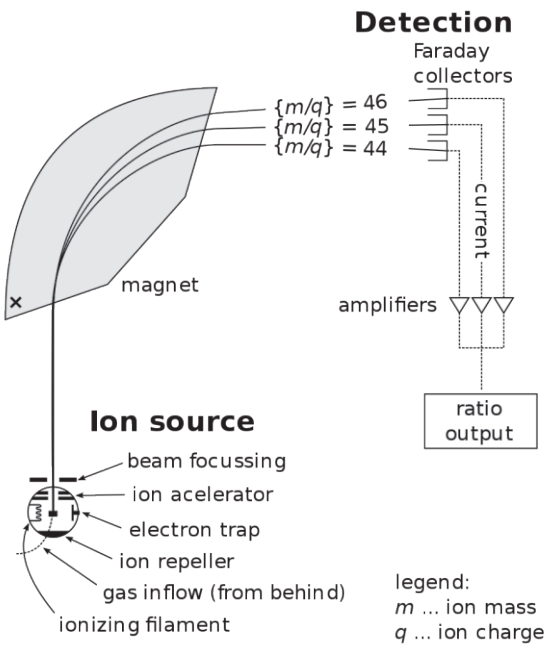

Figure 1.5. A schematic overview of the mass spectroscopy process. *A mass spectrometer has three major parts: an ion source, a mass analyser, and a detector. The mass spectrometer separates ionised samples and the mass analyser divides the amino acids from the samples by mass, charge and time of flight. The detector records the amino acids and a spectrum is created and evaluated when determining the proteins in the sample. The figure was retrieved from Wikipedia, Schematics of a simple mass spectrometer with sector type mass analyser.* ***https://en.wikipedia.org/wiki/Mass_spectrometry#/media/File:Mass_Spectrometer_Schematic.svg***

The ELISA method is the golden standard method for validation of protein expression (Figure 1.6). The sample is added to the 96-well plate and the protein binds to the bottom of the wells. The plate is washed and then incubated with antibodies (specific for the protein of interest) that is bound to a certain type of enzyme. Antibodies bind to the protein of interest. After washing, substrate (*e.g* horseradish peroxide, HRP, or alkaline peroxide) is then added and generates colour after interacting with the enzyme of the antibody. During the final analysis the ELISA plate is read in a plate reader and the colour intensity from the samples correlates directly with the amount of the protein (96).

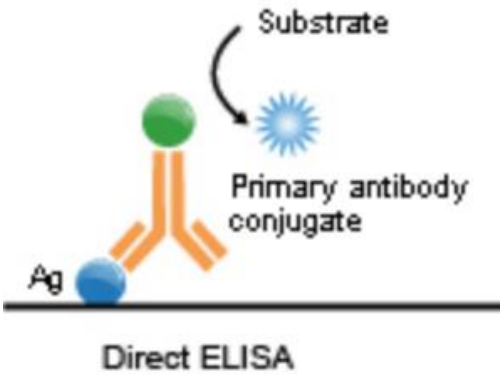

Figure 1.6. A schematic overview of the direct ELISA method*. The antibody (yellow) binds to the protein (blue) and the enzyme (green) reacts to the substrate and creates colour (light blue).The figure was retrieved from Wikipedia and modified, ELISA types, https://upload.wikimedia.org/wikipedia/commons/c/c9/ELISA_types.png*

2. Aims

The overall aim was to investigate long-term biological effects in thyroid tissue and plasma samples from rats after low-intermediate dose exposure to ^{131}I, by studying transcriptional and translational effects.

The specific aims of this work were to:

- Study the radiobiological effects on transcriptomic and/or proteomic expression data in thyroid and/or plasma from rats after injection of ^{131}I, in relation to non-exposed age-matched controls, depending on:
    - absorbed dose, for low-intermediate ^{131}I exposure (**Papers I** & **III**)
    - age, comparing data from rats that were young and adult at time of ^{131}I exposure (**Papers II-III**)
    - time after ^{131}I injection in plasma from young and adult rats, examined after 3, 6 and 9 months (**Paper II**)

- propose biomarker candidates for:
    - ^{131}I exposure (**Papers I-III**)
    - dose-response (**Paper I & III**)
    - age (**Papers II-III**)
    - thyroid function (**Papers I-III**)
    - thyroid cancer (**Papers I-III**)

  based on the expression of transcripts and proteins.

3. Material and Methods

Animal experiments (Papers I-III)

Male Sprague Dawley rats (Taconic Bioscience, Denmark**)** were i.v. injected with ^{131}I (0.5, 5, 50 or 500 kBq) or were mock treated at 5 (young) or 17 (adult) weeks of age (Figure 3.1). The rats had free access to water and standard rat chew and were under daily supervision. The animals were killed 3, 6, 9 or 12 months after injection and thyroid tissue and plasma were collected and stored at -80ºC for further analyses. Half of the thyroid was incubated in formalin, and then imbedded in paraffin. The imbedded tissues were cut in 4 µm slices and stained with haematoxylin and eosin. The morphology of the thyroid samples was evaluated by certified pathologists. The expression of transcripts and proteins were studied in remaining thyroid tissue and plasma, and data from exposed and age-matched non-exposed rats were compared.

All animal experiments were approved by the Ethical Committee on Animal Experiments in Gothenburg, Sweden (Permit Number: 146-2015).

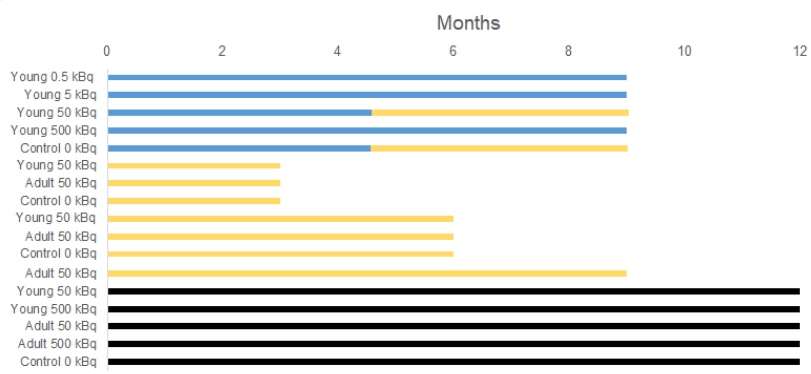

Figure 3.1. Overview of the rat studies. *A representation of the different test groups, including the injected activity and termination time point. The blue bars represent the test groups in* ***Paper I****, the yellow bars the test groups in* ***Paper II****, and the black bars the test groups in* ***paper III****. Note that the young 50 kBq 9 month group and the corresponding age matched control group are included in both* ***Paper I*** *and* ***Paper II,*** *and therefore coloured in both blue and yellow.*

Transcriptomic analyses

mRNA microarray analysis (**Papers I-II**)

Total RNA was extracted from homogenised thyroid tissue samples using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hiden, Germany). The RNA quality was evaluated using the Nanodrop ND-1000 and RNA 6000 Nano LabChip kit with Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) to determine RNA concentration, purity and integrity number (RIN). The RIN cut off value was set to 6. The RNA was hybridised using Agilent SurePrint G3 8x60K (Agilent, Santa Clara, CA, USA) at the Bioinformatics and Expression Analysis core facility at Karolinska institute. The Benjamini-Hochberg method was used for statistical analysis, and the fold change cut off values were set to >1.5 or <-1.5, and a FDR-adjusted p-value < 0.01.

RT-qPCR (**Paper I**)

cDNA was processed from mRNA samples, according to the manufacturer's instruction, using SuperScript™ VILO™ cDNA Synthesis Kit

(Invitrogen, 11754-050). Primers for selected genes and controls (*beta-actin, Gapdh* and *HPTR1* genes, Applied Biosystems) were run on a real-time 7500 HT sequence detection system (Applied Biosystems). The $\Delta\Delta C_t$ method was used to determine the relative expression of the analysed genes compared to the control genes (68).

Proteomic analyses

LC-MS/MS (Papers I-III)
Proteins from thyroid tissue and plasma were digested using trypsin and labelled with TMT 10-plex (Thermo Fisher Scientific, Waltham, MA, USA). Analysis was performed using LC-MS/MS at the Proteomics Core Facility at the University of Gothenburg (Gothenburg, Sweden) either for group-wise pooled thyroid and plasma samples or for individual thyroid samples. The fold change and p-value cut-offs were set to >1.5 and <0.05, respectively.

ELISA (**Paper I**)
Standards and triplicate samples were prepared and added into assigned wells of the ELISA plate (MyBioSource Cat no; MBS9324066) and HRP conjugate was added to each well. The plate was incubated at 37ºC, and then washed. Chromogen solutions were added, and then followed by stop solution. Absorbance was measured at 450 nm, and mean fold change values determined.

IPA analyses (**Papers I-III**)
Based on the differentially expressed genes or proteins, the changes in biological functions, canonical pathways and upstream regulators were analysed using the Ingenuity Pathway Analysis software (IPA; Ingenuity Systems, Redwood City, USA). Statistical analysis was made using Fisher´s exact test ($p<0.05$), when comparing the experimental data set with that in the Ingenuity Pathways Knowledge Base. The activation direction was determined by the z-score, where $z>2$ indicates activation and $z<-2$ indicates inhibition.

GO terms (**Papers I-II**)

For the transcripts, associated GO terms were obtained using the Nexus Expression software, and terms with $p<0.05$ (Benjamini-Hochberg method) were considered statistically significant. For the proteins, GO terms were received by the DAVID functional annotation tool (https://david.ncifcrf.gov/), using modified Fisher´s exact test ($p<0.05$). An in-house model was used for functional annotation based on Gene Ontology (GO) terms (97, 98). The model is based on parental GO terms, divided into 8 main categories (DNA integrity, gene expression integrity, cellular integrity, cell cycle and differentiation, cell communication, metabolism, stress response and organismic regulation) and 31 subcategories (99).

4. Results and discussion

Paper I

This paper considers the dose and exposure related long-term effects (9 months) on thyroid tissue and plasma from young rats injected with low or intermediate ^{131}I activity (0.5, 5, 50 or 500 kBq). The expression patterns of genes and proteins were studied in the tissues samples from radiation exposed rats and compared with corresponding data from non-exposed age matched controls.

Overall, much fewer transcripts than proteins were significantly regulated in the thyroid. A higher number of regulated proteins were seen for the thyroid tissue compared to plasma. Data from microarray analysis and LC-MS/MS protein expression were successfully validated by RT-qPCR and ELISA, respectively.

Few transcripts were seen in more than one of the groups, and only two transcripts were seen in all groups. In thyroid tissue, 2 proteins were identified in all groups and 7 in three groups, while only two were found in plasma and then only in three groups. Of these, the following dose-related expression was found for 2 proteins (all groups) and 7 proteins (three groups) in thyroid tissue, and two were found in plasma (three groups).

The expression patterns of the transcripts and proteins were related to absorbed dose. Also, potential relation to thyroid function and cancer were examined for the identified transcripts and proteins. Biomarker candidates were proposed for ^{131}I exposure (9; 2 transcripts and 7 proteins; Table 4.1), dose (4 proteins; Table 4.2) and thyroid function (4 proteins; Table 4.5).

The majority of obtained GO terms were related to metabolism, immune system and cell death. The canonical pathways from the IPA analysis were mainly related to actin cytoskeleton, B cell receptor, and hepatocyte growth factor (HGF), integrin and calcium signalling.

Paper II

In this paper, age- and time-related radiation-induced effects were examined in thyroid and plasma samples from young and adult rats 3-9 months after injection with 50 kBq ^{131}I compared with controls.

In thyroid tissue, fewer transcripts than proteins were statistically significant, and no common transcript was identified in all groups. The mass spectrometry analysis showed a larger number of proteins identified in the thyroid compared to plasma. From these data, biomarker candidates were identified: a) related to ^{131}I exposure (1 transcript; Table 4.1); b) related to age and time (4 proteins with unidirectional expression; Table 4.4); c) related to time but independent of age (3 with varying direction of expression; Table 4.4); d) related to age at a certain time-point (34 proteins and 1 transcript; Table 4.3); and e) related to thyroid function (1 transcript; Table 4.5). However, no single biomarker candidate related to age irrespective of time after exposure was identified. For this purpose, a panel of 20 proteins with the highest up-regulation in plasma was suggested.

From the GO term analysis profound effects were seen connected to the cell cycle and metabolism. From the IPA analysis of canonical pathways, the majority of the identified pathways were seen in only one group. However, the pathways that were in common for more than one group were related to the cell cycle, actin cytoskeleton, and to ephrin, integrin, paxillin and RhoA signalling.

Paper III

In this paper, age- and dose-related effects were investigated twelve months after ^{131}I injection (50 or 500 kBq) on protein expression in thyroid tissue of young and adult rats. The protein expression was compared with that in mock treated age matched control rats.

The LC-MS/MS analysis identified over 7,000 proteins and almost 1800 proteins were statistically significant, but ca 800 of them were seen in only

one of the groups. Biomarker candidates were proposed, related to ^{131}I exposure (a panel of 40 proteins; Table 4.1), age (10 proteins; Table 4.3), absorbed dose (5 proteins; Table 4.2), and thyroid function and thyroid cancer (9 proteins; Table 4.5).

The IPA analysis showed that the biological effects after exposure were most prominent for young individuals, related, e.g., to metabolism, and hormone synthesis and regulation.

Summary of Papers I-III

Altogether, the results showed differences in transcript and protein expression, 3-12 months after start of exposure. There were expression differences that could be related to dose, age at exposure, and time after exposure. In general, the data showed higher number of differentially expressed proteins than transcripts (**Papers I-II**). The higher number of significant proteins were expected, since 1) the regulation of transcripts generally occurs early after irradiation and reduces more rapidly than for proteins that are regulated at a later step in the regulation process, but also 2) the larger number of proteins that can be produced compared with the number of genes (65, 100). Also the higher number of proteins obtained in thyroid tissue than plasma was expected, since plasma contains fewer protein types in general compared to the thyroid (101). Furthermore, a larger number of significantly expressed transcripts and proteins were seen for low activities (5-50 kBq) compared to very low and intermediate activities (0.5 and 500 kBq) (**Papers I&III**). The same pattern of increased number of transcripts for the intermediate dose were seen in our previous studies on thyroid in mice and rats 24 h of ^{131}I injection (67, 68). No general time dependent trends in number of regulated transcripts or proteins were seen at 3, 6 and 9 months after exposure (**Paper II**).

Biomarker discovery is not easy, and it is especially difficult in the current application, where low expression differences might be expected for late response after relatively low absorbed doses. A practically useful biomarker should be found in plasma and have increased expression to be more easily identified by more clinically applicable biological methods such as ELISA, Western blotting, and qPCR, *etc*.

A biomarker related to ^{131}I exposure alone should be detected in all groups, and have uniformly increased or decreased expression levels. In **Paper I**, no protein in plasma fulfilled these criteria, but two transcripts and seven proteins in thyroid tissue were suggested as potential biomarker candidates for ^{131}I exposure (Table 4.1). No such candidate were seen in **Paper II** but the PTH protein in thyroid tissue had similar expression pattern irrespective of age. In **Paper III**, no single biomarker candidate for ^{131}I exposure was found, but a panel of the highest expressed 40 proteins was suggested.

For dose related biomarker candidates, the expression should ideally increase with increasing administered activity. Preferably, the biomarker should be present at all doses or in a defined dose interval. In **Paper I,** no biomarker candidate was seen in all four groups, but two proteins in plasma were suggested for the three lowest doses and two proteins in thyroid for the three highest doses (Table 4.2). In **Paper III,** five dose related biomarker candidates were seen in thyroid tissue.

Table 4.1. Exposure related biomarker candidates (transcripts (Tr) and proteins (Pr) in thyroid (Th) and plasma (Pl) (Papers I-III)

Exposure related						
Paper I		**Paper II**	**Paper III (panel)**			
Th, Tr	Th, Pr	Th, Pr	Th, Pr	Th, Pr	Th, Pr	Th, Pr
Afp, RT1-Bb	ARF3, DLD, IKBKB, NONO, RAB6A, RPN2, SLC25A5	PTH	WFDC2, B2M, Hemoglobin subunit beta-2, PRPF40B, GNL3L, LRPPRC, TUBB4B, AIF1L, FRYL, Ig gamma-2C chain C region	TNNT3, KRT5, SFN, DSG1, 3-ketodihydrosphingosine reductase, SERPINB5, ADA, HRNR, MYH8, RPTN	SLC25A46, PPP1R1A, WDFY2, GLRX5, SMPD1, KDSR, ASPH, HSPB3, BAG5, MRPL39	P3H1, SRSF4, PTGDS, TIE1, S100A9, ORM1, PRKAG3, KNG2, MYO18B, KNG1

Table 4.2 Dose related biomarker candidates (Papers I&III).

Dose related			
Paper I		**Paper III**	
Thyroid, proteins	Plasma, proteins	Thyroid, proteins low dose	Thyroid, proteins high dose
APRT, LDHA	DSG4, TGM3	PALM2, NME3	CLCC1, CPT2, HP

Age dependent biomarkers should be present in only young or adult rats at a certain time point. In **Paper II** and **Paper III,** 51 and 10 such protein biomarker candidates in thyroid and plasma were identified, respectively (Table 4.3). The criteria for age and time dependent biomarkers were that they should only be present in one of the age groups, but present at all time points with a time dependent expression pattern, ideally with unidirectional increasing or decreasing expression with time. In **Paper II,** 4 protein biomarkers, 3 in thyroid and 1 in plasma were proposed (Table 4.4). For age-dependent but time-independent biomarker candidates, the biomarkers should be present in only young or adult individuals at all or at certain time points. No single marker could be identified according to these criteria in **Paper II**, and therefore a panel of proteins in plasma were suggested. Time dependent but age independent biomarkers should be detected at all or several time points in both young and adult rats, and 3 biomarker candidates (2 proteins in thyroid and 1 protein in plasma) in **Paper II** fulfilled these criteria.

Table 4.3 The age related biomarker candidates (proteins and one transcript) (Papers II-III).

Age related				
Paper II				**Paper III**
Young		Adult		Adult
Thyroid, proteins	Plasma, proteins	Thyroid, proteins/ *transcript*	Plasma, proteins	Thyroid, proteins
FABP4, TKT, MLEC, PMAP, DECR1, CPT2, TUFM, GDI2, RAP1A, FABP5, KRT15, KRT4, HMMR, LGALS7	PVALB, FTL1, PLEC, F7, PF4, PROC, PKLR, GPD1, eEF1A1, CAPN1, CA1, RGS18	PVALB, HMGN2, EIF3J, KRT1, RPLP2, PTH, CPQ, HSPB6, DPYSL2, GOT1, MYBPC1, KRT13, *Vegfb*	HIST1H1E, MCPT1, FN1, HINT1, APOC4, eEF1A1, HIST1H4B, HSP90B1, F9, MYL6, LBP, FKBP1A	ALDH1A7, APOBEC2, ATPSCKMT, IGHG, LCN2, LGALS5, PRR33, SMB8, RT1-A1B, TAPC2

Table 4.4 Time- and age-related protein (Pr) biomarker candidates in thyroid (Th) and plasma (Pl) (Paper II)

Time and age dependent		Age dependent and time independent		Time dependent and age independent	
Young	Adults	Young	Adults	Young and adults	
Pl, Pr	Th, Pr	Pl, Pr	Pl, Pr	Th, Pr	Pl, Pr
CA1, FTL1, PVALB	HSPB6	ACTA2, ACTB, ANXA1, CA1, CA2, COPS4, CORO1A, CPS1, DSG4, LGALS5, NPM1, NRIF1, PFN1, PRDX4, PSBPC2, RGS18, RT1-AW2, SPRR1A, TPM4, XK	ANXA1, CMPK1, DHTKD1, EEF1A1, EZR, HIST1H1E, HIST1H2BA, Histone H2A type 3, Histone H3.1, HNRNPA2B1, HNRNPC, HNRNPK, LMNA, NPM1, NRIF1, PGAM1, PIGR, PPP1R7, PRDX4, VCP	KRT13, PTH,	eEF1A1

When it comes to biomarkers related to biological effects on thyroid function the biomarker should be thyroid specific and the level of expression should correlate with the thyroid function. In **Paper I**, four biomarker candidates were suggested, 2 for all activities and 2 for low activities (Table 4.5). For **Paper II** and **Paper III**, 1 and 9 proteins in thyroid tissue were related to thyroid function and/or thyroid cancer, respectively.

Table 4.5 Thyroid function and thyroid cancer related biomarker candidates (Papers I-III)

Thyroid function and thyroid cancer related		
Paper I	**Paper II**	**Paper III**
Thyroid, proteins	Thyroid, transcript	Thyroid, proteins
ACADL, SORBS2, TG, TPO	*Vegfb*	ADA, BAG5, BM2, HP, LCN2, S100A9, SERPINB5, SMPD1, TIE1

Interestingly, none of the suggested biomarker candidates in **Paper I** or **Paper II** were seen in **Paper III**. The only differences between the studies was the termination time point, where the data from **Paper III** was collected after 12 months, while the other were from the period 3-9 months, and that thyroid samples were pooled in **Papers I&II**, but not in **Paper III**. There are several potential reasons for this finding. Probably, the major reason is that only effects of low dose and relatively low dose rate exposure was studied, where no deterministic effects were expected. Then it is more probable that the individual cells respond differently, and give rise to multiple effects on expression at low level. Another factor is the long time interval after exposure, where differences between rats but also cells within a rat may develop differently. Pooling is often used to reduce biological variation, but this is under debate, and biological effects might be hidden in situations where individual differences are more probable as in the present data (102). It should be noted that validation of the expression of selected transcripts and proteins were successful in the present studies as well as in previous similar studies (68).

The expression data in **Papers I-III** were connected to several GO terms and canonical pathways, where most of them were seen in only one of the study groups. However, the effect on the metabolism was in common for almost all groups in all papers. This finding can be expected since one of the main tasks of the thyroid is to produce hormones and regulate metabolic processes (2).

5. General discussion

.In this thesis work, long term effects of low dose exposure to ^{131}I was studied. Human studies would have been preferred, but are not possible in an initial phase of explorative research, and the studies were performed on rats, with a reasonable size of the thyroid gland, compared with mice. Sprague-Dawley rats were chosen since they are commonly used in various toxicological studies (103, 104).

The studies was performed *in vivo* to be able to include the total response within the organism since we were interested in evaluating the total effects on transcript and protein expression as a basis for better understanding of corresponding situations in humans, especially after the Chernobyl accident. Such studies are not possible in *e.g.*in cell cultures, since radiation-induced systemic effects, related to, for example, hormonal, inflammatory and immune response, would be neglected.

In this work, rats were i.v. injected with ^{131}I, and thus all organs and tissues in the body were exposed, although to different absorbed doses. In general the thyroid received ca 40 times higher absorbed dose than the rest of the body (105). Still, the protein expression in plasma will most probably be influenced by proteins from other tissues, since the whole body is irradiated and the thyroid is small. Another factor to have in mind is the indirect effect of irradiation, since changes in hormone production of the thyroid can affect other organs, which in turn can affect the thyroid (106). The early radiobiological response, and to some extent also biodistribution of ^{131}I, is influenced by the circadian rhythm (66, 107). Furthermore, previous short-term studies on externally irradiated mice have demonstrated that early non-targeted and out-of-field responses on the transcriptomic level are complicated with different contributions from radiation and systemic factors (108). These factors were demonstrated in short-term studies and their influence on long-term effect are not yet understood.

The thyroid consists of both the iodine-accumulating thyrocytes and non-iodine-accumulating C-cells (109), beside cells in vessels and connective tissues. Due to the long range of emitted electrons from ^{131}I all cells in the thyroid were relatively homogenously irradiated (110). This means that the measured response includes not only that from thyrocytes, which explains, e.g. response related to Ca-signalling from the C-cells, and there might be methods to separate response from cell types (109).

The studies were performed on male rats only, in this case to avoid bias due to sex, since females in general are more sensitive to radiation compared to men (111). It is generally known that the sex of the animal can affect the gene and protein expression, and there might be large variations between different species, but also between different studies on the same species ((112), and references therein). Some of these differences were also dependent of age. Furthermore, thyroid diseases are more common in females than males, where one explanation is the higher amount of oestrogen in fertile females (113). From the thyroid cancer cases in Chernobyl cohorts, the gender difference is not as clear; an increased excess absolute risk and an excess relative risk of two was seen in females compared to males, but it was not statistically significant (114, 115).

One limitation in the age-related studies was that the rats were injected with the same ^{131}I activity, irrespective of age, and hence different body and thyroid masses. In the present studies, the young and adult rats weighed 100-150 g and 400-500 g, respectively, with corresponding thyroid weight around 17 and 24 g (116). Thus, the absorbed dose differed between the groups. The dosimetric estimations in **Papers II-III** were done based on biokinetic data from 180-200 g rats (thyroid weight 19 g) and the Medical Internal Radiation Dose formalism (105, 117). Therefore, the absorbed dose to thyroid will be less than 35 % higher in young rats than adult rats, since the relative thyroid weight decrease with age in this age interval (116). However, in **Paper I** only young rats were included and thus the relationship between the different doses will be the same as the differences in activity administered.

The proposed biomarkers from **Papers I-III** show little resemblance with data from our previous short-term studies in mice and rats 24 h after ^{131}I

exposure (66-68). This finding can be expected since an acute response in gene expression (that will not last over time) is seen a short time after irradiation, especially at higher doses, while long-term response is assumed to be persistent over time (100). According to my knowledge, there are no other study performed in rodents that concern long-term transcriptomic and proteomic effects in thyroid after low dose ^{131}I exposure. However, there are some studies of expression of transcripts and proteins performed in mice and rats (majority 6-10 weeks of age) for other organs and tissues (heart, kidney, liver, mammillary gland and testis), using whole body irradiation either by low-dose external irradiation (^{60}Co, ^{137}Cs, linear accelerator, x-ray and uranium tailings), or higher doses of X-rays (8 and 16 Gy) (118-124). These studies showed that the number of regulated genes and proteins were higher early after exposure (days-weeks) compared with data after months after exposure. Few genes and proteins (less than 100) with a fold change over 1.5 were seen more than two months after the exposure and the majority of these were down regulated. Also, the number of significantly regulated genes and proteins were in general higher for the lowest doses (0.05-0.5 Gy) and for the highest doses (8 and 16 Gy) seven and four months after irradiation, respectively. Data in **Paper II,** demonstrated no general time dependence of gene or protein expression. In **Papers I and III,** no general dose dependence was seen. However, the rats used in **Papers I-III** were 5 or 17 weeks of age, which is somewhat younger and older than the rodents used in the other studies, a difference might affect the results since young individuals are in general more sensitive to radiation (125, 126). However, the differences in tissue type and time after exposure is most probably the major contributing factors to the difference in results.

Several genes have previously been proposed as biomarkers for ionising radiation exposure (100, 127). Of these 53 genes, only one gene, *Vegfb* was seen in any of the present results, and then only for adult rats in **Paper II**. However, most of these 53 genes were identified from *in vitro* studies early after radiation exposure, which may cause a translational bias, as discussed above. Furthermore, 49 genes were suggested from short-term studies on mice and humans after external radiation exposure, and one protein,

ACTA2 (suggested age dependent biomarker in young rats) were seen in **Paper II** (128).

In an extensive review paper on radiation related protein expression in humans, 261 protein biodosimeters were suggested (129). Of these, 14, 33, and 6 proteins were identified in **Papers I, II** and **III**, respectively. Furthermore, the previously suggested biomarkers for thyroid malfunction (*TG*, and *TPO* genes, and TG, TSH, and TPO proteins) were identified also in the present long-term data, but only in few groups, probably due to differences in time after exposure (86). The resemblance was also low between the findings in **Papers I-III** and the previously suggested biomarkers from PTCs in the Chernobyl cohort, and very few candidates were in common (76). This lack of common results might be due to several factors, such as differences in absorbed dose and dose rate, normal or cancerous tissue, and translation bias when using rat instead of human material, since the consistency of genes is 62-88 % (50, 130).

6. Conclusions

In conclusion, radiobiological effects on the transcriptional and/or translational level were found in thyroid and/or plasma samples from irradiated rats versus non-irradiated age matched control rats, up to one year after ^{131}I injection. Effects were related to:

- absorbed dose, with a larger number of significantly expressed transcripts and proteins after exposure to low activities (5-50 kBq ^{131}I) compared to very low and intermediate activities (0.5 and 500 kBq ^{131}I) (**Papers I & III**)
- age, where, in general, a larger number of identified transcripts and proteins were seen in young compared to adult rats (**Papers II-III**)
- time after ^{131}I injection, but no general time dependent trend in expression of transcripts and proteins was seen (**Paper II**)

Based on the data from the transcript and protein expression analyses, biomarker candidates were proposed for various applications:

- As biomarker candidates for ^{131}I exposure, 2 transcripts and 7 proteins in thyroid tissue were proposed, based on the 9-month data from young rats (**Paper I). O**ne transcript in thyroid tissue was seen for adult rats **(Paper II**), and from the 12-month data from both young and adult rats, a panel of 40 proteins in plasma were suggested (**Paper III**). Exposure-related biomarkers could be of interest when evaluating if an individual has been exposed to ^{131}I or not.
- As biomarker candidates for evaluation of dose from ^{131}I exposure, 4 proteins were proposed, 2 in thyroid tissue and 2 in plasma, based on the 9-month data from young rats (**Paper I**).

- Several age-dependent biomarker candidates were proposed, 14 proteins in thyroid tissue and 11 proteins in plasma for young rats, and 1 transcript and 12 proteins in thyroid tissue, together with 12 proteins in plasma for adult rats 3-9 months after exposure (**Paper II**). In total,10 proteins in thyroid tissue were proposed for adult rats at 12 months after exposure (**Paper III**).
- ^{131}I exposure related biomarker candidates with known connection to thyroid function and/or thyroid cancer were identified. From the 9-month data from young rats, 4 proteins, 2 for all four dose levels, 1 for the two lowest doses and 1 for two other dose levels, were proposed (**Paper I**). In adults, 1 transcript was seen 3-9 months after irradiation (**Paper II**). In the 12-month data from young and adult rats, 9 proteins related to thyroid function or cancer were found in thyroid tissue (**Paper III)**.

7. Future perspectives

Today, there is still a general knowledge gap regarding the underlying molecular mechanisms behind radiation induced response in cells and tissues in vivo. Some short-term studies have been performed, while there are fewer long-term studies published. Furthermore, overall radiation induced effects and risks are to a high extent known for higher absorbed doses, while those for low and moderate absorbed doses and dose rates are less known. Better understanding of the radiation induced mechanisms and effects will lead to an increased understanding of the tissue response, which can be applied in future research on, e.g., medical applications and biota exposed at nuclear events.

This knowledge might aid in improving radiation therapy of cancer, both for increasing effects on tumour tissue and reduce side effects on normal tissues, and result in enhanced cure rate. With reliable early responding biomarkers reflecting risk of long-term side effects the treatment can be individualised and result in better quality of life. Further, with more cancer survivors, more radiation induced secondary cancer may occur, and radiation induced mechanisms and biomarkers may help in earlier detection of such tumours.

Altogether, the results from this work on radiation induced mechanisms and effects on thyroid tissue and plasma varied between the different studies, reflecting the complexity of long-term radiation-induced response after low doses and dose rates. Therefore, further *in vivo* studies on the radiation effects of the thyroid gland should be performed to better understand mechanisms and effects. Beside the factors included in the present work, activity of injected radionuclide (involving absorbed dose and dose-rate), age at exposure and time after exposure, other factors should also be considered, such as radiation quality and circadian rhythm. It would be beneficial to

repeat the studies in this thesis, with these factors in mind on a higher number of rats as well as using individual samples, both for validation and better statistical evidence.

When planning future studies and translation of knowledge to humans, it is important to consider that the present work include homogenous cohorts of animals and similar environmental conditions such as food, water, temperature, light, humidity, and social and environmental stimuli. In a human cohort there are differences in both genotype and phenotype, which probably will give larger differences in radiation induced response. In addition, the more rapid aging and aging effects of the animals need to be considered when planning long-term studies in humans.

Furthermore, direct studies on human tissues should be performed, since there is a transfer bias when translating data from animal studies, although the genome homology between rats and humans is rather high and rats are good first surrogates for this kind of studies. However, one of the main goals is to apply this knowledge and test proposed biomarkers on humans. Retrospective studies can be done on different cohorts selected after the Chernobyl and Fukushima accidents. Such studies have been done and show various important and realistic results from a group of patients. However, one limitation of these cohorts is the uncertainty in dosimetric estimations. Thus, more prospective short- and long-term studies should be initiated on well-characterised groups of patients exposed to ionising radiation from diagnostic or therapeutic procedures. Focus would most probably be on easily available tissue samples, and whole-genome and whole-proteome methods combined with Western blot, ELISA or qPCR techniques.

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